EVALUATION OF IMMUNE RESPONSES AND CYTOLOGICAL CHANGES IN *LUMBRICUS TERRESTRIS* AND *EISENIA FOETIDA* AS ASSAYS FOR XENOBIOTICS

DISSERTATION

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Abdolrahman Sadeghi Hariri, B.S., B. S.
Denton, Texas

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The earthworms, *Lumbricus terrestris* and *Eisenia foetida*, were used as non mammalian surrogate models to assess the immunotoxic potential of xenobiotic to mammals. Assays were developed and optimized for detecting spreading activity and phagocytosis of rabbit red blood cell (RRBC), bacteria, and yeasts by macrophage-like coelomocytes of *L.terrestris*. For phagocytosis, *Candida albicans* was found to be the most suitable cell. Among bacteria, *Bacillus thuringiensis*, because of its large size and Gram positive reaction, was the organism of choice for assaying phagocytosis. Exposure of *L. terrestris* to Polychlorinated biphenyl (PCB - Aroclor 1254), chlordane, and cadmium nitrate did not effect spreading activity and phagocytosis of RRBC and bacteria by the macrophage-like coelomocytes. However, contaminants in Superfund soils, significantly suppressed this non-specific immunity of *L. terrestris*. It is suggested that increasing the exposure time and exposing earthworms in soil be used in future assays of PCB's and heavy metals rather than the filter paper contact method. Bacterial

challenge of *L. terrestris* exposed to PCB caused the earthworm to become more susceptible to *Serratia marcescens* infection. However, *B. thuringiensis* remained non-pathogenic. Natural humoral antibacterial responses against *Bacillus megaterium* and *Aeromonas hydrophila* were measured in *E. foetida*. Antibacterial activity against *A. hydrophila* was significantly increased by injection of sub-lethal doses of *A. hydrophila;* it peaked 4 days after inoculation. The heavy metal content of refused drived fuel fly ash made *E. foetida* more susceptible to infectious agents, and significantly suppressed induction of the humoral antibacterial response of *E. foetida* against *A. hydrophila* when measured by in vitro assays. A preliminary stereological study of electron micrographs of epidermal tissue from *L. terrestris* exposed to copper sulfate demonstrated the sensitivity of this method in the quantitation of cellular and subcellular changes upon exposure to xenobiotics. This may be a promising area for future research. Determination of uptake of xenobiotics by coelomocytes using energy dispersive X-ray microanalysis of whole cells was unsuccessful.

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LIST OF ABBREVIATIONS

1.	ATCC	American Type Culture Collection
2.	ANOVA	Analysis of Variance
3.	CF	Coelomic Fluid
4.	CFU	Colony Forming Unit
5.	EDTA	Ethylene Diamine Tetraacetic acid
6.	EDX	Energy Dispersion X-ray
7.	EPA.	Environmental Protection Agency
8.	FA	Fly Ash
9.	LBSS	Lumbricus Balance Salt Solution
10.	OD	Optical Density
11	NA	Nutrient Agar
12.	NB	Nutrient Broth
13.	PCB	PolyChlorinated Biphenyl
14.	RDFF	Refused Drived Fuel Fly Ash
15.	RPM	Revolution Per Minute
16.	RPMI	Roswell Park Memorial Institude
17.	RRBC	Rabbit Red Blood Cell
18.	SEM	Scanning Electron Microscope
20.	SD	Standared Deviasion
21.	SRBC	Sheep Red Blood Cell
22.	TEM	Transmission Electron Microscope
23.	UNT	University of North Texas
24.	WBC	White Blood Cell

CHAPTER I

INTRODUCTION

Purpose

The problem confronting environmental toxicologists is the complex task of identifying the mechanism of action and toxic potential of environmental xenobiotics to public and environmental health. The assimilation of xenobiotic chemicals within organisms at sublethal or lethal levels may induce a sequence of biological effects. These range from molecular interference with biochemical mechanisms and interactions with cellular organelles, to pathological changes at the cellular, tissue, and organ levels. These result in an integrated functional or behavioral response, experienced at the whole organism level which may be reversible or irreversible. Though considerable work has been done and assays developed for studying acute toxicity, mutagenicity, carcinogenicity and teratogenicity of chemicals, less has been done on the effects of xenobiotics on immune function, especially with invertebrates. Among various invertebrates, much is known about the immune system in the earthworm and a number of assays of immune function have been developed. However, few studies have been made on the effect of environmental factors on immune function. The

purpose of this study was to evaluate the effect of xenobiotics on immune function of earthworms so that assays may be developed to determine the extent of environmental contamination. Specifically, the effects of chlordane, PCB, superfund soils, RDFF, and heavy metals (copper, cadmium) on non-specific and humoral immune responses were tested in *L. terrestris* and *E. foetida*. Assays evaluated included spreading, phagocytosis (RRBC, bacteria, yeasts), bacterial growth inhibition and immunity, and resistance to microbial challenge. In addition, stereologic studies of epidermal tissue and cells were conducted to determine the effect of copper sulfate on cellular morphology, chlordane exposed earthworms were examined by SEM for its effect on morphology of the coelomocytes, and attempts were made to determine the location of xenobiotics in cells using electron microscopy and energy dispersive X-ray (EDX) microanalysis.

Relevant Literature

Importance of Xenobiotics in the Environment

Pollution involves the releasing of some component(s) into the air, water, or soil which have a deleterious effect on the environment. Pollutants are not only poisons, they may also be nutrients released in such large concentrations that they have a harmful effect on the environment, or

substances that may suddenly appear in the environment in quantities that overwhelm natural recycling processes (154); e.g. crude oil. Toxic chemicals are poisonous by definition, and are, therefore counted as xenobiotics. There are some naturally occurring poisons but it is known that they help to preserve the balance of nature even when their mechanisms are not fully understood. But man-made toxic chemical wastes (xenobiotics) have no benefit to the natural environment. The development of modern technology has brought a dramatic increase in the production and consumption of chemicals. In a few cases, the benefits of chemical use have been accompanied by unexpected adverse environmental effects. Some examples of this are, the persistence and bioaccumulation of some heavy metals, polychlorinated biphenyls, kepone and dioxins.

Environmental Impact of Xenobiotics.

Some pollutants that are discharged in dilute concentrations into the general environment are re-concentrated by biological activity. Animals that graze large areas, and especially organisms at the lower end of the food chain, can contribute greatly to this biological amplification. A cow, for example, may graze over as much as 1,600 square feet a day (56). Many trace contaminants can build up in her milk in a relatively concentrated form.

Since milk constitutes a large fraction of the diet of infants and children, such a pollution pathway can be particularly destructive and dangerous. In some cases, the amount of biological amplification can be quite extraordinary. Clams, oysters, and other mollusks feed by filtering enormous volumes of water, and, while doing this, they separate some trace contaminants out of the water. Some freshwater mollusks can concentrate manganese by a factor of 300,000 and chlorinated hydrocarbon insecticides by up to 70,000 times (56). While humans could drink the water without suffering ill effects, the poisons concentrated in the flesh of the mollusks can pose real dangers.

In the past, the dangerous by-products of manufacturing have been buried on land or at sea, or released at concentrations so dilute that they appeared to pose no hazard. That legacy in recent years has come to haunt us. The best examples are Love Canal, New York (dioxin), Times Beach, St. Louis (dioxin) (62), Minamata Japan (mercury) and Itai Japan (cadmium). These have come to symbolize the pollution problem to many people (116).

Sources of Toxicants and Importance in Human Health.

Metals are widely distributed in the environment and are frequently essential to plant and animal health when consumed in minute quantities. However some metals, when

concentrated above natural levels constitute a serious threat if inhaled or ingested. Sixty years ago, arsenic was the only metal known to be a carcinogen (56). Today, it has been established that cancer can be caused by lead, cadmium, chromium, cobalt, iron, nickel, selenium, titanium, beryllium, copper, and zinc (56, 88). Unlike radioactive wastes, toxic metals have no half-life and are removed only through slow processes that operate over geologic time (56). As wastes, they cease being troublesome only when they are buried so deep in sediment that they are unlikely to be disturbed. As a category of pollutant, the heavy metals are rather comprehensive in their potential attacks on the human body. For example, certain forms of mercury and lead attack the central nervous system (56, 2), nickel and beryllium, the lungs (56); cadmium, the kidneys (56) and antimony, the heart (56).

Mercury, which is used in a wide variety of manufacturing processes ranging from the production of electrical equipment to dental supplies, is released into the environment by the combustion of fuels and the high-temperature processing of some minerals (53). It is a by-product of various manufacturing processes, is distributed widely in biocides, and can be released directly from discarded consumer products (2, 53).

Another heavy metal, lead, has long been recognized to cause major disorders and even death when large quantities of it are ingested (56, 75). More recent evidence shows that very low concentrations of lead, particularly in children, can adversly affect enzymes, various organs, and especially the nervous system (56, 75). Now that lead has been eliminated from paints in many countries, the greatest problem comes from using leaded gasoline in automobiles.

A variety of nonmetalic toxic substances can also pose serious problems, especially those that have a tendency to become concentrated through the food chain. The best known examples of bioconcentratable and biological amplification occur with the organochlorine insecticide DDT, chlordane (lesser degree than DDT), and polychlorinated biphenyls (PCBs) (44, 56, 89, 152). When DDT is sprayed on an area, it remains effective for a long period of time. During this period, because it is readily absorbed by living organisms, it can work its way up the food chain. When ingested by an animal, it tends to accumulate in the fatty tissue instead of being excreted. When one fish eats another, most of the meal is digested and then excreted, but much of the DDT is retained. As small fish are eaten by larger ones, which in turn are finally eaten by birds, the ratio of DDT to body weight increase steadily at each stage. At high concentrations, the chemical impairs reproductive functions

in birds, causes liver damage, and results in neurological disorders (56, 89).

PCBs are a family of chemicals that have long been known to be dangerous, and are normally introduced into the environment only in a very dilute form. More than 200 types of PCBs have been manufactured. Different kinds have different properties, and are used in products ranging from electrical insulators and plastics to pesticides and hydraulic fluids (152). It is not clear which PCBs pose serious dangers to humans or at what levels these dangers become acute. In 1968, some Japanese rice oil was contaminated by PCBs that accidentally leaked from a heating system. People who ate the rice oil suffered skin ailments, liver damage, swollen arms and legs,and other symptoms (56). Similarly DDT, PCBs are very stable compounds that accumulate in the fatty tissues of organisms, and are capable of extreme bioamplification (56, 89, 152). A few studies have also shown that these chemicals can interfere with the overall growth of plants when applied at high concentrations (152).

Previous and Current Methods for Assaying Xenobiotics

Chemical Analysis.

The use of land for treatment, repositories for agricultural pesticides and disposal of various municipal and industrial wastes has resulted in a desire for more

information concerning the effects of these materials on the soil and water ecosystem. Identifying and characterizing toxic chemicals in the environment was and is a key element of toxic substances research. New measurement methods and systems capable of detecting toxic materials over a wide range of concentrations and in complex environmental situations are continuing to be developed.

One of the oldest environmental contamination analyses was based on gravimetric and colorimetric methods (62). Crone Hugh (62) in the mid-1950s measured the lead content of motor fuel by converting the lead compounds to lead chromate, combusting all of the hydrocarbons, then weighing the lead chromate eluted from a packed column or paper chromatogram (62). Conventional column or paper chromatography are not accurate analytical methods, but are separatory methods which allow components to be measured by techniques which do not work on crude mixtures. Colorimetric methods used in conjunction with chromatographic separations give relatively good quantitative measurements (62).

Older analytical methodologies included infrared and ultraviolet spectroscopy. Newer methods include, gas chromatography, mass spectrometry, electron spin resonance, nuclear magnetic resonance, atomic absorption spectrophtometry, and thin layer chromatography. By the

1960s these techniques were established as general laboratory methods (62), and much effort was expended to improve them.

In the 1980s, the sophistication of the instrumentation associated with these techniques has increased greatly, improving sensitivity, selectivity, accuracy and the ease of use of the instruments (62). Also new techniques have been developed, for example, high performance liquid chromatography complemented gas chromatography as the two methods of choice for the analytical chemist, particularly in the detection and analysis of trace constituents in environmental pollution control.

The ultimate method for trace analysis now is the combination of gas chromatography as a separatory technique combined with mass spectrometry as a means of detection and identification (abbreviated as GC-MS) (62). In GC-MS, the analyst receives a crude sample which may be a post mortem sample of human or animal tissue, or a sample of soil suspected of being contaminated (62). As a first step, the analyst extracts the sample and brings it into a homogeneous form with a liquid solvent known to dissolve the compound being sought. After this, the sample may be concentrated by evaporation of the solvent and crudely fractionated by liquid chromatography (62). If the chemicals of interest are reasonably volatile, they may now be injected into the GC-MS.

Relatively nonvolatile components must be changed to volatile derivatives before injection.

The sensitivity of GC-MS varies according to the nature of the sample and the skill of the operator. The instrument can be expected to detect and quantify 0.1 to 1 ng in one sample injection (62). One important advance in instrumentation is the development of computerized data-processing equipment to go on the output end of the instrument (62). Computerization increases the sensitivity of the assay and allows the operator to store, sort and compare data with libraries of information (62).

Electron Microscopy

Stereology. Recently biologists, toxicologists, and pharmacologists have used stereological techniques to quantitate structural changes in tissues and cells exposed to a toxicant (7, 31, 127). Stereological techniques allow the extrapolation of structural information from a number of two-dimensional micrographs to form an average three-dimensional structural picture of cells or tissue (32). Weibel (1979) described the technique as follows: "Stereology is a body of mathematical methods relating three dimensional parameters defining the structure to two-dimensional measurements obtainable on sections of the structure." Because stereology allows one to measure cell structure, the assimilation of

data from a large number of micrographs is easily accomplished without author bias (32). These techniques also make it possible to detect very small cytological differences between populations of cells. Mathematics enters into the picture because the appearance of profiles depends on the chance of cutting the objects in a certain way, and since the data are quantitative, differences can be subjected to statistical analysis for assumption of correctness (32, 157). Thus, stereology describes the structure of an average cell in the population of sample cells. Average cell structure can be defined in three dimensional terms using such features as organelle volume, membrane surface area, number of organelles per unit volume, and/or other descriptors (32, 157). Stereological measurements use a test system such as grids to measure the area or the number of line intersections with various profiles of interest in a micrograph. The devices may be as simple as a series of lines or points on a transparent sheet, or as complex as computerized digitation tables and digitizing videocoms (32).

Microanalysis. Yet another way of investigating the presence of toxic ions in the cell is elemental microanalysis with the help of the electron microscope; i.e.the electron microscope can provide other data besides important morphological information (9). Electron microscopes used to identify or characterize the chemical nature of components

found in biological tissues, are said to be analytical electron microscopes (9). When a high energy beam of electrons interacts with a specimen, the atoms of the specimen may cause the electrons to decelerate. If this occurs, the kinetic energy associated with the electron is then converted into various types of X-rays, visible light, and heat. Some electrons may be transmitted through the specimen with the loss of some energy (inelastically scattered) or no loss of energy (elastically scattered). Other electrons may be given off from the top of the specimen as high energy (backscattered) electrons or lower energy (auger, secondary) electrons (9). The nature and the spectrum of the energy liberated, as well as the images formed by the new trajectories of the electron, may be captured using special detectors fitted to the electron microscope. Such detectors may reveal the atomic composition of the specimen struck by the beam of electrons (9). Energy dispersive X-ray (EDX) detectors are the predominant types used in biological studies. "The sensor consists of a disc-shaped semiconductor manufactured from a single crystal of silicon into which some lithium atoms are diffused (to correct for impurities and imperfections in the silicon crystal structure). When an X-ray strikes the semiconductor crystal, the absorbed energy alters the ability of the crystal to conduct a charge. Since the crystal is maintained

at a bias voltage of 100 to 1,000 volts, an increase in conductivity of the crystal can be readily detected and quantitated. Since the energy of the X-ray is directly proportional to the increase in conductivity in silicon crystal, it is possible to collect and measure the conducted current over a period of time and determine the intensity of the X-ray emission"(9).

In general, microanalysis is the easiest method (and sometimes the only one) for analysing microscopic samples. It has other advantages as well. It is sensitive to low concentrations - minimal detection limits (MDLs) are about 0.1% in the best cases and typically less than 1%; and its dynamic range runs from the MDL to 100%, with a relative precision of 1% to 5% throughout the range. Furthermore, the technique is practically nondestructive in most cases, and requirements for sample preparation are minimal (149).

Biomarkers - Animal Test Systems.

When a soil or groundwater contamination problem is discovered, the attention of the public and of regulatory agencies focuses on the potential effects on human health. However, human exposure to a chemical at some minimal concentration must occur before an adverse effect on human health is realized. For this reason, most damage data are derived from toxicity tests in which a xenobiotic of known

concentration is delivered to an individual or group of receptor animals under controlled environmental conditions, usually in the laboratory. Traditionally, laboratory animals for toxicity tests are small mammals such as mice, rabbits, or ginea pigs.

The introduction of animal testing in the U.S. in the 1920's was a major advance in toxicity testing (48). The functions of toxicity tests are to identify the symptoms and effects of xenobiotics on laboratory animals, to identify the toxic limits used as criteria in survey research and in environmental control, and to confirm field experience of pollution damage (116). The reactions of these mammals to different xenobiotics are currently the best available predictors of the effects the substances will have on the human body (48). The exposure-response relationship describes the likelihood of an organism to develop a particular adverse biological response as a function of its exposure to the xenobiotics. Toxicity tests of different xenobiotics on experimental animals have been done in two phases: acute, and chronic. In acute toxicity tests animals are exposed to relatively high concentrations of a xenobiotic for predetermined periods of time in a controlled environment (e.g. constant temperature,light). The xenobiotic is usually supplied in the medium (i.e. food or water or air) either as a single dose or at graded concentrations throughout the

experiment. The simplest expression of acute toxicity is the ranking of animals in comparison with the response of a standard species. The dose of a toxicant which is lethal for 50% of a group of animals (LD_{50}) is a classic example of an acute toxicity and dose-response test that measures acute lethality. It provides a statistically accurate measure of the amount of a chemical that will produce 50 percent mortality in a population of animals. Comparison of LD_{50} values for different agents gives a measure of relative toxicity. A variation on the LD_{50} test is the ED_{50} (for "effective dose"), which measures the amount of a chemical that will produce a deleterious effect other than death in 50 percent of the population (48). For greater precision, the threshold concentration for a given response of predetermined magnitude may be discovered by exposure of receptor animals over a standard period of time. For instance, laboratory mammals or fish are often tested for the $LC_{50/48\ h(or\ other\ periods\ of\ time)}$ value, which means the least concentration of the xenobiotic required to kill 50 per cent of a test population in 48 hours of exposure (116). Toxicity data from acute toxicity trials may be extrapolated to the external environment with confidence only where the conditions of exposure are strictly identical (e.g. same doses, temperature, animal health etc) (116). With each deviation from test conditions margins of error greatly increase. For

instance, acute toxicity data for fish may seriously underestimate damage in the field (e.g. actual toxicity is 0.4 - 0.6 of anticipated $LC_{50/48\ h}$ (depending on the xenobiotic involved) because of the greater sensitivity of some stages of the animal (e.g. young) and subtle interference with feeding and breeding (116).

The accumulation of persistent xenobiotic residues within an animal may be studied in chronic exposure tests. Chronic toxicity tests are essentially the same as those utilised for acute toxicity tests. The main difference is the animals are exposed to relatively low concentrations of xenobiotics over longer periods of time. The results depend very much on the criterion of chronic toxicity adopted, the concentration of the chemical used and the duration of exposure. The basic objectives in chronic toxicity tests are to determine the level of residues within a receptor that constitutes a threshold for a damaging response, and the rate at which accumulations of residues occur in response to exposure.

Frequently, chronic lethal toxicity tests are merely extensions of acute trials; they may extend knowledge of the lethal time/concentration function (116). In some cases it is suspected that different mechanisms of toxicity may be involved in acute and chronic lethal exposures (e.g. the toxicity of cadmium to fish) (116). Greater attention is now

being paid to the study of lethal and sub-lethal responses to very long-term exposure, even for a period equivalent to the life span of the animal. Such tests provide information on mechanisms of actions, dose/response relationships, target organs, accumulation in certain tissue, symptomatology, and carcinogenicity as well as lethality (48, 116).

It is clear from this recitation of procedures that complete toxicological evaluation of environmental xenobiotics, and even one chemical using a mammalian system is complicated, time consuming, expensive, and entails the sacrifice of thousands of laboratory animals which in turn causes socially controversial problems by animal rights groups. For these reasons, scientists are now trying to develop a new methodology known as *in vitro* toxicity testing. *In vitro* testing includes a battery of living systems - bacteria, cultured animal cells, fertilized chicken eggs, frog embryos - that can be employed to evaluate the toxicity of chemicals in human beings (48). But *in vitro* toxicity testing itself is a controversial issue, because it can not answer many questions; e.g., how can *in vitro* tests evaluate toxicological responses that involve, for instance, immunological processes, how can they evaluate chronic toxicity or recovery from toxic insults or how can exposure by ingestion, inhalation or topical contact be simulated ?

These problems must be solved if *in vitro* tests are ever to replace *in vivo* testing.

Since mammalian toxicity testing is costly and controversial, and *in vitro* testing for environmental toxicity testing is out of the question, a cost-effective and non-controversial whole animal toxicity test using a non-mammalian system probably is the best answer to the problem.

The Earthworm as a Monitor

Aristotle called earthworms the "intestines of the soil" (58). Earthworms eat their way through soil, salvaging bits and pieces of organic matter (11). Charles Darwin, in his classic work *The Formation of Vegetable Mound Through The Action of Worms*, said that "an earthworm can ingest its own weight in soil every 24 hours", and he estimated that from 10 to 18 tons of dry earth per acre pass through their intestines annually and are brought to the surface (58). The complexity of soil fauna is a complicating factor when biological assays are used to evaluate the impact of wastes on the soil ecosystem. Soil microorganisms and soil arthropods are very diverse, difficult to identify, and difficult to use in biological and environmental toxicity testing. However, earthworms, a major constituent of the soil biota, are relatively easy to obtain in large numbers, have a significant biomass, and are often studied as a

representative organism of the biological component of soils that may be affected by changes in soil environment (84). Therefore they can be used as possible indicators of the impact of man's activites on the soil ecosystem.

For the following reasons earthworms are an ideal, cost effective and socially non-controversial monitoring system for environmental toxicological assessment :

1. Earthworms' biological characteristics have been known for many years.
2. Many of their immunological features have been studied or are currently under investigation. Their immune systems appears to be sufficiently analogous to those in mammals, including human, so that they can be used to assay xenobiotics for potential immunotoxicity and for evaluating modes of toxic action in mammals (16, 17, 18, 20, 21, 129).
3. They are soil inhabitants.
4. They feed on soil.
5. They are cheap and easy to maintain in the laboratory.

Because of the possible argument about the equivalancy and analogy/homology of the earthworm to mammalian systems, especially humans, the purpose of the earthworm immunoassay screening system is not to replace the existing mammalian immunoassay, but to complement it.

Use of The Immune System

Vertebrate immune system

There are three major features which characterize the vertebrate immune system: specificity, memory, and tolerance. These features are carried out by a well developed immune system composed of (1) humoral or antibody mediated immune response (HMI), (2) cell-mediated immune response (CMI), and (3) non-specific immune components (NSI).

HMI involves the production of soluble factors which include a large class of specific protein molecules called immunoglobulins produced by plasma cells that developed from B lymphocytes, and several enzymes and chemical substances, which act as non-specific antimicrobial agents. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the foreign antigen that induced them. Binding by antibody inactivates viruses and bacterial toxins by blocking their ability to bind to receptors on target cells. Antibody binding also marks invading microorganisms for destruction, either by making it easier for a phagocytic cell to ingest them or by activating a system of blood proteins, called complement, that kills the invaders.

CMI based on T-cells production (cytotoxic, helper, suppressor T-cells), which participate in the recognition and neutralization of abnormal, damaged or foreign cells and

antigens in the body. The reacting cell, for example, can kill a virus-infected host cell that has viral proteins on its surface, thereby eliminating the infected host cell before the virus has replicated. In other cases the reacting cell secretes chemical signals that activate macrophages to destroy invading microorganisms.

NSI, primarily phagocytosis by macrophage and neutrophils, is the first and one of the most important mechanisms which defend the host against infection by ingesting invading microorganisms. Macrophages also play an important part in scavenging damaged cells, cellular debris and foreign perticles. Phagocytosis is usually carried out by actively amoeboid motile cells which are chemotactically attracted to invading microbes. Some aspects of the chemotactic process involve the action of the complement system and lymphocytes. Certain enzymes can also be considered part of the NSI system. The best known enzyme involved in defense is lysozyme, which is found in tears, nasal secretions, saliva, mucus, and tissue fluids of most vertebrates.

Invertebrate immune systems.

It is generally accepted that invertebrates are capable of protecting themselves from toxic foreign substances or infectious agents by well defined non-specific immunity and

some kind of humoral and cell mediated immunity which are analogus to vertebrate systems. Important differences between immunity in vertebrates and invertebrates are the apparent absence in the latter group of definitive lymphoid tissues and lymphoid cells, and the lack of specificity, as defined by antibody-antigen complementarity. Historically, knowledge of cellular immune responses in animals was first obtained from studies by Haekel and Metchinkoff of phagocytosis in invertebrates (91). Cellular mechanisms are mediated by blood cells which, in various groups, are termed amoebocytes, leukocytes, coelomocytes, or hemocytes.

As with vertebrates the effectiveness and outcome of immune responses to infection are determined by numerous factors, including the physiological state of the host and pathogen, the intensity of infection, and the pathogenicity of the invading organism (92).

The earthworm immune system.

The greatest amount of existing data on invertebrate cell-mediated and humoral immunity is derived from the study of annelids (3, 21, 38, 59). The common earthworm is a major representative of the class Oligochaeta in the phylum Annelida which belong to the eucoelomate protostome branch of the animal kingdom (58). Cell mediated immunity in annelids possesses a striking resemblance to that found among the

vertebrates (25). However, humoral immunity in annelids does not include antibodies such as those found in vertebrates (25).

The immune system of the earthworm is located in the body cavity or coelome which is filled with coelomic fluid composed of two main types of immune cells or coelomic cells : (1) amoebocytes (phagocytic coelomocytes), which are phagocytic amoeboid cells derived from the parietal and epithelial lining of coelomic cavity, are responsible for mediation of the non-specific and specific immune response (26, 37, 60, 137, 139, 140, 141), and (2) eleocytes, which come from the chloragogen cells that originate from the epithelium covering the viscera and blood vessels (37, 140). Chloragogen cells are important in glycogen and lipid storage (26, 136), and are responsible for synthesis and release of hemolysins (36, 142, 145).

Cell mediated immune response

Non-specific response. Earthworms similarly to vertebrates possess an efficient non-specific immune response executed primarily by phagocytic coelomocytes. There are numerous phagocytic coelomocytes in the coelome which search out foreign particles, and bacteria and destroy them by phagocytosis (128). The dorsal blood vessel of the earthworm has "valves" which are primarily sites of budding amoeboid

cells, and only secondarly perform a mechanical function in the circulation (85). Similarly, haemal glands adhering to this blood vessel in the midgut region of earthworms produce the coelomic fluid, phagocytes and non-phagocytic coelomocytes (85). Phagocytes seem to be capable of discriminating between different materials injected into the coelomic cavity (25, 85). For example, mammalian sperm or sperm from a worm of a different species are phagocytosed after an interval of about 20 hours, but allogeneic sperm are not; this suggests the absence of strong antigenic differences within a given genus and species (25, 85). Carbon and carmine particles and a variety of bacteria are engulfed more rapidly than foreign sperm or erythrocytes (85).

Specific response. The cell mediated immune response can be best demonstrated by specific skin transplantation. Autografts of an earthworm's own tissue heal promptly and show no signs of rejection, and allografts made between individuals of the same species (e.g. in *Lumbricus terrestris* are rejected, but in earthworms the alloimmune response is weak and slow (16, 17, 18, 20, 22). Xenografts exchanged between earthworms of different species (e.g. *Lumbricus* and *Eisenia*) heal at first but are always eventually destroyed (16, 17, 18, 20, 22).

Humoral immune responses.

Immunoglubulins. Although earthworms clearly show cell-mediated immune reactions, no immunoglobulins are present. However, the coelomic fluid of some earthworms (e.g. *Eisenia* and *Lumbricus*) contains a number of humoral factors (36, 110, 111, 129, 142, 144, 145, 158). These factors, although not structurally related to mammalian antibody, are proteins that exhibit analogous functional properties. In studies of which factors play an important role in earthworm defence mechanisms; agglutinating, hemolytic, and bacteriostatic activities have been detected in the coelomic fluid of *E foetida* and, except for bacteriostatic activity, also in *L. terrestris* (36, 110, 111, 129, 135, 136, 142, 144, 145, 158).

Agglutinins. The earthworm's coelomic fluid contains natural agglutinins which not only agglutinate a number of vertebrate erythrocytes but also a number of bacterial strains (28, 129, 131, 132, 144, 157) suggesting that they have an immune function. Coelomic fluid of *L. terrestris* contains hemagglutinins against a number of vertebrate erythrocytes, with the highest titers for rabbit erythrocytes (129). Induction of agglutinins have been shown in *Lumbricus* by injecting different vertebrate erythrocytes (157). Induced agglutinins may result from synthesis, secretion, or the activation of preformed materials, but when compared to

vertebrate antibody, they are produced more rapidly. Wojdani et al. (157) have shown that in *Lumbricus* induced agglutinin production occurs not only by injecting vertebrate erythrocytes, but also by injecting bovine serum albumin (BSA), carbohydrates, thymidine and amino acids. Coelomocytes capable of sythesizing and secreting hemagglutinins can be identified by their ability to form rosettes with various vertebrate erythrocytes. Studies have shown that at least three and possibly four hemagglutinins are present in induced fluid, whereas normal fluid contains only one or possibly two in detectable amounts (129, 131).

Coelomic fluid of *Lumbricus* contains agglutinins aggainst both Gram-positive and Gram-negative bacteria (131). These agglutinins, while active against a wide variety of bacteria, are also selective in their activity (131). Stein et al. (131) demonstrated that bacteria also induce agglutinin production, although the levels of induced agglutinins vary with the type of bacterium used as inducing agent. Agglutinins function to aggregate foreign material and may serve as an opsonin providing an efficient mechanism for phagocytosis (128). Although hemagglutinin studies have indicated that induced *Lumbricus* coelomic fluid contains three or four different proteins, bacterial absorption analyses suggest that there may be more (129, 131, 132, 158).

Lytic Factors. In addition to agglutinins, earthworm coelomic fluid also contains lytic factors. Hemolytic and proteolytic activities were detected in all *E. foetida* coelomic fluids and in about 10% of L. *terrestris* coelomic fluid samples tested by Ludmila et al. (135). Valembois et al. (24, 146) recorded hemolytic activity titers of 2 to 16 for *L. terrestris* instead of 42,660 for *E. foetida andrei* with sheep red blood cells (SRBC). Coelomic fluid of *E. foetida* after immunoelectrophoresis demonstrates a hemolyic factor as one of five protein components (28). This cytolytic factor lyses SRBC, and it was defined as a lipoprotein whose activity can be inhibited by 15 min heating at 56°C (28, 146). Using a modified Jerne's plaque assay, indirect fluorescence, transmission electron microscopy using peroxidase labeling, and scanning electron microscopy all indicated that both chloragogen cells, rich in granular endoplasmic reticulum, and senescent cells (eleocytes), synthesize and release hemolysins (142, 145, 146). By SDS-PAGE Roch et al. (111) characterized hemolysins and found two bands of 40 and 45 kd which were lipoprotein in nature. Recent chromatofocusing experiments showed high quantities of glutamic acid and glycine residues present in these proteins (146). Hemolytic activity is not inhibited by zymosan, inulin or lipopolysaccharide, nor by hydrazine or methylamine, suggesting that earthworm hemolysins are not

related to C3 or C3b complement components (114). In mammals, these nucleophiles dissociate the internal thioester bond of the complement key component C3, leading to a loss of functional activity (65). The hemolytic activity involves the binding of hemolysin molecules onto target cell membranes (116). Such binding has been reported as being partially inhibited by acetylate or methylated sugars (N-acetyl-D-glucosamine, alpha-methyl-D-mannopyranoids) (28, 111).

These hemolysin molecules are also capable of bacteriostatic activity directed against bacteria pathogenic for earthworms (e.g. *B. thuringiensis*, *B. megaterium*, and *A. hydrophila*) (142). These bacteria have different degrees of pathogenicity. LD_{50} values indicate that *E. foetida andrei* is 100 times more susceptibe to *A. hydrophila* than to *B. megaterium* (78). The bacteriostatic factors are also involved in the clotting of coelomic fluid (147). Because of its strong bacteriostatic activity in *E. foetida*, the lysin has been renamed as *Eisenia foetida andrei* factor or EFAF (28). The activity of EFAF seems important in earthworm defense against potential pathogens (29, 30, 76, 77, 78). Lassegues et al. (78) reported that injection of *A. hydrophila* and *B. megaterium* at LD_{50} doses induced enhancement of antibacterial activity and this increased activity did not discriminate between the two pathogenic bacterial species, regardless of the Gram staining (77). Whereas Cotuk et al.

(19) noted that injection of various bacteria did not induce marked bacteriolytic activity (30).

A lysozyme like enzyme was also recently identified and purified from the *Eisenia* coelomic fluid (30, 76, 77). One of the best known antimicrobial lysins is lysozyme, a bacteriolytic enzyme for Gram positive bacteria, specifically directed against the cell wall mucopeptide N-acetyl muramic acid-N-acetyl glucosamine (NAM-NAG). Lysozyme is a basic low molecular weight monomeric protein with intrachain disulfide bonds; its lytic activity is stable when heated in acidic medium and disappears when heated in an alkaline one (76). Studies of Cotuk and Dales (30) in 1984 and Kauschke et al. (68) in 1987 showed that activity of the coelomocyte extracts agree with the criteria for a lysozyme but the normal level in coelomic fluid is very low. However, Lassalle et al. (76) reported the existence of active and significant lysozyme-like activity against *Micrococcus lysodeikticus* in *E. foetida andrei* coelomic fluid using basically the same method. Lassalle et al. (76) isolated the active protein by fast protein liquid chromatography and characterized it as lysozyme because of its activity against *M. lysodeikticus* cell wall, heat stability at acidic pH and lability at basic pH and mw. of about 20,000. Lysozyme activity can be non specifically enhanced by injection of various cells, e.g. Gram-positive and Gram-negative pathogens, non-pathogenic

bacteria, as well as SRBC (76). Enhancement represents a rapid and transient phenomenon with a peak at 4-5 h after injection (76).

CHAPTER II

MATERIALS AND METHODS

Source of Organisms

Earthworms. Two different species of earthworms, *Lumbricus terrestris* and *Eisenia foetida* were obtained from Carolina Biological Supply, Burlington, N.C..

Microorganisms. *Bacillus thuringiensis* (ATCC 10792), *Aeromonas hydrophila* (ATCC 13447), and *Bacillus megaterium* (ATCC 10778) were obtained from American type culture collection, Rochville, MD. *Serratia marcescens* and *Saccharomyces cervisiae* came from the University of North Texas stock culture collection. The *Shigella* SPP.was isolated from a lesion of a dead earthworm and *Candida albicans* was an isolate obtained at the clinical laboratory of Dallas Childrens Hospital; both were identified by standard microbiological procedures

Maintenance of Earthworms.

L. terrestris was maintained at 10°C in continuous darkness in glass containers (50 cm x 25 cm x 30 cm) filled 1/3 full with peat moss moistened with water. Commercial high protein dry powdered baby cereal (Gerber Product, Fremont, MI) was layered on the surface as a source of nutrient and was replenished as needed. *E. foetida* was

maintained in cow manure moistened with water in small plastic containers (30 cm x 17 cm x 15 cm) filled 1/3 full and kept in continuous darkness at 20°C in an environmental chamber. Earthworms, were acclimated for a minimum of 14 day prior to examination, and checked daily. Fresh moistened peat moss and cow manure were prepared upon arrival of every new batch of worms. Unhealthy individuals were immediatly discarded. Sexually mature adults with well-developed clitella and masses of 2-6 g (*L. terrestris*) and 0.3-0.8 g (*E. foetida*) were used for all experiments.

Culture Conditions For Microorganisms and Standardization of Cell Suspensions.

All bacterial cultures were maintained on 1.5% nutrient agar (NA) (Difco Laboratorries, Detroit, Mich.) slants and kept at 4°C refrigerator until use. For working cultures, cells from slants were inoculated into 250-ml flasks, containing 50 ml half strength nutrient broth (NB) (Difco Laboratories, Detroit, Mich.) and incubated at 30°C for 24 h on a shaker agitated at 100 RPM. Yeasts were grown on Sabouraud dextrose agar plates (Difco Laboratories, Detroit, Mich.) at 30°C for 24 h.

Cell numbers were determined by the spread plate method on serial 10-fold dilutions. For each dilution five plates of NA were spread with 0.1 ml and incubated at 30°C for 24 h after which colonies were counted and the mean number of

bacteria correlated with the O.D. at 650 nm of the original suspension (1 cm light path). Standard suspensions were prepared by diluting to the approprate O.D. to give the cell concentration desired. For experimental purposes, bacterial suspensions (10 ml) were washed once in LBSS by centrifuging at 3200 x g for 10 min and then resuspending with LBSS to the desired concentration.

Collection and Preparation of Coelomocytes

Extrusion method - *L. terrestris*. During each assay period, coelomocytes from experimental and control worms were obtained using a non-invasive extrusion method developed to obtain large numbers of immunoactive cells (40).

Brifly, worms were removed from their 10° C incubator, rinsed with water, placed on a paper towel moistened with saline (0.85% w/v NaCl) and fecal matter removed by gently massaging the posterior end of the worms. Worms were then placed in a Petri dish containing 3 ml of extrusion medium consisting of saline (0.85% w/v NaCl) and 5.0% (v/v) ethanol, 10 mg of the mucolytic agent per ml, guaiacol glyceryl ether, (Sigma Chemical Co.,St. Louis Mo.)supplemented with 2.5 mg EDTA per ml (MCB Manufacturing Chemists, Inc., Cincinati, Ohio.), and adujusted to pH 7.3 with 1 N NaOH. Worms were bathed for a maximum of 3 min in this extrusion medium at room temperature. The lavage, cloudy with cells, was transferred to a test tube containing 10 ml of ice cold

saline or LBSS depending on the test to be performed. The cell suspension was centrifuged in a small serological tube at 80 x g for 5 min, washed once with saline (or LBSS) and resuspended in 1 ml of saline (or LBSS).

Puncture method - *L. terrestris*. To harvest coelomocytes with the puncture technique, coelomic fluid containing cells was collected by inserting a sharpened Pasteur pipette into the coelom posterior to the clitellum and allowing it to fill by intra-coelomic pressure (129). Leukocytes were then mixed gently with saline and centrifuged at 80 x g in a Beckman TJ-6 centrifuge for 5 min (40). The supernatant was removed and leukocytes resuspended in 0.5 ml of calcium free LBSS. If unwashed cells were needed, one drop of puncture fluid was placed on a slide or a coverslip.

Electrical shock method - *E. foetida*. Coelomic fluid samples were harvested by placing 20-30 worms in a glass funnel (8 cm) and attaching wires from the positive and negative poles of a 6V battery to the worm mass. Such electrical stimulation induced ejection of coelomic fluid through the dorsal body wall pores (143). The coelomic fluid was centrifuged at 11,000 x g, and the cell free supernatant sterilized by passing it through a Nalgene 0.2 μm cellulose acetate syringe filter (Nalge Company, A Subsidiary of Sybron Corporation, Rochester, NY), before storing at -20°C until use.

Determination of Coelomocyte Viability and Concentration.

Viable and non-viable cells were differentiated by staining with 0.4% trypan blue dye (Sigma Chemical Co., St Louis Mo.). Live cells, which do not take up the trypan blue dye, were differentiated from dead ones, and then the viable cell count performed by standard methods (Kirk et al.,1975) using an improved Neubauer hemocytometer. Four WBC's sides of the chamber were counted, and the average multiplied by 10^4 to get the cell concentration. The cell concentration was then adjusted to $1x10^6$/ml with LBSS.

Immunological Assays

Spreading.

Three hundred µl of coelomocyte suspension ($1x10^6$/ml) was placed on a sterile coverglass and incubated for 2, 3, 4, and 5 h at 10°C in a chamber with 100% humidity. The coverslips were removed, very gently rinsed three times in saline to get rid of non-adherent cells, stained in modified Wright's stain (Sigma Chemical Co. St Louis, Mo.) for 15 s, and air dried before observation under the light microscope (Nikon, x1000). The percentage of spreading cells was calculated from the number of spreading cells found in a total population of 500 cells.

Phagocytosis

Phagocytosis of RRBC. Coelomocytes, collected by the extrusion method, were adjusted to $1x10^6$ cells/ml. For each

test, 300 µL of the cell suspension was mixed with 300 µl of 2% (v/v) RRBC in a 3 x 100 serological test tube and then spread over a glass slide and incubated at 10°C for various periods of time in a chamber with 100% humidity. At the end of the incubation period slides were taken out of the chamber, washed four times in saline to get rid of any excess non-ingested RRBC and/or non-adherent coelomocytes, stained in modified Wright's stain for 15 s and air dried before observation under the light microscope. Percent phagocytosis was calculated from the number of coelomocytes that ingested RRBC in a total population of 500 cells.

Phagocytosis of bacteria. Bacterial cultures of *B. thuringiensis* or *S.marcescens* at a concentration of 1 x 10^9 CFU/ml in LBSS containing 2.5 mg/ml of EDTA were prepared as previously described. Coelomocytes, collected by the extrusion method were, counted and the cell number adjusted to 1 x 10^6 cells/ml. Two hundred µl of the bacterial suspension were then mixed with an equal volume of coelomocytes in a small serological tube and incubated at 10°C for various periods of time. One drop of this suspension was put on a glass slide, air dried, stained with Gram stain and examined with the light microscope (Nikon x 1000) for bacteria ingested by coelomocytes.

Phagocytosis of yeasts

A - In-vivo phagocytosis A few colonies of *S. cerevisiae* or *C. albicans*, grown in Sabouraud dextrose agar, were scraped from the culture plate and suspended in LBSS to make a cloudy suspension. One tenth ml of this suspension was injected with a Precision Glide 27 1/2 gauge needle (Becton Dickinson and Company, Rutherford, NJ) in several punctures into the coelom of *L. terrestris* posterior to the clitellum. Earthworms were incubated for 2 h at 10°C chamber after which they were punctured for their coelomic fluid. One drop of fluid was immediately placed on a microscope slide and mixed with a drop of saline. The percentage of phagocytosis was calculated from the number of coelomocytes that ingested the yeasts in a total population of 500 cells.

B. In-vitro phagocytosis. 1. Puncture method. A suspension of an overnight culture of *S. cerevisiae* or *C. albicans* were prepared in LBSS as described above. One drop of suspension was placed on a glass slide and mixed with 100 µl of coelomic fluid that was collected by the puncture method. Slides were incubated for 2 h at 10°C in the 100% humidified chamber. At the end of the incubation period slides were taken out of the chamber, the fluid part, gently removed with a pasteur pipette, was replaced with one drop of saline, and percent phagocytosis determined microscopically as described previously.

2. Extrusion method - An overnight culture of *C.albicans* was prepared and suspended in LBSS as described earlier. Coelomocytes of *L. terrestris* were collected by the extrusion method, washed once with saline, and resuspended in LBSS with no EDTA. One drop of *Candida* suspension mixed with one drop of coelomocyte suspension was incubated for 2 h at 10°C in 100% humidity. At the end of the incubation period the fluid was replaced with one drop of saline, and the percent phagocytosis by coelomocytes determined.

Bacterial Pathogenesis

Pathogenicity to *L. terrestris* An overnight growth of bacterial cultures of *B. thuringiensis*, *S. marcescens*, or *Shigella* Spp. were adjusted to concentrations of $1x10^6$, 10^7, 10^8, and 10^9 CFU/ml in LBSS. Injections of bacterial suspensions were performed with a sterile T.B. syringe (Becton Dickenson and Company, Rutherford, NJ) with a Precision Glide 27 1/2 gauge needle (Becton Dickinson and Company, Rutherford, NJ). A total volume of 0.1 ml was inoculated into the coelom, with 3-4 separate punctures into different segments of the body posterior to the clitellum. The final number of inoculated into the earthworm in each batch of worms was $1x10^5$, 10^6, 10^7, 10^8 cells. Controls recieved only 0.1 ml of LBSS. Fifteen worms were used for each cell concentration and the control. Earthworms were

returned to their 10°C chamber and checked daily over a period of five day for dead worms.

Pathogenicity to *E. foetida*. Overnight culture of *B. megaterium* or *A. hydrophila* at concentrations of $1x10^6$, 10^7, 10^8, 10^9, and 10^{10} CFU/ml were prepared in LBSS. Worms were removed from their 20°C chamber, rinsed in the water, and placed on ice for 1 h. Five batches (30 worms/batch) were injected with the bacterial suspensions using a sterile T.B. syringe and 27 1/2 Gauge needle to deliver 10 µl in the segments immediately posterior to the clitellum. Final levels of bacteria in each batch of worm would be $1x10^4$, 10^5, 10^6, 10^7, and 10^8 CFU respectively. Controls recieved only 10 µl of LBSS. The earthworms were returned to the incubation chamber, and checked daily for 4 days for dead worms.

Exposure of Whole Earthworms to Toxicants

Filter paper contact method. Exposure of *L. terrestris* utilized a 5 day contact test on filter paper previously treated with different toxicants at various exposure levels. The protocol, based on methods described by Roberts and Dorough (1984) for earthworms (109) and general standardized bioassay techniques recommended by EPA (1985) (97), has been shown to be effective and reproducible. Sublethal concentrations of PCB (Aroclor 1254) 10 $\mu g/cm^2$, cadmium nitrate 2.5 $\mu g/cm^2$, and chlordane 1 $\mu g/cm^2$ were used in these assays. A 9 cm diameter filter paper (Whatman No.

1) was placed at the bottom of 0.47 liter glass jars; a constant volume of 1 ml of acetone containing the approprate amount of PCB, Or 1 ml of double deionized water containing cadmium nitrate or chlordane were spread evenly on the filter paper. In the case of PCB, solvent was completly evaporated under the hood prior to exposure, after which each jar was moisted with 1 ml deionized water. Earthworms were washed with deionized water, and were placed in individual jars. The jars were sealed with lids and ventilated every 24 h when lids were removed to check for worm survival. Exposure was conducted under continuous darkness at 10°C throughout the 5 day exposure period.

Artifical soil-RDFF method. For immunotoxicological assays, *E. foetida* was exposed to 3 different concentrations of RDFF using an artificial soil protocol (93). RDFF, obtained from a test burn of densified refused drived fuel (dRDF) produced by a resource recovery plant in Minnesota (150). RDFF toxicity is based on the parent component, mainly heavy metals Cd, Cu, Cr, Zn, Ni, Pb (150). Tests were conducted in 3.7 liter glass jars containing 1000 g soil of 3 concentrations 30, 50, 70% of RDFF in artificial soil. Each jar, containing up to 100 worms per concentration, was placed in a 20°C environmental chamber with continuous light for 5 days. Controls were treated the same but without toxicant. Worms were examined daily and dead worms removed.

Superfund soil. *L. terrestris* was exposed directly to the complex mixture of xenobiotics in the Superfund soil. Superfund soils which contained mostly chlorinated organic pesticides (mainly chlordane) and heavy metals were collected by Clarence Callahan (EPA) from a Superfund site in Massachusetts. For the first of four Superfund soil experiments, worms which had been exposed for two days to four different levels of contaminated soil were recieved from the EPA. For the second, third, and fourth experiments earthworms were exposed to a 5% concentation of superfund soil in artificial soil for 5 days in the UNT environmental effects group laboratory. Soils for these three experiments were supposedly collected from the same site but from different areas than those in the first experiment.

Antimicrobial Activity of *E. foetida* Coelomic Fluid

Immunization. Animals, selected on the basis of having a recently developed clitellum, were maintained for 1 h in crushed ice before injection. *A. hydrophila* grown overnight in nutrient broth at a sublethal concentration of 10^8/ml was prepared in LBSS with no free calcium (2.5 mg EDTA/ml). Ten µl (10^6 cells) were injected in the segments immediately posterior to the clitellum with 27 1/2 gauge needle and T.B. syringe. Worms were returned into their 20°C chamber and incubated for 2 and 4 days before extrusion of

coelomic fluid by electrical shock for their *in vitro* antimicrobial activity.

***In-vitro* growth inhibition.** Bacteriostatic activity of *E. foetida* coelomic fluid was determined by incubating 50 µl of 10^9/ml of *A. hydrophila* and 50 µl of coelomic fluid for 30 min at room temperature (25°C). Serial ten fold dilution were plated out and emerging colonies counted after 24 h of incubation at 30°C. Normal control growth consisted of bacteria incubated with sterile saline alone. Each determination was done on 5 plates on 3 distinct pools of coelomic fluid. Percent inhibition was calculated by comparing survival in experimental animals versus the controls.

Electron Microscopy

Elemental analysis (EDX) *L. terrestris* exposed for five day by the filter paper contact method to sublethal levels of PCB (Aroclor 1254, 10 µg/cm^2), and cadmium nitrate (2.5 µg/cm^2) were used. In two other sets, lethal levels of Aroclor 1254 (100 µg/cm^2), and copper sulfate (2.55 µg/cm^2) were used and incubated for 3 days. Both extrusion and puncture methods were used to collect coelomocyte for these assays.

Extruded coelomocytes were washed twice by centrifugation (80 x g, 5 min) with 0.2 M sodium phosphate (pH 7.3). The supernatant was discarded and the cells placed

on Thermanox tissue culture cover slips (Nunc, Inc. Naperville, IL.) which contain no chlorine. The cells were air dried and the coverslip mounted on a carbon stub, and carbon coated in a JEE-4X Vacuum Evaporator (JEOL, Japan). The specimens were analyzed for the presence of elemental chlorine, cadmium, and copper with a Tracor-Northern TN 5500 energy dispersive X-ray (EDX) analyzer attached to a JSM-T 300 scanning electron microscope (JEOL Japan)

To prepare cell free coelomic fluid, coelomic fluid was collected by the puncture method, placed in a small conical centrifuge tube, and centrifuged at 6000 x g for 3 min. The cell free supernatant was placed on a Thermanox coverslip, air dried and the elemental composition determined by EDX analysis. In some assays single worms were used, and in others pooled samples were examined. Also from this prepration washed and unwashed coelomocytes were examined for the presence or absence of the above elements.

Morphological studies of coelomocytes by SEM. Three methods were used in this study. All the experimental earthworms (*L. terrestris*) were exposed to 1 µg of chlordane per cm^2.

1. Controls and exposed worms were extruded and washed once with saline. One drop of coelomocyte suspension was placed on a polylysine coated coverglass, and incubated for 3 h. At the end of the incubation period the coverglass was

rinsed three times with physiological saline and the cells fixed for 1 h in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Cells were dehydrated in 30, 50, 70, 90, and 100% x2 ethanol for 15-30 min in each except 100% ethanol which was for 1 h. Coverslips were removed, critical point dried , mounted on EM stubs, sputter coated with gold in a ES 100 unit (Polaron Instruments Inc.), and examined with JSM-T 300 scanning electron microscope at 10 Kv.

2. A few drops of control and exposed coelomocyte suspensions were placed on regular microscopic slides, incubated for 3 h, and washed three times with saline. The saline washes containing non-adherent cells were centrifuged for 5 min at 80 x g. The supernatant was discarded and sedimented cells placed on a polylysine coated coverglass and fixed with 4% glutaraldhyde. Cells were dehdrated, critical point dried, gold coated, and examined with the SEM.

3. One drop of extruded coelomocytes from control and exposed (chlordane) were mixed with RRBC, as for the phagocytic assay, was placed on a polylysine coated coverglass and incubated for 24 h. At the end of the incubation period the coverslip was removed, washed three times in saline, fixed in 4% glutaraldehyde, dehydrated, critical point dried, gold coated, and examined with the SEM.

SEM study of attachment of *B. thuringiensis* to coelomocytes. Extruded coelomocytes mixed with *B. thuringiensis* in a siliconized tube, were incubated for 3 h as for the phagocytic assay and washed three times with saline. One drop was placed on a polylysine coated coverglass and prepared for SEM analyses as described for the morphological study of coelomocytes by SEM.

TEM Study of Phagocytosis of *B. thuringiensis* by Coelomocytes of *L. terrestris* Extruded coelomocytes were mixed with *B. thuringiensis* in a siliconized tube, incubated for 3 h as for the phagocytic assay, washed three times with saline by centrifugation (80 x g 5 min). The supernatant was discarded and the cells prepared for TEM examination as follows: Coelomocytes were fixed immediately with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 30-60 min, washed three times with 0.1 M cacodylate, enrobed in 1.5% agar (50/50 - one drop cells, one drop agar) and cooled. The agar enrobed cells cut into approximately 0.5 mm blocks, were postfixed with 1% OsO_4 in 0.1% cacodylate, washed three times with 0.1 M cacodylate (15 min/wash), dehydrated in 30, 50, 70, 90, and 100% x2 acetone for 15-30 min (final 100% acetone for 1 h). Cells were embeded in Epon plastic, and 65 nm sections made with a diamond knife using a MT 6000 microtome (Sorvall Instrument, DuPont). Sections were stained, with uranyl acetate and lead citrate, and

examined with TEM 100CX II Electron Microscope (JEOL, Japan) at 80 KV.

TEM stereological study of *L. terrestris* tissues

Earthworms were exposed to a lethal concentration of copper sulfate (2.55 $\mu g/cm^2$) for 3 days by the contact filter paper method. Sick worms were removed, pined downed on a dissecting tray, and with no anesthetics, various tissues dissected out, and immediatly placed in 4% glutaraldehyde.

Part of the intestine, ventral nerve cord, skin and muscle were dissected out from 5 segments posterior to clitellum. The dorsal blood vessel was embedded along with the intestine. Reproductive organs located anterior to clitellum close to the mouth were also removed. Following glutaraldehyde fixation, EM specimen preparation, sectioning, and staining were carried out as described above except for the agar enrobing. For this study, only photographs of epithelial tissue of controls and exposed earthworms were taken and examined; nuclear volume ratios were measured using standard stereological procedures (157).

Statistical Analysis

Statistical analysis were performed using the Macintosh StatView program (41), and methods discussed in Biostatistical Analysis by Jerrold Zar (159). Distribution of data was tested for normality to determine whether

parametric or non-parametric tests should be used. Where appropriate, the two tailed independent t-test or Wilcoxon 2-sample test was used to test for significance between two groups of data. When more than two groups of data were compared, the 1-way ANOVA, multiple way ANOVA, and multiple way ANOVA with repeated measurements was utilized. The one way ANOVA test was applied in combination with the two tailed Dunnett-t multiple range comparison test. The level of significance for multiple range comparison tests was 0.05. For all other statistical tests, differences were significant if the probability was less than 0.05 ($p \leq 0.05$). The results are reported as ± the standard deviation from the mean.

CHAPTER III

RESULTS

Before the effect of xenobiotics on the immune response could be tested, it was necessary to determine which tests would be suitable and the optimal condition for testing. Therefore, in this section, experiments to determine these parameters are reported first, followed by experiments to determine the efficacy of these assays for monitoring the effect of xenobiotics. For clarity, experiments with *Lumbricus terrestris* and *Eisenia foetida* are treated separately.

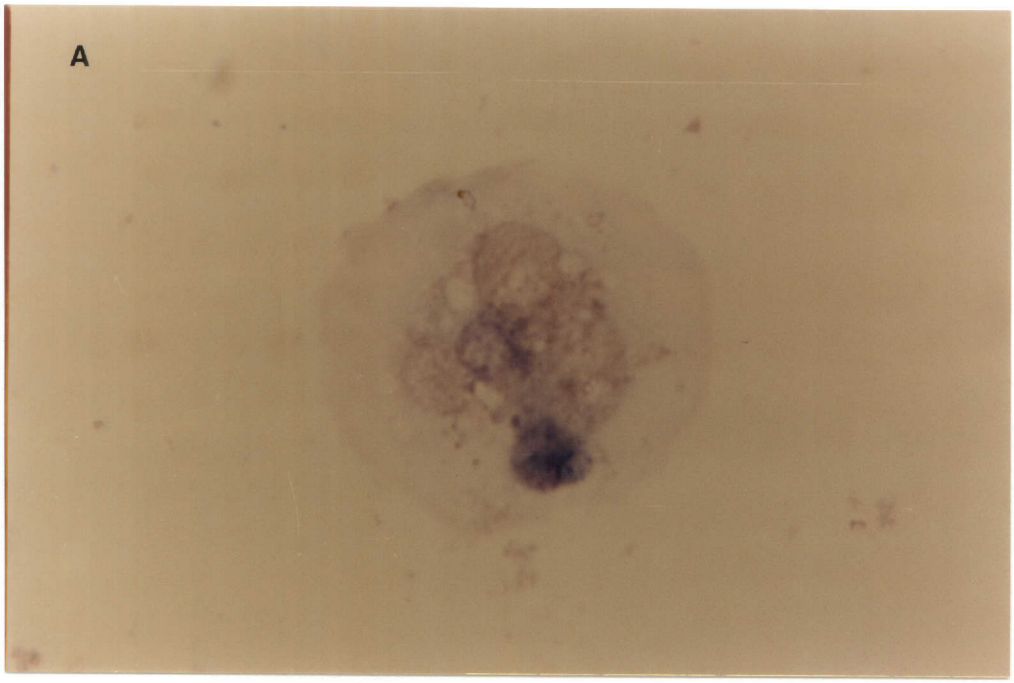
I. *Lumbricus terrestris*

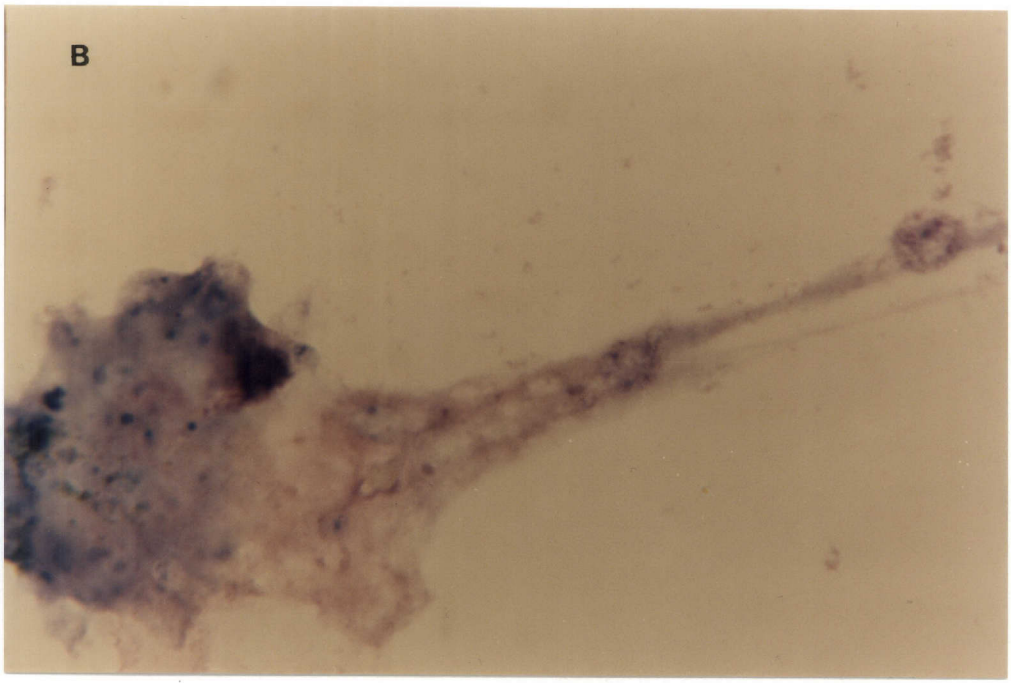
A. Methods Development

Spreading

Spreading activity is the first change in macrophage-like cells of the earthworm, *L terrestris* prior to phagocytosis. Macrophage-like coelomocytes are considered to be spread when they show a membrane occupying an area larger than that of unspread cells (appearing like a fried egg) (Fig. 1, A), and or showing pseudopod formation (Fig. 1, B). The effect of buffers (LBSS, RPMI 1640, physiological

FIG. 1. Photomicrograph of spreading macrophage-like coelomocytes stained with modified Wright's stain. (A) Fried egg appearance. (B) Pseudopod formation. Magnification, x1092.


A


B

saline), 5% CO_2, EDTA, incubation time and lysolecithin were tested for their effect on spreading activity.

Lysolecithin at concentrations of 1.0, 2.5, 5.0, 7.5, 10, 15, and 20 µg/ml and 5% CO_2 had no observable effect on coelomocyte activation. Removing cations by addition of EDTA reduced clumping of the cells. The combination of EDTA with LBSS gave the best result as a buffer system.

Using this system, the mean percent spreading activity of 5 different incubation times were 17.2 7.6, 20.1 ± 9.2, 23.2 ±9.6 25.4.± 10.9, and 32.6 ± 10.5 for 2, 3, 4, 5, and 24 h of incubation, respectively (Fig. 2).

In-vivo and *In-vitro* Phagocytosis/Association of RRBC, Bacteria and Yeasts

RRBC. Macrophage-like coelomocytes of *L. terrestris* can phagocytize and non-specifically ingest foreign particles including RRBC (rabbit red blood cells) (Fig. 3). In this study the effect of buffer composition (LBSS, RPMI 1640, physiological saline), EDTA, fetal calf serum, washing procedures, and incubation time were tested for their effect on the degree of phagocytosis of RRBC. Fetal calf serum was used to provide more nutrient for the cells. However, the high viscosity of fetal calf serum made it difficult to wash off non-adhering coelomocytes and excess RRBC. Stimulation of phagocytic activity by lysolecithin at the same

FIG. 2. Spreading activity of coelomocytes of *L. terrestris* in LBSS buffer. Error bars indicate standard deviation of the mean for 3 replicates. Ten earthworms were used for each incubation time.

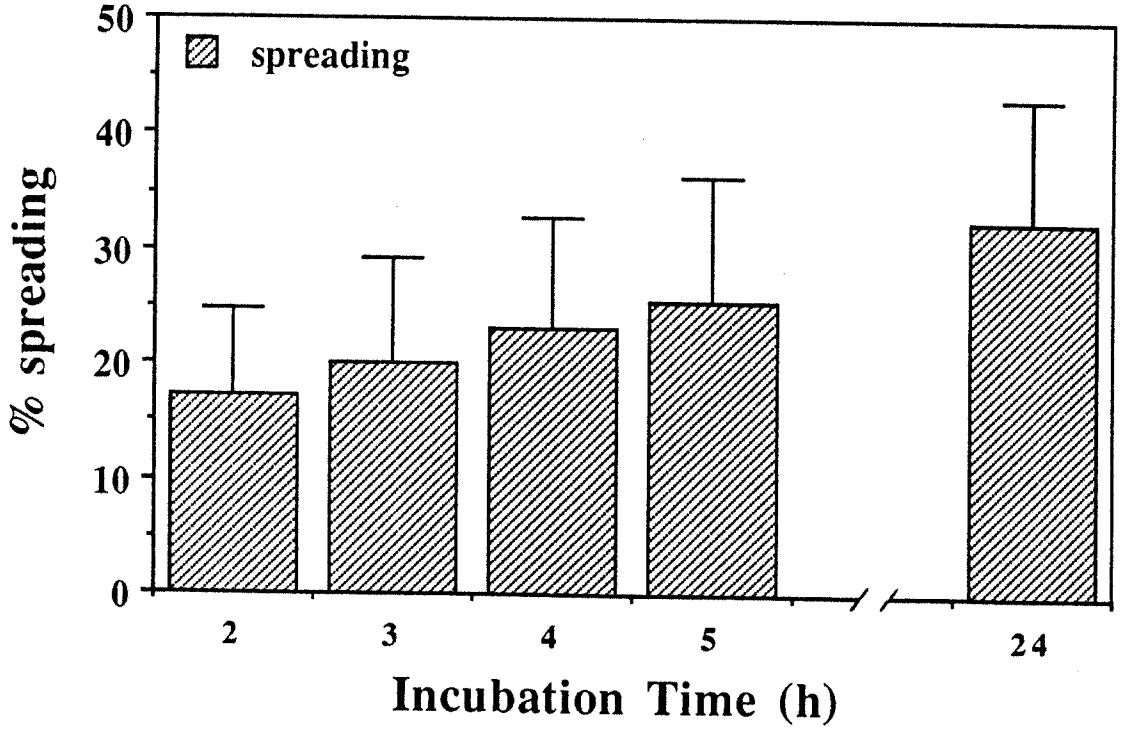
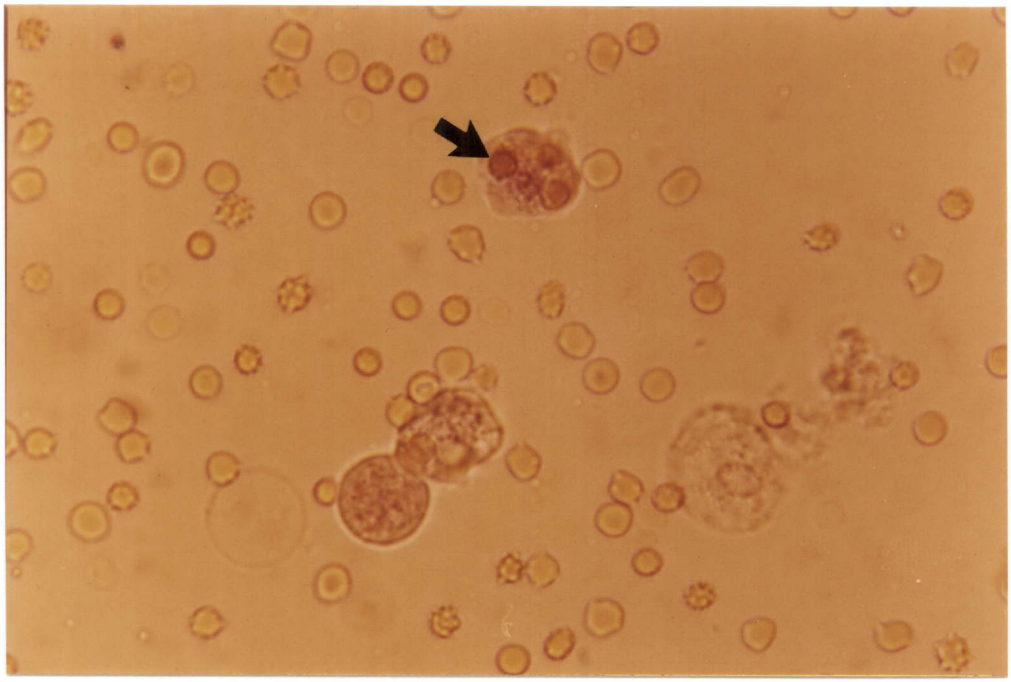

50
40
30
20
10
0
spreading
% spreading
2
3
4
5
24
Incubation Time (h)

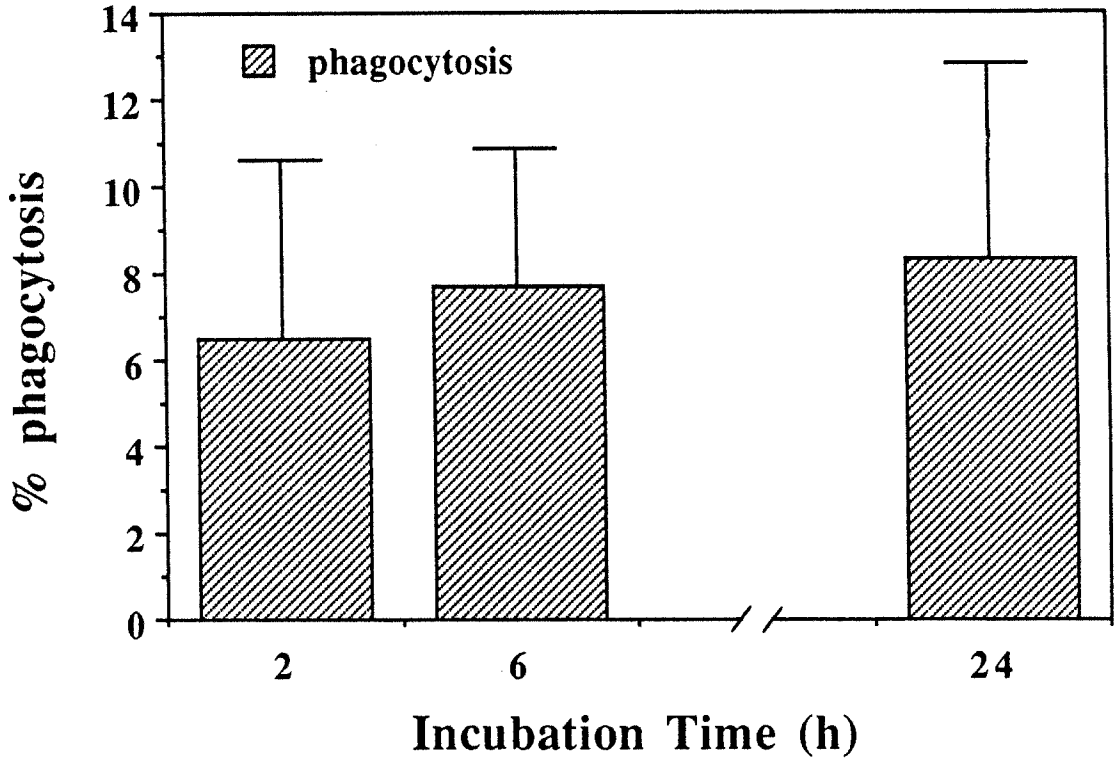
FIG. 3. Photomicrograph of a macrophage-like coelomocyte with ingested RRBC. Magnification, x672.



concentrations that were used for the spreading assay did not increase phagocytic activity. LBSS with no free calcium (LBSS + EDTA) gave the best result, although addition of EDTA did not completly eliminate clumping of the coelomocytes. The best procedure for washing coelomocytes was to use one wash at 80 x g for 5 min in 10 ml of saline. For the slide method, three washes after the incubation period was sufficient for removal of most of the non-ingested RRBC and non-adherant cells. The mean percent phagocytosis of RRBC by coelomocytes for 2, 6, and 24 h of incubation were 6.5 ± 4.1, 7.7 ± 3.2, and 8.3 ± 4.5, respectively (Fig. 4).

To dissociate the clumping of coelomocytes, which makes determination of phagocytosis difficult, trypsin (GIBCO Laboratory, Life Technologies Inc., NY.) and non-enzymatic cell dissociation solution (Sigma Chemical Co., St. Louis, MO.)were tested. Both had a limited effect on dissociating clumped cells for incubation periods up to 10 min. After 10 min individal cells were adversely affected. It seemed that the effect of trypsin was harsher on the cells than the non-enzymatic cell dissociation solution. I found out that if such a treatment is desired, it should be used before any incubation with particles to be phagocytized, since once the cells are clumped, it is difficult to dissociate them without damage being done to the cells.

FIG. 4. Phagocytosis of RRBC by coelomocytes of *L. terrestris* in LBSS buffer. Error bars indicate standard deviation of the mean for 3 replicates; 10 earthworms were used per incubation time.


14
12
10
8
6
4
2
0
% phagocytosis
phagocytosis
2
6
24
Incubation Time (h)

Bacteria. To determine the optimal conditions for phagocytosis of bacteria, the effect of assay tubes (conical and round bottom), incubation time, cell number, washing procedures (number of washes, volume, centrifugation speed) and use of lysozyme to remove non-phagocytized cells of *M. lysodekticus* were studied. Also, a comparison was made of Wright's stain and the Gram stain to detect phagocytosis. The results of the comparison showed that percent phagocytosis increases with time. When assays were performed in small conical tubes, percent phagocytosis was much lower than when regular small serological tubes were used. It was also observed that clumping of coelomocytes occurred more in conical tubes. Since distinguishing external bacteria from internalized bacteria was difficult, the term association rather than phagocytosis was used in later experiments. Phase contrast microscopy and a modification of washing techniques (six washings, 15 ml of wash, 50 x g centrifugation speed) helped some, but did not eliminate the clumping problem completely. Also silicon coating of tubes and pasteur pipets helped to prevent macrophage-like cells from attaching to the assay tubes. In comparing the Wright and Gram stain, the Gram stain permitted better visualization of bacteria. Because *S. marcescens* cells are Gram-negative and resemble some cytoplasmic inclusions (granules) in some coelomocytes, it was hard to determine phagocytosis with this

organism. The use of lysozyme to destroy non-phagocytized cells of *M. lysodiekticus* to enhance detection of phagocytised cells proved to be unsatisfactory because of entrapment in clumped coelomocytes. The small size of *M. lysodiiekticus* also made it difficult to detect phagocytosis. The best results were obtained with *B. thuringiensis* which are large Gram-positive cells and easily detected (Fig. 5). A typical phagocytosis assay of *B. thuringiensis* by coelomocytes of *L. terrestris* shows the percent phagocytosis for 0, 2, 4, 6, 8, 10, 21, 23, and 30 h of incubation time were 0.0 ± 0.0, 5.5 ± 1.2, 7.5 ± 2.1, 12.0 ± 2.4, 13.0 ± 3.8, 20.0 ± 3.9, 22.0 ± 5.8, 21.5 ± 4.9, and 25.0 ± 8.3, respecticely (Fig. 6) The results of percent association of bacteria with coelomocytes (Fig. 7) for 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 24.00, and 48.00 h of incubation time in a typical bacterial association assay were 1.9 ± 2.9, 10.3 ± 3.8, 10.0 ± 4.5, 14.0 ± 5.4, 13.3 ± 3.0, 18.0 ± 9.9, 21.3 ± 7.4, 21.7 ± 3.0, 23.3 ± 4.7, 27.6 ± 4.6, 34.7 ± 5.6, and 36.3 ± 6.2, respectively. Each number represents the mean of 3 assays, and 6 tubes per time of incubation. Using phagocytosis as the assay measure, the percent phagocytosis begins to level off at about 20% after 10 h. Using association, 20% association is reached in 3 h but peaks at about 35% after 24 h.

FIG. 5. Photomicrograph of Gram stained macrophage-like coelomocyte with ingested *B. thuringiensis* (arrow). Magnification, x672.

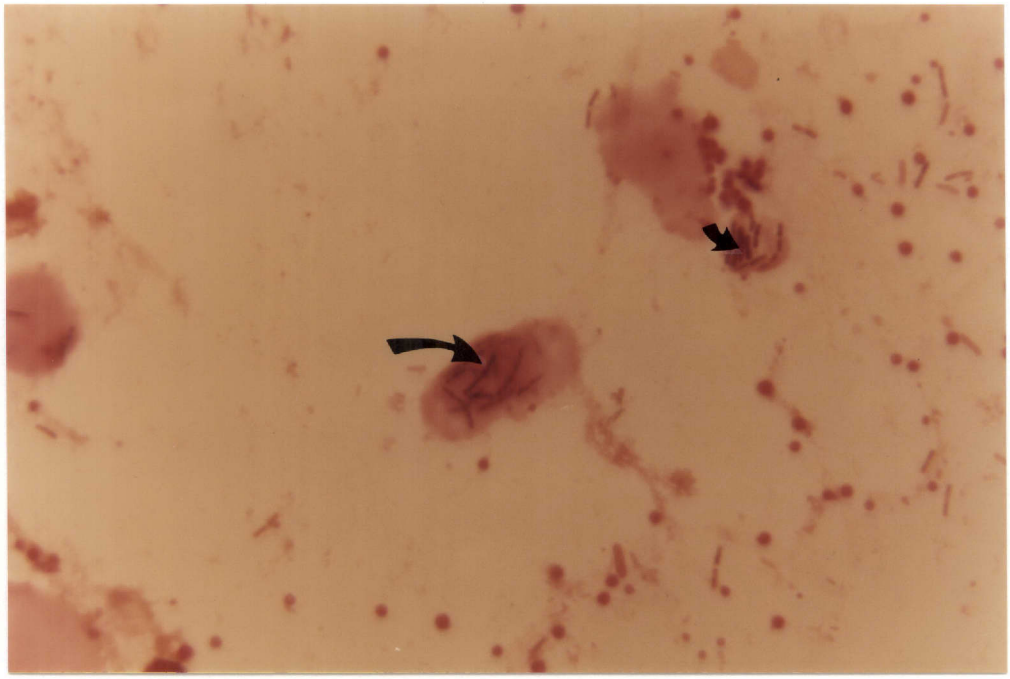


FIG. 6. Phagocytosis of *B. thuringiensis* by *L. terrestris* coelomocytes. Each column represents the average of 4 assays, and 6 tubes per time of incubation. Error bars denote standard deviation of the means.

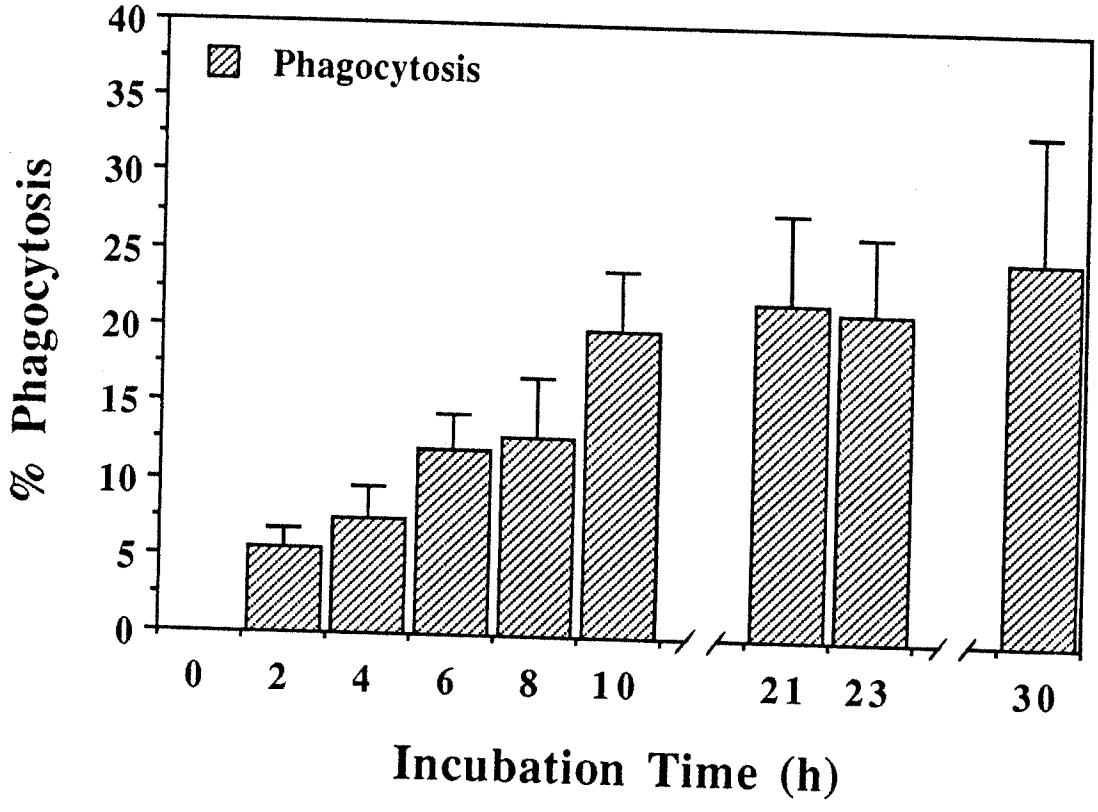
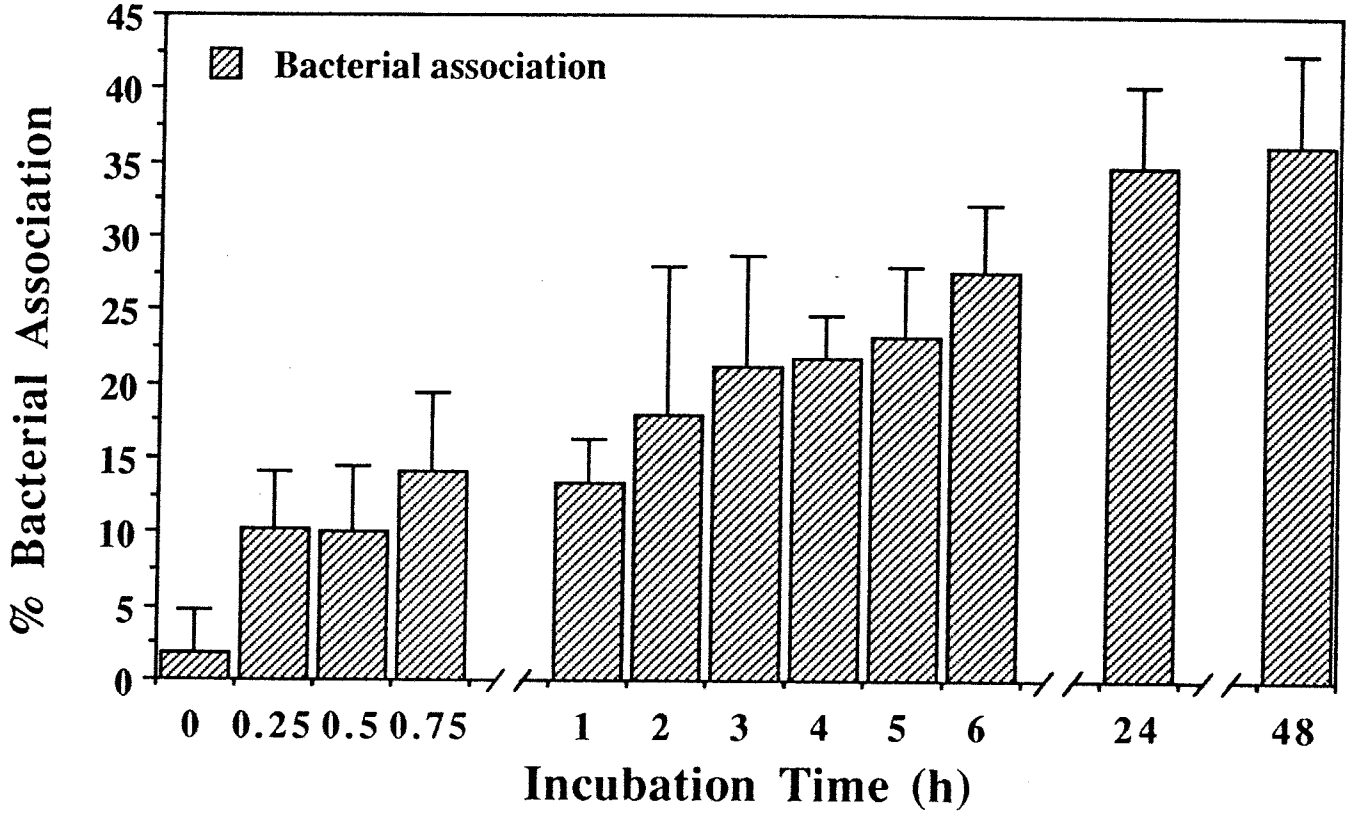

Phagocytosis
% Phagocytosis
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
21
23
30
Incubation Time (h)

FIG. 7. Association of *B. thuringiensis* with *L. terrestris* coelomocytes. Each column represents mean results of 3 assays, and 6 tubes per time of incubation. Error bars indicate standard deviation of the means.


Bacterial association
% Bacterial Association
45
40
35
30
25
20
15
10
5
0
0
0.25
0.5
0.75
1
2
3
4
5
6
24
48
Incubation Time (h)

Yeasts. Phagocytosis of the yeasts, *S. cerevisiae* and *C. albicans* (Fig. 8), gave the best results in terms of being easy to read.

1. *In-vivo* assay. The mean percent phagocytosis of *S. cerevisiae* and *C. albicans* by the *in vivo* method at 10°C and 2 h incubation were 4.5 ± 2.4, and 15.7 ± 6.4, respectively. There was a significant difference between mean percent phagocytosis of *S. cerevisiae* and *C. albicans* (2-tailed t = 8.694, p = 0.0001), total N =30 (Fig. 9).

2. *In-vitro*.assay - a. Puncture method. Coelomocytes obtained by the puncture method, phagocytized both *C. albicans* and *S. cerevisiae* (Fig. 10). However, there was a significant difference between phagocytosis of *Candida* and *Saccharomyces* with a mean percent phagocytosis of 13.3 ± 6.9, and 7.5 ± 3.9 for *Candida* and *Saccharomyces* respectively (2-tailed, unpaired t = 4.003, p = 0.0002).

b. Extrusion method. For this method only *C. albicans* was tested. Results of *in vitro* phagocytosis of *C. albicans* by the coelomocytes that were obtained by extrusion, showed a lower percentage of phagocytosis than the other two methods with a mean percent phagocytosis of 11.2 ± 5.2 after 2 h incubation at 10°C.

When phagocytosis of *C. albicans* by *in vivo*, *in vitro*-puncture, and *in vitro*-extruded methods were compared the results indicated a decrease in activity from *in vivo* (15.7 ±

FIG. 8. Photomicrograph of *C. albicans* (arrow) phagocytized by a macrophage-like coelomocyte. Magnification, x672.

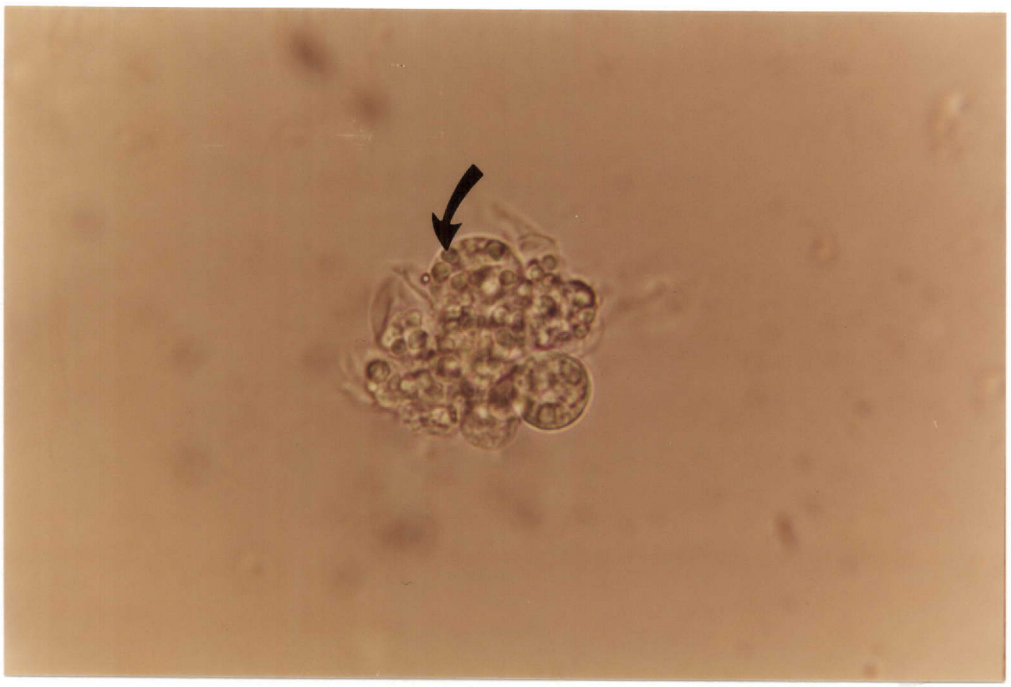
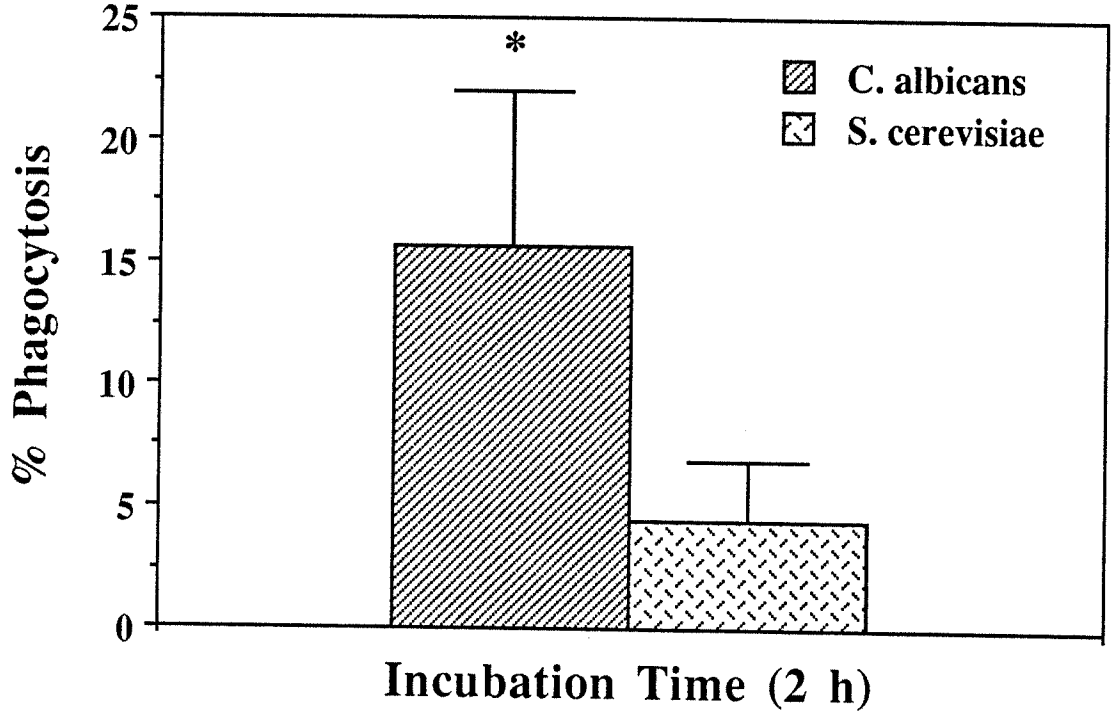
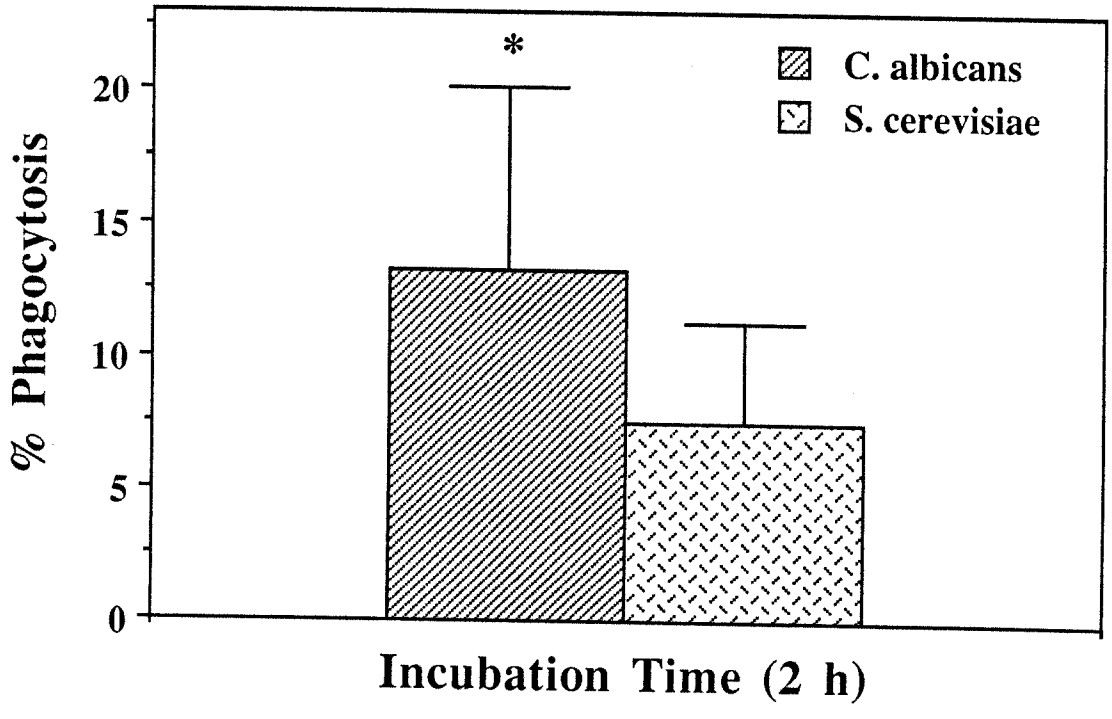


FIG. 9. *In vivo* phagocytosis of *C. albicans* and *S. cervisiae* by coelomocytes of *L. terrestris*. Each column represents the mean of 30 slide readings in 3 replicates for 2 h at 10°C temperature. Error bars indicated standard deviation of the means, and * denotes statistical significance at $p \leq 0.05$ (2-tailed $t = 8.964$, $p = 0.0001$).

FIG. 10. *In vitro* phagocytosis of *C.albicans* and *S. cerevisiae* by coelomocytes of *L. terrestris* obtained by the puncture method. Each column represents the mean reading of 30 samples in 3 replicates which were incubated for 2 h at 10°C. Error bars represents standard deviation of the mean, and * denotes statistical significance at $p \leq 0.05$ (2-tailed unpaired $t = 4.003$, $p = 0.0002$).


25
20
15
10
5
0
% Phagocytosis
*
C. albicans
S. cerevisiae
Incubation Time (2 h)


20
15
10
5
0
% Phagocytosis
*
C. albicans
S. cerevisiae
Incubation Time (2 h)

6.4) to *in vitro*-puncture (13.3 ± 6.9) and *in vitro*-extruded (11.2 ± 5.2) One factor ANOVA demonstrated a significant difference between percent phagocytosis by these three methods ($F = 3.832$, $p = 0.254$). A multiple range comparison Dunnett-test demonstrated that there is a significant difference between the *in vivo* and *in vitro*-extrusion methods (Fig. 11).

Electron Microscopy.

A morphological study of coelomocytes by SEM indicated that most of the cells have the ability to adhere to the glass slide and only disrupted and damaged cells were washed off. SEM studies failed to show attachment of *B. thuringiensis* to the surface of coelomocytes. However, examination of thin sections of phagocytic coelomocytes by TEM showed actual internalization of *B. thuringiensis* by macrophage-like coelomocytes (Fig. 12).

Bacterial Pathogenicity.

Pathogenicity of *B. thuringiensis*, *S. marcescens*, and *Shigella* SPP for *L. terrestris* was determined (Table. 1). Bacterial concentrations for all the experiments ranged from 1×10^5 to 1×10^8CFU/worm. Pathogenicity was determined after 5 days incubation. None of these microorganisms was found to be pathogenic at the bacterial concentrations used.

TABLE 1. Pathogenicity of bacteria to *L. terrestris*

	No. of surviving earthworms after 5 days				
	Saline control	Bacterial Conc. in 0.1 ml injection/worm			
microorganisms	0.1 ml/worm	1×10^5	1×10^6	1×10^7	1×10^8
B. thuringiensis	15	15	15	15	12
S. marcescens	15	15	15	15	14
Shigella SPP.	15	15	15	15	14

FIG. 11. Comparison of phagocytosis of *C. albicans* by coelomocytes of *L. terrestris* using the *in vivo*, *in vitro*-puncture (P) and *in vitro*-extrusion (E) methods. Each column represents 30 samples in 3 replicates. Error bars indicate standard deviation of the mean, and * denotes statistical significance between the *in vivo* vs. *in vitro* assays at $p \leq 0.05$ (one way ANOVA, $F = 3.832$, $p = 0.0254$).

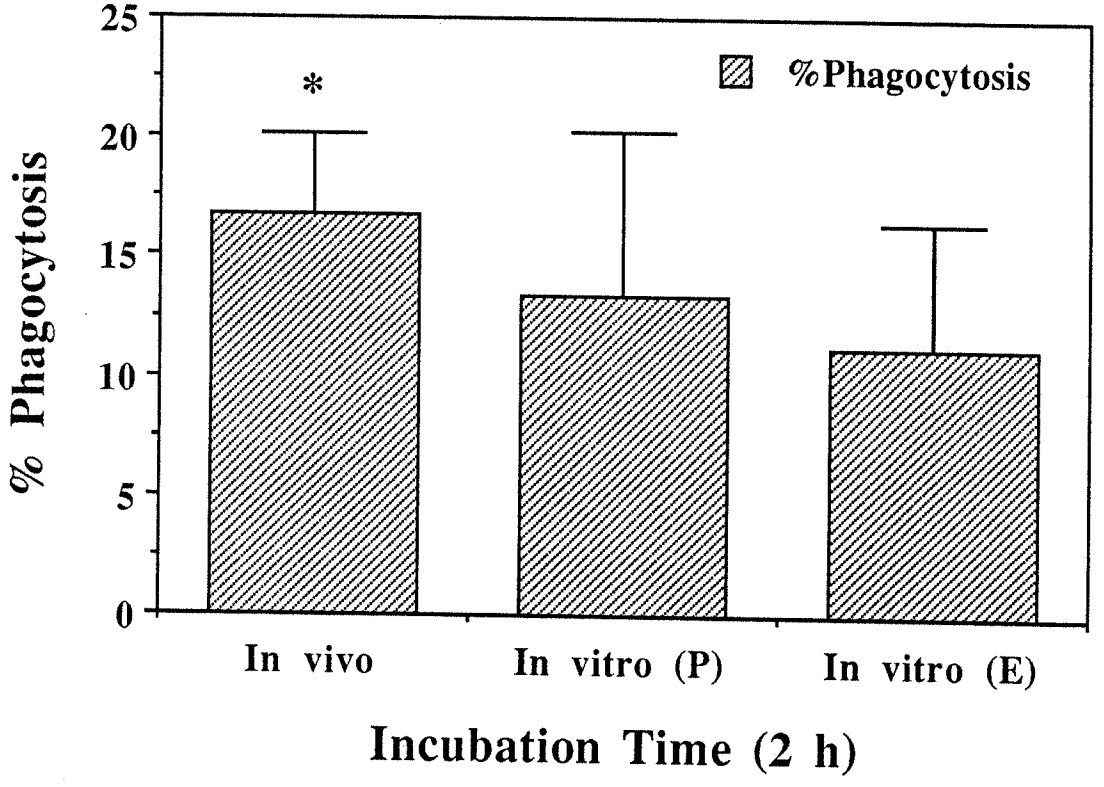
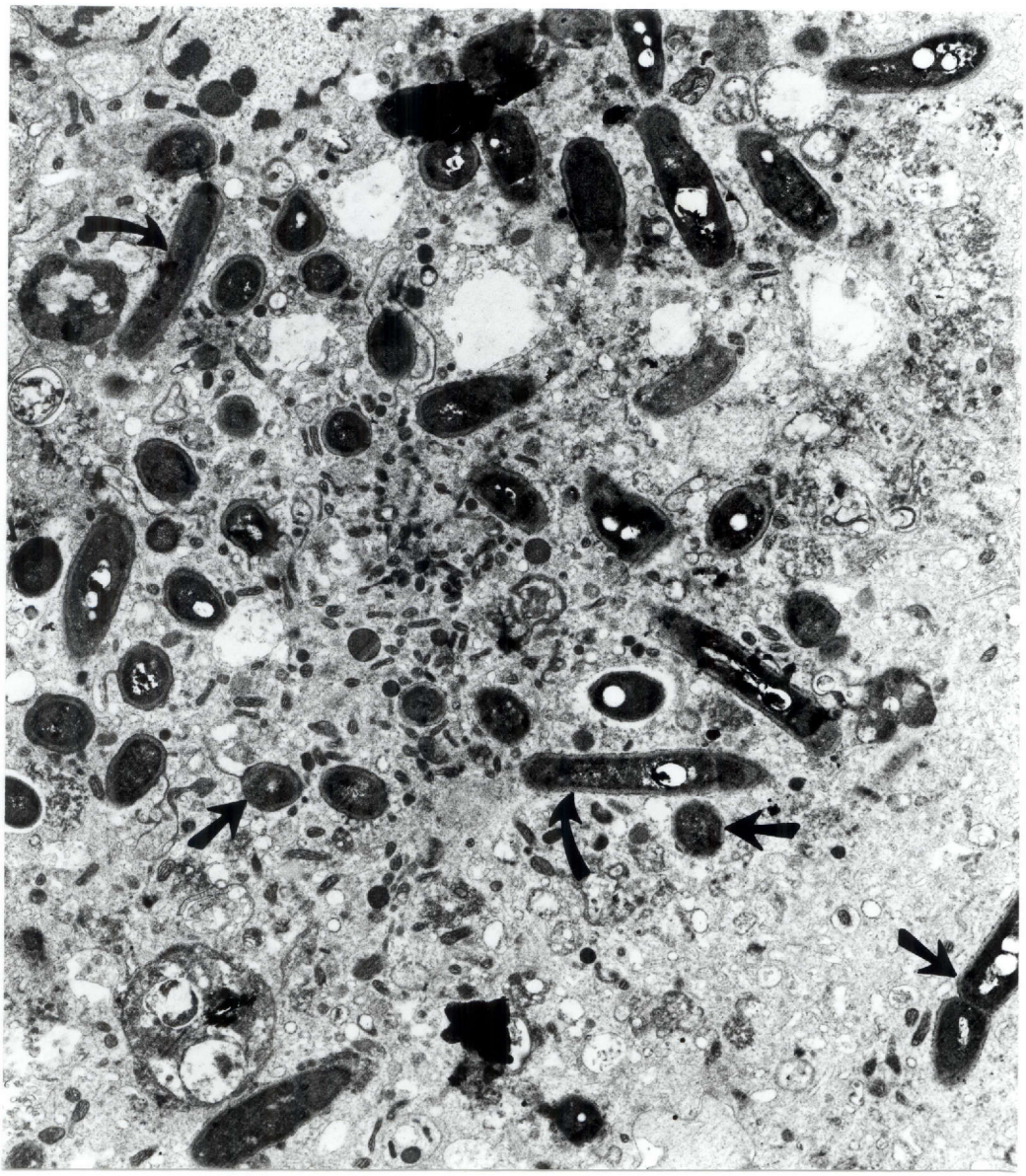

25
20
15
10
5
0
*
%Phagocytosis
% Phagocytosis
In vivo
In vitro (P)
In vitro (E)
Incubation Time (2 h)

FIG. 12. Transmission electron micrograph of a macrophage-like coelomocyte containing ingested cells of *B. thuringiensis* (arrow). Magnification, x7250.



B. Effect of Xenobiotics

Chlordane.

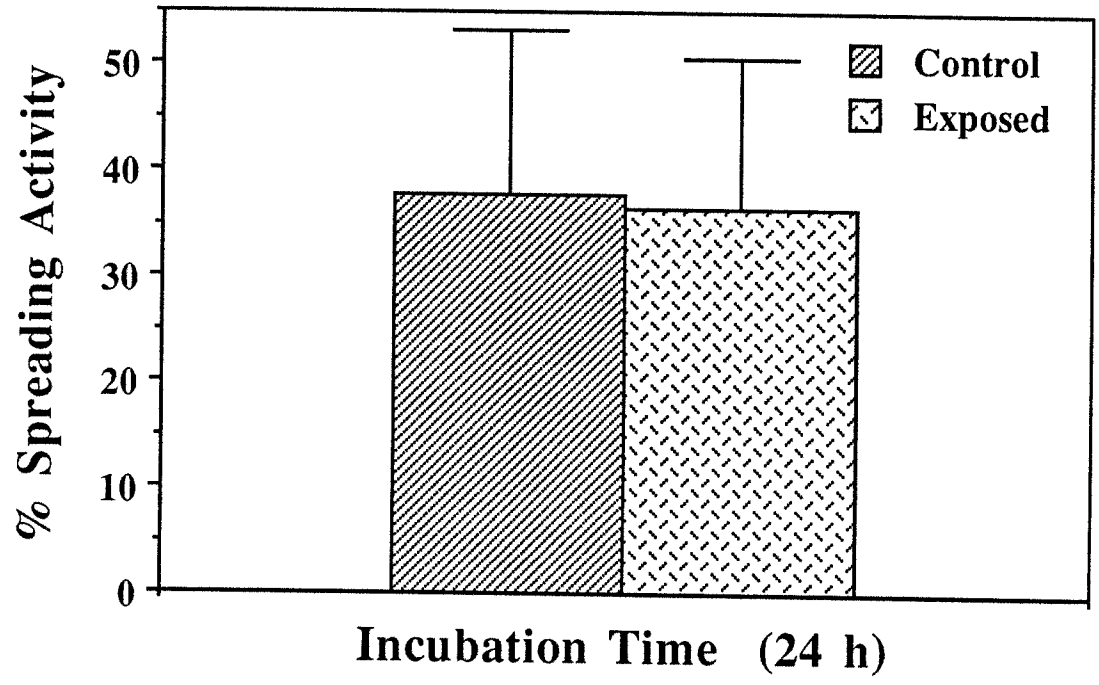
Exposure of *L. terrestris* to 1 µg of chlordane/cm^2 for five days apparently had no effect on spreading activity and phagocytosis of the coelomocytes, even though, the worms were contracted and obviously under stress. The mean percent spreading activities after exposure for 24 h were 37.9 ± 15.4, and 36.5 ± 14.3 for controls and exposed earthworms, respectively (Fig. 13). Phagocytosis of RRBC followed the same pattern with the mean percent phagocytosis of 8.35 ± 5.2, and 8.4 ± 5.9 for controls and exposed samples, respectivly (Fig.14). There was no significant difference for spreading or phagocytosis between controls and exposed earthworms. Using the unpaired t test for spreading t = 0.439, p =0.6617 with N = 96 and for phagocytosis t = 0.018, p = 0.9857 with N = 96 and total of 4 replicates (n = 12 per assay).

Cadmium nitrate.

Exposure of *L. terrestris* to a sublethal concentration of cadmium nitrate (2.5 µg/cm^2) for 5 days showed no effect on spreading activity and phagocytosis of RRBC by the coelomocytes. The mean percent spreading activities after 5 days exposure using 5 replicates, were 29.0 ± 16.6, and 27.6 ± 16.7 for controls and exposed worms, respectively.

FIG. 13. Effect of chlordane on spreading activity of coelomocytes of *L. terrestris*. Columns represents the mean percent spreading activity of coelomocytes from control and exposed earthworms for 4 replicate assays after 24 h incubation. Error bars indicate standard deviation of the mean.

FIG. 14. Effect of chlordane on phagocytosis of RRBC by coelomocytes of *L. terrestris*. The columns represent the mean percent phagocytosis of coelomocytes from control and exposed earhworms for 4 replicate assays after 24 h incubation. Error bars indicate standard deviation of the mean.


% Spreading Activity
50
40
30
20
10
0
Control
Exposed
Incubation Time (24 h)

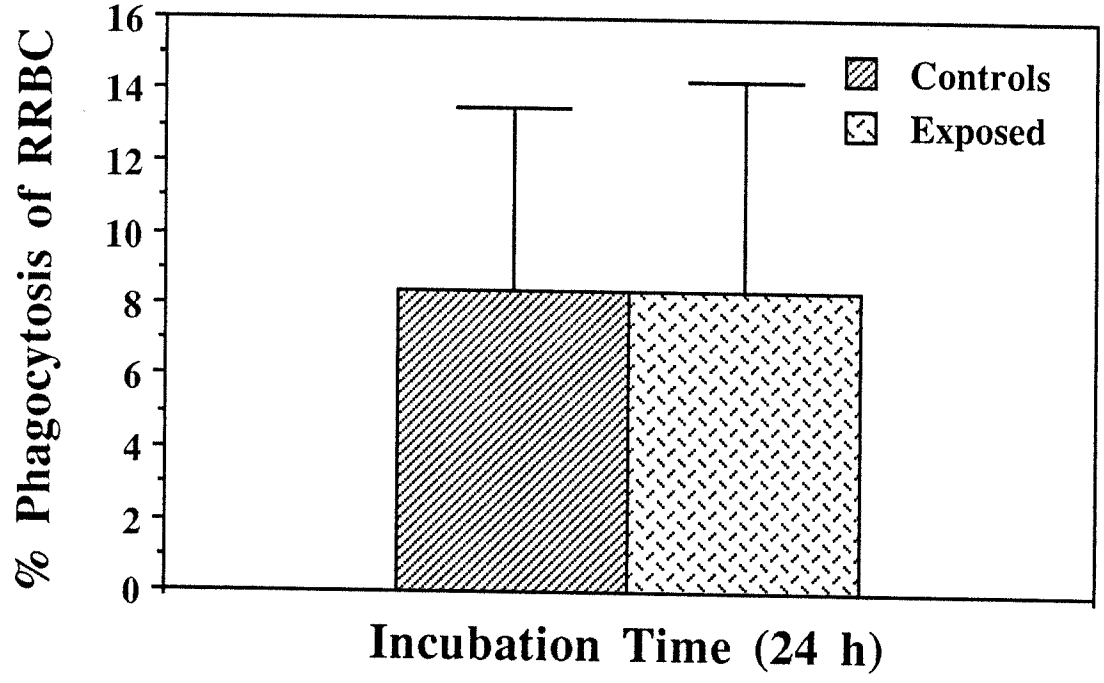
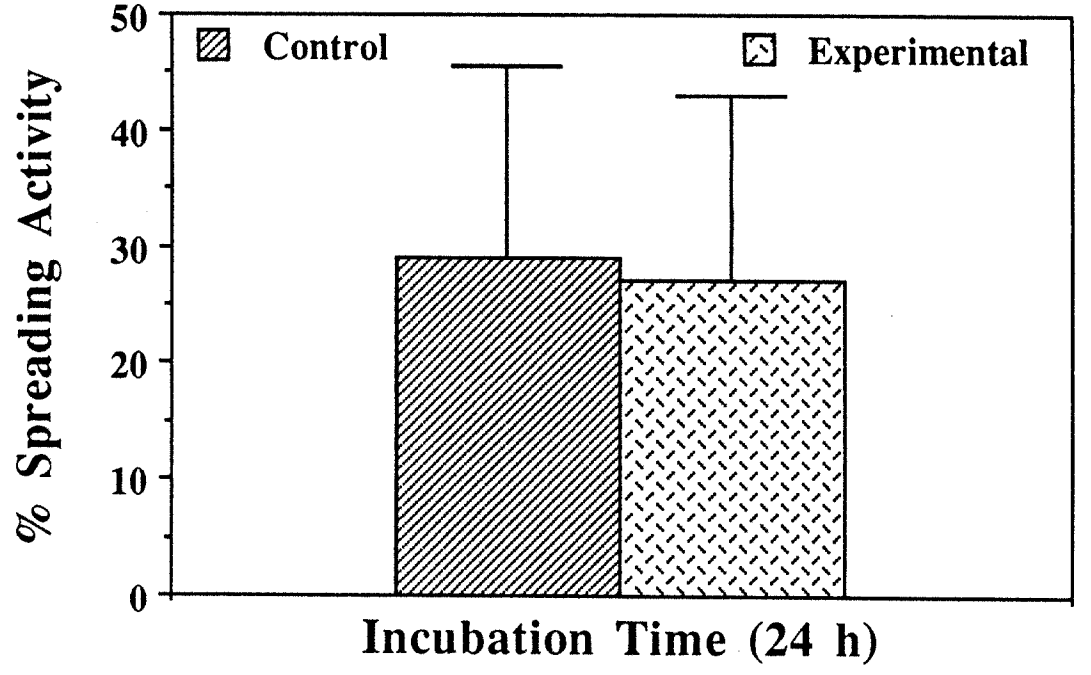
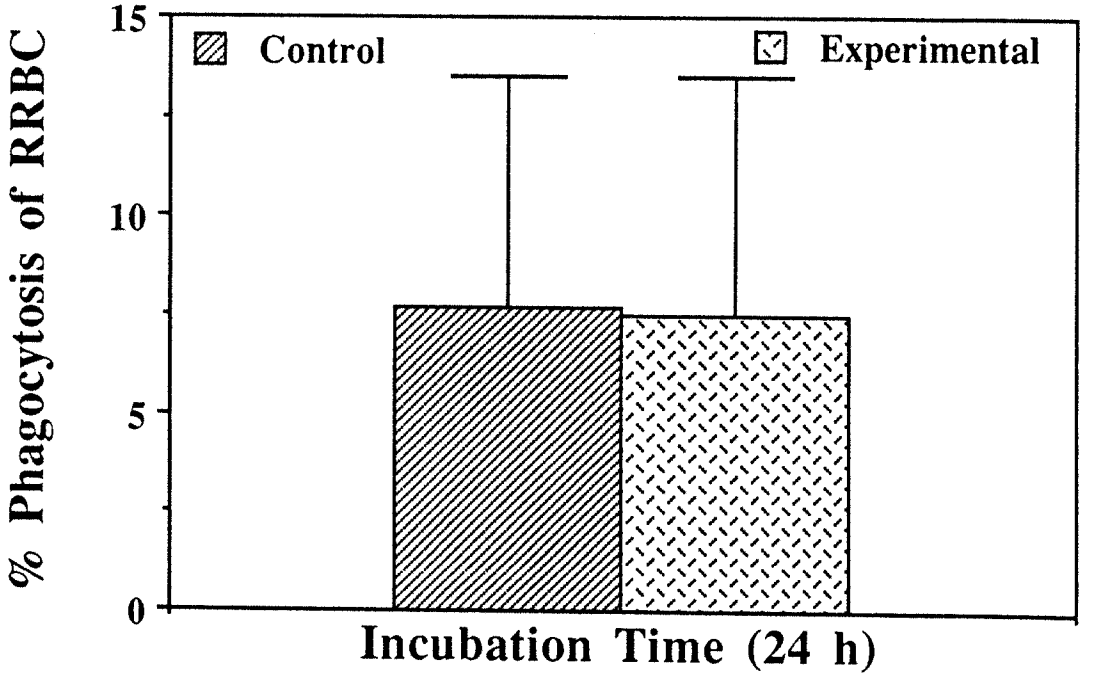

% Phagocytosis of RRBC
16
14
12
10
8
6
4
2
0
Controls
Exposed
Incubation Time (24 h)

FIG. 15. Effect of cadmium nitrate on spreading activity of coelomocytes of *L. terrestris*. Earthworms were exposed to 2.5 µg of cadmium nitrate/cm^2 for 5 days and incubated for 24 h. The columns represent the mean percent spreading activities for controls and exposed earthworms of 5 replicates (n = 12 for each run). Error bars indicate standard deviation of the means.

FIG. 16. Effect of cadmium nitrate on phagocytosis of RRBC by the coelomocytes of *L. terrestris*. Earthworms exposed to 2.5 µg of cadmium nitrate/cm^2 for 5 days and incubated for 24 h. The columns represent the mean percent phagocytosis of RRBC for the controls and experimentals of 5 replicate assays (n = 12 for each run). Error bars indicate standard deviation of the means.


50
40
30
20
10
0
Control
Experimental
% Spreading Activity
Incubation Time (24 h)


15
10
5
0
Control
Experimental
% Phagocytosis of RRBC
Incubation Time (24 h)

(Fig. 15). The mean percent phagocytosis of RRBC by the coelomocytes after 5 days exposure and 5 replicates were 7.7 ± 5.9 and 7.5 ± 6.0 for controls and experimental worms, respectively(Fig. 16). N for both spreading or phagocytosis assays was 120. There were no significant differences between controls and exposed earthworms in either assay. Using two tailed unpaird t test for spreading $t = 0.476$, $p = 0.6347$, and $t = 0.0169$, $p = 0.866$ for the phagocytosis.

Superfund soils

Experiment #1. In this experiment the earthworms, exposed to four different contamination levels at the EPA site and shipped to UNT, were in very bad health. Some had died during transportation to UNT and some soon after arrival. Since there were not enough samples for statistical analysis, results with worms from level 3 contamination were eliminated from the analysis and results of level 1 and 2 were combined. The combined levels of 1 & 2 are refered to as level A and level 4 as level B.

Spreading activity. The results indicate suppression of this activity (Fig. 17 and table 2), although, one way ANOVA showed no significant difference between control and experimental mean spreading activities at 30 min incubation ($F = 0.827$, $p = 0.471$). Differences in mean percent spreading activity for experimentals and the controls were significant at 60 and 120 min of incubation with $F = 5.457$,

p = 0.0166; F = 4.563, p = 0.0283 for 60 and 120 min, respectively by one way ANOVA. However, using a 2-tailed Dunnett multiple range comparison (control vs. experimental), for the 60 min incubation time, spreading activity was only significant for Superfund B. In the case of 120 min, both levels demonstrated a significant reduction using the Dunnett test.

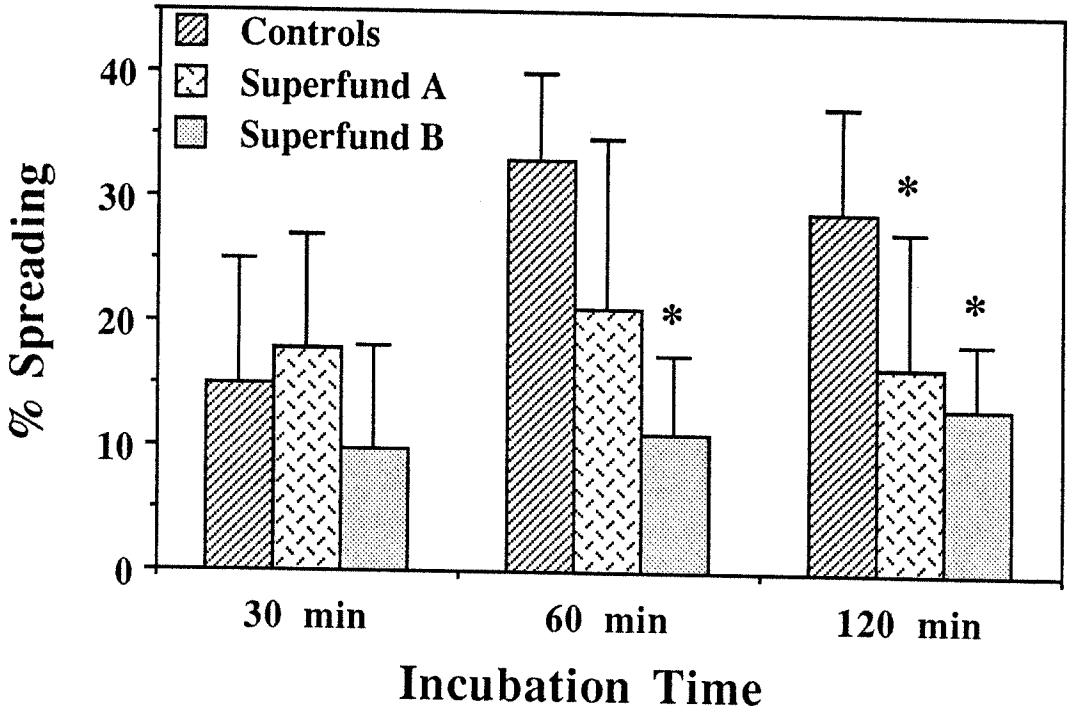
Phagocytosis of RRBC. Results of this assay show suppression of phagocytic activity after 48 h *in situ* exposure to Superfund soil. Results presented in Fig 18 and table 3 show percent phagocytosis of RRBC at 3 different incubation periods by coelomocytes of *L. terrestris*. One way ANOVA showed a significant difference in percent phagocytosis of RRBC by the coelomocytes as follows: 30 min incubation F = 4.253, p = 0.0402; 60 min incubation F = 8.128, p = 0.0041; 120 min incubation F = 8.532, p = 0.0034. The two tailed Dunnett multiple range comparison showed significant reduction in phagocytosis of RRBC for 30 and 60 min in both Superfund levels A & B compared to controls. However, for 120 min the 2-tailed Dunnett only level B was significantly different.

Superfund soils

Experiments #2 & 3. In these experiments earthworms were exposed to a 5% concentration of Superfund soil in

FIG. 17. Effect of Superfund soil #1 (levels A & B) on spreading activity of *L. terrestris* coelomocytes. Each column represents percent spreading activity of coelomocytes from control and experimental worms for 30, 60, and 120 min of incubation at 10°C. Error bars indicate standard deviation of the means, and * denotes statistical significance at $p \leq 0.05$.

FIG. 18. Effect of Superfund soil #1 (levels A & B) on phagocytosis of RRBC at 3 incubation period by coelomocytes of *L. terrestris*. Each column represents percent phagocytosis of RRBC by coelomocytes from control worms and worms exposed to Superfund soils. Each error bars indicate standard deviation of the means and * denotes statistical significance at $p \leq 0.05$.


Controls
Superfund A
Superfund B
% Spreading
40
30
20
10
0
*
*
*
30 min
60 min
120 min
Incubation Time

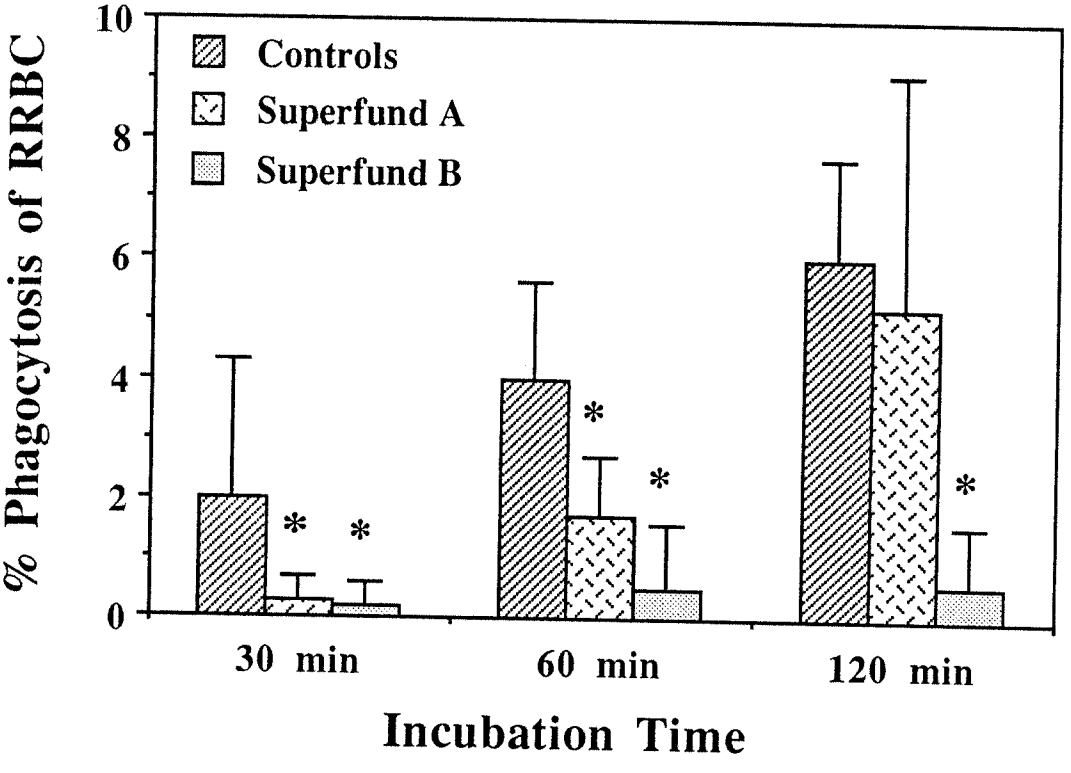

Controls
Superfund A
Superfund B
% Phagocytosis of RRBC
10
8
6
4
2
0
*
*
*
*
*
30 min
60 min
120 min
Incubation Time

TABLE 2. Effect of Superfund soils #1 (levels A & B) on spreading activity of coelomocytes of *L. terrestris*

	Percent spreading activity		
Incubation time (min)	30	60	120
controls	14.8 ± 11.2	32.8 ± 7.8	29.0 ± 9.4
Level A	17.8 ± 10.5	21.1 ± 14.6	16.5 ± 11.9
Level B	9.7/± 9.2	11.2 ± 7.0	13.3 ± 5.6

±SD

N = 15 for each group

TABLE 3. Effect of Superfund soils #1 (levels A & B) on phagocytosis of RRBC by coelomocytes of *L. terrestris*

	Percent phagocytosis of RRBC		
Incubation time (min)	30	60	120
controls	2.4 ± 2.3	3.8 ± 1.8	5.8 ± 1.9
level A	0.25 ± 0.5	1.7 ± 1.1	4.8 ± 3.5
level B	0.17 ± 0.4	0.5 ± 1.2	0.57 ± 1.3

±SD

N = 15 for each group

artificial soil for 5 days. The results of the individual experiments were combined.

Spreading. The mean percent spreading activity of coelomocytes in control and experimental earthworms at incubation times of 1, 6, and 24 h indicate suppression of spreading activity (Fig.19, table 4). There was a significant difference between controls and experimentals when they were compared by 2-tailed, unpaired t-tests for each time of incubation as follows : 1 h incubation, t = 3.15, p = 0.0033; 6 h incubation, t = 4.128, p = 0.0002; 24 h incubation, t = 4.886, p = 0.0001.

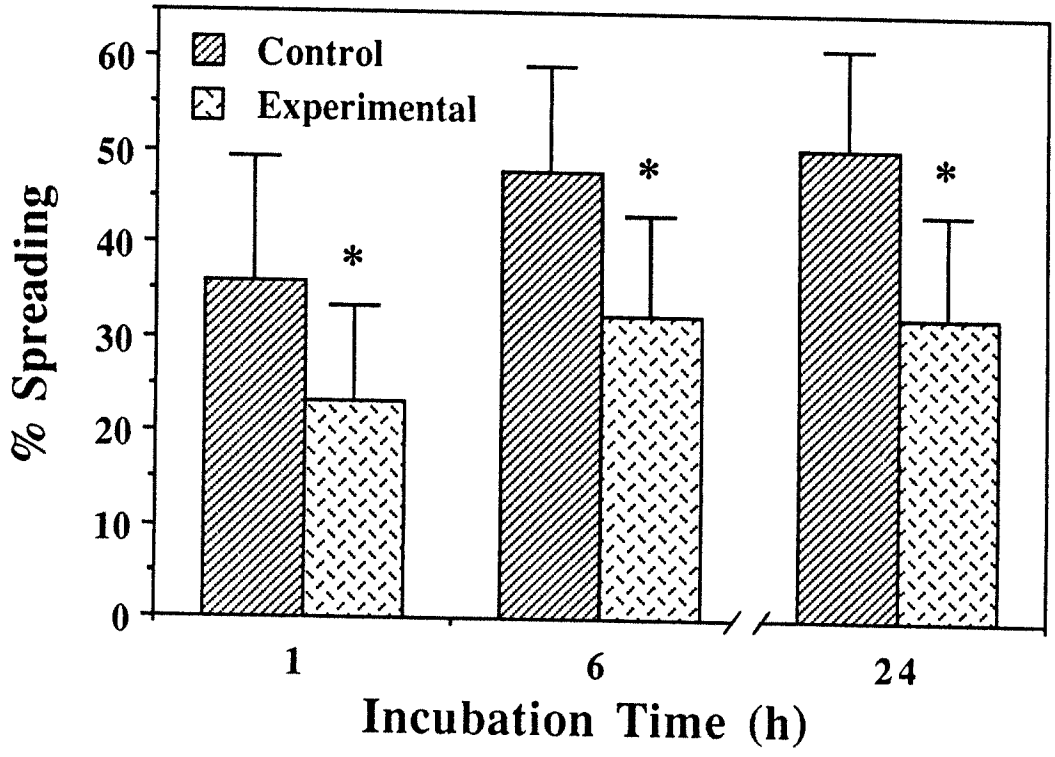
Phagocytosis of RRBC. Data for the phagocytic assays also indicated significant reduction in this activity of coelomocytes after 5 days of exposure to 5% Superfund soils. Mean percent phagocytosis of RRBC by coelomocytes of *L. terrestris* are presented in table 5 and Fig. 20; there were significant differences between controls and exposed earthworms in their coelomocyte phagocytic activities. Two tailed, unpaired t-tests for 1, 6, and 24 h of incubation were : t = 4.554, p = 0.0001; t = 6.0, p = 0.0001; t = 6.89, p = 0.0001, respectively.

Superfund soil.

Experiment #4. The exposure conditions for this experiment were the same as experiments 2 & 3, except that the soil sample came from a different area of the same site.

FIG. 19. Effect of 5% Superfund soils (Experiments 2 &3) on spreading activity of *L. terrestris*. Each column represents mean percent spreading activity of coelomocytes of the combined results of experiments 2 & 3. Error bars indicate standard deviation of the means and * denotes statistical significant at $p \leq 0.05$.

FIG. 20. Effect of 5% Superfund soils (2 & 3) on phagocytosis of RRBC by coelomocytes of *L. terrestris*. Each column represents mean percent phagocytosis of RRBC by the coelomocytes of controls and exposed worms. Error bars indicate standard deviation of the means, and * denotes statistical significance at $p \leq 0.05$.


Control
Experimental
% Spreading
60
50
40
30
20
10
0
*
*
*
1
6
24
Incubation Time (h)

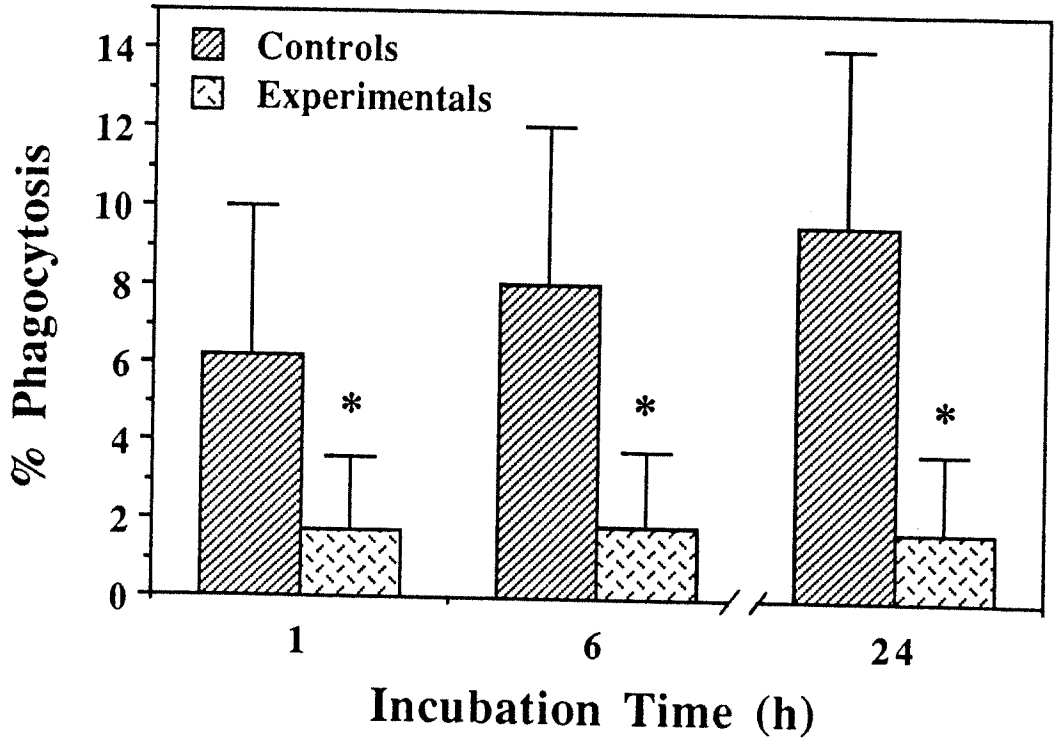

Controls
Experimentals
% Phagocytosis
14
12
10
8
6
4
2
0
*
*
*
1
6
24
Incubation Time (h)

TABLE 4. Effect of 5% Superfund soils (2 & 3) on spreading activity of *L. terrestris* coelomocytes

	Percent spreading activity		
Incubation time (h)	1	6	24
controls	35.9 ± 13.8	48.0 ± 11.5	50.6 ± 10.9
experimentals	23.2 ± 10.6	32.6 ± 10.9	32.3 ± 11.5

± SD

N = 37 for each group

TABLE 5. Effect of 5% Superfund soils (2 & 3) on phagocytosis of RRBC by coelomocytes of *L. terrestris*

	Percent phagocytosis of RRBC		
Incubation time (h)	1	6	24
controls	6.2 ± 3.9	8.1 ± 4.2	9.6 ± 4.6
experimentals	1.7 ± 1.9	1.9 ± 1.9	1.8 ± 2.1

± SD

N = 37 for each group

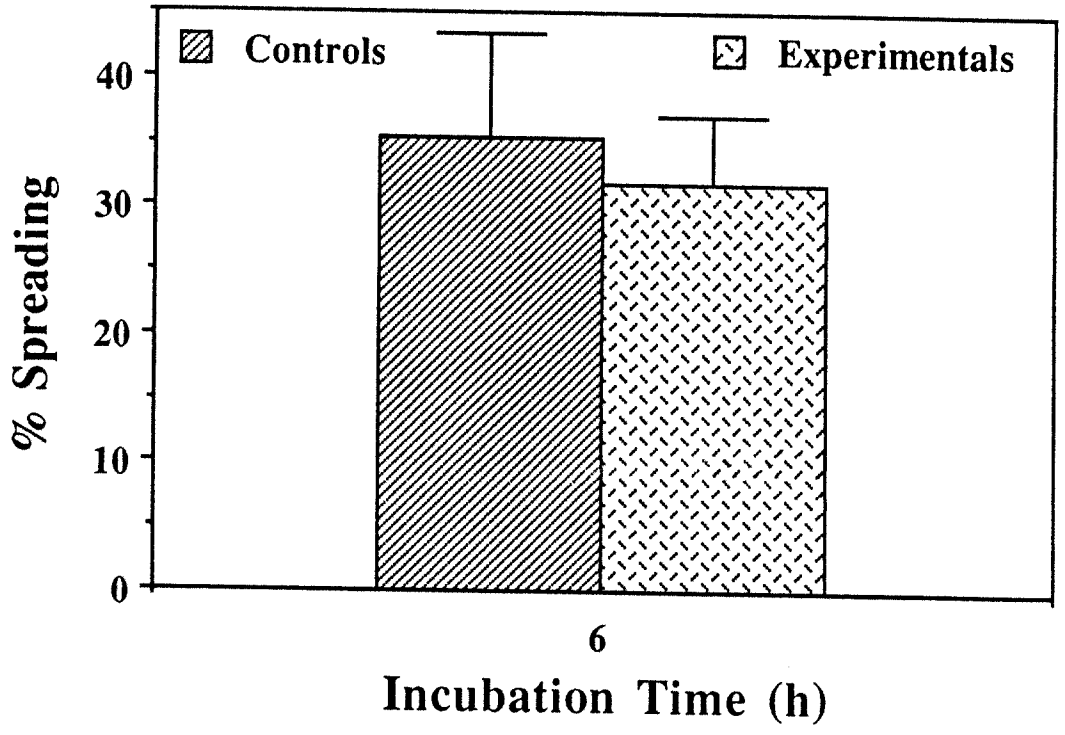
Also, spreading and phagocytic assays were run using the tube method for 6 and 24 h incubation times and the slide method for 6 h. At the end of 5 days exposure the worms had no lesions on their bodies but were under stress as evidenced by protracted contraction. There were no significant differences between controls and experimental worms in either percent spreading or percent phagocytosis of RRBC when data were analyzed by 2-tailed, unpaired t-tests (Figs. 21, 22). The mean spreading activity for 6 h incubation was 35.5 ± 8.2 for the controls, and 31.5 ± 5.5 for the experimentals (t = 1.4, p = 1.755). The mean percent phagocytosis of RRBC for 6 h incubation (tube method) were 8.4 ± 4.9 and 7.8 ± 5.6 for the controls and the experimentals, respectively (t = 0.269, p = 0.7901). The mean percent phagocytosis of RRBC for 24 h incubation (tube method) were 9.8 ± 8.6, and 9.4 ± 5.2 for controls and experimentals, respectively (t = 0.143, p = 0.8874). For the 6 h incubation using the slide method, the results were 6.7 ± 5.5 and 6.2 ± 4.5 for controls and exposed earthworms, respectively.

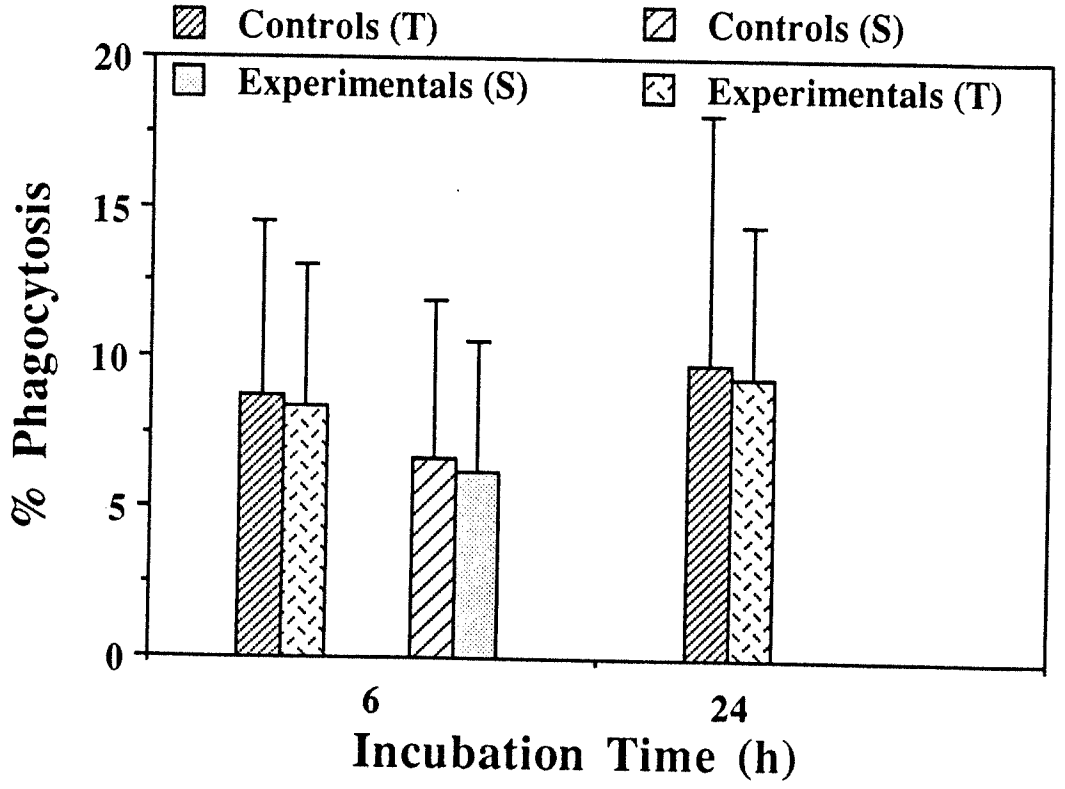
PCB (Aroclor 1254).

Pathogenicity. Exposure of *L. terrestris* to 10 µg of PCB/cm^2 for 5 days did not effect the immune response of earthworms against *B. thuringiensis* inoculation (Fig. 23, table 6). Twelve of 15 worms survived a challenge of 10^8

FIG. 21. Effect of Superfund soil (#4) on spreading activity of *L. terrestris* coelomocytes. Each column represents the mean percent spreading activity of 12 specimens for both control and experimentals. Error bar denotes standard deviation of the means.

FIG. 22. Effect of Superfund soil (#4) on phagocytosis of RRBC by coelomocytes of *L. terrestris*. Two methods of assay were used; the tube method (T) using 6 and 24 h incubation time and the slide method (S) using only a 6 h incubation time. Each column represents the mean percent phagocytosis of 12 specimens for both control and experimentals. Error bars denote standard deviation of the means.


Controls
Experimentals
% Spreading
40
30
20
10
0
6
Incubation Time (h)


Controls (T)
Controls (S)
Experimentals (S)
Experimentals (T)
% Phagocytosis
20
15
10
5
0
6
24
Incubation Time (h)

bacteria. However, the same amount of Aroclor 1254 suppressed the worm's immune response against *S. marcescens* (Fig.24, table 6). This suppression could be seen at bacterial concentrations of 1 x 10^7 and 1 x 10^8 CFU/worm. The number of deaths at the challenge level of 10^8 bacteria, was statistically significant. Results of analysis using the two tailed, unpaired t-tests for 10^7 and 10^8 bacterial concentrations were: t = 2.44, p = 0.712; t = 40, p = 0.0001 respectively. Fifteen earthworms were used per bacterial concentration plus 15 for the control making a total of 75 earthworms per assay. Each mean in table 8 and Figures 23 & 24 represents 3 replicates.

Phagocytosis of bacteria. Exposure of *L. terrestris* by the filter paper contact method to 10 µg of PCB (Aroclor 1254)/cm^2 for 5 days, did not alter the ability of the coelomocytes to phagocytize *B. thuringiensis*. Two tailed, unpaired t-test showed that there were no significant differences between controls and treated animals in phagocytic activity of their coelomocytes (t = 0.121, p = 0.905). Data presented in Fig. 25 and table 7, represent the mean phagocytosis of *B. thuringiensis* as a function of time. Four replicate assays were run with a total N = 48.

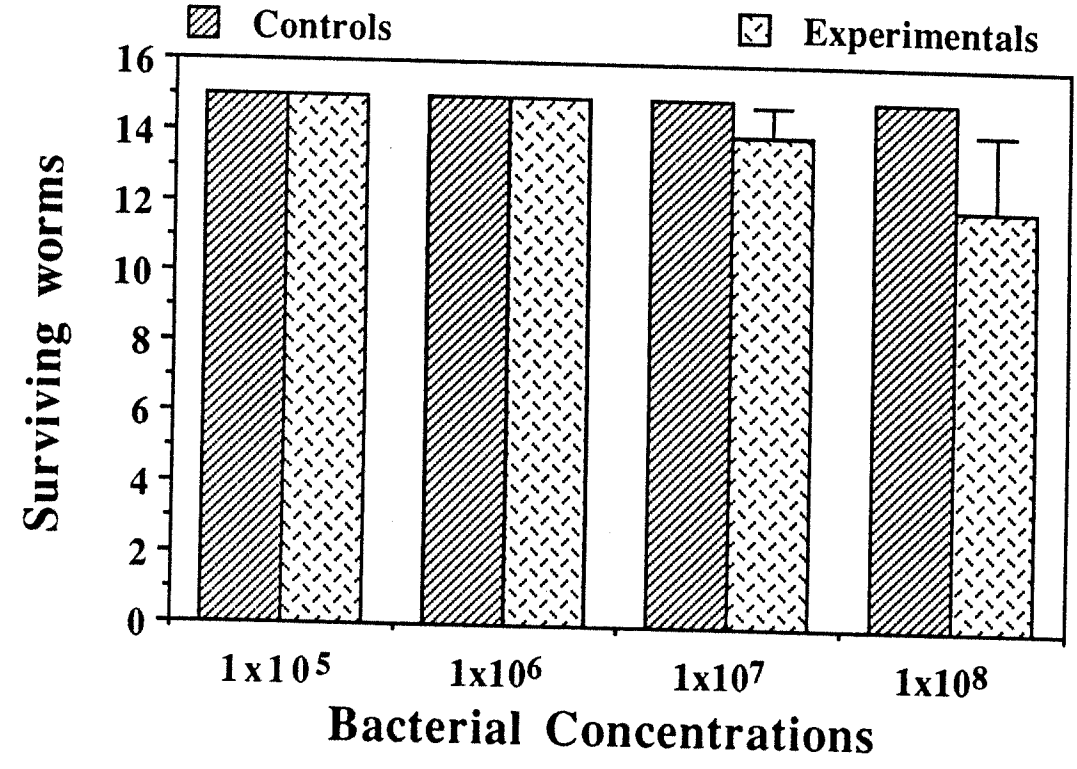
TABLE 6. Effect of 10 µg PCB (Aroclor 1254)/cm^2 on pathogenicity of bacteria to *L. terrestris*

Bacterial concentration per earthworm	Surviving earthworms after exposure and inoculation	
	B. thuringiensis	*S. marcescens*
0.0 (controls)	15.0 ± 0.0	15.0 ± 0.0
1 x 10^5 CFU	15.0 ± 0.0	15.0 ± 0.0
1 x 10^6 CFU	15.0 ± 0.0	14.7 ± 0.5
1 x 10^7 CFU	14.0 ± 0.8	9.7 ± 3.8
1 x 10^8 CFU	12.0 ± 2.2	1.7 ± 0.6

±SD

FIG. 23. Effect of PCB (Aroclor 1254) on pathogenicity of *B. thuringiensis* to *L. terrestris*. Earthworms were exposed to 10 $\mu g/cm^2$ by the filter paper contact method for 5 days at 10°C. Animals were inoculated with 1×10^5, 10^6, 10^7, and 10^8 CFU/animal.

FIG. 24. Effect of PCB (Aroclor 1254) on pathogenicity of *S. marcescens* to *L. terrestris*. The conditions used were the same as those indicated in Fig 23. A * indicates a significant difference.


Controls
Experimentals
Surviving worms
16
14
12
10
8
6
4
2
0
1x10 5
1x10 6
1x10 7
1x10 8
Bacterial Concentrations

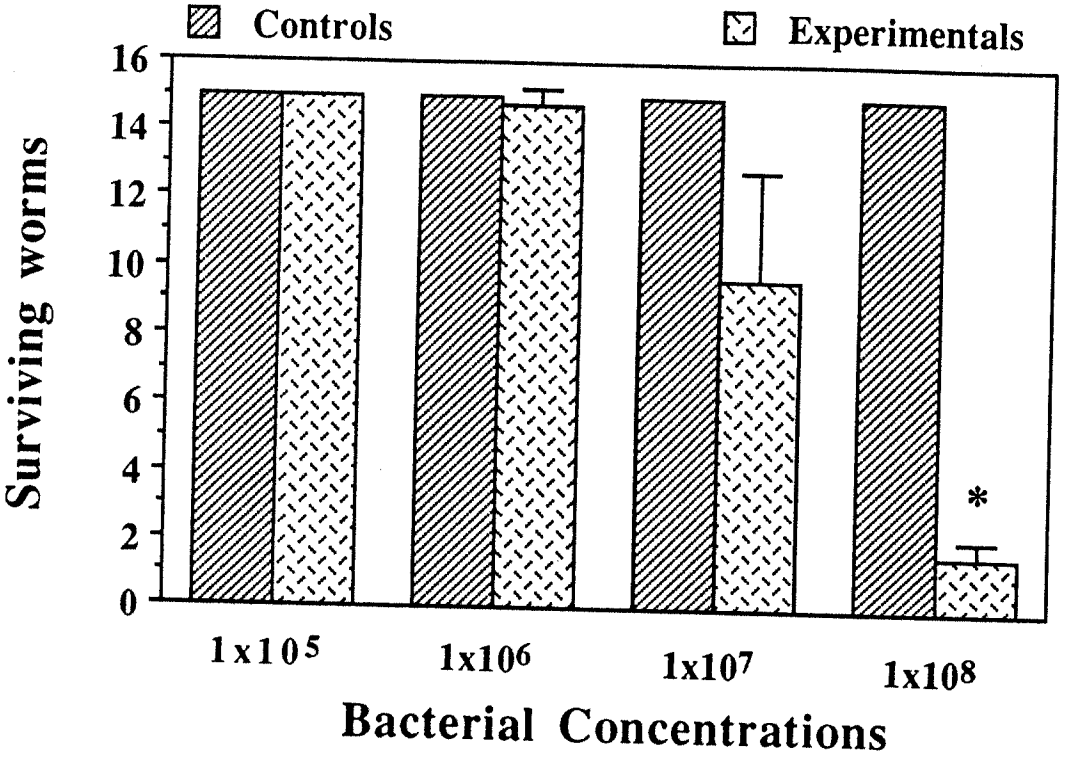

Controls
Experimentals
Surviving worms
16
14
12
10
8
6
4
2
0
*
1x10 5
1x10 6
1x10 7
1x10 8
Bacterial Concentrations

FIG. 25. Effect of PCB (Aroclor 1254) on phagocytosis of *B. thuringiensis* by coelomocytes of *L. terrestris*. Earthworms exposed to 10 µg of PCB/cm^2 by the filter paper contact method for 5 days. Each column represents the mean percent phagocytosis of 4 samples with 4 replicate assays. Error bars indicate standard deviation of the means.

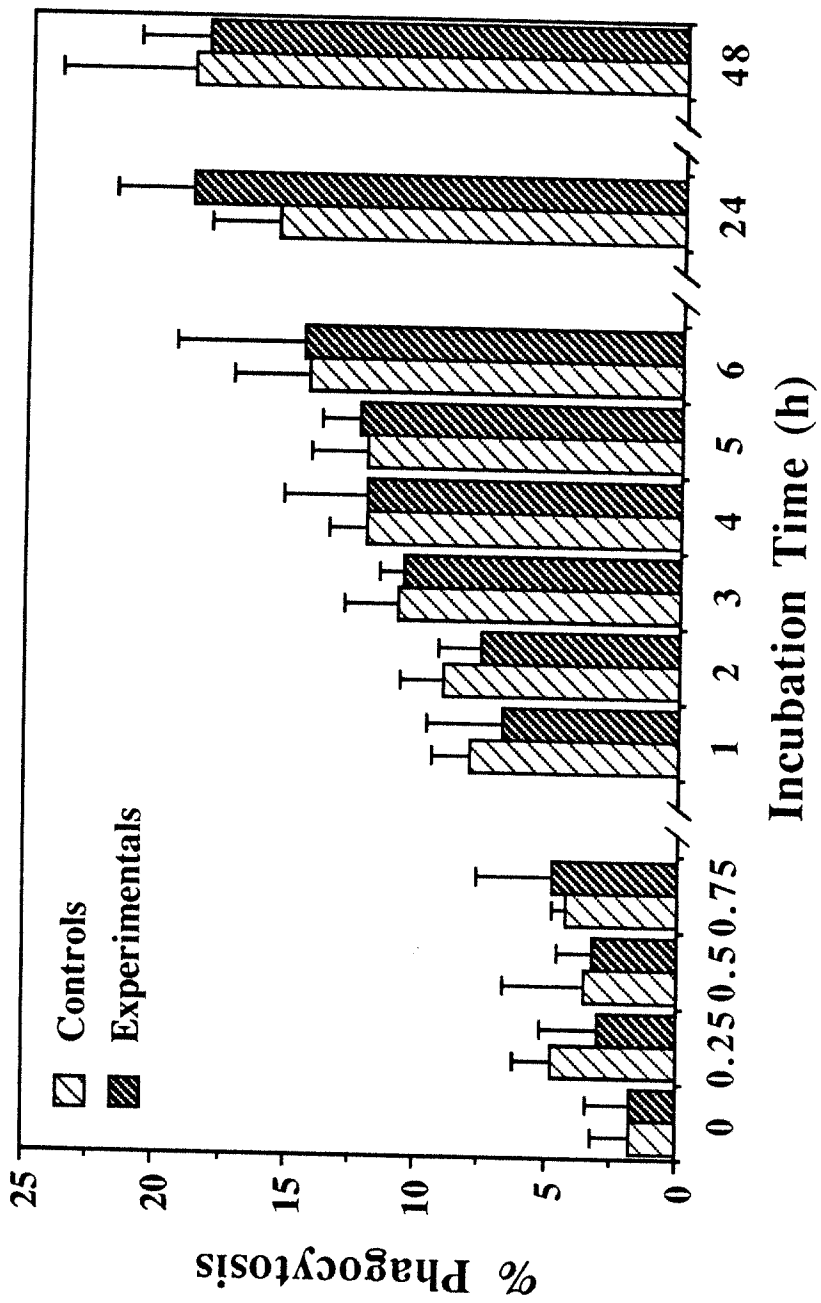

% Phagocytosis
25
20
15
10
5
0
Controls
Experimentals
0 0.25 0.5 0.75 1 2 3 4 5 6 24 48
Incubation Time (h)

TABLE 7. Effect of PCB (Aroclor 1254) on phagocytosis of *B. thuringiensis* by the coelomocytes of *L. terrestris*

Incubation Time (h)	Percent phagocytosis	
	controls	Exposed
0.00	1.75 ± 1.5	1.75 ± 1.7
0.25	4.75 ± 1.5	3.00 ± 2.2
0.50	3.50 ± 3.1	3.25 ± 1.3
0.75	4.25 ± 0.5	4.75 ± 2.9
1.00	8.00 ± 1.4	6.75 ± 1.5
2.00	9.00 ± 1.6	7.50 ± 1.7
3.00	10.75 ± 2.1	10.50 ± 1.0
4.00	12.00 ± 1.4	12.00 ± 3.2
5.00	12.00 ± 2.2	12.25 ± 1.5
6.00	14.25 ± 2.9	14.50 ± 4.8
24.00	15.50 ± 2.6	18.75 ± 2.9
48.00	18.75 ± 5.1	18.25 ± 2.6

± SD

N = 48 for each controls and experimentals

Electron Microscopy

SEM. SEM examination of coelomocytes from exposed and non-exposed earthworms to 1.0 μg of chlordane/cm^2 indicated no morphological alterations in coelomocytes after exposure of worms for 5 days by the filter paper contact method. Results of 3 methods used are as follows :

1. Electron micrographs of coelomocytes from exposed earthworms after 3 h incubation on a polylysine coated coverglass demonstrated no morphological differences between the controls and the experimental worms (Figs. 26-A & B).

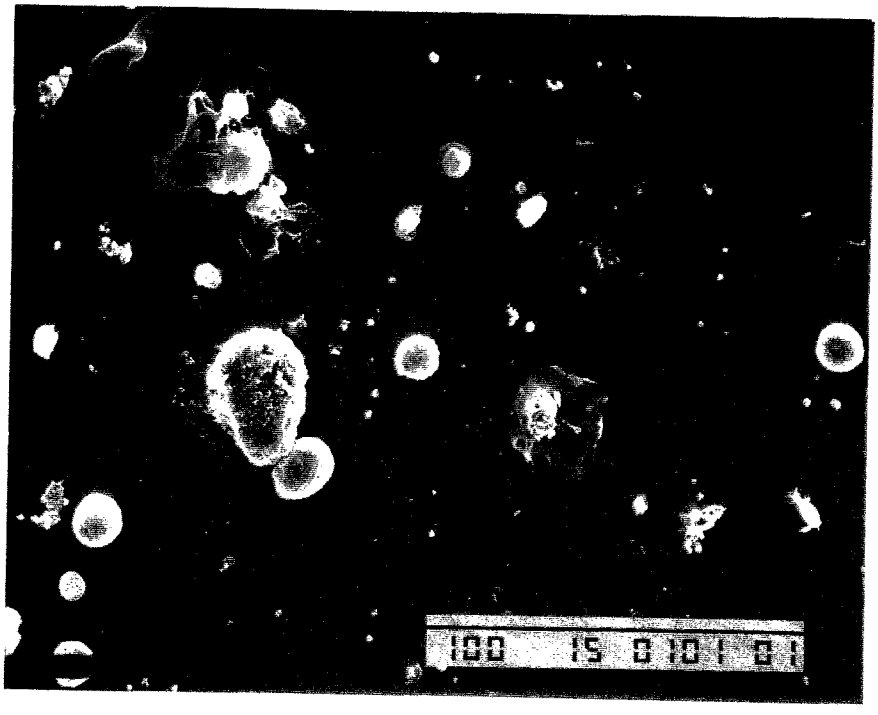
2. Electron micrographs of cells from exposed worms that were set up for 24 h phagocytosis of RRBC, show no obvious changes in morphology of the coelomocytes (Figs. 27-A & B)

3. Electron micrographs of non adhering cells washed off slides with saline, show that most of the control cells were distorted and damaged (Fig. 28-A), whereas those from exposed animals showed different types of cells which were morphologically unchanged (Fig. 28-B).

These results can be taken as an observation, since there were not enough data to determine statistical significance.

Elemental analysis (EDX). Elemental analysis by EDX of earthworm coelomocytes and coelomic fluid exposed to lethal and sublethal levels of PCB (10, 100 μg/cm^2), sublethal

FIG. 26. Scanning electron micrographs of coelomocytes which adhere to polylysine coated slides. (A) Control. (B) Chlordane exposed. Magnification, x500.


100 15 0 10 1 01

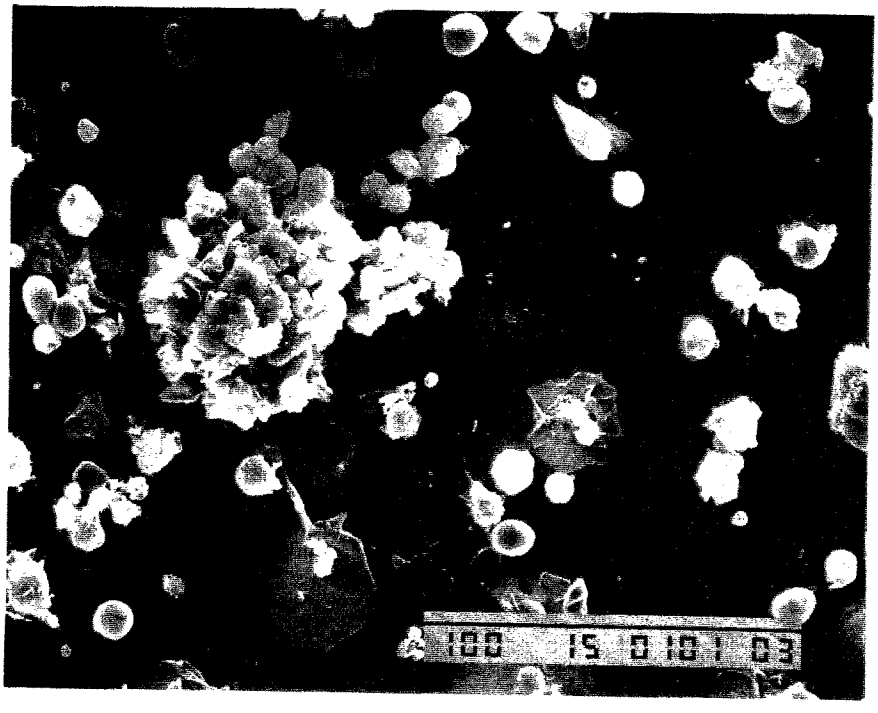

100 15 0 10 1 03

FIG. 27. Scanning electron micrographs of a mixture of coelomocytes and RRBC on polylysine coated slides, incubated for the phagocytosis assay. (A) Control. (B) Exposed to chlordane. Magnification, x500.

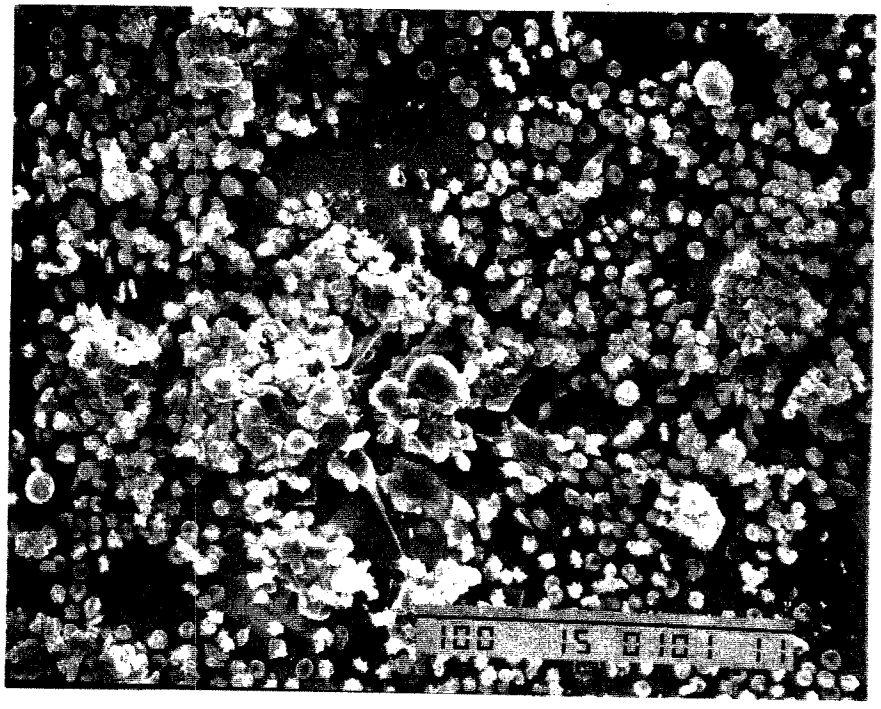
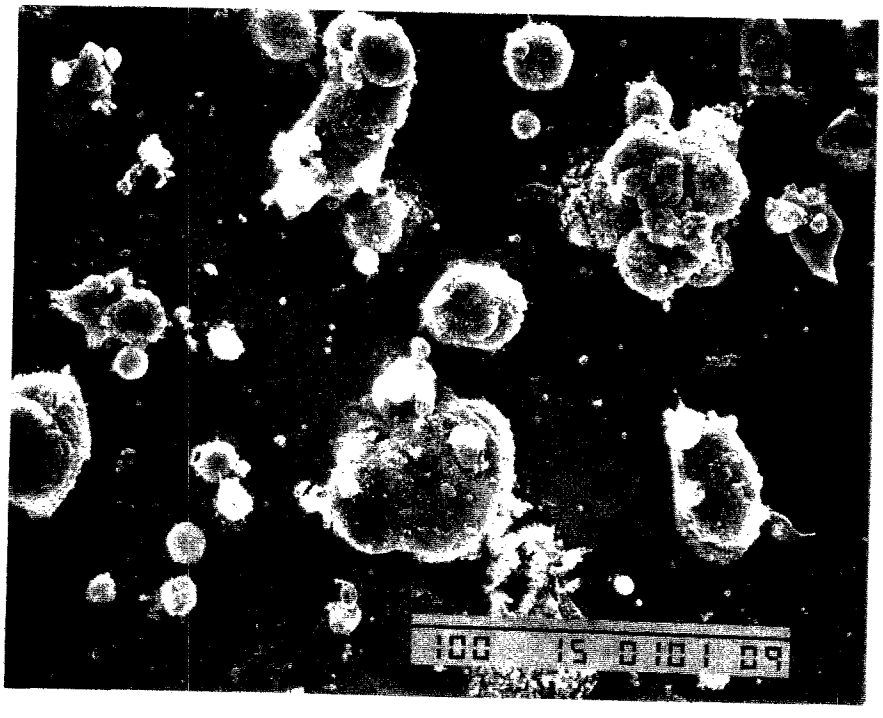


FIG. 28. Scanning electron micrograph of non-adherent coelomocytes. (A) Control. (B) Chlordane exposed. Magnification, x500.


100 15 0 10 1 88


100 15 0 10 1 89

level of cadmium nitrate (2.5 μg/cm^2),and lethal levels of copper sulfate (2.55 μg/cm^2) demonstrated highly variable results with low reproducibility from one experiment to another. Cells and fluids collected by puncture and extrusion methods followed almost the same pattern of results, even though counts from the puncture method tended to be higher than those obtained by extrusion. Analysis were performed on different cells: small, medium, large, smooth, spread, single cells, clusters of cells, chlorosomes, and coelomic fluid. Figs 29, 30, 31, and 32 are examples of spectra from cells from worms exposed to PCB. Two other toxicants, copper sulfate and cadmium nitrate showed the same high variability.

Stereological study. Exposure of *L. terrstris* to a lethal concentration of copper sulfate (2.55 μg/cm^2) for 3 days caused significant reduction in nuclear volume ratios of epidermal tissue (Figs. 33, 34, 35)). The mean nuclear volume ratio of control animals were 6.8 ± 2.2, whereas exposed earthworms had 4.4 ± 2.6. There was a significant difference between controls and experimental animals using two factor repeated measures ANOVA (F = 11.105, p = 0.029), with N = 84 for 3 earthworms, 2 blocks,and 14 repeated measurements (14 pictures of each with approximatley 2000 points).

FIG. 29. EDX analysis of a sample of coelomocytes from an earthworm exposed to 10 μg of PCB (Aroclor 1254)/cm^2. The spectrum shown here is a background analysis of the Thermanox cover slip showing no chlorine present.

FIG. 30. EDX analysis of a sample of coelomocytes from a control earthworm. The spectrum is from an large control cell on a Thermanox cover slip. No chlorine was detected.

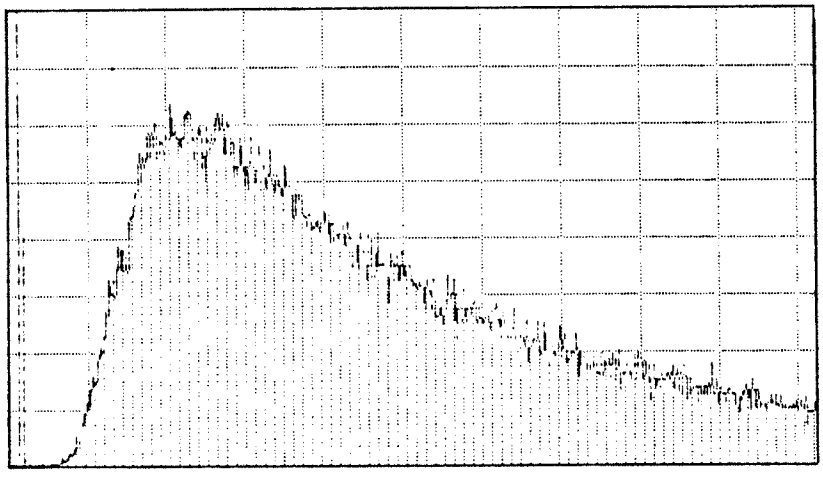
QUALITATIVE ELEMENT IDENTIFICATION

SAMPLE ID:EXP. BACKGR NEXT TO CELL, SLOW 1,5000X

POSSIBLE IDENTIFICATION

PEAK LISTING
ENERGY AREA EL. AND LINE

TN-5500 North Texas State University THU 14-SEP-89 08:30
Cursor: 0.130keV = 0

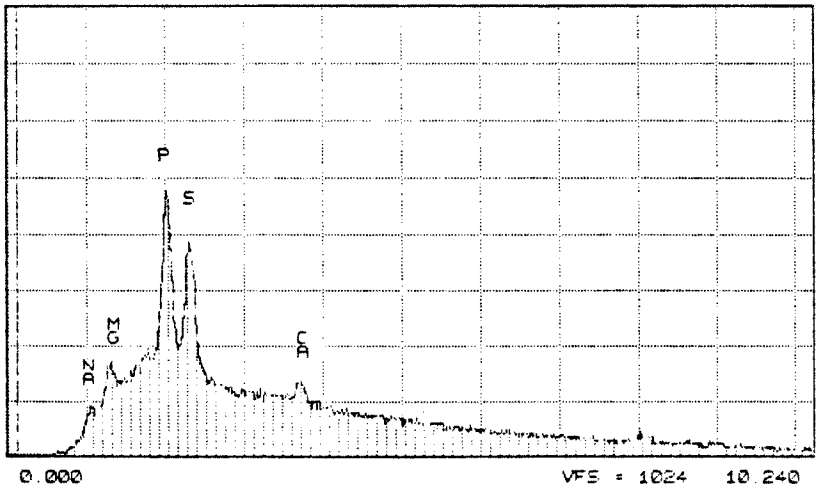


0.000 B- 5 VFS = 256 10.240
100 EXP. BACKGR NEXT TO CELL, SLOW 1,5000X

PEAK LISTING

	ENERGY	AREA	EL. AND LINE
1	1.030	423	NA KA
2	1.298	1137	MG KA OR AS LA?
3	2.016	5316	P KA OR ZR LA?
4	2.317	4095	S KA OR MO LA?
5	3.710	714	CA KA

TN-5500 North Texas State University THU 10-AUG-89 10:08
Cursor: 0.120keV = 0 ROI (2) 0.000: 0.000



0.000 VFS = 1024 10.240
100 CONTROL-I-ONE CELL-L- 3500X

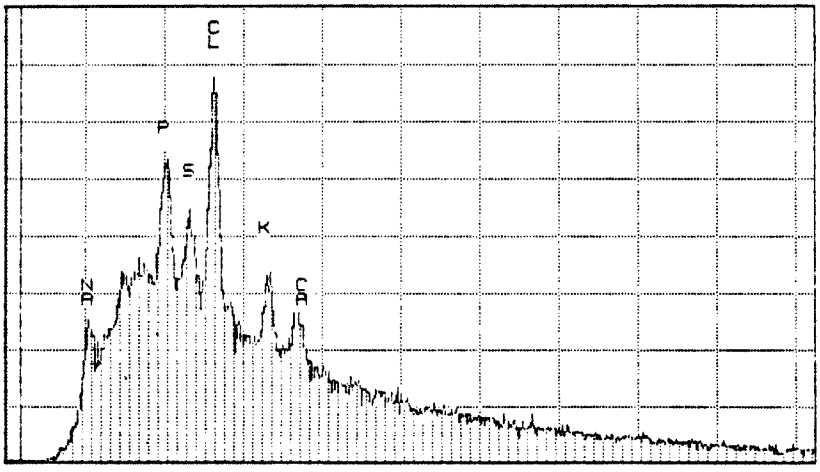
FIG. 31. EDX analysis of a sample of coelomocytes from an earthworm exposed to PCB (Aroclor 1254) at 10 $\mu g/cm^2$. The spectrum shown is from a large experimental cell on a Thermanox cover slip showing a high level of chlorine.

FIG. 32. EDX elemental analysis of a sample of coelomocytes from an earthworm exposed to PCB (Aroclor 1254) at 10 $\mu g/cm^2$. The spectrum shown is from a experimental cell on a Thermanox cover slip showing very low levels of chlorine compared to other elements detected in the cell.

PEAK LISTING

	ENERGY	AREA	EL. AND LINE
1	1.039	1043	NA KA
2	2.014	2141	P KA OR ZR LA?
3	2.318	1178	S KA OR MO LA?
4	**2.626**	**3788**	**CL KA**
5	3.317	1208	K KA OR IN LA?
6	3.695	819	CA KA

TN-5500 North Texas State University FRI 21-JUL-89 09:30
Cursor: 0.180keV = 0 ROI (2) 0.290: 0.300

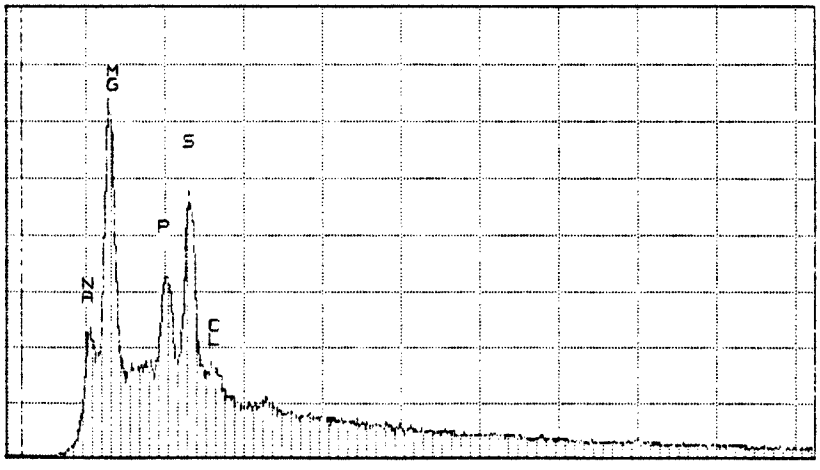


0.000 B- 5 Kα = 0.19keV VFS = 512 10.240
100 EXTRUSION EXP_2_PH _5000X

PEAK LISTING

	ENERGY	AREA	EL. AND LINE
1	1.038	1468	NA KA
2	1.300	9023	MG KA OR AS LA?
3	2.013	3139	P KA OR ZR LA?
4	2.314	6077	S KA OR MO LA?
5	2.643	595	CL KA OR RH LA?

TN-5500 North Texas State University THU 14-SEP-89 08:22
Cursor: 0.190keV = 0



0.000 B- 5 Kα = 0.19keV VFS = 1024 10.240
100 CELL, EXP, SPOT, 5000X

FIG. 33. Transmission electron micrograph from epidermal tissue of a normal control *L. terrestris*, showing a number of nuclei (N). Magnification, x4750

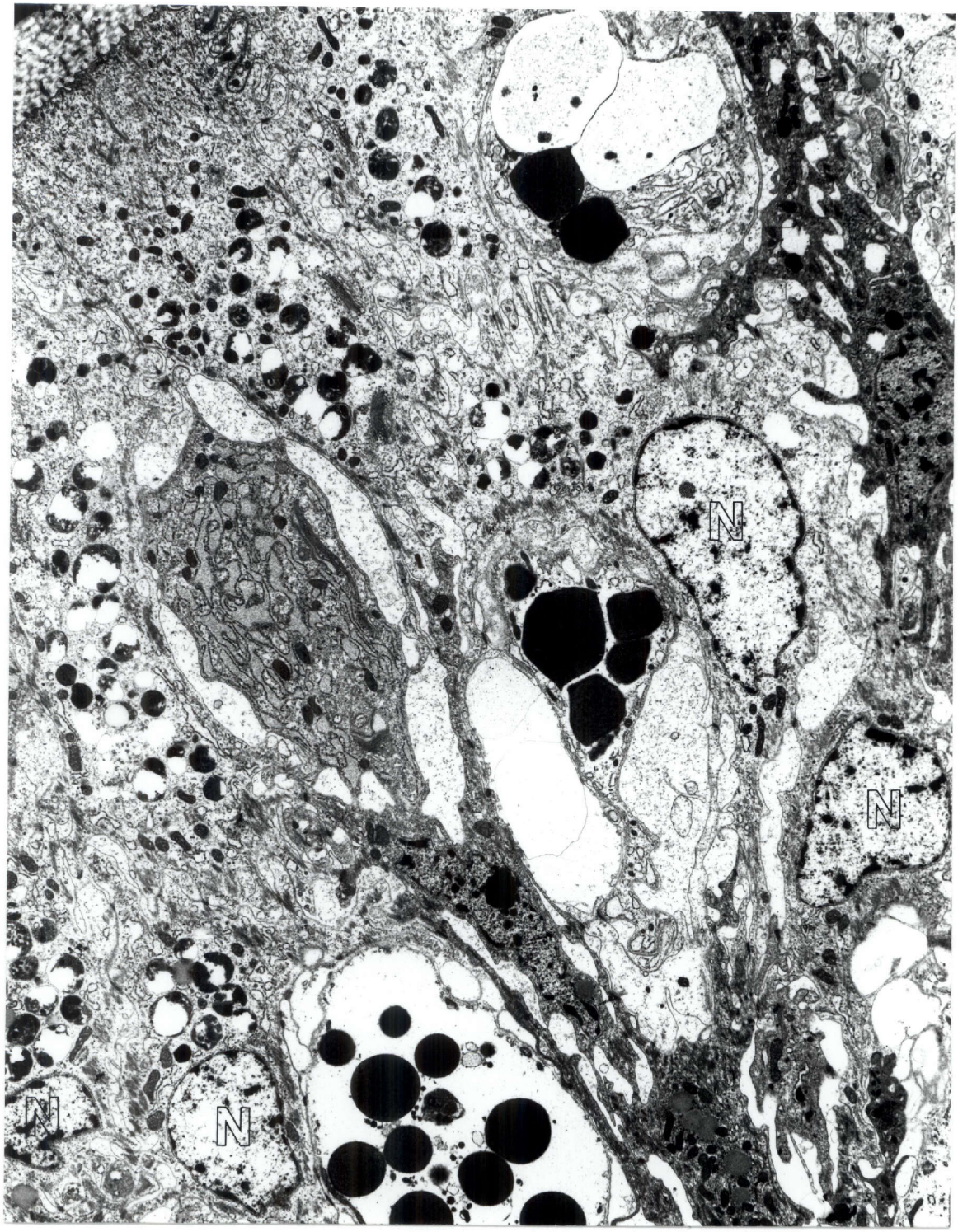

N
N
N
N
N

FIG. 34. Transmission electron micrograph from epidermal tissue of a *L. terrestris* exposed to copper sulfate, showing a number of nuclei (N). Magnification, x4750.

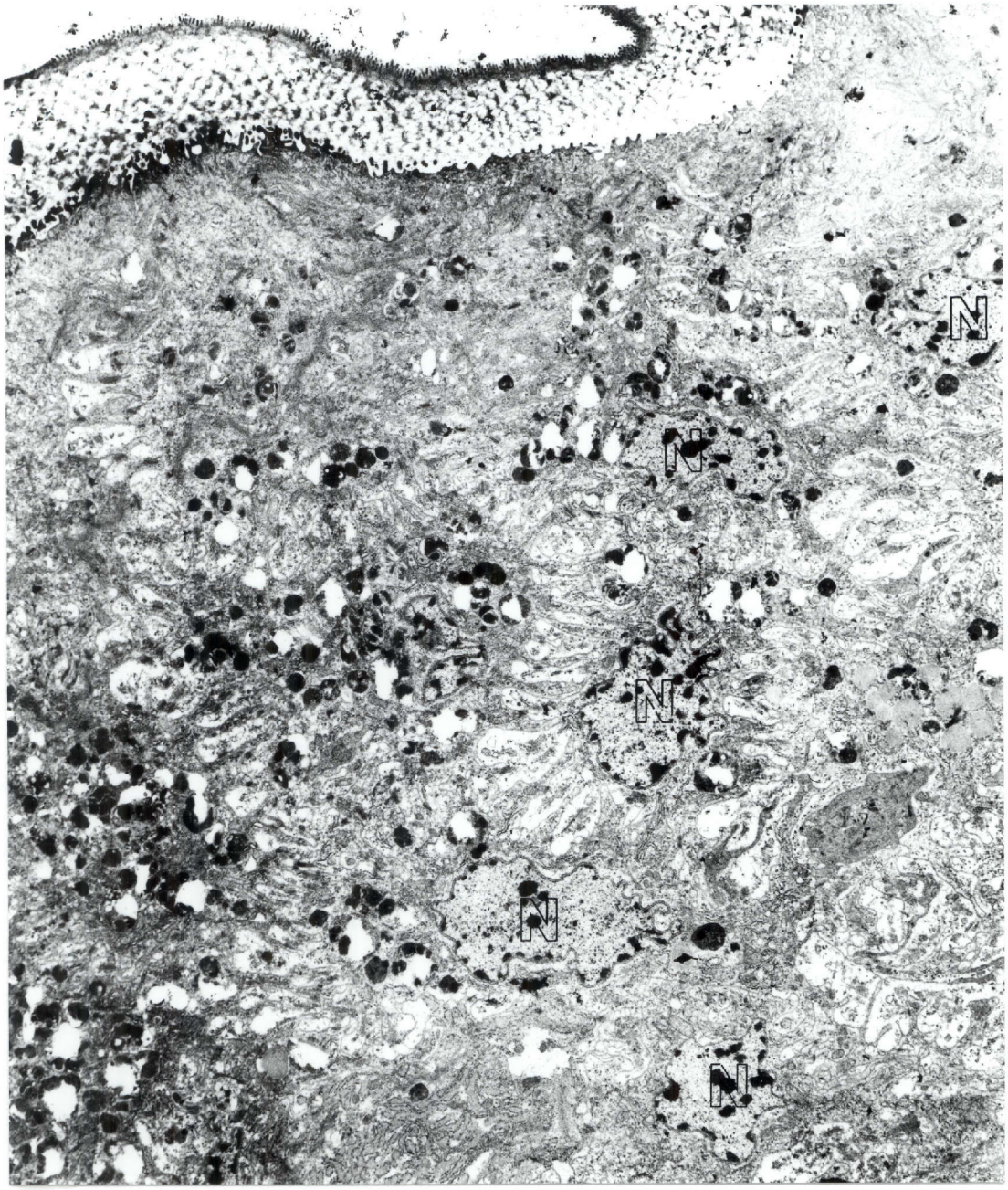
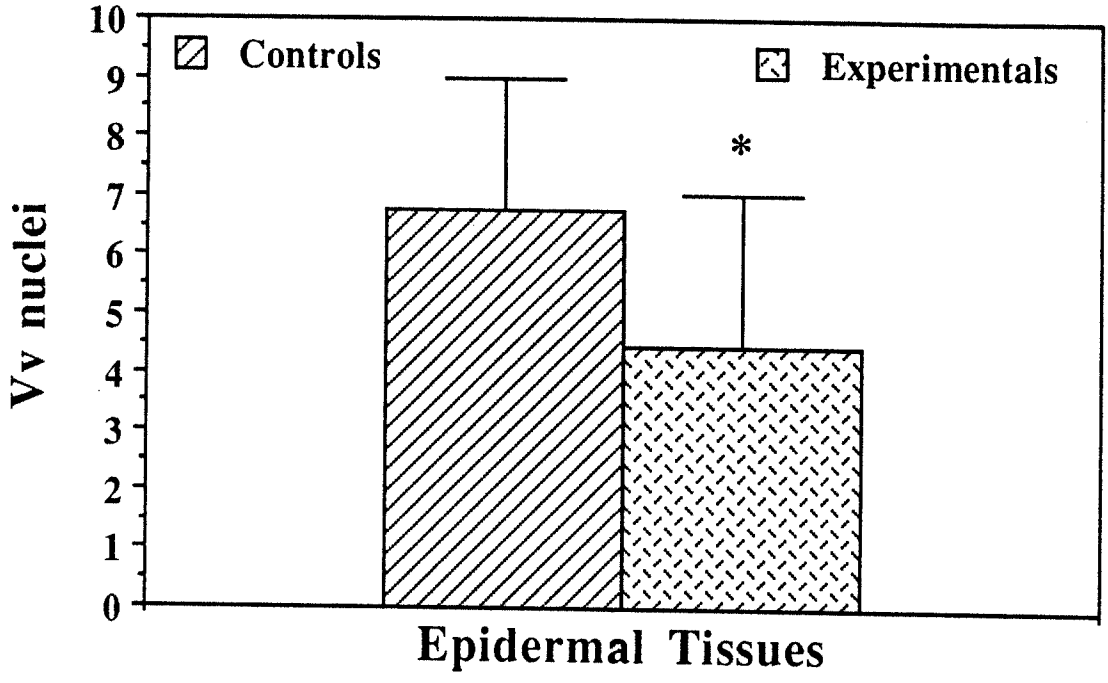

N
N
N
N
N

FIG. 35. Effect of copper sulfate at 2.55 $\mu g/cm^2$ on nuclear volume ratio in epidermal tissue of *L. terrestris*. Each column of control and experimental tissue represents a mean nuclear volume ratio of 3 specimens, 2 blocks, and 14 pictures with approximatley 2,000 points for both control and exposed earthworms. A * indicates a significant difference.


10
9
8
7
6
5
4
3
2
1
0
Vv nuclei
Controls
Experimentals
*
Epidermal Tissues

II. *Eisenia foetida*

A. Methods Development

Bacterial pathogenicity. Two bacteria, *B. megaterium* and *A. hydrophila*, were tested for their pathogenicity for *E. foetida*. *B. megaterium* was not pathogenic when inoculated at a level of 1 x 10^8 CFU/earthworm. However, *A. hydrophila* was found to be a potential pathogen for *E. foetida*, at concentrations of 1 x 10^7 CFU/worm and higher. The mean earthworm survivals after injection of *A. hydrophila*, were 51.0% ± 1.7 (LC_{50}), and 1.0% ± 0.5 for bacterial concentrations of 1 x 10^7 and 1 x 10^8 CFU/earthworm, respectivly (table 8).

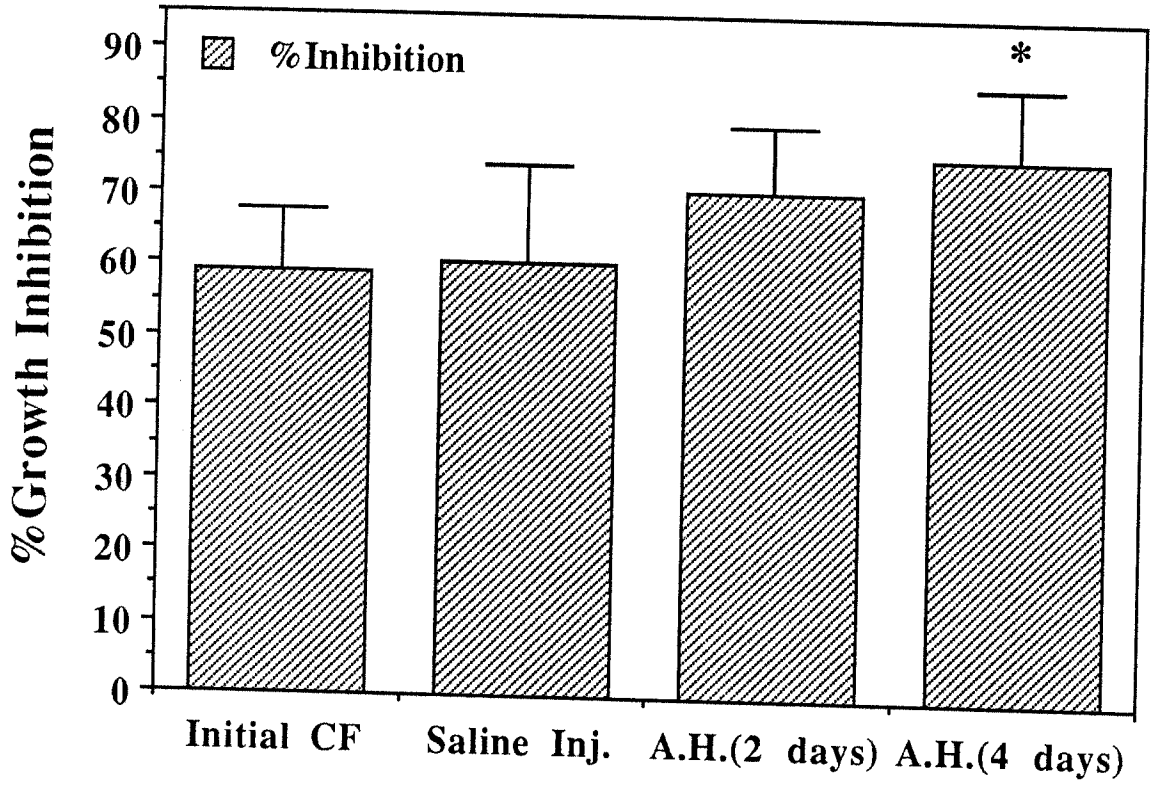
Antimicrobial activity of coelomic fluid (CF). In vitro antimicrobial activity of CF was tested with *A. hydrophila*. Initial CF (before any injection of bacteria) showed 59.1% ± 8.7 growth inhibition of *A. hydrophila* compared to saline controls. Injection of sub-lethals level of *A. hydrophila* (10^6 CFU/earthworm) enhanced this activity. The mean percent growth inhibition of the saline control, 2 and 4 days post injection of *A. hydrophila* were 60.5 ± 13.5, 70.8 ± 9.1, and 75.8 ± 9.7, respectively (Fig. 36). There was a significant difference in antimicrobial activity of CF 4 days after immunization compared to initial and saline injected controls. One way ANOVA showed significance for 4 days post *Aeromonas* injection

TABLE 8. Pathogenicity of bacteria to *E. foetida*

Bacterial conc./worm	Percent earthworm survival at 4 days of injconction	
	B.megaterium	*A. hydrophila*
1×10^4	100 0 ± 0.0	100.0 ± 0.0
1×10^5	100.0 ± 0.0	100.0 ± 0.0
1×10^6	100.0 ± 0.0	100.0 ± 0.0
1×10^7	95.7 ± 1.2	51.0 ± 1.7
1×10^8	87.7 ± 2.1	1.0 ± 0.5
Controls 10 µl LBSS	100.0 ± 0.0	100.0 ± 0.0

± SD. Total for each bacterium = 480 earthworms, 30 worms per concentration. Experiments were done in 3 replicates.

FIG. 36. Antimicrobial activity of non-immunized and immunized *E.foetida* coelomic fluid against *A. hydrophila* (A.H.). Each column represents the mean percent growth inhibition of 30 pooled samples with 3 replicates. Error bars indicate standard deviation of the mean and * denotes statistical significance at $p \leq 0.05$.


% Growth Inhibition
90
80
70
60
50
40
30
20
10
0
% Inhibition
*
Initial CF
Saline Inj.
A.H.(2 days)
A.H.(4 days)

(F = 3.42, p = 0.0411). Dunnett multiple range comparison showed both initial vs. 4 days post *Aeromonas* injection, and saline injected vs.4 days post *Aeromonas* injection to be significantly different.

B. Effect of Xenobiotics

RDFF. Exposure of *E. foetida* to 3 different concentrations of 30, 50, and 70% RDFF for 5 days, before any injection of infectious agents resulted in a dose response lethal effect compared to control artificial soil exposure. The mean percent earthworm survivals were 100.0 ± 0.0, 96.5 ± 3.0, 81.0 ± 5.7, and 69.7 ± 9.4 for controls, 30, 50, and 70% FA exposure respectively (Fig. 37). A total of 3440 earthworms in 3 replicates were used in this assay.

Suppression of Immune response by RDFF. At the fifth day of exposure to RDFF, the remaining live earthworms were removed from the jars and injected with *A. hydrophila* (10^6 CFU/earthworm) and saline controls and incubated for 4 days at 20°C. Suppression of the immune system as indicated by a dose response lethal effect of *Aeromonas* injected animals (Fig. 38) as compared to controls. Mean percent earthworm survival after saline injection were 100.0 ± 0.0, 99.0 ± 1.4, 91.9 ± 2.1, and 89.3 ± 3.3 for controls, 30, 50, and 70% FA exposed, respectively. Mean percent

FIG. 37. Effect of RDFF on survival of *E. foetida* after challenge with *A. hydrophila*. Control column (non-exposed) represents 720 earthworms and for 30, 50, and 70% FA columns, there were 720, 1100, and 900 earthworms, respectivly. Error bars represents standard deviation of the means, and assay was ran in 3 replicates.

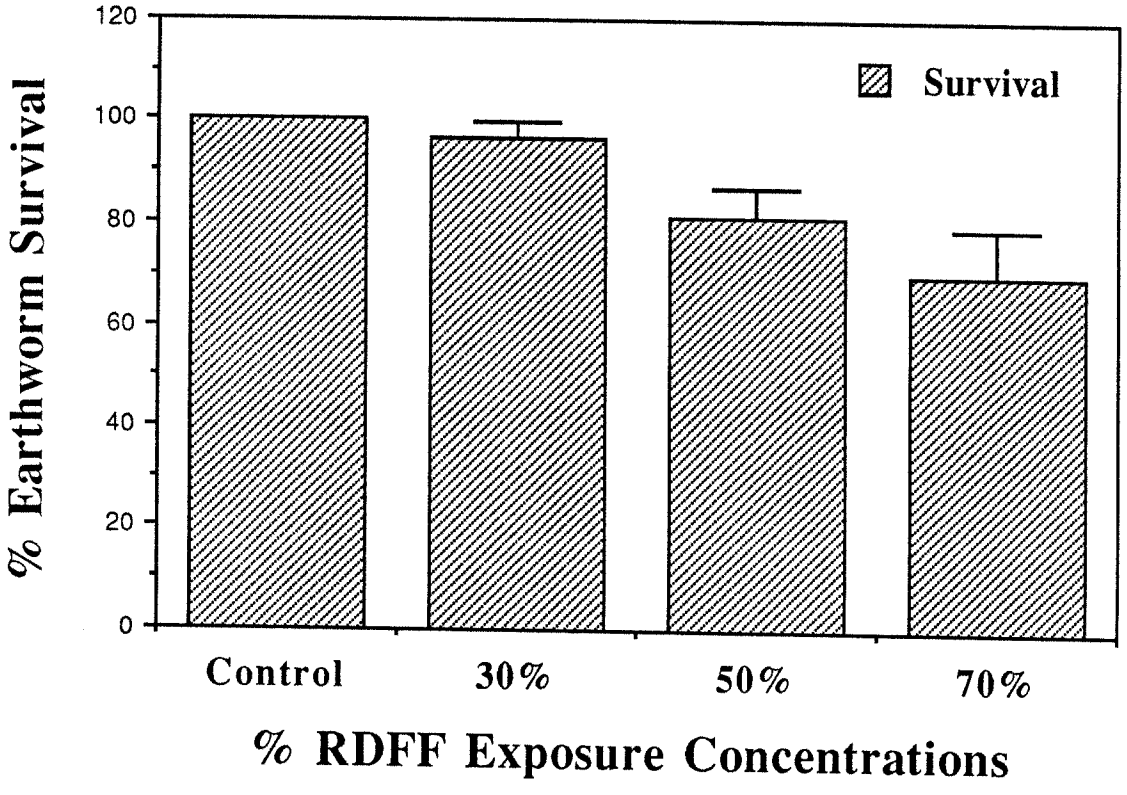
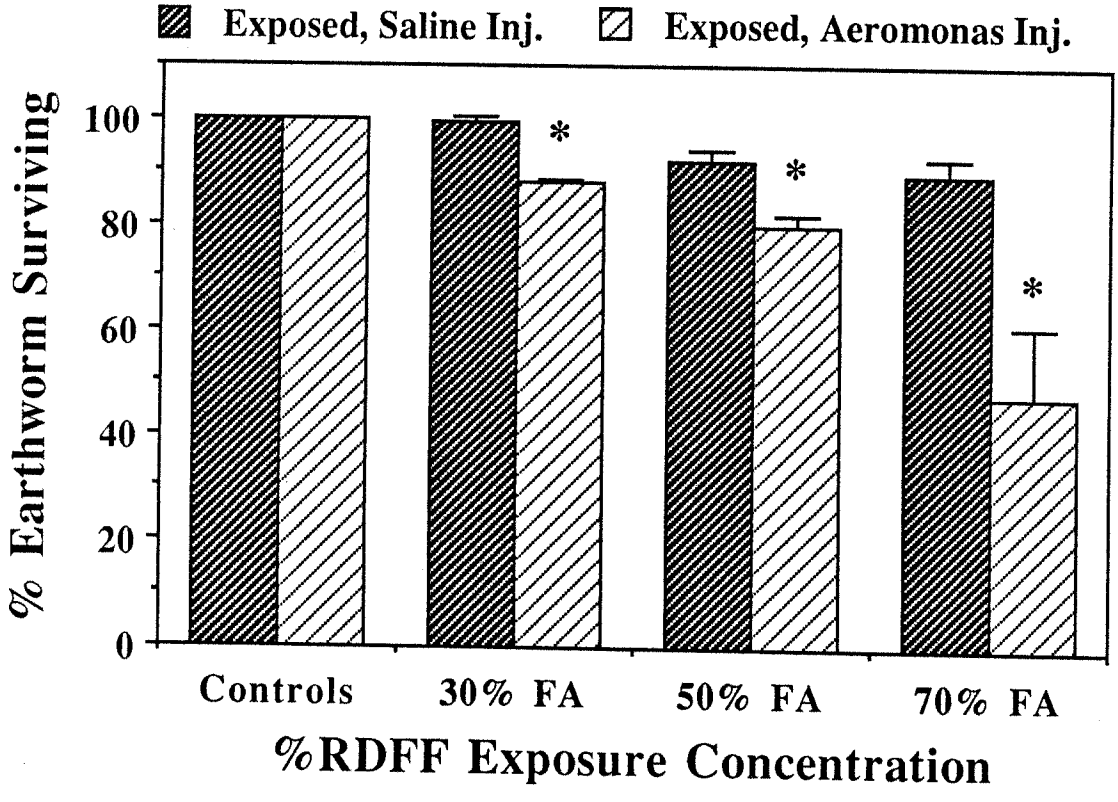

% Earthworm Survival
120
100
80
60
40
20
0
Survival
Control
30%
50%
70%
% RDFF Exposure Concentrations

FIG. 38. Suppression of *E. foetida* immune system to challenge with *A. hydrophila* by 5 days exposure to RDFF. Total number of surviving earthworms (see Fig. 37) used for 3 replicates were 2937 which were divided into groups of 720, 688, 886, and 643 worms for non-exposed, 30, 50, 70% FA exposure, respectively. Error bars indicates standard deviation of the means, and * denotes statistical significant at $p \leq 0.05$.


Exposed, Saline Inj.
Exposed, Aeromonas Inj.
% Earthworm Surviving
100
80
60
40
20
0
*
*
*
Controls
30% FA
50% FA
70% FA
%RDFF Exposure Concentration

earthworm survival after *Aeromonas* injection were 100.0 ±0.0, 87.8 ± 0.8, 79.7 ± 2.1,and 47.7 ± 13.3 for controls, 30, 50, 70% FA exposure, respectively. Two way ANOVA showed a significant difference between survival in saline controls and *Aeromonas* injected samples (F = 31.328, p = 0.0001). Also when each group (30, 50, 70% FA) analyzed by 2-tailed, unpaired t-tests, showed a significant difference between saline injected controls and *Aeromonas* injected samples as follow: t = 9.571, p = 0.007; t = 6.542, p = 0.0012; t = 4.3, p = 0.0126 for 30, 50, and 70% FA exposures, respectively.

Suppression of induction of antimicrobial activity of CF. Coelomic fluid from the remaining live earthworms were collected and assayed for *in vitro* antimicrobial activity. Fig. 39, and table 9 show the antimicrobial activity of CF from uninoculated, saline injected, and *Aeromonas* injected earthworms after 5 days of exposure to 30, 50, and 70% FA. There was a significant difference between the control uninoculated coelomic fluid antimicrobial activity and the exposed uninoculated worms by one way ANOVA (F = 3.504, p = 0.0465). Dunnett multiple range comparison indicated a significant difference only between controls and 70% FA exposure. However, there was a significant difference between all *Aeromonas* injected groups analyzed by one way ANOVA (F = 9.499, p = 0.0028). The

FIG. 39. Effect of 5 days exposure of *E. foetida* to RDFF on antimicrobial activity of CF. Each column represents the mean percent growth inhibition of *A. hydrophila* by the CF. Error bars shows standard deviation of the means.

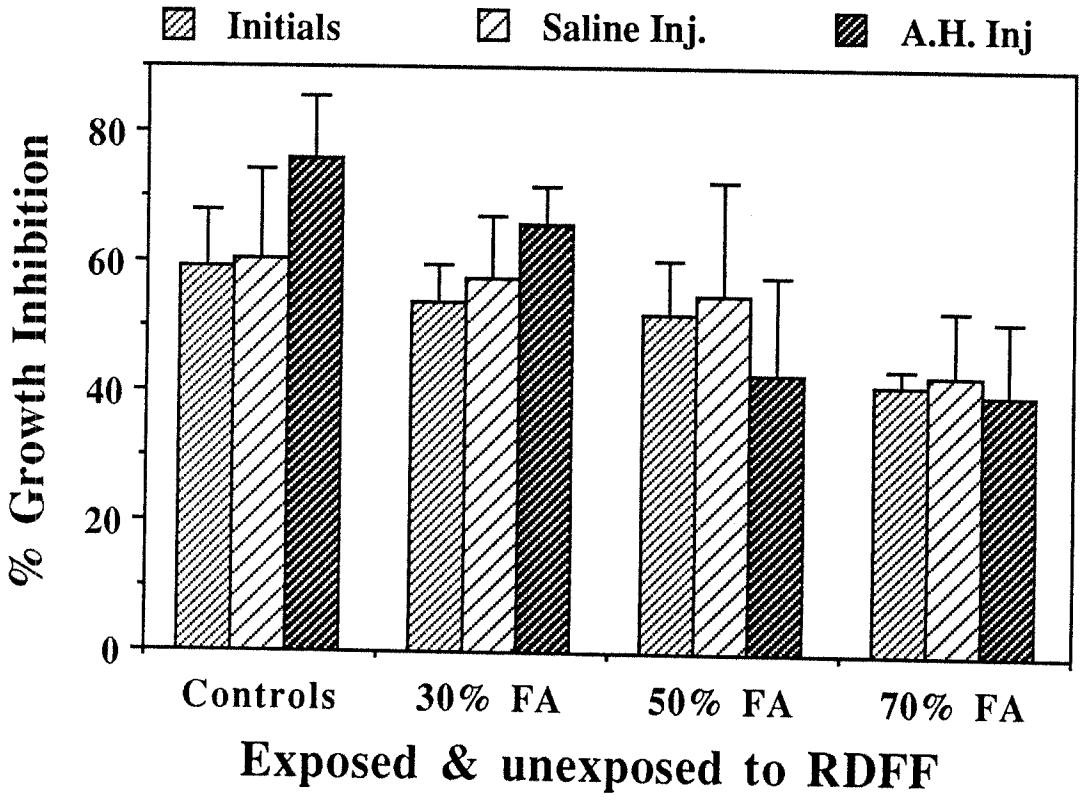

Initials
Saline Inj.
A.H. Inj
% Growth Inhibition
80
60
40
20
0
Controls
30% FA
50% FA
70% FA
Exposed & unexposed to RDFF

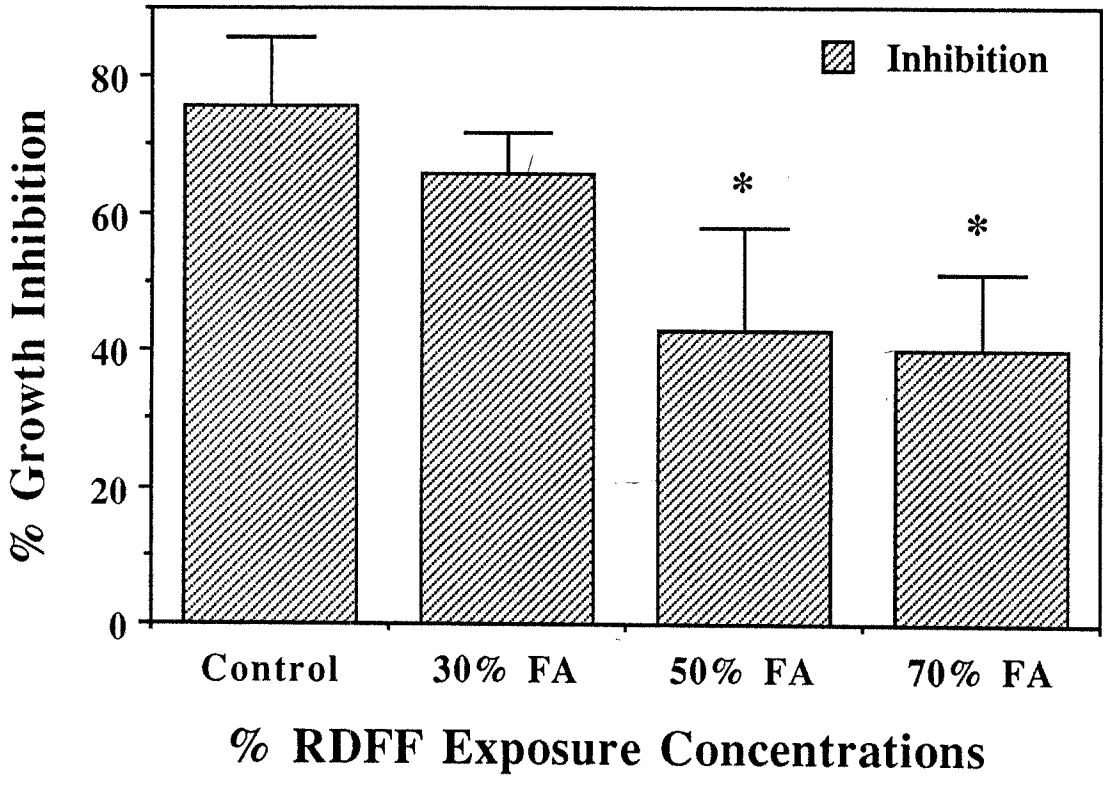
TABLE 9. Effect of RDFF on antimicrobial activity of *E. foetida*'s CF

Exposure	Percent growth inhibition		
	uninoculated CF	saline injected	*Aeromonas* injected (10^6/worm)
Controls	59.1 ± 8.7	60.5 ± 13.5	75.8 ± 9.7
30% FA	53.7 ± 6.0	57.7 ± 9.4	65.7 ± 9.7
50% FA	52.0 ± 8.6	55.2 ± 17.1	43.0 ± 14.8
70% FA	41.3 ± 2.5	43.0 ± 9.9	40.0 ± 11.1

± SD

Dunnett multiple range comparison showed the differences were between controls and 50% and 70% FA exposures. There was no significant difference between controls and 30% FA exposure (Fig. 40).

FIG. 40. Suppression of induction of *in vitro* antimicrobial activity of *E. foetida* coelomic fluid by 5 days exposure to RDFF. Each column represents mean percent growth inhibition of *A. hydrophila* by the CF. Error bars show standard deviation of the mean, and * indicates statistical significant at $p \leq 0.05$.


Inhibition
% Growth Inhibition
80
60
40
20
0
*
*
Control
30% FA
50% FA
70% FA
% RDFF Exposure Concentrations

CHAPTER IV

DISCUSSION

Lumbricus terrestris

Methods Development

Spreading of macrophage-like coelomocytes of *L. terrestris* on glass slides upon incubation (Fig. 2), is indicative of a primary functional characteristic of macrophage-like coelomocytes. This activity, comparable to that of mammalian and fish macrophages (94, 155, 156), has been proposed as a pre-requisite step for the development of ingestion activity of macrophages (94). The percentage of spreading cells in a total population of mixed coelomocytes shows that a large number (32.6 ± 10.5 SD) of coelomocytes have the ability to spread at 24 h; indicates the importance of this type of coelomocyte in the immune response of *L. terrestris*. This finding was also backed up by the SEM study of washed and unwashed slides which showed that a large number of cells have the ability to adhere to glass surfaces and are not washed off the slide.

Unlike mouse macrophages (94), treatment of the cells with lysolecithin and incubation with CO_2 had no observabale effect on the spreading and phagocytic activities of coelomocytes. In mammals, some reports indicate a slight

increase of phagocytic activity in the presence of CO_2. Lysophosphatidylcholine has been shown to induce spreading of murine macrophages and to stimulate the ingestion of IgG - but not IgM-coated erythrocytes by these cells (94, 122). No activation by lysolecithin in earthworms, can be explained either by a difference in the specificity of immune cells toward different chemical stimuli or a technical problem in the spreading and phagocytosis protocols which prevent enough stimulation of these cells to be detected by light microscopy. Possibly higher concentrations (>20 µg/ml) and longer incubation times in the presence of lysophosphatidylcholine (>2 h) would enhance stimulation of spreading.

Clumping upon incubation and centrifugation was a major problem for both the spreading and phagocytic assays. The presence of Ca^{++} in the medium, although it increases phagocytic activity, also increases cell-to-cell adhesion in mammalian systems (122). Clumping is reduced by addition of EDTA which chelates cations (especially Ca^{++}) and results are comparable to those obtained in mammalian systems.

Time course assays show that the spreading activity of macrophage-like cells of *L. terrestris* start as early as 2 h of incubation. Evaluation of buffers for the spreading and phagocytosis assays, including LBSS, RPMI 1640, and saline, show that LBSS with the addition of EDTA is the best system.

Fetal calf serum is known to increase the percent phagocytosis. However, because of its high viscosity which made it difficult to wash off non-adherent and non-ingested RRBC, it was not included in the final protocol.

It was found that washing the coelomocytes after extrusion should be as gentle as possible. The maximal number of washings should be once for 5 min at 80 x g, since more washing and centrifuging forces the macrophage-like cells together and enhances clumping. Such clumping can not be effectively reversed even after treatment of the cell suspension with trypsin and non-enzymatic cell dissociation solution.

The earthworm's macrophage-like coelomocytes actively phagocytize RRBC, bacteria, and yeasts. RRBC is the classic suspension used for phagocytic assays and has been used many times by different investigators. In tube assays of phagocytosis of bacteria, the use of large round bottom serological tubes was preferred because there was more contact between the cells and the bacteria resulting in a higher percentage of phagocytosis than in small conical tubes. Also, in conical tubes cells were more tightly packed and clumping was more evident than when serological tubes were used.

M. lysodekticus and *S. marcescens* because of their small sizes, and the resemblance of *S. marcescens* to some

cytoplasmic inclusions were not useful in phagocytic assays. To eliminate any possible bias in the experimental design, these bacteria were not used for toxicological experiments.

It was much easier to visualize and count phagocytized bacteria especially Gram-positive ones using the Gram stain rather than Wright's stain. With Wright's stain, both bacteria and the cytoplasm of coelomocytes stain blue which makes it hard to distinguish them from other elements of the cells, but with the Gram stain the cytoplasm of coelomocytes stains pink and Gram-positive bacteria stain purple. *B. thuringiensis,* due to its large size (1.0 - 1.2 µm width, 3.0 - 5.0 µm length) and Gram positive reaction, was a good candidate for bacterial phagocytosis. Phagocytosis is a step by step process involving attachment of the particle to the cytoplasmic membrane, invagination of the membrane, and the formation of a phagocytic vacuole consisting of the particle surrounded by the cytoplasmic membrane (phagosome) (46, 96). When evaluating phagocytic indicies, it is often difficult to determine under a light microscope whether small particles (like bacteria) are actually ingested or are merely attached to the cell. A particle or a bacterium on the surface of a cell may appear to be ingested when it is only adhering to it. This leads to errors in evaluating ingestion during phagocytosis. For this reason the term assocation rather than phagocytosis was used in some experiments. Even though

percent association shows a higher count than percent phagocytosis, the pattern of increase as a function of time is similar for both end points. For this reason I used phagocytosis rather than association in experiments assaying the effect of xenobiotics. Time course phagocytosis/association of bacteria (Figs. 6, 7) shows greater phagocytic activity of macrophage-like coelomocytes against bacteria than RRBC (Fig. 4), probably because ingestion of small particles is easier and faster. However, the possibility that it is an indication of antigen specificity of these phagocytic cells can not be ruled out.

Identity of macrophage-like coelomocytes and phagocytosis of *B. thuringiensis* were confirmed by transmission electron microscopy. After incubation with *B. thuringiensis*, numerous coelomocytes contained intracellular bacteria (Fig. 12). However, SEM studies failed to show attachment, probably because during the preparation procedure which involved fixation, washing, dehydration and critical point drying, the bacteria were washed off the cell's surface.

Even though phagocytosis is considered a non-specific immune response, phagocytosis of *S. cerevisiae* and *C. albicans* by 3 methods (*in vivo*, *in vitro*-puncture, and *in vitro*-extrusion; Figs 9, 10, 11) suggests specificity of earthworm coelomocytes toward these yeasts. The percent

phagocytosis of *C. albicans* was significanty higher than *S. cerevisiae*. *In vivo* assays showed the highest percentage of phagocytosis among the 3 methods which suggests that it is the method of choice for detecting the effect of xenobiotics on the non-specific immune cells. However, if one wants to use those earthworms in sequential assays to follow the depuration process in subchronic immunotoxic studies (40), this method can not be used, because earthworms die after the puncturing. Differences between percent phagocytosis by *in vivo* and *in vitro* assays(with *in vivo* being higher) suggests the presence of possible opsonizing factors in the coelomic fluid of *L. terrestris*. This result is similar to those of Laulan et al.(1988) who showed IgG and the complement C_3 fragment of human serum (opsonizing factors) increased phagocytic activity of *L. terrestris* coelomocytes toward SRBC (79). These results support the hypothesis that *L. terrestris* coelomic fluid possesses certain components which share some common function and structure with vertebrate humoral immune reaction components.

Evaluation of yeasts for use in phagocytic assays was made after testing the effect of xenobiotics on phagocytosis of *B. thuringiensis* and RRBC. However, data suggested that using *C. albicans* has several advantages over RRBC, bacteria, and *S. cerevisiae*: they are smaller than RRBC (easier to ingest thus giving higher percentages of

phagocytosis), but much larger than bacteria and therefore easier to see; there is no need for staining (a simple wet mount using 10X or 40X objective lenses of a microscope is sufficient); because of their shiny appearance under the microscope and larger size, it is much easier to differentiate actual ingested yeasts from attached ones; they are easy to culture; it is an opportunistic pathogen for human, so that challenge assay results can be related to the human system. Both *in vivo* and *in vitro* phagocytosis of *C.albicans* can be utilized together to evaluate the effect of xenobiotics on phagocytic cells and production of opsonizing factors.

Pathogenicity testing of bacteria was attempted because it could be used as an assay in challenge experiments with different toxicants. To be useful, an increase in virulence of bacteria for earthworms exposed to toxicants should be proportional to the level of toxicant. In pathogenicity testing all humoral, cellular, and non-specific immune response are involved in the defense mechanisms. Three microorganisms *B.thuringiensis*, *S. marcescens*, and *Shigella* SPP. were tested. *B. thuringiensis* and *S. marcescens* are soil flora and the *Shigella* SPP. was isolated from a body lesion of an earthworm. None of them were found to be potential pathogens for the earthworm at levels up to 10^8 CFU/worm. *B. thuringiensis'* pathogenicity for *L. terrestris*

is a controversial issue among some investigators. Smirnoff (125) showed 100% mortality after 2 months of feeding the animals with 50 x 10^9 bacteria per gram of soil. However, Genz (5) provided experimental data that shows *B. thuringiensis* is not pathogenic for *L. terrestris*.

In summary, methods were optimized for assaying coelomocyte spreading and phagocytosis as well as a method for determining pathogenicity of bacteria toward earthworms. In assaying coelomocyte spreading and phagocytosis, washing of the coelomocytes after extrusion should be as gentle as possible and excessive washing and high speed centrifugation should be avoided. LBSS buffer is a good buffer system and with the addition of EDTA helps to minimize the problem of clumping of coelomocytes. Lysolecithin at the concentrations and incubation times used did not stimulate macrophage-like cells spreading or phagocytic activity. The percent spreading activity and SEM adherence study indicates that a large proportion of the total population of coelomocytes are phagocytic in nature which indicates the importance of these cells for the animal's immune response. In general, earthworms, like other animals, possess an efficient, non-specific mechanism for disposing of foreign non-self material, which is as efficient as equivalant responses in humans. Earthworm macrophage-like coelomocytes are phagocytically active; engulfing RRBC, bacteria, and yeasts.

For the bacterial phagocytic assay, *B.thuringiensis* because of its Gram-positive reaction and long rod shape, is the candidate of choice. Among RRBC, bacteria and yeasts that were studied, I found several advantages for using *C.albicans* rather than RRBC or bacteria. Of 3 microorganisms tested for pathogenicity none were found to be pathogenic for *L. terrestris*.

Xenobiotic Effects

In earthworms exposed to a PCB (Aroclor 1254) the pathogenicity pattern of *S. marcescens* changed from non-pathogenic to pathogenic at concentrations of 10^7 and 10^8 CFU/worm (Fig. 24). However, the pathogenicity pattern of *B. thuringiensis* did not change significantly, even though the number of surviving worms started to decrease at the highest bacterial concentration used. This difference between the two bacteria is probably due to virulence differences of the bacteria themselves as well as to inhibition of the immune response of earthworms exposed to the PCB. For the PCB to cause any immunological effect, a sufficient amount would have to reach those cells that are actively involved in the defense mechanisms. The immune response of birds and mammals can be suppressed by PCBs (120, 121, 123, 133), and in fish, several studies suggest that chronic exposure to PCBs can affect the incidence of fish disease (50, 67, 90, 120, 121). PCBs are lipophilic compounds and compartment bioavailability

is dependent upon lipid distribution. Since the cellular component of coelomic material constitutes the major lipid source (plasma membrane, vacuoles and granules), cellular distribution of PCB should be expected to take place. Of the two main types of immune cells (coelomocytes and chloragogen cells), multifunctional chloragogen cells, which play an important role in the immune system and detoxification mechanisms (36, 142, 143, 145), store protein, glycogen and lipid (26, 27, 138) with lipid levels as high as 61% (27). Rodriguez (115) reported no detectable level of PCB in coelomic fluid using GC-MS analysis. However, in this study, EDX microanalysis of CF showed the presence of PCB in the CF although the results were highly variable. This indicates absorbance of PCB through the skin and possibly intestine and distribution and release of PCB during translocation from the outside environment to the inside. Valemobois et al.(1982) suggests that secretions from chloragogan cells have bacteriostatic activity (143). Therefore, if a considerable amount of PCB's accumulated in the chloragogan cells, they could alter the normal function of the cells and disrupt the secretory process. PCBs modulate mammalian immune cells by binding the cytosolic aromatic hydrocarbon (AH) receptor (143). PCBs form a receptor ligand complex which is translocated into the nucleus, where it binds with the specific DNA sequence which results in depression of some

structural genes and increased production of numerous biologically active products of the AH complex (123). Among these products are molecules that have immunoregulatory effects which act either by altering the normal metabolism of lymphoid cells or by actually interfering with cell-cell communication during an immune response (123). The AH receptor has yet to be identified in earthworms.

Exposure of *L. terrestris* to Aroclor 1254 did not show any suppression on phagocytosis of *B. thuringiensis* by the macrophage-like coelomocytes (Fig. 25). Since chloragogen cells may also play an important role in PCB translocation, it is possible that not enough PCB reaches and is incorporated into the phagocytic cells from the chloragogen cells to affect their normal activities. 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 (27). Translocation of PCBs from the carcass to chloragogen cells may initially take place during migration of maturing chloragogen cells. When mature, some chloragogen cells gain access to the coelomic fluid, where they are frequently called eleocytes (81). Subsequently, eleocytes undergo degeneration, and release their granules into the coelomic cavity (81). Some free chloragogen granules (chlorosomes) are known to be incorporated into leukocytic coelomocytes

(27). It is possible that in chronic exposure, there is enough incorporation of PCB into phagocytic cells so that phagocytosis of bacteria is altered. Rodriguez reported highly variable suppression of phagocytosis of RRBC by phagocytic coelomocytes, and suggested that those inconsistencies do not support a demonstration of a suppresive effect of PCB on phagocytosis (27).

Although exposure of earthworms to PCB by the filter paper contact method did not change the macrophage-like coelomocytes spreading and phagocytic activities, susceptibility to infection by *S. marcescens* was changed (Fig. 24). Thus it appears that the humoral factors of the immune system of *L. terrestris* are more sensitive to PCB exposure than the non-specific cellular immune responses. In immunity against bacterial infection, humoral responses rather than non-specific immunity, are the major factor in the defense mechanism. This change in susceptibility upon exposure to PCBs indicates that this system could be used as an assay for environmental contamination.

The results of chlordane exposure showed no significant effect on spreading and phagocytosis of RRBC. Chlordane is one of the cyclodienes which can be absorbed through intact skin (44). The mechanism of action of chlorinated hydrocarbon insecticides in mammals and insects has never been clearly elucidated, although it is apparent that these

compounds are neurotoxins. This neurotoxicity effect stimulates earthworm muscles which stay in a state of sustained contraction (tetany) so that the worms look very short and fat. This effect itself can cause reduction of surface area exposure of animals to more chlordane. Chlordane is soluble in body fat and indeed, its fat solubility and slow degradation is the basis for its prolonged retention and accumulation in adipose tissues. Spyker-cramer et al.(1982) reported suppression of contact hypersensitivity responses in the absence of any effect on humoral immunity or symptoms of toxicity in 14 1/2-week old offspring of female mice dosed with 8 mg chlordane/Kg during gestation (126). In another study, feeding of rats with a related chlorinated hydrocarbon (carbamate) showed some alteration of non-specific immunity as seen by decreases in the serum complement level, phagocytic index and percentage of active nutrophils (95). Further research with earthworm exposure to chlordane should focus on duration and method of exposure, It may be necessary to replace the filter paper contact method with artificial soil mixed with chlordane and increase the exposure time.

In chlordane exposure there was an observation with no statistical value, using SEM analysis to detect any morphological changes of coelomocytes upon exposure to chlordane. Most of the results show no morphological

differences between control and experimental coelomocytes which adhere to polylysine coated slides. However, the majority of control cells adhered to polylysine coated slides and only disrupted cells washed off whereas, coelomocytes exposed to chlordane lost their ability to adhere but not their morphological properties. Thus, chlordane appears to affect the surface properties of cells without altering the morphology.

Cadmium nitrate did not alter the spreading activity and phagocytosis of RRBC of *L. terrestris* phagocytic cells after 5 days of exposure. Several studies of cadmium effects on mammalian macrophages that were done either by *in vivo* injection of cadmium salt into the experimental animals or direct contact of cadmium and cells in *in vitro* systems report different results. Variables normally implicated in these opposite effects are exposure route, dose, duration of exposure, strain and species differences. Most reports support suppression rather than activation. Loose et al. (1978) reported a significant impairment in the phagocytic capacity of alveolar macrophages, peritoneal macrophages, and PMNs incubated in a medium containing either cadmium chloride or cadmium acetate (83). A previous study by the same investigator had demonstrated that cadmium (chloride or acetate) decreases the respiratory burst in phagocytic cells (82). Since the respiratory burst is concomitant with

phagocytosis (117), the phagocytic step is also impaired by cadmium. However, Koller and Roan (1977) reported activation of macrophages instead of suppression of their activity (72). We should keep in mind that these methods can not represent uptake of heavy metals like cadmium in actual environmental conditions. Negative spreading and phagocytosis results (neither suppression nor activation) of earthworms exposed to cadmium can be explained by the exposure methodology used and length of exposure. Even though the filter paper contact is an easy way to expose earthworms to a toxicant, uptake of toxicant can not be the same as the soil contact method in which the whole surface area of earthworms is covered. Therefore, for future work, to get a valid result with the filter paper contact method, the length of the exposure should be prolonged, since it has been proven that earthworms absorb and accumulate cadmium and lead from contaminated soils (148).

All experiments, except one, with Superfund soils, which contain a mixture of heavy metals and organochlorinated pesticides (mainly chlordane), showed suppression of both spreading activity and phagocytosis of RRBC. In the first experiment, which was conducted with undiluted Superfund soils, suppression of spreading activity and phagocytosis of RRBC was significantly different from controls, although the results were highly variable. However, when the assays were

conducted with 5% diluted superfund soils (Experiments 2 &3 - exposure 5 days with no lethal effect), suppression of both spreading and phagocytosis at all 3 incubation periods were highly significant with lower variability. None of the worms showed any sign of the sustained contraction which I observed in worms exposed only to chlordane. Although it is not exactly known how different heavy metals disrupt the normal function of macrophage-like cells, *in vivo* and *in vitro* experiments with mammals show different toxic effects upon exposure to different heavy metals. Of all the heavy metals, most studies in mammalian system were done with Pb, and Hg. Cadmium is usually categorized with Hg and Pb as a very toxic heavy metal. *In vitro* studies of heavy metals on mammalian macrophages have shown decreased phagocytic activity upon exposure to Cd, Cr, Mn, Ni (49). Cadmium also decreased the oxidative burst during phagocytosis (83, 82) and inhibited O_2 consumption during phagocytosis (47). *In vitro* exposure to Hg and Pb has been shown to inhibit macrophage oxidative metabolism of mice (33), and Pb has been reported to inhibit phagocytosis (69) and/or antigen processing or some other accessory macrophage function (8) in C57B 1/6 and BDF_1 mice. If a single heavy metal or few with different cellular or subcellular targets have a suppresive effect on macrophage-like coelomocyte activity, it is logical to assume that the toxic effect by mixtures of those heavy metals would be

magnified. Furthermore, the effect might be even more if additional organochlorinted compounds are present. These effects might be either additive or synergistic.

In the last experiment on Superfund soil, there was no suppression of either spreading activity or phagocytosis of RRBC. Although the earthworms looked stressed and contracted, they were generally healthier looking than the animals in the earlier experiments. The sustained contraction of the worms is the same as that observed when worms were exposed to chlordane and might be indicative of a high concentration of this compound in this soil sample. The healthier looking worms might reflect less heavy metal or pesticide (other than chlordane) contamination in this soil which was collected from a different area at the same Superfund soil site. However, no heavy metal or pesticide analysis of these samples was available to confirm this hypothesis. Lack of any significant effect of this soil (as opposed to soil from other sites) on spreading activity and phagocytosis shows that these assays are sensitive enough to detect differences in levels of environmental contaminants. As with chlordane exposure, for the toxic effect of this soil to take place it may need an exposure time longer than 5 days. In this experiment, it was found that the tube method is more efficient than the slide method, probably because of more surface contact of coelomocytes and RRBC in tubes.

Microanalysis (EDX) of coelomocytes and coelomic fluid from earthworms exposed to sublethal concentrations of Aroclor 1254 and cadmium nitrate, and lethal levels of Aroclor 1254 and copper sulfate, gave highly variable results and is not a suitable technique for assaying uptake by the method used. Ion distribution in normal as well as in pathological cell systems of both plants and animal specimens can be studied routinely by using this technique (55). The minimal detection limit for EDX is 0.1 - 0.2% of an element in a mixture, and the smallest amount of element that can be detected in an ultrathin section is ~10^{-19}g under favorable conditions (55). In comparison the sensitivity of GC-MS is 0.1 - 1.0 ng in one sample injection of an extracted sample. The greatest advantage of EDX over GC-MS is there is no need for extraction of elements from cells or a tissue, and whole cells can be analyzed using an air dried specimen. However, specimen condition, topology, presence of contaminating elements from buffers or media, and uneven distribution of elements reduces the sensitivity and affects the detection of elements of interest in a biological specimen. EDX detects (in the system used) the characteristic X-rays from all the elements above atomic number 11 (sodium) in the analyzed area of a specimen and calculates the percentage of elements present in the analysed area. Because of the detection limits of elements in a mixture, the presence of a large

amount of other elements can cover up the minute amount of an element from, for example, a xenobiotic containing chlorine. If the location of an element in the cytosol or subcellular organelle was known, there would be a better chance of detecting the element of interest with higher precision and lower variability by performing the microanalysis only on that organelle.

As a preliminary study for quantitation of heavy metal toxicity at the cellular and subcellular level, the changes occuring in epidermal tissue of *L. terrestris* during a 2 day exposure to a lethal level of copper sulfate was determined by stereological analysis. The earthworm is covered by a thin, transparent cuticle secreted by the epidermis beneath it and this cuticle is composed of layers of parallel collagen fibers in a matrix which also forms an epicuticle (34, 87, 98, 107). The epicuticle and matrix are chiefly polysaccharide (124, 153). The epidermis which lies beneath the cuticle, covers the body wall musculature and consists of supporting cells, satellite cells, basal cells, secretory cells, and setigerous cells. The epidermis also includes sensory cells, and nerve endings (12, 63, 106). The earthworm epidermis has three types of gland cells; large granular, orthochromatic mucous cells; reticulate, metachromatic mucous cells; and small granular, proteinaceaus cells (99-105). Since the epidermis beneath the cuticle is

the first cellular structure of the body that is in contact with a toxicant in the earthworm's environment, for any toxicant to reach any other tissue it has to pass through this tissue.

Copper, used as the toxicant in this stereological analysis, is essential to human life and health. However, like all heavy metals, at elevated levels it is also toxic to mammalian systems. For example, pathological studies of copper toxicosis in sheep liver shows the hepatocytes with cytoplasmic vacuolation and necrosis, and lobules containing clusters of dead cells with fragmented nuclei (64, 66). However, pathological observation by itself is not a quantitative measurement. Stereology is of interest to a systems analysis approach because it can be used to attach quantitative values to complex biological structures identified in light and electron micrographs. Cells and their organelles can be characterized in terms of volume ratios (Vv), surface area to volume ratios (Sv), length to volume ratios (Lv), frequencies, shapes, etc. upon exposure to a toxicant or uptake of a chemical or a drug. When combined, the structure and functional data can be used to assemble relatively simple, yet surprisingly powerful, information networks; e.g. to pinpoint the specific cellular response to one or several xenobiotics. In my study the stereological approach was done only with the nuclear volume ratio (V_v) of

epidermal tissue of exposed and unexposed earthworms. The results show that this approach may be useful since there was a reduced nuclear volume of experimental earthworms as opposed to non-exposed control worms. This could be due to disruption of normal mitosis or inhibition of cytokenesis resulting in an increase in cytoplasmic volume. For future investigations, this study could be extended to different tissues including immune cells, to pinpoint the exact target cell and possibly organelle of certain xenobiotics and quantitate the damage or changes caused by those xenobiotics.

In summary, PCB exposure suppressed the immune system, allowing *S. marcescens* to become opportunistic pathogen. However, *B. thuringiensis* remained non-pathogenic. PCB, chlordane, and cadmium nitrate did not affect phagocytosis and spreading activity of macrophage-like coelomocytes, probably because exposure times were too short so that not enough toxicant reached the phagocytic cells to disrupt their normal activity. It is suggested that longer exposures and use of artificial soil exposure rather than the filter paper contact method, would be optimal. Superfund soils, because of their high content of heavy metals as well as chlordane, suppressed the immune system. Both spreading activity and phagocytosis of RRBC were significantly reduced by exposure to 2 days in undiluted soil and 5 days in diluted soils. One

soil sample which apparently had more chlordane than heavy metal, did not suppress phagocytic cells.

Although microanalysis is a good and sensitive way of detecting trace elements in biological specimens, highly variable results were obtained on analysis of whole cells due to uneven distribution and presence of large amount of other elements which prevented the detection of traces of toxicants. Use of this technique for further toxicological experiments was not pursued.

Quantitation by stereological analysis of damage in epidermal tissue after exposure of *L. terrestris* to copper sulfate was shown to be a promising technique for future experiments to assess the effect of environmental xenobiotics at the cellular and subcellular levels. Nuclear volume ratios of epidermal tissue were significantly reduced in worms exposed to copper sulfate.

Eisenia foetida

METHODS DEVELOPMENT

Of the two bacteria *B. megaterium* and *A. hydrophila*, tested for their pathogenicity on *E. foetida*, *A. hydrophila* was found to be pathogenic at 1 x 10^7 CFU/worm and higher by intracoelomic injection of a bacterial suspension. *A. hydrophila* is a Gram-negative rod whose normal habitat, cow manure, is the same as that for *E. foetida*. *A. hydrophila* is

a pathogen for cold blooded animals including fish, frogs, and snakes (10). It is also a pathogen in humans causing gastrointestinal disease, traveler's diarrhea and occasionally wound infection and septicemia especially in chidren, old people and immunosuppressed patients (4, 61). Most of these disease conditions are due to production of a cytotoxin (endotoxin) by *A. hydrophila* (4, 61).

The coelomic fluid of *E.foetida* naturally exibits antibacterial activity against some Gram-positive and Gram-negative bacteria. In experiments reported here, coelomic fluid from unchallenged worms of *E. foetida* inhibited the *in vitro* growth of *A. hydrophila*. One injection of a sublethal concentration of this bacterium resulted in increased humoral bacteriostatic activity which peaked 4 days after injection. This induction of bacteriostatic activity compared to both the initial CF and saline injected control CF was significantly higher (Fig 36). Roch et al. (1987) identified at least three antibacterial molecules from *E. foetida*'s coelomic fluid (46), suggesting the existence of a complex humoral defense system in this worm. It is not known if the positive response to the injected bacteria is due to an inceased synthesis of antibacterial proteins normally found in the CF, or to synthesis of a new molecular species.

Effect of Xenobiotics.

Exposure of *E. foetida* to 3 concentrations of 30, 50, and 70% RDFF in artificial soil showed a dose response lethal effect (Fig 37). At 70% RDFF, 30% of the earthworms died after 5 days of exposure. The toxicity of RDFF to *E. foetida* is due to its heavy metal content (150). Hartenstein et al. (1981) studied *E. foetida* grown in activated sludge treated with various concentration of heavy metals and noticed no effect from Pb, Mn, Cr even at the highest level of treatment, whereas Co, Hg, Cu, Ni, and Cd specifically inhibited growth of earthworms even caused mortality (54, 86). There is always a possibility that the collective effect of different compounds or chemicals on a biological system could be higher than individual ones. Although I did not study the physiological effect of heavy metals on earthworms, in this situation it is a probable that not only the immune system but also the overall physiology of the earthworm is affected. However, suppression of immune defense mechanism upon 5 days exposure to RDFF was strongly implicated after injection of bacteria. Injection of sublethal levels of *A. hydrophila* caused a dose response lethal effect which was significant in all levels of RDFF exposure compare to saline controls (Fig 37). In mammals, the majority of heavy metal studies have been done on lead and cadmium. Most of these studies indicate increased

susceptibility of experimental animals to infection with Gram-negative bacteria that contain endotoxin. Selye et al. (1966) reported that a single intravenous injection of lead acetate increased the sensitivity of rats to endotoxins of various Gram-negative bacteria (118). This was followed by similar studies in mice and chickens (108, 118, 134) demonstrating that lead acetate enhanced responsiveness to bacterial endotoxins. In addition to lead, cadmium induced similar changes in rats exposed to *Salmonella enteriditis* endotoxin (13, 15) and *E. coli* infection (14). Differences between Gram-positive and Gram-negative bacterial challenge was reported by Cook et al. (1975). They found that an acute dose of lead acetate enhanced the susceptibility of rats to an intravenous challenge with *E. coli* by approximately 1,000 fold whereas, an equal dose of the Gram-positive *Staphylococcus epidermidis* failed to produce mortality, although a dose 10-times higher gave more deaths than observed in the non lead treated animals (15). Lead exposure also reduced hepatic phagocytosis in mice (43, 119) and primary and secondary antibody response to pseudorabies virus in rabbits (70). Suppression of the humoral response in rats by nickel and chromium was also reported by Figoni and Treagan (1975) as measured by injection of *E. coli* bacteriophage T-1 into rats (42).

Immune response toward an antigen in mammals involves 3 main types of cells: T-cells, B-cells, and macrophages. In earthworms, based on morphological characteristics by flow cytometry and microscopy (52), and functionality by E (erythrocyte) and S (secretory) rosette formation (82) and phagocytosis, there are at least 3 or 4 types of coelomocytes. E-rosette formation is a capability exhibited by both human T-cell and a type of *L. terrestris* coelomocytes. S-rosette formation is caused by an agglutinin factor of the humoral response of *L. terrestris*. Macrophages and macrophage-like coelomocytes are both capable of phagocytizing invading infectious agents (i.e. bacteria and yeasts) and various foreign particles including RRBC. Because the immune system of the earthworm is analogus to mammals, it is logical to assume that the heavy metal content of RDFF causes death after immune suppression, although, several factors, either alone or in combination, may elicit a cascade of reactions. Immune suppression permits uninhibited bacterial growth, resulting in the production of lethal quantities of endotoxin, impairment of the endotoxin detoxifying properties of chloragogan cells (in mammals - hepatic paranchymal cells) and macrophage-like coelomocytes, impairment of the production of antibacterial substances, reduced phagocytic activity and antigen processing or

presentation by macrophage-like coelomocytes, and reduction in production of opsonizing factors.

To confirm the *in vivo* suppression of the immune system and to show the direct involvement of humoral responses of *E. foetida* by RDFF, earthworms were injected with sublethal levels of *A. hydrophila* and then the *in vitro* antimicrobial activity of CF of exposed and unexposed earthworm was measured. Before injection of any bacteria, the antibacterial activity of CF was measured. Suppression of antimicrobial activity of CF from uninoculated samples showed a reduction in this activity at all levels of exposure, although only at levels of 70% RDFF, was suppression statistically significant (Fig 39). After inoculation, all the results suggest a dose response impairment of induction of this activity with a significant difference at the 50 & 70% RDFF levels. The ability of heavy metals to render the earthworm more susceptible to infectious agents suggests a possible reduction in humoral immunity, and an indication that cells responsible for production of antimicrobial molecules were inhibited by heavy metals. In mammals, Hg and Pb have been shown to inhibit humoral immunity (antibody production) in some animals (73, 74). Koller and Brauner (1977) suggested that Pb decreased B-cell responses (71), and Blakey and Archer (1981) have suggested that the ability of Pb to inhibit humoral immunity is due to the inhibition of

macrophage accessory functions with T-cell involvement being limited (6). All of this suggests that in addition to macrophage-like coelomocytes, there must be a type of coelomocyte similar to B-cells of mammals in terms of certain functional characteristics such as the production of antimicrobial molecules in earthworm which are suppressed by heavy metals present in the RDFF. It has been shown that macrophage-like coelomocytes are not responsible for both phagocytosis and production of antimicrobial molecules (humoral response) (143). Whether heavy metals inhibit antigen processing of macrophage-like coelomocytes or directly effect antimicrobial producer cells, overall suppression by heavy metals is detrimental to the animal.

Im summary, *E. foetida*'s immunity has some specificity toward foreign infectious agents, *B. megaterium* was found to be non-pathogenic whereas *A. hydrophila* is pathogenic when injected at levels $>10^7$/worm. Coelomic fluid of *E.foetida* naturally exibits antibacterial activity against *A. hydrophila*. This activity can be induced to increase by a sublethal injection of *A. hydrophila* with the increase peaking 4 days after inoculation. The high level of heavy metals in 70% RDFF (150) has a lethal effect on *E. foetida* after 5 days exposure, causing sufficient suppression of the immune system that sublethal levels of *A.hydrophila* for unexposed worms become lethal. It is possible that the

mixture of heavy metals in RDFF cause this suppression by disrupting normal function of multiple immune cells; e.g. macrophage-like coelomocytes and antibacterial secreting cells. The *in vitro* antimicrobial assay was found to be an efficient and sensitive method for detecting the effect of xenobiotics on the immune response of *E. foetida*.

Conclusions

The results of this study have shown that:

1. Immunoactive coelomocytes, which can be obtained by extrusion, puncture or electrical shock, can be used in immunoassays of the effect of xenobiotics on earthworms.

2. PCB's and RDFF suppressed the immune system of earthworm to the extent that pathogenesis of bacteria is enhanced. In *E. foetida* a two log reduction in bacterial challenge of *A. hydrophila* caused significant killing of RDFF exposed worms over control worms. With *L. terrestris*, treatment of worms with PCB allowed *S. marcescens* to become pathogenic.

3. Heavy metals have a strong immunosuppressive effect on antibacterial activity (humoral factors) of *E. foetida* coelomic fluid against *A. hydrophila* and phagocytic activity of macrophage-like coelomocytes of *L. terrestris*.

4. To detect the effect of chlordane and PCB on spreading and phagocytic activities of *L. terrestris* macrophage-like coelomocytes, use of a prolonged exposure time in artificial soil rather than the filter paper contact method is suggested.

5. *C. albicans* has several advantages over RRBC and bacteria for phagocytosis assays.

6. Preliminary stereological studies of thin sections of epidermal tissue in worms exposed to copper sulfate indicate this technique would be useful in studying and quantitating cellular changes in animals exposed to different xenobiotics.

7. Use of microanalysis (EDX) would only be useful in determining uptake of xenobiotics if the subcellular localization of the xenobiotics were known.

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