Effect of copper fungicide on earthworm, *Lampito mauritii*

ABSTRACT

Copper such as copper oxychloride has wide use as a fungicide/bactericide which prevents infection in plants. The recommended dose of copper oxychloride for rice fields is 3 g/l which contains 50% copper i.e 1.5 g copper/l or 1500 ppm. The earthworms that play a major role in soil physical, chemical and biological improvement of soil are exposed to the copper fungicide. During the suspension culture the epi-anecic earthworm, *Lampito mauritii* (Kinberg) could not survive beyond 30 ppm. So, the earthworms were exposed to lethal levels of copper i.e., 0, 10, 20 and 30 ppm of copper and the effect on growth, feeding, respiration, excretion and regeneration was found to be significantly deleterious. On exposure to a sublethal dose of copper oxychloride the respiration increased but there was a marked reduction in growth, feeding, excretion and regeneration. The positive contribution of the earthworm was hampered but it continued to utilize energy from the system and this was the major finding of this work.

KEYWORDS

Sublethal dose, copperxychloride, epi-anecic earthworm, growth, feeding, respiration and excretion

1. INTRODUCTION

Soil is a habitat for many micro and macro-organisms. The microorganisms include archaea, bacteria, actinomycetes, fungi, algae, protozoa and macrofauna like springtails, mites, nematodes, earthworms, ants, insects and larger organisms like burrowing rodents. Among all soil biota, earthworms are the key biotic components that plays a vital role in maintaining the chemical, physical and biological properties of temperate and tropical soils. Earthworms (macrofauna) belong to the taxonomic phylum Annelida and family Lumbricidae.

Aristotle called the earthworm “the Intestine of the earth”. By ingesting soil debris, earthworms have been shown to enhance the bioavailability of soil nutrients such as carbon, nitrogen, and phosphorous. While burrowing earthworms ingest a large amounts of soil and are therefore exposed to contaminants through their intestine as well as through their skin. As a result, substances are concentrated from the soil into their body making them ideally suited for assessing the bioavailability of many harmful substances [1].

Earthworms host millions of decomposers (bio degrader) microbes in their gut and excrete them in soil along with nutrients like nitrogen and phosphorus in their excreta called ‘vermicast’ [2]. Edward and Fletcher [3] showed that the number of bacteria and ‘actinomycetes’ contained in the ingested material increased up to 1,000 fold while passing through the gut. The numbers, biomass, and activity of microbial communities in the earthworm gut have also been shown to be different from that of uningested soil [4]. Singleton et al. [2] studied bacteria associated with the intestine and casts of earthworms and it included *Pseudomonas*, *Paenibacillus*, *Azoarcus*, *Burkholderia*, *Spiroplasm* and *Actinobacterim.*

Lampito mauritii is one of the anecic earthworms found widely in Odisha and especially in Western Odisha, where this work has been conducted. The main contribution of the anecic earthworms is fragmentation of litter and its transportation to the lower layers of soil. It also produces mucus and helps in macrostructure formation. It ingests and disperses the soil biota and controls the population of some soil biota including microbes and mineralization process is facilitated [5].

To increase the yield, pesticides are used to decrease the yield loss by the pest. In addition to destroying the pests, the pesticides have a detrimental effects on non-target organisms like earthworms which play important role in soil fertility. The epigeic and epi-anecic earthworms are exposed to pesticides when it is spread. Earthworms have been selected as a suitable representative soil organism as they contribute to the overall productivity of agricultural soils through their feeding, casting and burrowing activities [6]. Generally, pesticides cause toxicity in earthworms but indirectly can cause the reduction of the population by decreasing the organic matter input and weed coverage [7], [8]. Growth, reproduction and avoidance behavior are also affected by pesticides [9].

Copper oxychloride (50% WP), trade name blue copper is a protective wettable fungicide having the double effect of systematic, contact and prevention action. Copper can be used on numerous crops against fungal and bacterial diseases. It can serve as a fatal enemy of major obstinate disease in the growing period of the crop. Its active molecule penetrates with high performance. It can enter the in vivo of the plant rapidly, and expand in the ailing plant, sterilizing inside and outside. It is used as a fungicide to control Scab, Anthracnose, Downy mildew, Early blight, and Late blight diseases of various crops, vegetables, potato, tomato, tea, coconuts, spices, tobacco, fruits like grapes, bananas and nuts etc.

Copper as copper oxychloride is a protectant fungicide/bactericide which prevents infection in plants. Its mode of action is by interfering with the enzyme system of spores and mycelium, a process that is usually irreversible. It forms a chemical barrier against fungal attacks and is a foliar fungicide with preventative action.

There are several reports that the pesticides used for the reduction of loss of yield due to pests also result in the reduction of non-target organisms like earthworms which play important role in soil fertility [10], [11], [12], [13]. Most of the studies show the lethal effect the various pesticides. In this study, an attempt has been made to find the effect of a widely used fungicide copper oxychloride on the anecic earthworm, *Lampito mauritii* at subtoxic level. Although at a subtoxic level the organism is not killed, its metabolic parameters like growth respiration, excretion, etc. are hampered and these changes may also be considered as the bioindicators of the toxic substances entering into the ecosystems.

2. MATERIAL AND METHODS

2.1 Copper fungicide

Copper oxychloride is used as a fungicide and the recommended dose for rice fields is 3 g/l .where copper present is 50% i.e. 1.5g copper/l. so the recommended dose is 1500mg/l i.e. 1500 ppm of copper**.**

2.2 Lampito mauritii (Kinberg)

Figure 1. Image of earthworm *Lampito mauritii*

Lampito mauritii (Kinberg) is one of the endemic species classified under epi-anecic species. It is widely distributed in India. It can sustain a temperature range of 25-26ºC with soil moisture of 10-20 % and pH of 5.82 – 7.2 where its growth and reproduction are favored [14]. The species live in vertical burrows about 1-2 meters into the soil profile and extend to the surface burrows are channels for the preferential flow of air and water and are often lined with organic matter especially protein-rich mucus.

They are light greyish to brownish body colored but deeply pigmented at their head and tail region. Their body is of medium size and clittelum covers 14^{th} to 17^{th} segment. Male pores on slightly raised areas of 18^{th} segment; paired female pores on 14^{th} segment. Length is about 95mm -155mm with body live weight is usually 1 g. This species has the capability to produce 12-45 per year cocoons on average by an adult [15].

Immature *Lampito mauritii* earthworms were utilized for conducting the survivility experiments. Gut contents were cleaned by keeping them in distilled water for three hours [16]. Earthworms were exposed to 0 to 1500 ppm of copper as copper oxychloride solution. After 240 hours, the earthworms exposed to beyond 30 ppm of copper solution could not survive. So the sub-lethal dose of 10, 20 and 30 ppm of copper was used for the study and control sets were maintained at 0 ppm copper.

2.3 Growth

The biomass of earthworms reflects the physiological requirements of tissue. In the present study the change in the biomass of the earthworms i.e., the change in weight of earthworms at different concentrations of copper solution i.e., 0 ppm 10 ppm, 20 ppm and 30 ppm has been studied.

For this purpose 500 g substate (300g soil and 200 g cow dung) was air dried, passed through 2mm sieve and placed in in polythene packets. Moisture was maintained at 20 ± 2 g % by adding distilled water for contro treatment and respective copper solution in experimental sets i.e. 10, 20, 30 ppm in 10 replicates of each concentration. Approximately 1.5 g of earthworm was introduced in each experimental packet after 5 days of moisture addition and microbial activation. Soil temperature was maintained at 25 ± 2˚c.

The change in the weight over initial weight was observed after 10, 20 and 30 days and the percentage change in weight of earthworms over zero-day culture was estimated.

2.4 Feeding (Stable Aggregate Formation)

Earthworm conserves nutrients by forming stable aggregates. As the formation of stable aggregates is directly proportional to feeding. The formation of stable aggregates is governed by the percentage of clay, amount of organic matter in the soil, bacterial mucilage, fungal mycelia and colloid contribution of macrofauna [17], [18].

In the present study, the effect of 0, 10, 20, and 30 ppm of fungicide on the stable aggregate formation has been observed. The earthworms were inoculated in zero days and the estimation of stable aggregates was done at an interval of 10 days. After 10 days culture sets were sieved with water. The stable aggregates formed were collected, air-dried and weighed. The carbon content of the aggregate formed was estimated by Walkley and Black [19] titration method. The amount of carbon by energy conversion has been calculated and according to available standard values energy conversion for carbon is 41.44 kJ, g^{-1}, dry wt. [20].

2.5 Respiratory rate

The respiratory rate was quantified by the Alkali absorption method [21]. Carbon dioxide evolution was measured at 25 ± 2˚c and expressed as mg of CO_2, g^{-1} live worm tissue, hr^{-1}, kg^{-1} soil.

500gm (300 g soil and 200 g cow dung) 2mm sieved, air dried soil was taken in plastic jars with moisture content maintained at 20±2 g% by addition of distilled water in control sets i.e. '0' ppm and respective copper solution in experimental sets i.e. 5, 10 and 20 ppm. 10 replicates of each concentration were taken and 5g of earthworm was inoculated into them. The jars were kept undisturbed for 5 days for microbial activation. The respiratory metabolism was maintained at 0, 10, 20 and 30 days.

2.6 Excretion

Estimation of ammonia on exposure to the copper fungicide was done by Kaplan method [22]. *Lampito mauriti*, the anecic earthworms were collected from an uncontaminated site and inoculated in culture packets which are prepared by mixing soil and cow dung in 3:2 ratio.

40 replicas were taken, 10 for each ppm of fungicide (0, 10, 20, 30). 20±2 g% Moisture level was maintained by giving distilled water to 0 ppm replica (control), 10 ppm fungicide to 10ppm and 20 and 30 ppm fungicide to 20 and 30 ppm replica respectively. Approximately 1.5 g earthworms were inoculated in each replica consisting of a mixture of 300 gm of sieved pasture soil and 200 gms of sieved cow dung.

After inoculating the earthworms, 1.6 gm of moist soil (dry wt.1 gm) was taken from each replica of 0ppm, 10 ppm,20 ppm, and 30 ppm. The amount of ammonia was estimated by measuring OD at 655 nm. This process was repeated every 10 days interval. And 0 day, 10 days, 20 and 30 days readings were observed. The ammonia excretion was determined.

2.7 Statistical Analysis

Statistical analysis, two-way ANOVA was performed for inferring the data obtained [23] to find whether the subtoxic level of copper oxychloride has any significant effect on the earthworm. These are depicted in table 1-4.

3. RESULTS AND DISCUSSION

3.1 Growth

Growth trends of *Lampito mauritii* when exposed to a sublethal dose of copper oxychloride are shown in Figure 2.

During 30 days of observation, it was noticed that the weight of earthworm gradually increased in control over 0 days. But growth was seen to be decreased in 10, 20 and 30 ppm exposure to copper. The weight decreased by 21.02%, 45.74%, and 44.20% over initial weight on exposure to 10, 20 and 30 ppm copper respectively by 30 days. Two Way ANOVA showed a significant impact of copper on the growth of *Lampito mauritii* at 0.01 and 0.001 levels of significance to duration (F_{cal} = 9.34, n_1=3 n_2= 9) and dose (F_{cal} =162.24, n_1=3 n_2= 9), respectively (Table 1).

Biomass is a good indicator of the physiological requirements of tissue and material cycling [24]. In the present study, significant reduction in biomass was found on exposure to a sub-lethal dose of copper. The rate of growth is gradually decreased. Similar results were reported by Khan *et al.,* [25]. They found a significant reduction in earthworm biomass after exposure to different concentrations of copper chloride and concluded abnormal functioning of major physiological systems such as digestion and absorption. Helling *et al.,* [26] found that the growth and reproduction of *Eisenia fetida* were significantly decreased in consecutive weeks after exposure to copper fungicide. Bart et al., [27] also reported a reduction in the growth of enchytraeids (pot worms) in the laboratory on exposure to copper oxychloride. Reduction in biomass was also found in the earthworm on exposure to pesticides by various studies [28], [29], [30], [31]. Zhang et al., [33] found a reduction in the growth of *Acrobeloides nanus*, *Pristionchus pacificus*, and *C. elegans* by mancozeb. Kreutzweiser et al. [34] reported that Imidacloprid reduced earthworm growth. Dichlorovos application leads to a reduction in earthworm biomass [35]. According to Capowiez and Berard, [36] and Gomez-Eyles et al., [37] reduction in body weight may reflect the reduction in feeding by the earthworms. They also opined that worms excavate less when exposed to imidacloprid, which means that they feed less and have fewer intestinal contents. Loss in body weight may also be attributed to the reduction of enzyme activities like cellulase activity which compromises the feeding efficiency of exposed organisms and it ultimately results in lower weight gain [38].

3.2 Feeding

The stable aggregate formation of *Lampito mauritii* under the impact of copper is presented in Figure 3.. In control, there is an increase of 30.42% in stable aggregate formation was observed after 30 days. But when earthworms were exposed to 10, 20 and 30 ppm of copper after 30 days of culture there was a decrease of about 4.06%, 13.18% and 17.24% in stable aggregate formation on exposure to 10 , 20 and 30 ppm respectively. Two Way ANOVA showed that significant impact of copper in energy content of stable aggregate formation by the earthworm at .001 and .05 levels of significance to duration (F_{cal} = 334.7, n_1=3 n_2= 9) and dose (F_{cal} = 168.6, n_1=3 n_2= 9) (Table 2).

The feeding habit of earthworms results in the physical aggregation of the soil. Earthworm activity creates structures, casts and galleries that modify the circulation and accumulation of water [39]. In the present study, significant reduction in stable aggregate formation which is directly proportional to feeding on the application of different doses of copper through fungicide is studied. Gunston et al. [40] also reported a negative impact of pesticides on earthworm feeding and cast production. Weight loss and reduction in feeding on exposure to toxicants in soil have been reported by various researchers [41], [42], [43].

The degree of stability of casts depends on the food and behavior of the worms [44]. Arthur, [45] noticed that the bacterial population in the earthworm gut was about 1000 times more than the surrounding soil. The production of polysaccharides like levan and dextran by many bacteria binds quartz and clay particles. Soil particles are cemented by calcium humate produced by the interaction of ingested organic matter and calcite excreted by calciferous glands of earthworms hypothesized by Meyer [46]. A significant reduction in earthworm cast on ground surface on the application of fungicides like carbendazim, benomyl, etc was observed by Keogh and Whitehead [47]. Reduction in surface casting, an abundance of earthworms, biomass and litter accumulation when benomyl and related fungicides were sprayed [48]. Reduction in cast production was also reported in *L.terrestris* and *Aporrectodea caliginosa* on exposure to Imidacloprid and Chlorpyrifos, respectively [49], [50]. Larink and Sommer [51] also found that feeding behavior was reduced by imidacloprid and cyfluthrin.

Burrows and Edward [52] correlate the reduction in feeding as a natural strategy for survival i.e., reducing food intake to avoid toxins. Such a strategy was reported to be commonly used in earthworms to avoid poisoning with heavy metals [51] but also organic chemicals such as pesticides [53], [54].

3.3 Respiration

The rate of respiratory of *Lampito mauritii* under the impact of copper is shown in Figure 4.. It was noticed that the rate of respiration was increased with duration and treatment. i.e. after 10 days of exposure the rate of respiration was found to be 3.93, 7.04, 7.04, and 8.11 mg of CO_2 evolved/g of live tissue, hr-1, kg^{-1} at 0, 10, 20, 30 ppm of copper, respectively. At 20 days 15.98%, 28.99%, and 51.49% increase in respiration was observed in 10, 20, and 30 ppm exposure of copper respectively. After a month there is an increase of about 13.40%, 45.49%, and 65.78% was observed under 10, 20 and 30 ppm of exposure to copper. ANOVA showed a significant impact of copper on the respiratory metabolism of earthworms at 0.001 level of significance to duration (F_{cal} = 296.01, n_1=3 n_2= 9) and dose (F_{cal} = 135.34, n_1=3 n_2= 9) (Table 3).

Energy loss of an organism is represented by respiratory metabolism. Most of the organism's energy is utilized in respiration. Soil respiration is being used for the estimation of biological activity [55]. Earthworms on exposure to carbaryl and endosulfan showed an increased value of CO_2 evolution. Temperature stress results in an increase in oxygen consumption reported by Senapati and Dash [56]. Bolton [57] measured the respiration of earthworm species *Dendrobaena rubida* and *Lumbricus castaneus* at 10ºC and reported values between 75 and 100 µl CO_2 $h^{-1}g^{-1}$. In the present study enhancement in the respiratory metabolism when exposed to different concentration of copper fungicide has been observed which results in the loss of more energy utilized by the earthworms. Similar results were also observed by Panda and Patnaik [10] where exposure of earthworms, *Perionyx excavates* to cartap hydrochloride increased respiration. It is suggested that exposure to toxicants like heavy metals or other pesticides impedes food consumption, while at the same time, energy was required to eliminate the toxicant [58], [59]. The increase in respiratory metabolism might be attributed to higher muscular activities [60], [61] and energy utilization to overcome oxidative impairment [62]. According to Maboeta et al. [63] energy budget model describes the underlying physiological processes as the catabolisation of tissue under stress conditions, with rates of weight loss accompanied by the increase in maintenance rates.

3.4 Excretion

In Figure 5 the impact of copper on the ammonia excretion of *Lampito mauritii* (Kinberg) was demonstrated. In control ammonia excretion was gradually increased whereas in 10, 20 and 30 ppm of exposure to copper, excretion was decreased up to 13.33%, 26.66%, 46.66% respectively. After a month excretion decreased drastically i.e 53.60%, 88.14% and 97.42% in 10, 20 and 30 ppm exposure of copper respectively. ANOVA test shows a significant impact of copper on ammonia excretion at 0.001

level of significance in laboratory culture to duration (F_{cal} = 174.7, n_1=3 n_2= 9) and dose (F_{cal} = 275.24, n_1=3 n_2= 9) (Table 4).

Mucous protein and nitrogenous metabolic by-products like ammonia, urea and free amino acids are the main nitrogenous compounds excreted by earthworms [64]. In the present study, the alteration in ammonia excretion of earthworms was found on exposure to a sub-lethal dose of copper fungicide. The rate of excretion of earthworm *Lampito mauritii* increases day-wise and decreases with an increase in copper concentration .

On exposure to sublethal dose of copper, the organism remains in the ecosystem but it fails to contribute its positive role to the system as a result of the toxicity of copper. A decrease in excretion of earthworms has been reported on the application of a sublethal dose of malathion by Senapati *et al,* [65]. Reduction in ammonia excretion on exposure to copper fungicide has been reported by Patnaik and Senapati, [66].

The maximum channelization of the energy towards the respiratory metabolism may be one of the major reasons for the decrease in the excretion of earthworms under stress conditions.

4. CONCLUSION

Copper fungicide which is most frequently used by farmers does have a detrimental effect on non-target organisms like earthworms. At sub-lethal doses although the earthworms are not eliminated from the system, their positive contributions like growth, aggregate formation, and nitrogen contribution towards soil in form of ammonia are drastically decreased. At the same time, most of the energy is channelized towards the maintenance of the body on exposure to copper. On exposure to the sub-lethal dose, it remains in the system utilizing the resources but does not contribute positively towards the system. So, there should be a study of the effect of sublethal doses of pesticides and alternate biopesticides should be chosen for use in the croplands for reduction of yield loss due to pest. The sublethal effects like effects on the parameters like growth, feeding, respiration and excretion can also be used as indicators for the presence of toxic substances. Further research work should include effective biopesticides for the protection of crops against pests that will not be detrimental to the soil fauna.

Table 1. Two way ANOVA for growth of *Lampito mauritii* earthworm under the impact of copper with respect to treatment and duration.

Source of Variation	*Sum of squeres*	*df*	*MS*	*F-cal*	*P-value*	*F tab*	*Significance*
Between duration	1.38	3	0.46	9.34	0.01	7.0	Significant
Between Treatment	23.95	3	7.98	162.24	0.001	13.9	Significant
Interaction	15.22	9	1.79	34.37	0.05	1.95	Significant
Within	7.08	144	0.05				
Total	47.63	159					

Table 2: Two Way ANOVA for energy content of stable aggregate formed by earthworm *Lampito mauritii* under the impact of copper with respect to treatment and duration

Source of Variation	*Sum of Square*	*df*	*MS*	*F-cal*	*P-value*	*F tab*	*Significance*
Between Duration	0.295985	2	0.147993	334.7241	0.001	27.0	Significant
Between treatment	0.2237	3	0.074567	168.6523	0.001	23.7	Significant
Interaction	0.062995	6	0.010499	23.74646	0.5	2.18	Significant
Within	0.04775	108	0.000442				
Total	0.630431	119					

Table 3: Two way ANOVA for respiration of *Lampito mauritii* earthworm under the impact of copper in laboratory culture.

Source of Variation	***Sum Square***	***df***	***MS***	***F cal***	***P-value***	***F tab***	***Significance***
Betweeen duration	**875.59**	**2**	**437.80**	**296.01**	**0.001**	**27.0**	**Significant**
Between treatment	**406.02**	**3**	**135.34**	**91.51**	**0 .001**	**23.7**	**Significant**
Interaction	**58.91**	**6**	**9.82**	**6.64**	**0.05**	**2.18**	**Significant**
Within	**159.73**	**108**	**1.48**				
Total	**1500.26**	**119**					

Table 4. Two way ANOVA for Ammonia excretion of *Lampito mauritii* earthworm under the impact of copper with respect to treatment and duration.

Source of Variation	*Sum of square*	*df*	*MS*	*F tab*	*P-value*	*F cal*	significance
Between duration	**0.10**	**2**	**0.05**	**174.70**	**0.001**	**27.0**	**Significant**
Between treatment	**0.24**	**3**	**0.08**	**275.24**	**0.001**	**23.7**	**significant**
Interaction	**0.11**	**6**	**0.02**	**65.35**	**0.05**	**2.18**	**significant**
Within	**0.03**	**108**	**0.01**				
Total	**0.48**	**119**					

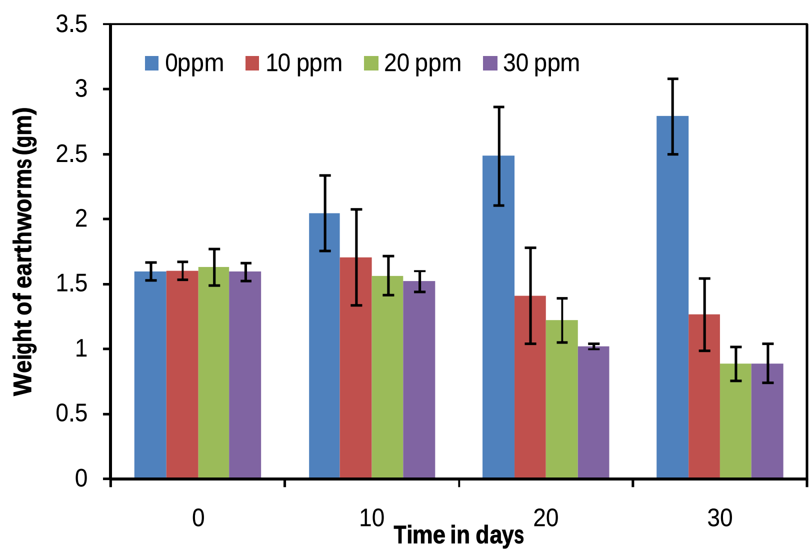

Figure 2: Change in weight of *Lampito mauritii* (Kinberg) on exposure to copper.

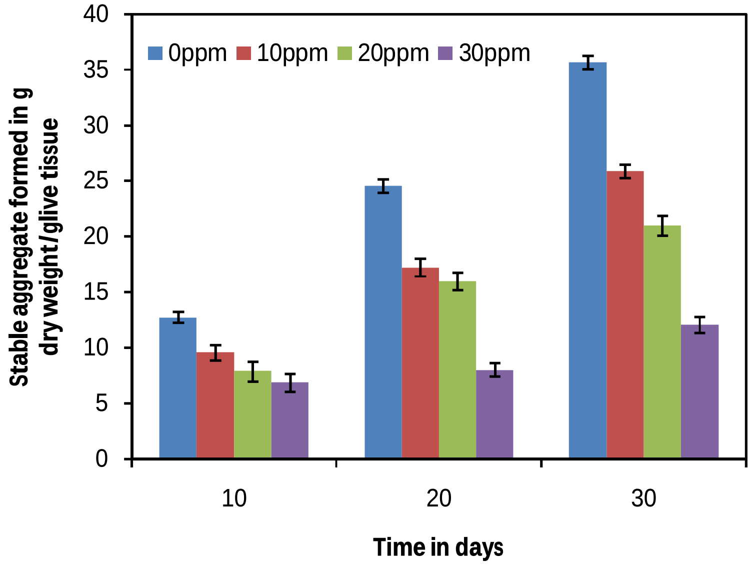

Figure 3. Change in weight of stable aggregate formed by *Lampito mauritii* (Kinberg) on exposure to copperoxychloride.

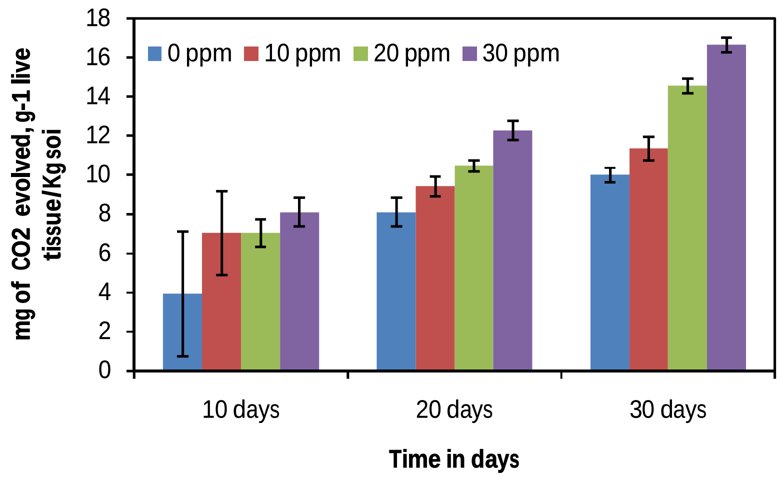

Figure 4. Respiratory rate of *Lampito mauritii* under the impact of copper in laboratory culture.

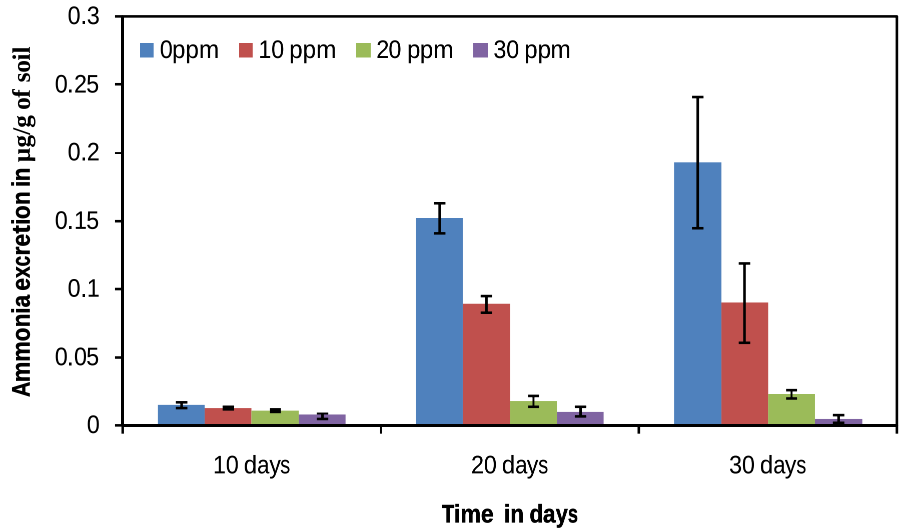

Figure 5 Ammonia excretion of *Lampito mauritii* (Kinberg) under the impact of copper

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