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Toxicity of thiacloprid (Insecticide) on biomass, gut microbial population and digestive enzymes activity of an Indian Earthworm, *Lampito mauritii* (Kinberg) (Annelida: Oligocheta)

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Abstract
Earthworms are important soil organisms. Now a days many pesticides and chemical fertilizers are applied in the agricultural field. They are slowly eradicating earthworm population in soil. Thiacloprid is commonly used as insecticides in Tamilnadu. Hence in the present study was selected to observe toxicity of thiacloprid on biomass, gut microbial population and digestive enzymes activity of an Indian earthworm, *L. mauritii.* After exposure to thiacloprid low (1/10th of 96 h LC50 - 0.03 ppm kg−1) and high sublethal concentration (1/5th of 96 h LC50 - 0.03 ppm kg−1), *bacterial and fungal population, digestive enzymes such as*cellulase, α amylase, β amylase, protease and lipase level were decreased at 5th, 15th, and 30th day of experiment compared with control. The biomass of *L. mauritii* was also decreased upto 90 days. The results reported that thiacloprid insecticide severely inhibited the earthworm.

Keywords: *L.mauritii*, thiacloprid, biomass, digestive enzymes, microbes

Introduction
Earthworms are most important organisms in soil invertebrate fauna, they are considered as ecosystem engineers due to their burrowing activities, ingestion of soil and production of castings etc. (Latif *et al*., 2009) [17]. Pesticides are applied in agriculture fields, may affect the non-target soil organisms including earthworms and significantly damage the ecosystem (Lavelle and Spain 2002) [18]. Pesticides are either directly applied to soil to control soilborne pests or are deposited on soil as runoff from foliar applications. Hence, earthworms are exposed to pesticides through skin contact and by feeding on contaminated litter in soil. The effect of pesticides on earthworm may be either due to soil or foliar application at high concentrations or low, accumulation of low levels of persistent pesticide residues (Correia and Moreira 2010) [8]. when earthworms are preferred as food by amphibians, reptiles, birds and mammals there is a possible risk of these pesticides reaching higher trophic levels (Marino *et al*. 1992) [19]. Several researchers have advocated the use of earthworms as ecotoxicological model for risk assessment and bioassay of pesticides (Edwards and Bohlen 1992; Cikutovic, *et al*. 2010) [9, 7]. Earthworms have a relationship with soil microorganisms ranging from commensalism to species-specific mutualism. Parle (1963) [27] first reported the presence of microbes in the earthworm gut and several researchers followed the study in earthworm gut microbes using direct culture methods (Karsten and Drake, 1995). Garg *et al*. (2006) [10] suggested that vermicomposting is a waste management technique by production of organic fertilizers from organic wastes. Earthworms are crucial drivers of the vermicompost process. They cause fragmentation of the ingested material through muscular action thereby increasing the surface area for microbial activities (Edwards, 1988 and Lazcano*et al.*, 2008). In the earthworm gut has variety of intestinal microorganisms which produce digestive enzymes such as amylase, proteases, lipases, and cellulasesto enhance the biodegra- dation of organic matter (Aira*et al.*, 2006). Pizl and Novokova (1993) reported the establishment of different kinds of relationship between earthworms and microbes. They aremicrobes form a part of food for earthworm, proliferated in the gut and vermicomposts, earthworms help in the distribution of microbes and together with earthworm, the microbes mineralise humifies organic matter and facilitates chelation of some metal ions. The role of microbial activity in the gut and cast of earthworms and soil is very essential for degradation of organic wastes to release of nutrients. The activity of the earthworm gut is a miniature composting tube that mixes, conditions and inoculates the residues. Vermicast is rich in microbial activity, plant growth regulators and pest repellents while organic materials pass through earthworm gut. Intestine of L. mauritii earthworm has important role in conversion of organic wastes into nutrient rich vermicompost. So in this study, *L. mauritii* gut was selected for observation and enumerating of bacterial and fungal population and estimation of digestive enzymes activity.

Thiacloprid (Alanto) is a chemical class of neonicotinoid insecticides. It is an effective tool for the control of a broad spectrum of pests.Thiacloprid, due to its rain-fastness property, is stable even under conditions of heavy rains and sunlight providing longer persistence. It covers broad range of targeted pests, *viz*., aphids, whiteflies, thrips, and lepidopterans. In our area most of the farmers (Kumbakonam, Tanjore District) are using the insecticide (Thiacloprid) whereas *L.mauritii* (South Indian earthworm species) is widely distributed in our place. Various toxicological studies were carried out by many researchers in earthworms. Chakra Reddy and Venkateswara Rao (2008) [5] had been studied that the biological responses of earthworm, Eiseniafoetida (savigny) to organophosphorus pesticide, profenofos. Sherwan Taeeb Ahmed (2013) [34] had studied that the impact of four pesticides (cyren, ridomil, triplen and mamba) on the earthworm *Lumbricusterrestris*(annelida; oligochaeta). Vaidya (2016) [36] had studied that mercuric chloride significantly decreased protein level in ovary and testis tissues of *Perionyx excavates.* Rishikesh *et al.* (2019) [31] had observed that the effect of chlorpyrifos and cypermethrin and their combination on acute toxicity and biochemical responses of *Eudriluseugeniae.* But anyone not attempt in Thiacloprid effect on biomass, gut microbial population and digestive enzymes activity on *L.mauritii.* Hence in the present study is aimed to observe toxic effect of thiacloprid (insecticide) on biomass, gut microbial population and digestive enzymes activity of Indian earthworm, *Lampitomauritii.*

Materials and Methods

Materials

L. mauritii, clay loam soil and fresh cowdung were collected from agricultural farm and dairy yard in Asoor village, Kumbakonam. Thiaclopridwas purchased from agrocenter. The sun dried and powdered cowdung (earthworm's nitrogen rich natural food) was mixed with soil (low in nitrogen) in the ratio of 1:3 (vol/vol) and used as soil substrate throughout the study.

Chronic Toxicity Study

Long-term toxicity test (for 90 days) were designed to assess the effect of sublethal concentrations of thiacloprid on growth of *L. mauritii*. The author's earlier acute toxicity evaluation of Thiaclopridon *L. mauritii* indicated that the 96 h LC_{50}value was 0.3 ppm kg^{-1}. From 96 h LC_{50} value as one lower sublethal concentration (0.03ppm kg^{-1}) (T1) and one higher sublethal concentration (0.06 ppm kg^{-1}) (T_2) have been selected (as followed by many researchers in toxicology studies). Three plastic troughs (diameter 35cm x height 12cm) each filled with 1 kg of soil substrate were designated as C (control), T_1 and T_2. The control was mixed only with water. The required quantity of thiacloprid for T1 and T2 was mixed with soil substrate using 300 ml of water to ensure homogenous mixture and required moisture. Three replicates were maintained for C, T_1 and T_2. 10 non-clitellate (about 30-40 days old) *L. mauritii* were introduced after taking initial weight into each experimental media. The troughs were covered by nylon net, and maintained at room temperature 28±2°C with 50-60% moisture. The wet weight of earthworm were recorded once in every 10 days upto 90 days.

Microbial Study

Earthworms were removed from C, T1, and T2 on day 5, 15, and 30 and sacrificed to study their microbial composition. The dilution plate technique was used to study the bacterial and fungal population in the gut of L. mauritii. The gut contents (3–4 cm of gut spanning 20–30 segments) were dissected using sterile scissors and placed in sterile test tubes containing 1 ml of sterile saline (1 g $NaCl_2$ in 100 ml distilled H_2O). The tubes containing the gut contents were shaken vigorously and used as inocula. Using a micropipette, 0.01 ml of the inocula was spread using a standard platinum loop onto nutrient agar plates for bacterial growth and Sabouraud Dextrose Agar plates for fungal growth. The plates were incubated for 18–24 h at 37 °C and 4–7 days at 28 °C for bacterial and fungal growth, respectively. The Colony Forming Units (CFU) on the media were estimated using a colony counter and expressed as CFU × 10^3cm−1 for bacteria and CFU × 10^4 cm−1 for fungi as per a previously described protocol (Baron *et al*., 1994) [3].

Gut Digestive Enzymes Analysis Cellulase activity

A total of 1g of frozen earthworm gut tissue was ground with 20ml of 1/15M dibasic sodium phosphate (K_2HPO_4) in a mortar maintained at 5°C with crushed ice and cellulase activity determined according to the method of Nokrans (1957) [24, 26].

Total amylase (α and β) Activity:

Enzyme extract was prepared by grinding 1g of the earthworm gut tissue with 20ml 1/10M sodium acetate buffer pH 5.0 in a mortar maintained at 5°C with crushed ice and the buffer extract centrifuged at 18.000g for 30 minutes at 2°C. The α amylase activity was determined according to the method described by Wilson (1971) while β amylase activity was determined according to the method of Swain and Dekker (1966) [35].

Lipase Activity

Enzyme extracts were prepared in the same way as those of total amylase activity. Lipase activity in the extracts was determined using the method of Yong and Wood (1977) [41].

Proteinase Activity

Enzyme extracts were prepared in a manner similar to those of total amylase activity except that 20ml of 0.05M sodium phosphate buffer pH 6.0 were used as the extracting buffer. Proteinase activity in the enzyme extracts was determined using the method of McDonald and Chen (1965) [20]. Statistical analysis.Statistical analysis The statistical significance between treatments and control was analyzed using one way analysis of variance (ANOVA) with the help of the computer package SYSTAT.

Results and Discussion

Biomass Study

The effect of lower and higher sublethal concentration of Thiacloprid exposure on the biomass of L. mauritii are presented in table 2. In control the initial weight (3.03 ±0.09) of L. mauriti increased continuously upto 90 days. In sublethal concentration of Thiacloprid mixed soil substrate (T1 and T2). The weight of the worms increased slowly (compared to control) and on 19th day recorded 5.23 ±0.09 in T1 and 3.37 ±0.09 in T2. In the experiment the biomass was significantly decreased ($p<0.01$) over control in all the periods of study. The weight gain was more in lower concentration (T1) than in higher concentration (T2). (Table 1 and Fig.1).

There is adequate evidence that sublethal concentrations of pesticides can affect earthworm growth and reproduction (Reinecke and Venter, 1985; Neuhauser and Callahan, 1990) [29, 23, 25]. After exposed to endosulfanIn, an isopod had decreased growth rate. It was explained by a reduced feeding that may have been a strategy to avoid the pesticide (Ribera *et al*., 2001) [30]. Gobi Muthukaruppan *et al*. (2005) [21] observed glandular cell enlargement in the intestinal region of perionyxansibaricus when exposed to butachlor and suggested that which may massively affect food intake and which in turn may indirectly inhibit the earthworms' growth and reproductive capacity. Kavitha*et al*., (2008) observed that organophosphorus insecticide monocrotophos highly inhibited growth and reproduction of L. mauritii. Sherwan Taeeb Ahmed (2013) [34] was studied that the impact of four pesticides on the earthworm Lumbricusterrestris. The Results indicated that there are loss of weight, signs and symptoms of toxicity such as coiling, selling of the body swollen, sluggish movements and discharge of coelomic fluid.

Many investigators have reported a high toxicity of Chlorpyrifos and Cypermethrin insecticides on many species of earthworms in different countries, the effects ranging between moderate mortality to Perionyxexacavtus (Chakravorty and Kaviraj 2010) [6] and decrease in body weight of Eiseniafetida (Yasamin and Dsouza 2010). Booth *et al*. (2000) [4] observed loss ofweight of *Aporrectodeacaliginosa*when treated with organophosphate pesticides in field and laboratory also. Farrukh and Ali (2011) [37] clearly showed that dichlorovos caused a decrease in the weight of all groups of earthworms, when they were exposed to different concentrations of dichlorovos fumigant insecticide.

Table 1: Biomass of *Lampito mauritii* after exposed to lower and higher sublethel concentration of thiacloprid for 90days

Exp. Period (days)	Biomass of *Lampitomauritii* ($g^{-10)}$				
	Control	T1	%	T2	%
1	3.03±0.09	3.17±0.09	4.62	2.97±0.09	1.98
10	3.60±0.12	2.87±0.09	20.27	2.40±0.06	33.33
20	4.07±0.15	3.37±0.09	17.19	2.20±0.12	45.94
30	4.77±0.09	3.63±0.12	23.89	2.17±0.09	54.50
40	5.30±0.06	4.00±0.06	24.52	2.23±0.15	57.92
50	5.73±0.09	4.10±0.06	28.44	2.70±0.06	52.87
60	6.50±0.06	4.27±0.09	34.30	3.10±0.06	52.30
70	7.00±0.06	4.60±0.06	34.28	3.20±0.12	54.28
80	7.43±0.09	5.10±0.06	31.35	3.30±0.06	55.58
90	8.10±0.06	5.23±0.09	35.43	3.37±0.09	58.39

Values shown are the means of 3 experiments ± standard error.
C - control (soil substrate alone).
"%" indicates percent decrease over control (C).
T1 - 1/10th of the LC50 after 96 h, 0.03 ppm kg-1 (LC).
T2 - 1/5rd of the LC50 after 96 h, 0.06 ppm kg-1 (HC).

Table 2: Effect of sublethal concentration of thiacloprid on bacterial population of *L. mauritii* gut

Exposure period (days)	Bacterial population (CFU X 10^{-3} /3-4 cm of L. mauritii gut)				
	Control	T1	%	T2	%
5th day	324.33±3.48	199.33±11.02	38.54	154.00±14.73	52.51
15th day	327.00±3.78	202.33±11.46	38.12	155.66±15.01	52.39
30th day	329.33±4.05	204.33±11.46	37.95	157.66±15.01	52.12

Values shown are the means of 3 observations ± standard error.
C - control (soil substrate alone).

"%" indicates percent decrease over control (C).
T1 - 1/10th of the LC50 after 96 h, 0.03 ppm kg-1 (LC).
T2 - 1/5rd of the LC50 after 96 h, 0.06 ppm kg-1 (HC).

Table 3: Effect of sublethal concentration of thiacloprid on Fungal population of *L. mauritii* gut

Exposure period (days)	Fungi CFU X 10^{-4} / Lampitomauritii gut 3-4 cm				
	Control	T1	%	T2	%
5th day	206.67±7.31	141.33±8.82	31.61	107.67±6.23	47.90
15th day	208.33±7.26	143.33±8.82	31.20	109.67±5.70	47.35
30th day	210.33±7.26	145.00±9.07	31.06	111.67±5.70	46.90

Values shown are the means of 3 observations ± standard error.
C - control (soil substrate alone).
"%" indicates percent decrease over control (C).
T1 - 1/10th of the LC50 after 96 h, 0.03 ppm kg-1 (LC).
T2 - 1/5rd of the LC50 after 96 h, 0.06 ppm kg-1 (HC).

Table 4: Sublethel effect of thiocloprid on digestive enzymes level of *L. mauritii* gut

Experiments	experimental period	Enzyme activity (mg/g)				
		Cellulase	Aamylase	Amylase	Protease	Lipase
Control	5	46.58±0.03	7.48±0.02	7.65±0.03	8.4±0.02	1.78±0.01
	15	47.00±0.02	8.01±0.03	7.48±0.01	8.6±0.03	1.65±0.02
	30	47.24±0.04	7.67±0.02	7.91±0.04	8.78±0.01	1.69±0.03
T1(LC)	5	18.21±0.01	2.72±0.02	2.92±0.02	4.21±0.01	0.61±0.01
	15	21.32±0.03	5.22±0.03	2.71±0.02	4.91±0.02	0.71±0.02
	30	23.45±0.04	6.17±0.03	3.2±0.01	5.52±0.01	0.83±0.03
T2(HC)	5	16.24±0.02	1.91±0.04	1.47±0.01	2.01±0.02	0.21±0.01
	15	14.17±0.03	3.22±0.03	2.54±0.02	3.25±0.01	0.42±0.02
	30	15.43±0.02	4.47±0.01	3.01±0.03	4.52±0.02	0.59±0.01

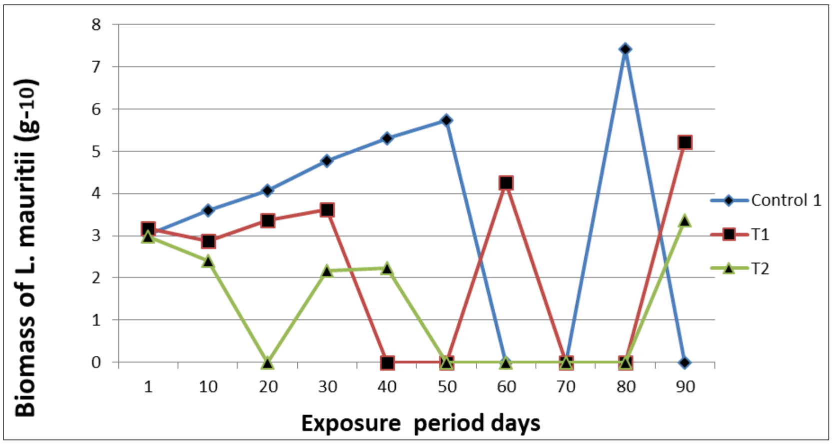

Fig 1: Biomass of *Lampitomauritii* after exposed to lower and higher sublethel concentration of thiacloprid for 90days

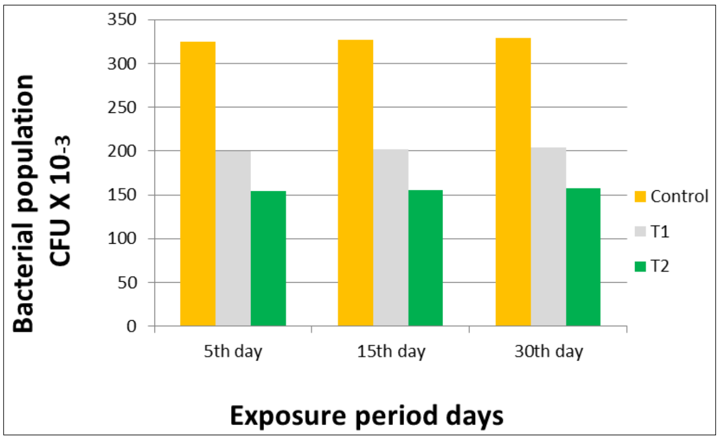

Fig 2: Effect of sublethal concentration of thiacloprid on bacterial population of *L. mauritii* gut

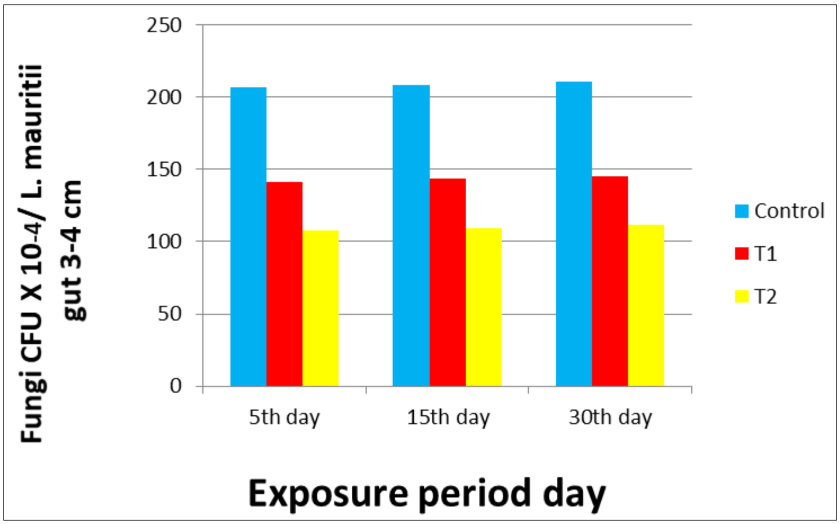

Fig 3: Effect of sublethal concentration of thiacloprid on Fungal population of *L. mauritii* gut

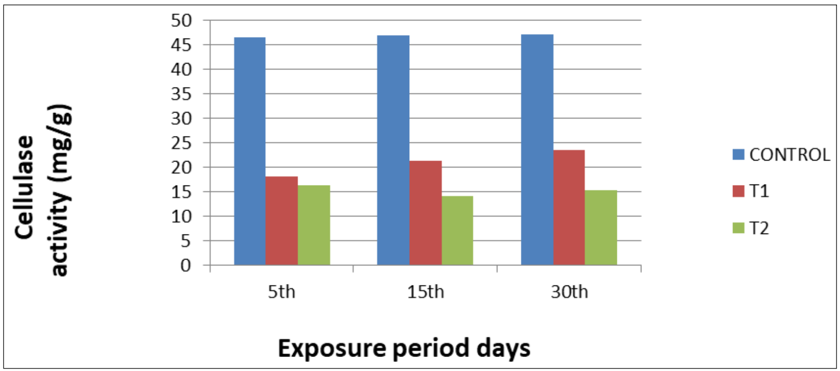

Fig 4: Sublethal effect of thiacloprid on Cellulase of *L. mauritii* gut

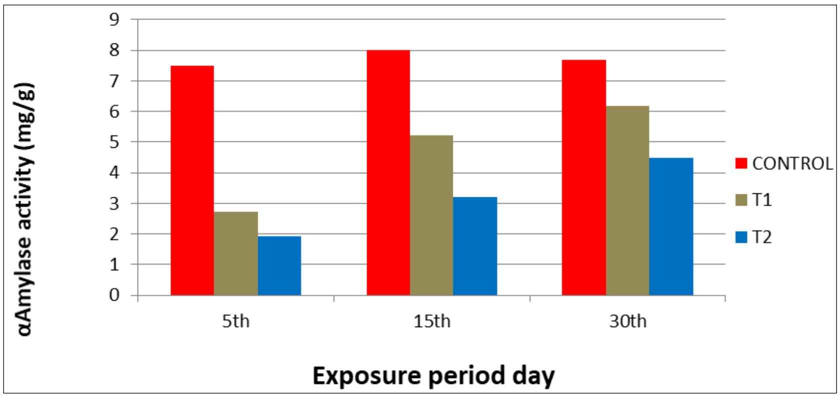

Fig 5: Sublethal effect of thiacloprid on α Amylase of *L. mauritii* gut

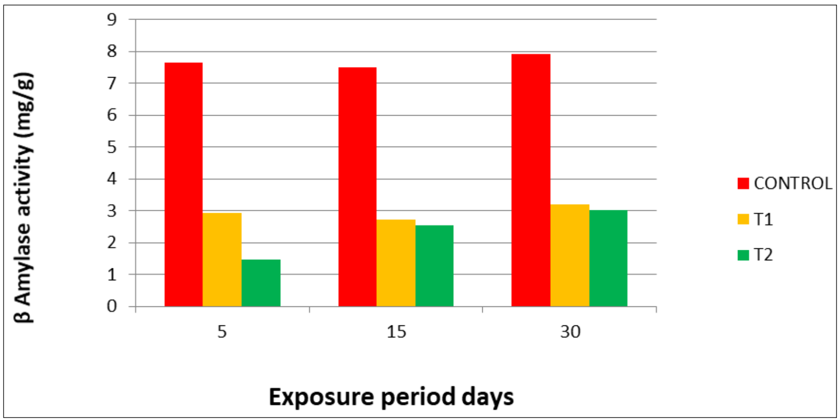

Fig 6: Sublethel effect of thioclopride on β Amylase of L. mauritii gut

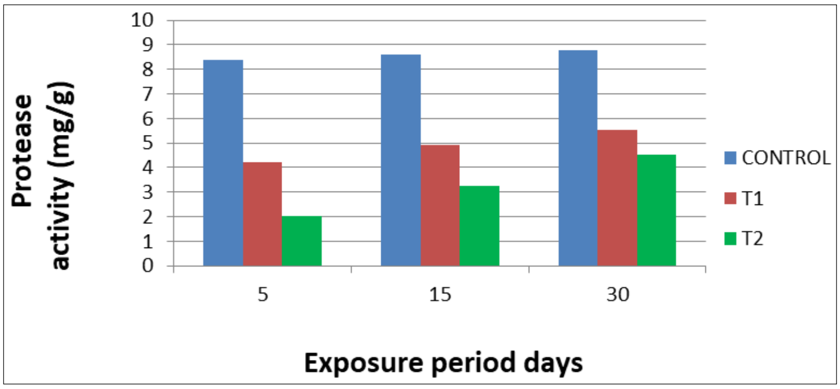

Fig 7: Sublethal effect of thiacloprid on Protease of L. mauritii gut

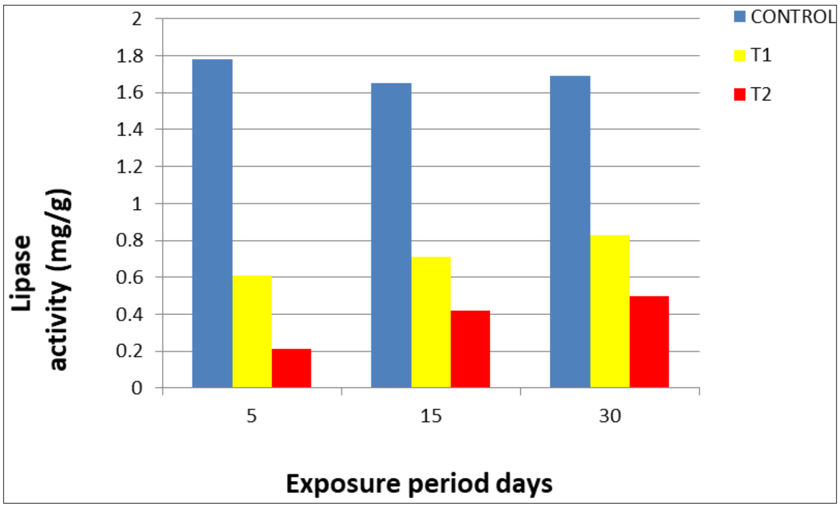

Fig 8: Sublethal effect of thiacloprid on Lipase of *L. mauritii* gut

Enumeration of the Microbial Population

Using the lower sublethal concentration of thiacloprid (T1), there was no significant change in the bacterial population (3%) on day 1. The population significantly decreased by 23% and 34% as compared to the control on days 5, 15 and 30 respectively.Similarly, using the higher sublethal concentration (T2) the bacterial population did not change significantly on day 1. The population significantly decreased by 39% and 49% on days 5, 15 and 30 respectively. Upon exposure to the lower sublethal concentration (T1) of thiacloprid, the fungal population significantly decreased by 23%, 27%, and 31% on days 5, 15 and 30 respectively, as compared to that in the control. Using the higher sublethal concentration (T2), fungal populations significantly decreased by 26%, 31%, and 36% on days5, 15 and 30 respectively. The gut bacterial and fungal populations of *L. mauritii* were reduced in the 5th, 15th and 30thday exposed T1 and T2 as compared to control earthworms (Table 2 and 2: Fig. 2 and 3).

The bacterial and fungal populations were comparatively less in the high concentration (T2) than low concentration of thiacloprid (T1). Earlier studies [Kalam and Mukherjee, 2001] have also reported that the total microbiota were adversely affected at high concentrations of hexaconazole persisted in the soil for up to 21 days. Thus, it can be concluded that microbial proliferation is suppressed by thiacloprid in a dose-dependent manner. Certain pesticides inhibited bacterial growth (Nawab*et al.*, 2003). A study previously reported the reduction of fungi upon organophosphorus pesticide treatment [Ambrogioni, 1987] [1]. The use of six insecticides in a cotton field reduced the soil fungal population by 75%. The bacterial population in this treated field returned to the levels as seen in the control field after 20 days (Vig*et al.*, 2008). Sub-lethal doses of cypermethrin significantly affected reproduction and alkaline phosphatase activity of epigeic earthworm, *Eiseniafetida* (Rupa Dasgupta*et al.*, 2015) [32]

Gut Digestive Enzymes Study

The results of the gut digestive enzyme activities of *L. mauritii* after exposure to thiacloprid is presented in Table 3. Cellulase enzyme level in control was 46.58 47.00 and 47.24 mg/g of protein for 5^{th,} 15th and 30th day of experiment period. In T1, it was 18.21, 21.32, and 23.45 mg/g of protein whereas in T2, 16.24,14.17 and 15.43 mg/g of protein. Comparedwith control highly decreased level of cellulase was observed in T1 and T2 7.48,8.01 and 7.67 mg/g of α amylase was observed in control. But in T1 and T2 decreased level such as 2.72, 5.22, 6.17 and 1.91, 3.22 and 4.47 mg/g was observed. The similar trend of results were noted for β amylase, protease and lipase level for T1 and T2 at the period of 5th, 15th and 30th day of thiacloprid exposure. Lower and higher concentrations of thiacloprid highly inhibited the digestive enzymes level in gut of *L. mauritii* than control. All the digestive enzymes level was highly decreased in T2 than T1 and control. (Table 4)

In the present study, digestive enzymes such as cellulase, α amylase, β amylase, protease and lipase activity were significantly decreased in T1 and T2 at 5th, 15th and 30th day of experiment than control. When earthworms exposed to thiacloprid, avoided food consumption due to presence of toxic effect of thiocloprid in media. It was reason for less enzymes level in gut. Corroborate the finding was Sanyal*et al*. (2016). They were suggested that amylase activity in *Perionyxexcavatus*was decreased than control on 7th, 15th and 30th day of herbicide, Pretilachlor exposure due to prevented food consumption because of the herbicide persistence in food. Digestive enzymes were secreted by earthworm *E. eugeniae* in association with gut microflora, and they might be involved in cleaving the macromolecules in the feed mixtures. The enzymes (cellulase, protease, dehydrogenase) were analysed in the earthworm gut during the vermicomposting process in the different treatments (Ravindran, 2015). Bamidele*et al.* (2014) have reported that more microbial diversity was observed in sawmill vermicompost. The results were strongly proved that during the degradation of organic wastes the digestive enzymes activity was increased by the action of microbes in gut. Organic matter sources of carbon as feed for microbes.

Chakra Reddy and Venkateswara Rao (2008) [5] have been reported that organophosphorus pesticide, profenofos inhibited AChE activity, morphological alterations and histological changes in the body wall of earthworm, E*sseniafoetida.*The organophosphorus and carbamate insecticides are known to inhibit acetylcholinesterase which plays an important role in neurotransmission at cholinergic synapses by raid hydrolysis of neurotransmitter acetylcholine to choline and acetate (Kwong, 2002; Kavitha and Venkateswara Rao, 2007) [16, 11]. Kavitha and Anandhan (2018) [15] reported that atrazine herbicide affected histrology of intestine of *L. mauritii.*Kavitha and Anandhan (2015) [14] was studied that carhayl intoxicated *L. mauritii* showed elevated level of LP and lower activity of antioxidants upto 15 days.

Conclusion

Earthworms are major macrofauna in terrestrial ecosystem.They are secondary decomposer, while feeding on partially degraded (by microbes) organic matter, make them further get fragmented by the gizzard in the alimentary canal. By this mechanical action of gizzard, particle size is decreased thereby increasing the surface area available for growth of microbes. This promotes further breakdown of organic matter by the microbes on the one hand and provide greater supply of food (microbes) for worms on the other hand. Now a days, many chemical fertilizers and pesticides are used in agricultural field to control pest and boost up the crop yield. But the pesticides and chemical fertilizers have ability to destroy farmer's friend of earthworms and microbes in soil. The present study also proved that thiacloprid insecticide highly inhibited biomass, gut bacterial and fungal population and digestive enzymes level in L.mauritii.

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