Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(5): 2043-2048

Received: 18-07-2020
Accepted: 23-08-2020

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Impact of Gyphosate and pendimethalin herbicides on qualitative and quantitative parameters of coelomocytes in earthworm *Eisenia fetida*

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Abstract
In stress conditions immune defense mechanism is well developed in earthworms which guard them from pathogens in the environment. Coelomocytes are the immune cells of the earthworms and maintain the innate immune system of these lower animals. Extensive use of herbicides decreases the coelomocytes number. An experiment was performed to test the effect of glyphosate and pendimethalin herbicides on coelomocytes of *Eisenia fetida.* The results revealed that these herbicides had interfered with the immunity of worms and there was significant decrease in the total number and types of coelomocytes. As compared with control, maximum reduction in total number of coelomocytes (11.9×1^{04}) was recorded in worms exposed to highest combined dose of glyphosate and pendimethalin @1.50+2.50 µl/kg, followed by (15.1×1^{04}) and (24.6×1^{04}) coelomocytes in pendimethalin @ 3.00 µl/kg and glyphosate 5.00 µl/kg, respectively.

Keywords: Coelomocytes, *Eisenia fetida*, Eathworms, glyphosate, immune cells, pendimethalin

1. Introduction
Earthworms are prominent invertebrates belonging to family lumbricidae and are dominant in the temperate and tropical soils. They are hermaphrodite but self-fertilization does not usually take place. During laying eggs, the sexually mature worms exhibit distinctive epidermal ring shaped area called the clitellum, which has gland cells that secrete material to form a viscid, girdle like structure known as cocoon.

The immune cells in lower coelomate animals are known as coelomocytes eg. in annelida, arthropoda and mollusca. Coelomocytes are the immune competent cells of the earthworms which originated from the mesenchymal lining of the cavity (Bilej *et al.*2010) [1]. These cells are specially developed in earthworms (Cooper and Roch 2003) [3], which guard them from pathogens in the soil. Coelomocytes are the type of leukocytes which maintain the innate immune system of these lower animals (Hostetter and Cooper, 1972 [8]; Engelmann, *et al.*, 2005) [6]. These cells types are studied because they provide information about mechanisms governing innate immunity. Cellular immunity of earthworm depends largely on cytotoxic, phagocytic response and activities of phenol oxidase and antioxidant enzymes (Gautam *et al.* 2018) [7]. Cellular aggregation is an important metabolic behavior of coelomocytes and is often influenced by chemical stressors.

So keeping in view the above mentioned concerns, present investigation was planned with following objective.

To assess the effect of herbicides on qualitative and quantitative parameters of coelomocytes in *E.fetida*

2. Materials and Methods
2.1 Collection of the test animal
The culture of earthworm species *E. fetida* was perpetuated to use the third generation of earthworms to avoid the pre-exposure or residual effects of agrochemicals at vermicomposting unit of Department of Zoology, CCSHAU, Hisar. Mature and completely clitellate earthworms were used for experimentation to evaluate the effect of herbicides on growth and reproductive potential.

2.2 Collection of Substrate
The cow dung, used as substrate was obtained from Biogas Plant of Department of Microbiology, CCS HAU, Hisar. To rule out the deleterious effect of harmful gases and increase in temperature during vermicomposting, cow dung was pre decomposed for 15 days prior to study.

2.3 Procurement of Herbicides
Two herbicides glyphosate and pendimethalin were procured from Kisan Seva shop Hisar.

Table 1: Three treatments were given to the earthworm along with control

Sr. No.	Treatment	Concentration (micro litre/kg substrate)
1.	Control	0.00
2.	Pendimethalin	1.50, 2.25, 3.00
3.	Glyphosate	2.50, 3.75, 5.00
4.	Pendimethalin+Glyphosate	0.75+1.25, 1.13+1.88, 1.50+ 2.50

2.4 Coelomocytes in *E. fetida*
Sexually mature earthworms were taken from the treated tubs after 90 days of chemicals exposure. To avoid contamination, the gut of earthworms was cleared from organic matter by filter paper feeding method.
Earthworms were washed in running water and were then placed on the filter paper to remove excess water droplets. Coelomic fluid was obtained by means of electric shock (Cooper and Suzuki, 1995) [2], earthworms were subjected to electric shock of 6 volt for a period of 1 minute. Due to this shock treatment the coelomic fluid was extruded out through dorsal pores. The fluid was then diluted 1:100 with HBSS (Hanken´s Balanced Salt Solution), which was used in the laboratory to maintain cell and tissue structure and physiologic integrity.

2.5 Quantitative analysis of Coelomocytes
The coelomocytes were counted in Neubauer haemocytometer by the chamber method. This gives the coelomocyte density, i.e., cell number per µl. Then, this coelomocyte density was multiplied by factor three (volume of extrusion of fluid), to get the cell number per animal.
Total number of coelomocytes = cell number per µl× 3
The formula used to determine the concentration of cells in the sample suspensions by Neubauer haemocytometer is:

$$\text{Number of coelomocytes } \mu l^{-1} \text{ of suspension} = X \times 400 \times 10 \times 1000 \times D$$

Where,
X = Average number of cells per small square
400 = Number of small squares
10 = Depth factor
1000 = Conversion factor for mm^3 to cm^3
D = Dilution factor

2.6 Qualitative analysis of coelomocytes
A drop of the coelomic fluid was placed on a slide in order to make a thin smear and then dried at room temperature for 24 hours. After the slide had dried, it was stained with leishman's stain. The percentages of each type of coelomocyte were calculated by analyzing the slides under light microscope on the basis of morphology of coelomocytes such as amoebocytes, eleocytes and granulocytes.

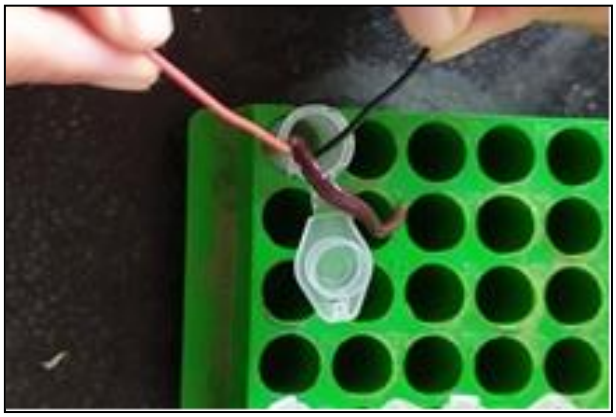

Plate 1: Electric shock given to earthworms

Plate 2: Coelomic fluid

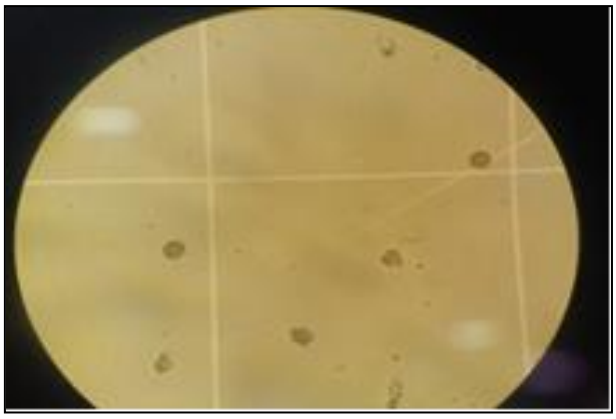

Plate 3: Coelomocytes of Eisenia fetida

3. Results and Discussion
Recorded data on the effect of different concentration of glyphosate and pendimethalin on the total number of coelomocytes of earthworms are presented in figure 1. The total number of coelomocytes decreased per unit (µl) of coelomic fluid after 90 days of exposure as compared to zero day of treatment. Mixed doses of glyphosate with pendimethalin exhibited synergistic toxic effect on total number of coelomocytes in comparison to individual exposure to both chemicals. The maximum reduction in total number of coelomocytes (11.9×10^4) was noted in worms exposed to highest combined concentration (1.50+2.50 µl/kg) of glyphosate and pendimethalin, followed by (15.1×10^4) Pendimethalin @3.00 µl/kg individually. Whereas, earthworms treated with glyphosate alone @2.50 µl/kg, 3.75 µl/kg and 5.00 µl/kg, the number of coelomocytes decreased up to 38.6×10^4, 35.2×10^4 and 24.6×10^4, respectively.

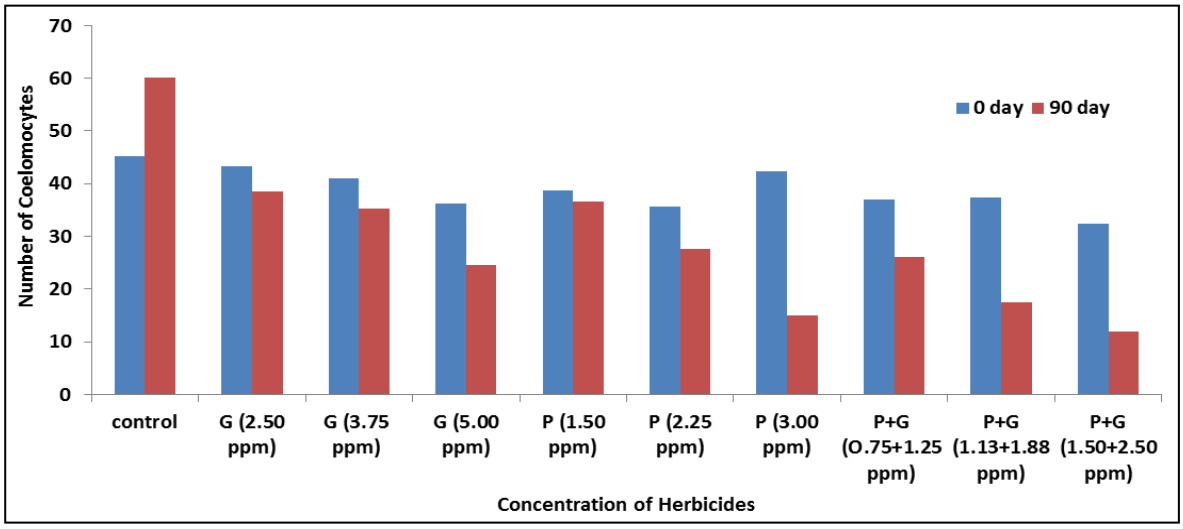

Fig 1: Effects of herbicides on total number of coelomocytes in *E. fetida*

Findings revealed that the coelomocytes of *E. fetida* are very sensitive and even at lowest sub-lethal doses of herbicides affected the count and type of coelomocytes and response was equal in dose dependent manner. Therefore, observations of the present study can be concluded in a way that coelomocytes can be used as a sensitive parameter for assessing the toxicity of herbicides. Reduction in number of amoebocytes imitate an exhaustion of propagation or formation of multicellular bodies encapsulating foreign bodies, being too large to be expelled during electro-stimulation for analyses. Our observations showed similarity with work of Muangphra *et al*, 2012 [9]. They also observed the comparative toxicity of glyphosate and paraquat. Their findings proved that glyphosate shows only aneugenic effects on coelomocytes of earthworms while paraquat shows both clastogenic and aneugenic, so paraquat was more toxic comparatively and both of these herbicides affected the microtubules and microfilaments of coelomocytes adversely.

3.1 Qualitative analysis of coelomocytes

Qualitative analysis of coelomocytes showed herbicides impacts on the total number of granulocytes-I, granulocytes-II, Amoebocytes-I, Amoebocytes II and eleocytes. Types and total number of coelomocytes decreased with exposure time and increased concentration of herbicides.

Chen *et al* 2012 [5] exposed worms to different concentrations of individual enantiomer of methamidophos for one day and their study concluded that the methamidophos could damage the immune system of the earthworms, as well as the neutral red retention times were significantly descended from 76.88 to 29.78 min with the increase in concentration of methamidophos. The lysosomes of healthy earthworms (control) can keep neutral red, but when the lysosomes membrane was injured, the neutral red was released and the coelomocytes became red. The neutral red retention average time of coelomocytes from healthy earthworms was 85.26 minutes. Whereas lysosomal membranes of worms was found significantly destroyed due to exposure to methamidophos.

3.2 Granulocytes-I

Granulocyte-I were lower in size and on their surface had fewer vesicles. Observations on the effect of glyphosate and pendimethalin on the total number of Granulocyte-I of earthworms exposed to doses of different concentration are presented in Fig. 2. The results showed that Granulocyte -I decreased with increase in concentration of herbicides. Doses of glyphosate and pendimethalin in combination were more toxic than individually, as the total number of Granulocyte-I, recorded on day one was 7.00×10^4 and it decreased up to 2.66×10^4 on the 90th day in worms exposed to highest concentration (1.50+2.50µl/kg) of pendimethalin+ glyphosate, followed by a decrease from 7.66×10^4 on zero day to 4.00×10^4 on 90th day in pendimethalin alone @3.00µl/kg. However, when worms were treated with glyphosate (5.00 µl/kg) the number of Granulocyte-I reduced from 8.00×10^4 on zero day to 5.33×10^4 on 90th day (Fig.2).

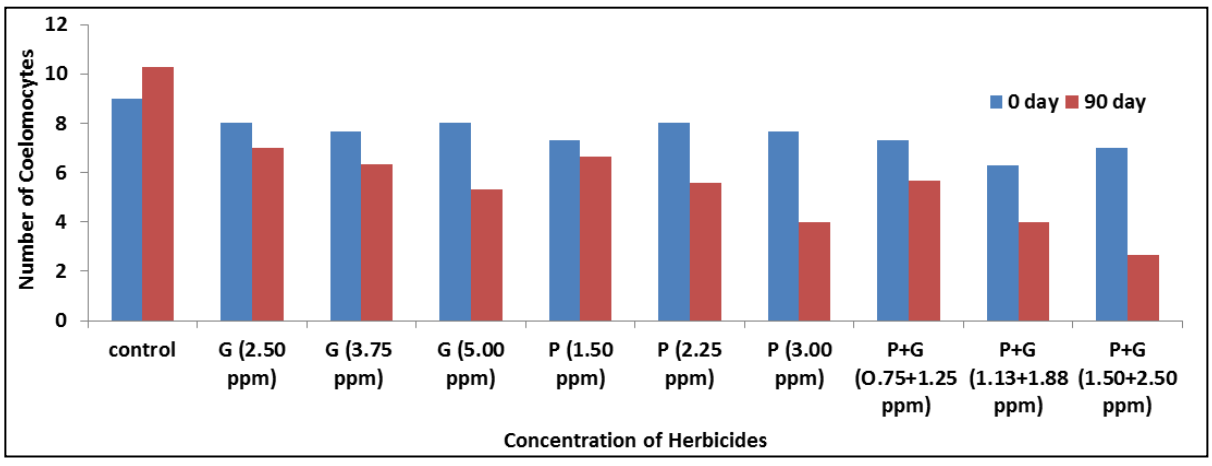

Fig 2: Effect of different concentration of herbicides on Granulocyte-I

3.3 Granulocytes -II

Granulocyte-II had characteristic vesicular structures on their surface. Observations on the effects of Glyphosate and Pendimethalin on total number of granulocyte-II of earthworms are presented in Fig. No.3. The Results showed that Graulocytes-II decreased with increase e in concentration of herbicides. Doses of Glyphosate and Pendimethalin in combination were more toxic than individually, as maximum reduction in counts of Granulocyte-II, (7.6×10^4) on zero day to the 2.00 $\times10^4$ on 90th day was noted in worms exposed to highest concentration (01.50+2.50µl/kg) of pendimethalin + glyphosate followed by a decrease in counts from 7.6×10^4 on zero day to 4×10^4 on 90th day on exposure to pendimethalin @ 3.00 µl/kg. The number decreased from 8.3×10^4 on the first day to 6.00 $\times10^4$ on 90th day on exposure to glyphosate @ 5.00µl/kg (Fig.3).

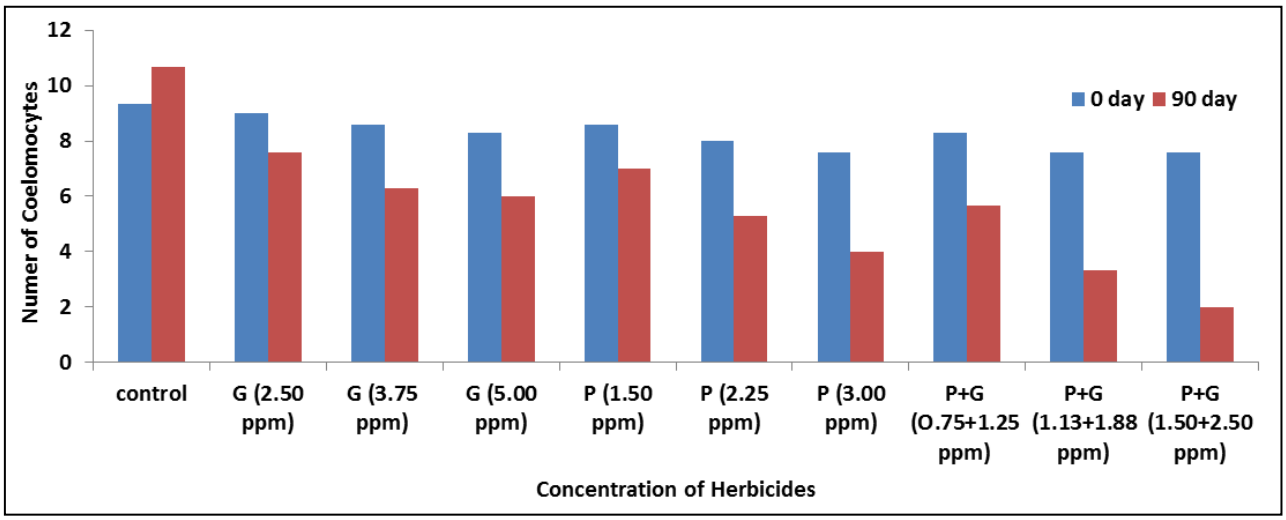

Fig 3: Effect of different concentration of herbicides on Granulocyte-II

3.4 Amoebocytes-I

Amoebocytes-I had pseudopodia distributed on the cell periphery. They had big, oval or sometimes slightly concaved nucleus which was centrally located and contained uncondensed chromatin with some heterochromatic lumps. Their cytoplasm had few lysosomes, many longitudinal or oval vacuoles and round vesicles. The results showed that number of Amoebocytes-I decreased with increase in concentration of herbicides. Doses of glyphosate and pendimethalin in combination were more toxic than individually, as maximum reduction in counts of Amoebocytes-II, (8.66×10^4) from zero day to the 2.30 $\times10^4$ on 90th day was recorded in worms exposed to highest concentration (01.50+2.50µl/kg) of pendimethalin + glyphosate followed by a decrease in counts from 8.00×10^4 on zero day to 4.33×10^4 on 90th day on exposure to pendimethalin @ 3.00 µl/kg and number reduced from 8.66×10^4 on zero day to 6.00 $\times10^4$ on 90th day on exposure to glyphosate @ 5.00µl/kg (Fig.4).

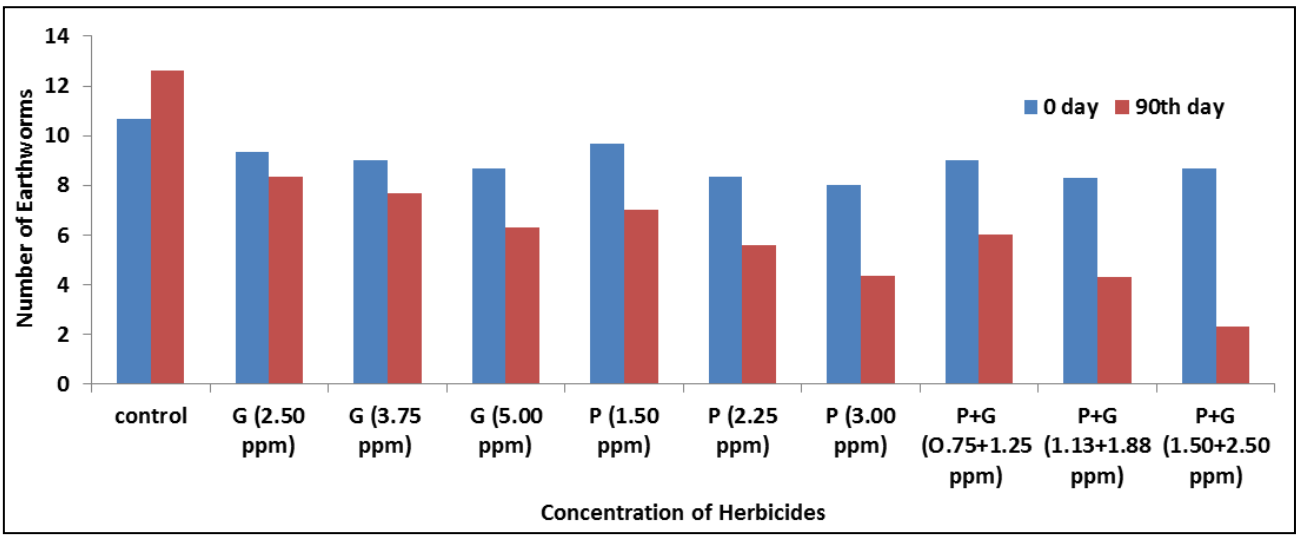

Fig 4: Effect of different concentrations of herbicides on Amoebocytes-I

3.5 Amoebocytes II

Type II amoebocytes had irregularly distributed pseudopodia, mostly concentrated on one pole of the cell. Nuclei were large and bean shaped having clearly visible nucleolus. Their cytoplasm was clearly differentiated in to homogenous ectoplasm and heterogeneous endoplasm.

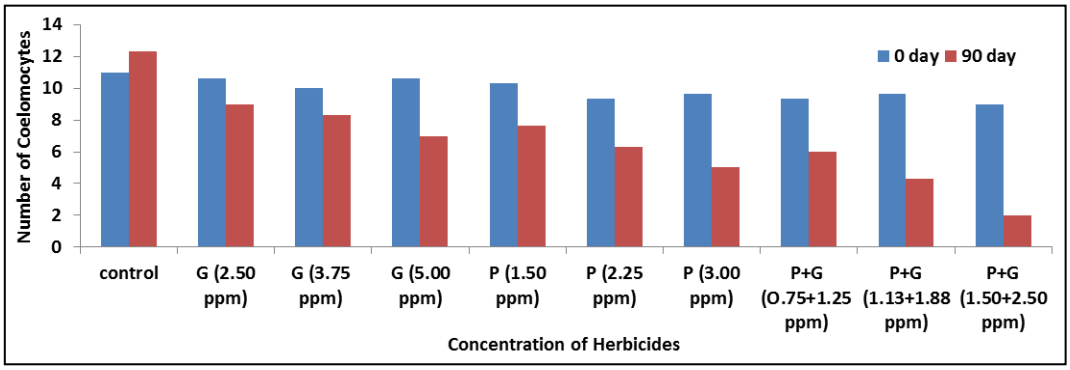

Fig 5: Effect of different concentration of herbicides on Amoebocytes-II

The endoplasm had many organelles such as mitochondria, lysosomes, phagosome vacuoles, free ribosomes and bundles of microtubules. The homogenous ectoplasm was lacking organelles but sometimes contains very few fine and electron light vesicles. The observation on effect of herbicides on Amoebocytes-II showed that as the concentration of herbicides increased, number of Amoebocytes-II decreased. Combined doses of glyphosate and pendimethalin were more toxic than individually, as maximum reduction in counts of Amoebocytes-II, (9.00×10^4) from zero day to the 2.00×10^4 on 90th day was noted in worms exposed to highest concentration (1.50+2.50µl/kg) of pendimethalin + glyphosate. A decrease in counts from 9.66×10^4 on zero day to 5.00×10^4 on 90th day on exposure to pendimethalin @ 3.00 µl/kg alone and from 10.6×10^4 on zero day to 7.00×10^4 on 90th day on exposure to glyphosate @ 5.00µl/kg (Fig.5).

3.6 Eleocytes

Eleocytes were round or oval and smaller than amoebocytes and granulocytes. They had low nucleocytoplasm ratio. The nuclei were small, spherical, eccentrically located and polymorphic granules were present in the cytoplasm. Latter was lack of organelles and clearly differentiated into homogenous endoplasm and heterogenous ectoplasm. The observation on effect of different concentration of herbicides showed that as the concentration of herbicides increased, the number of eleocytes decreased. Doses of glyphosate and pendimethalin in combination were more toxic than individual application of either of the herbicides. Maximum reduction in the counts of eleocytes (9.6×10^4) from zero day to 3.66×10^4 on 90th day was noted in worms exposed to highest concentration (1.50+2.50µl/kg) of pendimethalin + glyphosate followed. A decrease in counts from 9.66×10^4 on zero day to 7.00×10^4 on 90th day on exposure to pendimethalin @ 3.00 µl/kg alone and number reduced from 9.30×10^4 on zero day to 7.66×10^4 on 90th day on exposure to glyphosate @ 5.00µl/kg (Fig.6).

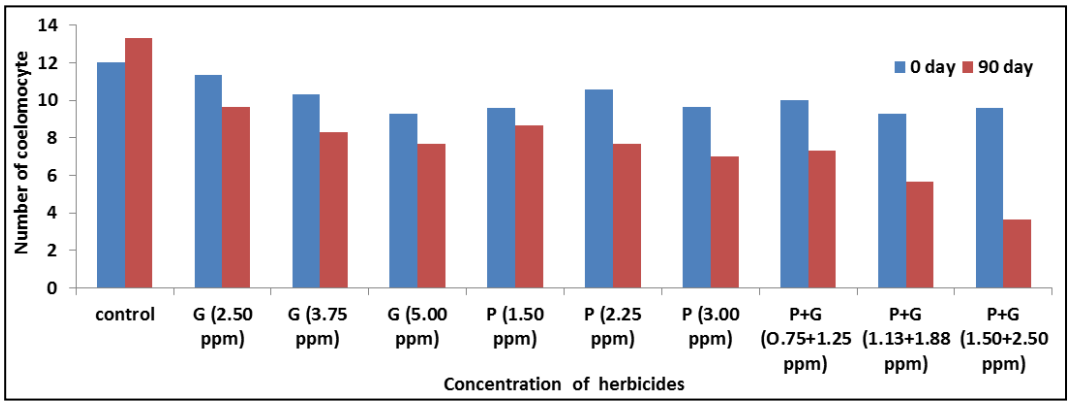

Fig 6: Effect of different concentration of herbicides on Eleocytes

The observation taken by Curieses *et al.* [4] in 2018 were similar to present investigation and they examined the toxic effects of two pesticides widely used at application rate recommended and over a non-target organism such as *E. fetida.* The results on viability response, DNA damage and trophic indexes showed that both pesticides exerted deleterious response in coelomocytes of *E. fetida* exposed *in vivo* and ex vivo.

Our findings are also supported by Ray *et al.* in 2019 [10], they investigated the toxicity of heavy metals (cadmium, chromium, lead, and mercury) on coelomocytes of earthworms and reduction in coelomocytes was reported by examining the amount of pro oxidants decrease in antioxidant enzymes activities which are the negative sign of immune compromisation of *M. posthuma.*

4. Conclusions

Total numbers of coelomocytes per unit volume (µl) of the coelomic fluid were calculated during present investigation and the results revealed that exposure of earthworms *(E. fetida)* to all concentrations of both herbicides caused a significant decrease in all type of coelomocytes i.e. Eleocytes, Amoebocytes and Granulocytes. Reduction in number of coelomocytes was dose and duration dependent. Maximum reduction in number of coelomocytes was recorded in combined dose of pendimethalin + glyphosate @1.50+2.50µl/kg followed by pendimethalin @ 3.00 µl/kg then followed by glyphosate @ 5.00 µl/kg in comparison to control.

The maximum reduction in total number of coelomocytes (11.9×1^{04}) was recorded in worms exposed to highest

combined dose of glyphosate and pendimethalin @ 1.50+2.50 µl/kg, followed by (15.1×1^{04}) and (24.6×1^{04}) coelomocytes in pendimethalin @ 3.0 µl/kg and glyphosate @ 5.0 µl/kg respectively.

5. Acknowledgement

The sincere vote of thanks is offered to the Department of Zoology, Chaudhary Charan Singh Haryana Agricultural University, Hisar for providing the essentialities timely for carrying out the research work.

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