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ORIGINAL ARTICLE

Effect of fungicide on cellulase activity of epigeic earthworm *Eisenia fetida* (Oligochaeta)

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ABSTRACT

In the present study, the epigeic earthworm, *Eisenia fetida*, selected as the test specimen were exposed to two fungicides, carbendazim and captan, in natural garden soil. The 96 hour LC_{50} values of the two selected fungicides were determined. According to the LC_{50} value of the two fungicides more toxic carbendazim used for the cellulase enzyme activity assessment. Feeding preference experiment was carried out and they showed maximum preference for *Anacardium oxidentale* (cashew) leaves. The test specimen was exposed to sub lethal doses of carbendazim, i.e. 25% of LC_{50} value and 50% of LC_{50} value, along with the control set. The enzyme activity measured on the 3rd, 7th, 15th and 30th day from the experiment .The values of the control set and two sub lethal doses, i.e. 25% of LC_{50} value and 50% of LC_{50} for 3rd, 7th, 15th and 30th day were 1.09 ± 0.40, 1.59 ± 0.50, 1.25 ± 0.45; 1.29 ± 0.48, 1.50 ± 0.50, 1.75 ± 0.55; 2.25 ± 0.85, 1.58 ± 0.50, 1.83 ± 0.60 and 2.75 ± 1.15, 3.05 ± 1.35, 3.05 ± 1.30 mg of glucose/minute/mg protein respectively. The enzyme activity was suppressed a little in between 7th and 15th day of the experiment. From the activities of the enzyme we can use as a potential biomarker to detect pesticide pollution in soil in agro ecosystem and can be further used in genotoxicity studies.

INTRODUCTION

Present day agricultural practices largely depend on agro-chemicals for enhancing productivity. There are 60,000 varieties of chemicals in use with several thousand being added annually (Maugh, 1978). Besides seeds, nutrients, water etc, use of pesticides including fungicides is indispensable. Alarming population growth throughout the globe necessitates more food and cash crops production results rapid growth of pesticide market (Ecobichon, 2001). Inspite of their benefits, increasing trend of fungicide application has deleterious effect on human environment and agro-ecosystem.

The Bordeaux mixture was the first fungicide to be used on a large scale world-wide (Schneiderhan 1933). Regular use of fungicides can potentially pose a risk to the environment, particularly if residues persist in the soil or migrate off-site and enter waterways (e.g. due to spray drift, run-off) (Kookana et al., 1998;Wightwick & Allinson, 2007; Kibria et al., 2010; Komarek et al., 2010). If this occurs it could lead to adverse impacts to the health of terrestrial and aquatic ecosystems. For instance, concerns have been raised over the long term use of copper-based fungicides, which can result in an accumulation of copper in the soil (Wightwick et al., 2008; Komarek et al., 2010).This in turn can have adverse effects on soil organisms (e.g. earthworms, microorganisms) and potentially pose a risk to the long-term fertility of the soil (Wightwick et al., 2008; Komarek et al., 2010)

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A greater proportion (80%) of biomass of terrestrial invertebrates is represented by earthworms which play an important role in structuring and increasing the nutrient content of the soil. Therefore, they can be suitable bioindicators of chemical contamination of the soil in terrestrial ecosystems providing an early warning of deterioration in soil quality (Culy and Bolger ,1995; Sorour and Larink, 2001; Bustos-Obregón, and Goicochea, 2002). This is important for protecting the health of natural environments and is of increasing interest in the context of protecting human health (Beeby, 2001) as well as other terrestrial vertebrates which prey upon earthworms (Dell'Omo et al., 1999). The suitability of earthworms as bioindicators in soil toxicity is largely due to the fact that they ingest large quantity of the decomposed litter, manure, and other organic matter deposited on soil, helping to convert it into rich topsoil (Reinecke et al.,1999; Sandoval et al., 2001). Moreover, studies have shown that earthworm skin is a significant route of contaminant uptake (Lord et al.,1980) and thus investigation of earthworm biomarkers in the ecological risk assessment (ERA) can be helpful (Sanchez-Hernandez, 2006)

In the present study, two fungicides carbendazim and captam were used for acute toxicity test but only carbendazim used to evaluate the toxic effects of the sub-lethal doses on the cellulase enzyme activity, of the epigeic earthworm *Eisenia fetida* (Table-1).

SOIL ORGANISMS

Soil organisms can be defined as organisms which spend at least a part of their life cycle in or on the soil (Hendrix et al., 1990). Morphologically, soil organisms range from less than 1 micrometer in diameter to several centimeters in diameter (Lee, 1985). In general soil fauna act as catalysts of microbial activity. Soil fauna can be subdivided on the basis of their size into microfauna, mesofauna and macrofauna

(Wallwark, 1970). The large and conspicuous soil animals are the macrofauna, which include amphipods, isopods, centipedes, millipeds, adult as well as larval insects, mollusks and earthworms. Microfauna include protozoa, Nematoda etc. and mesofauna like Acarids, Collembolans and Enchytraedes etc.

Among these the ecological importance of earthworms in the organic breakdown and soil formation processes is well established (Wallwork, 1970). These animals are very sensitive to environmental characteristics like pH, moisture, temperature etc. and their ecological importance depends upon the prevailing edaphic and climatic parameters.

Table.1 The fungicides used in the study with their respective RADs.

Chemical Name	Trade Name	RAD*(mg/kg)
Carbendazim	BAVISTIN	0.96
Captan	CAPTAF	4.80

*RAD- Recommended Agricultural Dose

Ecological Importance of Earthworms:

Aristotle was one of the first who pointed out the role of earthworms in turning over the soil and called them "The Intestines of the Earth". However, earthworms were considered as pests until the publication of Charles Darwin's book "The Formation of Vegetable Mould through the Action of Worms", in 1881, where he convincingly documented in great detail the importance of earthworms in the breakdown of organic matter and the formation and maintenance of soil structure. Since then, a vast amount of studies have documented that earthworms play an essential role in improving soil structure and fertility. The main contributions of earthworms in this respect as highlighted in these studies are**:**

i. Physical participation by feeding, fragmentation of leaf litter, aeration turnover and dispersion (Lee and Foster, 1991; Lavelle et al., 1997).
ii. Chemical participation by digestion of organic substances and by contributing nutrients to the soil through metabolic by-product and dead tissue.
iii. Grazing over microflora and altering soil micro floral composition (Brown, 1995; Scheu, 2003).

In recent years it has been stressed that the role of the earthworms does not stop below ground, they also affect the above ground subsystem, especially plant performance including growth, development and plant community composition (Scheu et al., 1999; Schimidt and Curry, 1999; Zeller and Arnune, 1999; Wurst et al., 2003).

In forest ecosystems earthworms, especially litter feeders such as *L. terrestris*, can consume all the litter deposited on the soil surface within a period of several weeks (Knollenberg et al., 1985) or months (Satchell, 1967). Incorporation of litter by earthworms in apple orchards can be an important mechanism for preventing outbreaks of scab fungus, spores of which are transmitted from litter to new foliage by spring rains. Raw (1962) found a high correlation between *L. terrestris* biomass and apple leaf litter incorporation, with over 90 percent of litter incorporated during the winter when this species was abundant. Incorporation of surface litter may be an important function of earthworms in no-tillage agro-ecosystems.

Introduction of earthworms to areas not previously populated has led to improvement of soil quality and productivity in New Zealand grassland, on drained Dutch polders (Van Rhee, 1977) in heath land in Ireland (Curry and Bolger, 1984), and in mining spoils in the U.S. (Vimmerstedt and Finney, 1973). Lumbricids in a pasture soil produced casts that contained 73 percent of the nitrogen found in the ingested litter, indicating importance of earthworms in incorporating litter nitrogen into the soil as well as the inefficiency of earthworm in digestion of nitrogen (Syers et al., 1979). Earthworms increase the amount of nitrogen mineralized from organic matter in soil. Because nitrification is enhanced in earthworm casts, the ratio of nitrate-N to ammonium-N tends to increase when earthworms are present (Ruz Jerez et al., 1988). Nitrogen-fixing bacteria are found in the gut of earthworms and in earthworm casts, and higher nitrogenise activity, meaning greater rates of N-fixation, are found in casts when compared with soil (Simek and Pizl, 1989).

Earthworms increase level of metabolic activity in soils, but often reduce nematode abundance and microbial biomass (Yeates, 1981; Ruz Jerez et al., 1988) because they reduce the amount of substrate available to other decomposers and they also ingest other decomposer organisms as they feed. This process would tend to accelerate nutrient cycling.

Earthworms as model test organisms in ecological risk assessment

Extensive use of insecticides in agricultural field produces several deleterious effects on soil ecosystems. Insecticides produce inhibitory effect on the macrofaunal, mesofaunal and microfaunal population of the soil and disturb the equilibrium of soil organisms. Since earthworms constitute about 92 % of the invertebrate biomass of the soil, researchers around the world have used earthworms as model organisms for soil toxicity testing. The inception, testing and standardization of the acute earthworm toxicity test by OECD (1984) and EPA (1996) (URL 1) have been the catalysts for the emergence of earthworms as one of the key organisms in environmental toxicology. Therefore, it is not surprising that a number of recommendations have focused on laboratory testing methods on ecotoxicology using earthworms as the test organisms. The first two International Workshops on Earthworm Eco- toxicology (IWEE) supported a role for the artificial soil acute toxicity test in screening chemical toxicity. This included not only classical toxicity studies (e.g. Callahan et al., 1994; Edwards & Bohlen, 1992), but also field soil assessment (Bierkens et al., 1998; Charrois et al., 2001; Dorn et al., 1998), remediation evaluation (Ducrocq et al., 1999) and evaluations of bioavailability (Amorim et al., 2002). However the third international workshop on earthworm eco toxicology (IWEE III) held in Denmark in 2001was less supportive of the role of acute toxicity tests and suggested reproductive tests with sub lethal doses instead.

Laboratory experiments also give little idea about the consequences of the toxicant on behaviour of the soil organisms for exposure and effects in the field. Various physical and chemical aspects of the soil that are not included in laboratory tests may have a direct or indirect impact on the degradation, persistence, bioavailability and exposure of the different chemicals applied to the soil. Posthuma (1998) concluded that factors such as temperature and soil moisture content explain only a small part of the difference in response observed in their laboratory and field studies. This also emphasizes the

importance of developing models to predict exposure and bioavailability under field conditions.

Systematic position of the selected specimen:

Phylum: Annelida
Class: Oligochaeta
Family: Lumbricidae
Order: Haplotaxide
Suborder : Lumbricina
Genus: *Eisenia*
Species: *Eisenia fetida*
Eisenia fetida (Savigny, 1826)

Distribution:

Eisenia fetida is also an epigeic earthworm and is known as the red wiggler worm. The worm is native to the palearctic and are also found in Europe, North and South America, some parts of Asia, Africa, Iceland and Australasia (Reynolds, 1997). They are commonly found under the bark of tree trunks or animal dung accumulation or decaying plant material throughout the European countries and have become successfully established throughout the world for vermicomposting (Kale et al., 1982; Reinecke et al., 1992; Yasmin and DSouza, 2010). This species is exotic to India. But it is extensively cultured in this country as a most favourite worm species in composting and organic gardening (Garg et al., 2006). The species has acclimatized well in Indian climatic condition and is now readily available from any composting pit in the country.

MATERIALS AND METHODS

Biology:

Total length, diameter and number of segments of the body of *Eisenia fetida* ranges from 35 to 130mm (generally >70mm), 3 to 5mm and 80 to120 segments respectively. The life span of the species is about 450 days. The life cycle of *Eisenia fetida* has been studied by several researchers including cocoon production (Hartenstein et al., 1979), effect of temperature on the reproduction (Reinecke and Kriel, 1981), incubation time and hatching rate (Tsukamato and Watanabe, 1977). T**he life span of *Eisenia fetida* is reported to range from 4 to 5 years (Bouche, 1977). The worms become clitellate and began to produce cocoons by 4-6 weeks and after about 27 weeks the rate of cocoon production declines (Hartenstein et al., 1979). Cocoons** of *E. fetida* are even smaller than a grain of rice, shaped like lemon and yellow-coloured. The incubation period of the cocoon is about 23 days. The cocoons gradually change its color from golden yellow to deep red; much like maroon as 4 to 6 embryonic red wiggler worms develop inside. Cocoons hatch at a temperature of 68° to 77° F (20° to 25° C). The juveniles emerge from the cocoons at about 3-4 weeks. Juveniles are about $^1/_2$ inch in thickness and do not have any genital markings or the clitellum. Once they hatch they readily become organic waste eating machines. About 40-60 days are required for the juveniles to develop into an adult. It develops the genital markings and clitellum. The clitellum contains their reproductive organ and can only be seen when *E. fetida* is ready to reproduce and the clitellums are orange in color (Bouche, 1977).

Eisenia fetida can be easily bred in the laboratory using variety of organic medium and has a short generation time. Therefore, the species is very appropriate for toxicity studies (OECD, 1984; 2004).

Collection and Culture of test specimens:

Specimens of *Eisenia fetida* were collected from the vermicompost unit, from Tamluk (West Bengal, India), that has never been used for any agricultural purpose and pest control. The specimens were brought to the laboratory and were cultured in large earthen pots. Finely grinded soil (collected from the same grasslands) and farmyard manure mixed in the ratio of 1:1 was used as the culture medium (Ismail, 1997). The culture pots were covered with fine meshed iron nets and kept inside BOD incubators at 28 ± 0.5ºC. An approximate level of 50% moisture was maintained by adding distilled water into the medium. Farmyard manure was added as feed every week during the entire period of culture(OECD 1984, 2004).

Experimental Procedures:

Studies were performed with age synchronized specimens (150-250 mg). Experiments were conducted in small inert polythene boxes (16 X 12 X 1 cm; total area, 192 cm2) containing soil, collected from grasslands, as the test medium. Soil samples were dried, grinded and sieved to get a particle size of 0.25 mm before filling in the experimental boxes. The moisture content of the soil was measured by Infrared Torsion balance moisture meter [Adair Dutt, Kolkata] (Joy and Chakravorty, 1991). Finally the experimental boxes were kept in an Environmental Chamber at a constant temperature of 28 ± 0.5°C and 60-65% relative humidity.

The physiochemical parameters of both the soil media, viz, pH and Organic carbon Content were measured and the temperature and moisture content were kept constant (Table 2).

Table.2 Physiochemical parameters of the natural soil used as medium in both the Acute toxicity test and Enzyme activity estimation.

Natural soil parameters	Values
P^H	6.90
Organic Carbon Content	1.18%
Moisture	61.2%

a. Acute Toxicity Test:

Different levels of the carbendazim and captan based on their recommended agricultural doses (RAD) (viz RAD, 1/2X-RAD,2X-RAD and 3X-RAD) were administered into the test boxes with a micropipette (Lofs-Holmin, 1983). The amount of a fungicide required was determined from the total area of the experimental box and was converted into mg per kg soil taking into consideration the total amount of soil (200 g) contained in one box. The experiment was setup with three replicates for each level of the fungicide and control. The boxes were then left undisturbed for about 30 min for uniform spreading of the chemical in the soil medium. Five numbers of age synchronized specimens of *Eisenia fetida* were then transferred into the boxes. Observations were made every 24 h. Those individuals, who showed no apparent sign of life, even when poked with a needle, were considered dead and were removed. The total mortality obtained after 96 h of exposure were subjected to probit analysis by EPA probit analysis program, version 1.5(US EPA 2006) to determine LC_{50} value (Table 3) and 95% confidence limit of each insecticide. The entire experiment was repeated three times (Dasgupta *et al.,* 2010).

Table.3 LC_{50} values of the two fungicides used in the Acute Toxicity study.

Chemical Name	Trade Name	LC_{50} Values
Carbendazim	Bavistin	5.38 mg/kg
Captan	Captaf	10.41 mg/kg

b. Determination of Feeding Preference of test organisms:

Open choice experiment was done on epigeic earthworm *Eisenia fetida* with five common tree species leaf litters viz., *Anacardium occidentale* (cashew), *Mangifera indica* (mango), *Shorea robusta* (shal), *Acacia auriculiformis* (Acacia) and *Eucalyptus citridora* (Eucalyptus), to study their food preference. The experiment was conducted in plastic trays containing five different randomly distributed leaf litter in pits in petri dishes inserted into a uniform layer sand bed (Maity and Joy, 1999a; 1999b). Fifty adult specimens of same size and age group were released in the centre of the plastic tray and they were to migrate among the litter types. Known amount of litter cuttings were used. Optimum moisture and temperature were maintained throughout the experimental period. The rate of migration and colonization of specimens were recorded by counting their number in each litter type at 15 days interval up to 90 days. Thus, cashew was selected as the source of food to be provided to the earthworms during the entire period of digestive enzyme estimation.

c. Estimation of Digestive Enzyme:

A very important aspect of the laboratory study was the quantitative estimation of the digestive enzyme cellulase (Sadasivam and Manickam, 2010) determined under laboratory conditions in natural garden soil (pH-6.90, organic carbon-1.18% moisture content-61.2%) by exposing the earthworms to sub-lethal dose of the fungicide, carbendazim, i.e., 25% and 50% of LC_{50} value. The specimen earthworms were kept inside inert polyethylene boxes of 192 cm^2 area each containing 250g of sieved garden soil along with 15 worms. Distilled water was added to maintain 60-70% moisture. The earthworms were provided with finely cut cashew leaf litter as food during the entire experimental period on a small petri-dish inside each box into a uniform layer of soil. The experiment was set following the procedure of open choice experiment as described by Maity and Joy, 1999a; 1999b. The food was contaminated with fungicide in the treatment boxes. The whole set up was kept inside an Environmental chamber and the temperature (28±0.5°C) and humidity (67%) was maintained. The determination of cellulase activity was performed on 3rd, 7th, 15th and 30th day from the setting of the experiment. The test specimens were kept in starvation before setting of the experiment.

RESULTS

The 96 hrs acute toxicity tests showed that Carbendazim with an LC_{50} value of 5.38 mg/kg soil was more toxic than Captan, LC_{50} value 10.41 mg/kg soil. The LC_{50} value of carbendazim is about five times higher than its RAD and in case of captan it is about two times higher than its RAD.

In the feeding preference experiment the earthworms showed maximum preference for *Anacardium occidentale* (cashew) leaves followed by *Mangifera indica* (mango), *Shorea robusta* (shal), *Acacia auriculiformis* (Acacia) and *Eucalyptus citridora* (Eucalyptus) (Fig A).

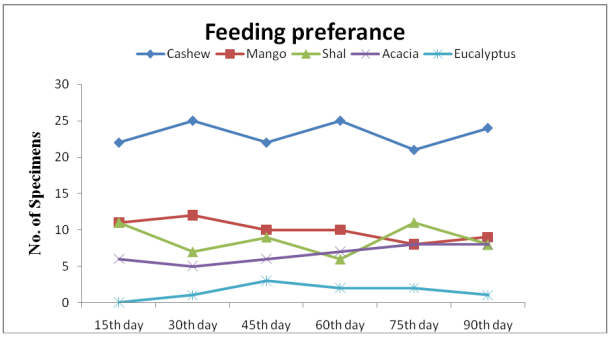

Figure: A Feeding preference of *Eisenia fetida* in the leaf litter of five tree species.

Due to more toxic LC_{50} value of carbendazim determined by the acute toxicity test, the chronic toxicity test was carry forword with only carbendazim.

In this experiment the cellulase enzyme activity in response to the carbendazim, of the test specimen was higher in both the sub lethal doses, 25% LC_{50} and 50% LC_{50} viz, 1.59 ± 0.50 mg of glucose/min./mg protein and 1.25 ± 0.45 mg of glucose/min./mg protein respectively and 1.50 ± 0.50 mg of glucose/min./mg of protein and 1.75 ± 0.55 mg of glucose/min./mg protein respectively than that of the control values viz, 1.09 ± 0.40 mg of glucose/min./mg protein and 1.29 ± 0.48 mg of glucose/min./mg protein on the 3rd and 7th day respectively after setting of the experiment. The activity of the enzyme diminished significantly than the control value (2.25 ± 0.85 mg of glucose/min./mg protein) on the 15th day of the experiment viz, 1.58 ± 0.60 mg of glucose/min./mg protein in both the sub lethal doses i.e. 25% of LC_{50} and 50% of LC_{50} value respectively. But on the 30th day of the experiment the activity of the enzyme increased to 3.05 ± 1.30 mg of glucose/min./mg protein and 3.05 ± 1.35 mg of glucose/min./mg protein in both the sublethal doses i.e. 25% of LC_{50} and 50% of LC_{50} respectively which are slightly higher than the control value i.e. 2.75 ± 1.15 mg of glucose /min./mg protein (Fig B).

One way ANOVA has been done using SPSS ver.16.0

DISCUSSION:

The LC_{50} value of carbendazim is higher than its RAD which indicates that this fungicide is ecologically safe in respect of short term (96 hours) acute toxicity. Studies of acute risk on *Eisenia fetida* after application of carbendazim in vineyards shows a LC_{50} value of 5.7 mg/kg. (URL 2).

Maximum colonization in Cashew and Mango with higher rates of degradation of these leaf litters can again be related to their lower antinutrient contents, viz polyphenol and tannin leading to higher palatability. (Hendriksen, 1990; Hobbie *et al*., 2006; Patricio, 2012; Johansson, 1995).

On the 3rd and 7th day of the experiment, cellulase activity of the earthworms somewhat significantly increased in both the sub lethal doses as compared to the control. This is probably because of the test specimen were unable to sense the fungicide contamination in the food and consumed it, as a result of keeping them in starvation before setting of the experiment. On the 15th day there was a little increase in the cellulase activity. This is because of that the earthworms were little bit affected by the fungicide but the enzyme activity didn't increase in the same rate as observed between 3rd and 7th day in sub lethal doses but in control the enzyme activity increased. On the 30th day the enzyme activity increased further in sub lethal doses and also in control compared to the 15th day's result of the experiment.

Probable cause of this increase in cellulase activity is that the fungicide is been degraded in food or the earthworms after sensing the fungicide become resistant to it. As the enzyme activity increased it can be said that the earthworms are not avoiding the food and restoring their enzyme activity to normal value.

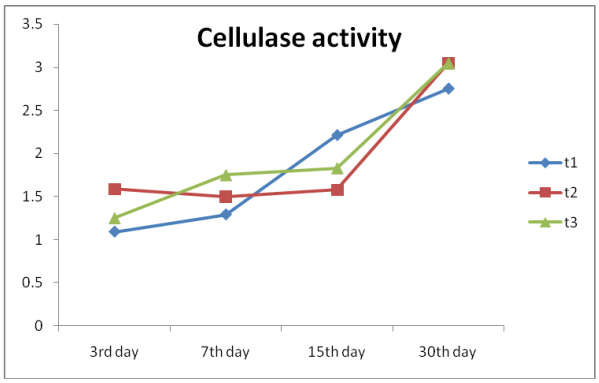

Figure: B Cellulase activity of *Eisenia fetida*, T1(Control), T2 (25% of LC_{50}) and T3 (50% of LC_{50}) Values of the enzyme are expressed in least significant difference, $p<0.05$ probability value.

Studies on the effect of carbendazim on the cellulase activity of *Eisenia fetida* has not been reported so far.

CONCLUSION:

From the above study it can be concluded that carbendazim shows less toxicity upon the earthworm after a certain period from the initial date of exposure, it does not have harmful effect when long term exposure is performed. In this regard carbendazim can be treated as an ecologically safe fungicide.

Last of all, it can be concluded that the enzyme cellulase can be used as a potential biomarker to detect pesticide pollution in agro ecosystem.

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CONFLICT OF INTEREST: None

REFERENCES

Amorim MJ, Sousa JP, Nogueira AJA, Scares AMVM, Bioavailability and toxicokinetics of C-14-Lindane (Gamma-HCH) in the enchytraeid *Enchvtraeus albiduf*, in two soil types: The aging effect. Arch. Environ. Contam. Toxicol, 2002, 43, 221-228.

Bierkens J, Klein G, Corbisier P, Van den Heuvel R, Verschaeve L, Weltens R, Schoeters G,. Compartive sensitivity of 20 bioassays for soil quality. Chemosphere, 1998, 37, 2935-2947

Beeby A, What do sentinels stand for? Environmental Pollution, 2001,112(2), 285–298.

Bouche MB, Strategies lombriciennes, In: Soil organisms as components of Ecosystems, Biol. Bull (Stockholm), 1977, 25, 122 – 132.

Brown GG, How do earthworms affect micro floral and faunal community diversity, Plant and Soil, 1995, 170, 209–231.

Bustos-Obregó E, Goicochea RI, Pesticide soil contamination mainly affects earthworm male reproductive parameters, Asian Journal of Andrology, 2002,4(3), 195–199.

Bostrom U, Lofs-holmin A, Testing Side effects of pesticides On Soilfauna Critical Literature Review, Report 12, Swedish Univ. Agri. Sci.,Uppsala, 1982,70.

Callahan CA, Shrazi MA, Neuhauser EF,Comparative toxicity of chemicals to earthworms, Environ. Toxicol. Chem., 1994, 13(2), 291-296.

Charrois JWA, McGill WB, Froese KL, Acute ecotoxicity of creosote-contaminated soils to *Eisenia fetida*: A survival-based approach. Environ.Toxicol. Chem., 2001, 20 2594-2603.

Culy MD, Berry EC, Toxicity of soil-applied granular insecticides to earthworm populations in cornfields. Down to Earth, 1995, 50, 20–25.

Curry JP, Bolger T, Growth, reproduction and litter and soil consumption by *Lumbricus terrestris* in reclaimed peat, Soil. Biol. Biochem., 1984, 16, 253-257.

Das Gupta R, Chakravorty PP, Kaviraj A, Studies on relative toxicities of six insecticides on epigeic earthworm, Perionyx excavates, Bull. Environ.Contam.Toxicol. 2010, 85, 83-86.

Dell'Omo G, Turk A, Shore RF, Secondary poisoning in the common shrew (Sorex araneus) fed earthworms exposed to an organophosphate pesticide, Environmental Toxicology and Chemistry, 1999, 18(2), 237–240.

Dorn PB, Vipond TE, Salanitro JP, Wisniewski HL, Assessment of the acute toxicity of crude oils in soils using earthworms, Microtox (R) and plants, Chemosphere, 1998, 37 845-860.

Ducrocq V, Pandard P, Hallier Soulier S, Thybaud E, Truffaut N, The use of quantitative PCR, plant and earthworm bioassays, plating and chemical analysis to monitor 4- chlorobiphenyl biodegradation in soil microcosms. Appl. Soil. Ecol., 1999 , 12,15-27.

Ecobichon DJ, Pesticide use in developing countries.Toxicol., 2001, 160, 27-33.

Edwards CA, Bohlen, PJ, The effects of toxic chemicals on earthworms, Rev. Environ. Contam. Toxicol., 1992, 125,23-99.

Garg P, Gupta A, Satya S, Vermicomposting of different types of waste using *Eisenia foetida*; A comparative study, Biores. Technol., 2006, 97, 391-395.

Hartenstein R, Neuhauser EF, Kaplan DL, Reproductive potential of the earthworm *Eisenia fetida.* Oecologia(Berl.), 1979, 43, 329-340.

Hendriksen NB, Leaflitter selection by detritivore and geophagus earthworms, 1990, vol 10, 17-19.

Hendrix PF, Crossley Jr. DA, Blair JM, Coleman DC, Soil biota as components of sustainable agroecosystems. In: Sustainable Agricultural systems, Soil and water Conservation Society, Ankeny, Lowa. 1990.

Hobbie SE, Reich PB, Oleksyn J, Ogdachi M, Zytkowiak R, Hale C, and Karlolewski P, Tree species effects on decomposition and forest dynamics in a common garden, Ecology, 2006 vol 87(9), 2288-97.

Ismail SA, Vermicology - The Biology of Earthworms, Orient Longman, Chennai, 1997.

Johansson MB, Berg B, and Meentenmeyer V, Litter mass loss rates in late stages of decomposition a climatic transect of pine forest Can. J. Bot., 1995, 1509-1521.

Joy VC, Chakravorty PP, Impact of insecticides on non target micro arthropod fauna in agricultural soil, Eco toxicol. Environ. Safety. 1991, 22, 8-16.

Kale RD, Bano K, Krishnamuoorthy RV, Potential of *Perionyx excavatus* for utilizing of organic wastes, Pedobiol, 1982, 23, 419-425.

Kibria G, Yousuf Haroon AK, Nugegoda D, Rose G, *Climate change and chemicals. Environmental and biological aspects,* New India Publishing Agency, Pitam Pura, New Delhi, 2010.

Knollenberg WG, Merritt RW, Lanson DL, Consumption of leaf litters by *Lumbricus terresteis* (Oligochaetea) on a Michigan Woodland and food plain Am. Midl. Nat., 1985, 113 (1), 1-6.

Komarek M, Cadkova E, Chrastny V, Bordas F, Bollinger JC, Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environment International,* 2010, 36, 138 – 151.

Kookana RS, Baskaran S, Naidu R, Pesticide fate and behaviour in Australian soils in relation to contamination and management of soil and water: a review. *Australian Journal of Soil Research,* 1998, 36 (5), 715 – 764.

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson P, Heal, OW, Dhillion S, Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur. J. Soil. Biol., 1997, 33, 159–193.

Lee KE, Earthworm: Their ecology and relationships with soils and land use, Academic press, Sidney, Australia, 1985.

Lee KE, Foster RC, Soil fauna and soil structure *Aus. J. Soil Res.,* 1991, 29(6), 745 – 775.

Lord KA,Briggs GG,Neale MC, Manlove R, "Uptake of pesticides from water and soil by earthworms," Pesticide Science, 1980, 11(4), 401–408.

Maity SK, Joy VC, Impact of antinutritional chemical compounds of leaf litter on detrivore soil arthropod fauna.Journal of Ecobiology, 1999a, 11(3), 193-202.

Maity SK, Joy VC, Soil micro arthropod feeding effect on nonnutrient chemicals of decomposing leaf litter, Indian Journal of Environment And Ecoplanning, 1999b, 2(3), 225-231.

Maugh TH, Science. 1978, 199,162.

OECD (Organization for Economic Co-Operation and Development), Guidelines for testing of chemicals No 207, Earthworm Acute Toxicity Test, OECD, 1984, Paris, Fance.

OECD (Organization for Economic Co-Operation and Development), Guidelines for testing of chemicals No 222, Earthworm Reproduction Test. (Eisenia fetida/andrei). OECD, 2004, Paris, France.

Patricio MS, Nunes LF, and Pereira EL, Litterfall and litter decomposition in chestnut high forest stand in northan Portugal, Forest Sys., 2012, vol. 21(2), 259-271.

Posthuma L, Application of body residues as a tool in the assessment of soil toxicity, In: Advances in earthworm ecotoxicology. SETAC press, 1998

Reinecke AJ, Vijoen SA, Saayman RJ, The suitability of *Eudrilus* e*ugeniae* , *Perionyx excavatus* and *Eisenia fetida* (Oligochaeta) for vermicomposting in Southern Africa in terms of their temperature requirement, Soil. Biol. Biochem, 1992, 24, 1295-1307.

Reinecke SA, Kriel J, temperature on the reproduction of the earthworm *Eisenia fetida* (Oligochaeta), S. Afr. J. Zool., 1981, 16, 96-100.

Reinecke SA, Reinecke AJ, Lysosomal response of earthworm coelomocytes induced by longterm experimental exposure to heavy metals. Pedobiologia, 1999, 43(6), 585–593.

Reynolds JD, International pesticide trade: Is there any hope for the effective regulation of controlled substances? Florida State University. J. Land Use Environ. Law, Retrieved on 2007-10-16, 1997, 131.

Ruz Jerez E, Ball PR, Tillman RW, The role of earthworms in nitrogen release from herbage residues. In: Jenkinson, D. S and Smith, K. A. (eds.), Nitrogen Efficiency in Agricultural Soils. (Publisher Unknown), 1988.

Sanchez-Hernandez JC, Earthworm biomarkers in ecological risk assessment. Reviews of Environmental Contamination and Toxicology, 2006, 188, 85–126.

Sadasivam S, Manickam A, Biochemical Methods. New Age International (P) Ltd.,Publishers New Delhi. 2010.

Sandoval MC, Veiga M, Hinton J, Klein B, Review of biological indicators for metal mining effluents: a proposed protocol using earthworms, in Proceedings of the 25th Annual British Columbia Reclamation Symposium, 2001, 67–79.

Satchell JE, Lumbricidae. In: Burges,A. and F. Raw (eds.), Soil Biology. Academic press. New York, N.Y. 1967.

Scheu S, Alphei J, Bonkowski M, Jentschke C, Soil food web interactions and ecosystem functioning: Experimental approaches with systems of increasing complexity. In: Rastin, N., Bauhus, J. (eds.), Going underground– Ecological studies in forest soils. Research Signpost, Trivandrum, 1999,1–32.

Scheu S, Effects of earthworms on plant growth: patterns and perspectives, Pedobiol., 2003, 47, 846–856.

Schmidt O, Curry JP, Effects of earthworms on biomass production, nitrogen allocation and nitrogen transfer in wheat-clover intercropping model systems. Plant and Soil, 1999, 214, 187–198.

Schneiderhan FJ, The discorvery of Bordeaux mixture: Three papers: I. Treatment of mildew and rot. / II. Treatement of mildew with copper sulphate and lime mixture. / III. Concerning the history of the treatment of mildew with copper sulphate. By Perre Marie Alexis Millardet 1885. A Translation,1933.

Simek M, Pizl V, The effect of earthworms (Lumbricidae) on nitrogenous soil. Biol. Fert. Serl., 1989, 7, 370-373.

Sorour J, Larink O, Toxic effects of benomyl on the ultrastructure during spermatogenesis of the earthworm Eisenia fetida. Ecotoxicology and Environmental Safety, 2001, 50(3), 180–188.

Syers JK, Sharplay AN, Keeny DR, Cycling of nitrogen by surface-casting earthworms in a pasture ecosystem. Soil. Biol. Biochem., 1979, 11, 181-185.

Tsukumoto J, Watanabe H, Influence of temperature on hatching and growth of *Eisenia foetida* (Oligochaeta, Lumbricidae). Pedobiol., 1977, 17, 338-342.

Van Rhee JA, A study on the effect of earthworms on orchard productivity. Pedobiol., 1977, 17, 107-114.

URL 1 EPA, 1996. http://www.epa.gov/nerleerd /stat2.htm accessed 2 Nov 2009

URL 2 www.inchem.org/documents/jmpr/jmpmono/v95pr19.htm

Vimmerstedt JP, Finney JH, Impact of earthworm introduction on litter burial and nutrient distribution in Ohio Strip mine spoil bands. Soil Sci. Sor. Am. Proc., 1973, 37, 388-391.

Wallwork JA, Ecology of soil animals, McGraw hill, London. 1970.

Wightwick A, Allinson G, Pesticide residues in Victorian waterways: a review. *Australasian Journal of Ecotoxicology,* 2007, 13, 91 – 112

Wightwick A, Mollah M, Partington D, Allinson G, Copper fungicide residues in Australian vineyard soils. *Journal of Agricultural & Food Chemistry*. 2008, 56, 2457 –2464.

Wurst S, Langel R, Reineking A, Bonkowski M, Scheu S, Effects of earthworms and organic litter distribution on plant performance and aphid reproduction. Oecologia(in press) 2003.

Yasmin S, DSouza D, Effects of pesticides on the growth and reproduction of earthworm: A Rev. Appl. Environ. Soil Sci., 2010, doi:10.1155/2010/678360.

Yeates GW, Soil Nematode population depressed in presence of earthworms. Pedobiol., 1981, 22 191-195.

Zaller JG, Arnone JA, Earthworm responses to plant species loss and elevated CO_2 in calcareous grassland. Plant and Soil, 1999, 208, 1–8.