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Original Article

Heavy Metal Pollution and Temperature Stress Induced Changes in Acetyl Choline Esterase Activity in Two Ecologically Different Earthworm Species

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Abstract

Heavy metal influx through applications of pesticides and chemicals to soils is alarming. Acetylcholinesterase is an enzyme participating in cholinergic system and breaks down acetylcholine into choline and acetate, terminating the neurotransmission process. Since earthworms are the first to encounter the metal stress, its important to study the alterations in neurotransmitter, acetylcholineesterase (AChE) activity. Inclusion of temperature in ecotoxicological studies is gaining importance due to its influence on organism's physiology. Hence, the present study highlights the influence of temperature on AChE activity in two ecologically different earthworm species viz., epigeic- *Eudrilus eugeniae* and aneic- *Lampito mauritii* when exposed to metal stress and temperature co-exposure . Both species exhibited an augmentation in the level of AChE was observed upon exposure to both metals (Zn^{2+} and Cr^{6+}). In *Eudrilus eugeniae* , Zn^{2+} co-exposures carried at 18^{o}C and 28^{o}C found to inhibit the activity on day 21 and 28. Chromium exposures carried out at 18^{o}C caused significant (p<0.05) augmentation in AChE activity on day 7, 14 and 21 and the observed % change found to be +95.75, +27.71 and +32.99 respectively; while a considerable suppression in activity was evident on day 28 (-29.17 %). In *Lampito mauritii*, significant (p<0.05) inhibition was evident in Zn^{2+} exposure at 18^{o}C and 28^{o}C explaining the influence of temperature in increasing the toxicity. Likewise, Cr^{6+} co-exposures carried out at 18^{o}C found to inhibit AChE activity on day 21 and 28 and similar, inhibition was evident in exposures made at 28^{o}C on day 7 and 28. Thus emphasising significant role of temperature in toxicological assessments.

Keywords: *AChE Activity, Eudrilus Eugeniae, Hexavalent Chromium, Lampito Mauritii, Temperature Toxicity, Zinc*

Introduction

Earthworms have direct contact with the soil and are frequently used in ecotoxicological investigations and are considered as useful sentinel creatures of soil contamination (Kammenga *et al*. 2000). Also, they play a significant role in soil formation and maintaining soil structure. Inoculation of earthworms could be a valuable strategy for accelerating soil formation and ecosystem development in degraded substrates (Pelaez-Sanchez *et al*., 2024). An essential nervous system enzyme, acetyl cholinesterase (AChE), is present at cholinergic synapses and neuromuscular junctions in the central nervous system. AChE is a member of the cholinesterase (ChEs) family of specialized carboxylic ester hydrolases that hydrolyze choline esters. It has been demonstrated that organophosphorus and carbamate pesticides target this site, irreversibly inhibiting AChE and causing an excessive build-up of acetylcholine, which in turn causes hyperactivity and damages the nervous and muscular systems. It has been demonstrated to stop nerve impulses by causing the neurotransmitter acetylcholine to hydrolyze into choline and acetate at the postsynaptic membrane and stop synaptic transmission by

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stopping nerve terminals from firing continuously. As such, it is necessary for the central and peripheral neural systems to function normally.

Few studies have shown the detrimental effects of heavy metals in inhibiting AChE activity in the following animal models, such as mussels (Najimi *et al*., 1997) and crustaceans (Diamantino *et al*., 2003). The majority of studies support AChE inhibition upon metal exposures in various groups of invertebrates. In earthworms, the primary cholinesterase is represented by acetyl cholinesterase. Only a few earthworm species have had its action discovered and biochemically characterized (Caselli *et al*., 2006). Propionylcholinesterase and non-specific ChE are the two forms of ChEs that have been identified in *Eisenia fetida* (Ribera *et al*., 2001). Calisi, Lionetto and Schettino (2011) report that the animal's pre-clitellar area had the greatest concentration of AChE activity, indicating a major function for this enzyme in the dorsal brain's functioning, which is situated close to the prostomium. The existence of AChE in *Eisenia andrei*, taking into account the substrate preference and the effects of various inhibitors was revealed by Caselli *et al*. (2006). Additional research by Rao and Kavitha (2004) showed that *Eisenia fetida* was exposed to two organophosphate pesticides, namely chlorpyrifos and azodrin, which resulted in a time-dependent inhibition of AChE. Similarly, research by Calisi, , Lionetto and Schettino (2009) showed that exposure to carbamate methiocarb insecticides significantly inhibited AChE in *Eisenia fetida*. Sanchez-Hernandez *et al*. (2018) reported changes in soil enzyme dynamics and AChE levels in earthworms as a result of chloropyrifos treatment. Temperature has an impact on Cu co-exposure, although Bednarska *et al*. (2017) observed no synergistic effect on AChE activity. In earthworms, AChE inhibition is currently AChE inhibition in earthworms is currently considered a significant early warning indicator of the harmful effects of pesticides (Booth & O'Halloran, 2001). It is also one of the biomarkers used to assess the early effects of pollutants on different groups of organisms in terrestrial ecosystems. Since earthworms are burrowing organisms and are subject to daily temperature fluctuations, it is important to take into account how temperature affects choline esterase activity. This study was started because the majority of ecotoxicological investigations show gaps in knowledge about the impact of temperature and how it modulates AChE activity.

Method

Earthworms

Eudrilus eugeniae were gathered from a vermicomposting plant in Dodaballapur, Bangalore which had no history of any chemical pollution. The species was chosen based on several criteria, including its size, abundance in reproduction, and tendency to live on the surface. *Lampito mauritii* is the most common species found in Bengaluru, and the specimens were taken from an area where pollution had never been present before. After being securely transported to the lab, the worms were allowed to acclimatise for one month in plastic culture boxes filled with a soil and farmyard manure combination (3:1) that had a pH range of 6.8 to 7.0 and a moisture content of 35 to 40%. The mature earthworms employed in this investigation had a fully formed clitellum. They were taken out of the culture boxes a day before using and let to empty their gut contents on Petri dishes with moist filter paper (in the dark). The selected worms were mature, clitellate and weighed between 400 and 600 mg and 1.0 and 1.2 g depending on the species (Basha & Latha, 2016).

Selection of metal concentrations and LC_{50}

The elements zinc (Zn^{2+}) and chromium (Cr^{6+}) are found in many soils and are essential to the proper operation of biological systems. When found in higher amounts, they may be toxicants. There are several oxidation states of chromium, but only two are stable in terms of biology and the environment: Cr^{3+}, which is non-toxic, and Cr^{6+}, which is poisonous (Ducros, 1992). For testing, a sublethal dose of 350 ppm Zn and 8 ppm Cr was chosen (Basha & Latha, 2016).

Preparation of experimental beds

The experimental beds were prepared by spiking control soil (sun-dried, urine free cowdung and air dried sieved soil in the ratio 3:1) with heavy metals Zn^{2+} and Cr^{6+}. Elemental composition of the soil bedding using atomic absorption spectroscopy revealed 1.29 ppm of Zn^{2+} and below detectable limits of Cr^{6+}. Soils were treated individually with solutions of potassium dichromate ($K_2Cr_2O_7$) and zinc chloride ($ZnCl_2$) to attain the necessary concentrations of 350 ppm Zn^{2+} and 8 ppm Cr^{6+}. Ten gut-extracted earthworms were put to each bed and then the beds were kept in an environment chamber at various soil temperatures (18℃, 24℃, and 28℃) and with a 14:10 light/dark cycle (Latha & Basha 2016). Temperatures were selected based on the annual reports of IMD (India meteorological Department) on variation in soil temperature in Bengaluru. According to OECD guidelines, six worms were chosen from random experimental boxes from each temperature and metal exposure for a further analysis at 7, 14, 21, and 28 days of exposure.

Acetyl choline esterase

Ellman *et al*. (1961) provided an estimate of acetylcholine esterase activity. A yellow 5-thio-2-nitrobenzoic acid anion is released when acetylcholine esterase hydrolyzes acetyl choline to produce acetic acid and thiocholine, which then quickly react with 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction mixture was incubated for 10 minutes at room temperature, comprising 3 ml of 0.1 M phosphate buffer (pH 8.0), 20 µl of 0.075 M acetyl thiocholine iodide, and 0.01 M DTNB. Tissue homogenate (0.1 ml) was then introduced and mixed by inversion. In a spectrophotometer, the color absorbance was recorded for five minutes at 412 nm, with 30-second intervals, and the linearity of the data was verified. The expression for the enzyme activity was "µmol of hydrolyzed substrate/min/mg protein."

Statistical analysis

Duncan's multiple range test (DMRT) with one-way analysis of variance was conducted using SPSS software (20.0 version) at (P<0.05) level of significance (Latha & Basha, 2016). Results were expressed as the mean ± standard error. Percentage change observed between control and treated groups are represented in parenthesis. "+" sign denotes increase and "-" sign denotes decrease with respect to control. The percentage change was calculated as:

$$Percentage\ change\ = \frac{Control\ -\ Experimental\ group}{Control} \times 100$$

Results

Data shown in fig. 1 illustrate the impact of temperature stress (18^{o}C, 24^{o}C, 28^{o}C) on the modulation of the heavy metal toxicity (Zn^{2+} and Cr^{6+}) in terms of changes occurred in the AChE activity in two ecologically different species viz., *Eudrilus eugeniae* and *Lampito mauritii* studied for different durations i.e., 7, 14, 21 and 28 days.

Eudrilus eugeniae

In *E. eugeniae,* the co-exposure of Zn^{2+} spiked soils at 18^{o}C found to have a significant (p<0.05) modulatory effect in augmenting the AChE activity on day 7 (+124.64 %) and marginal increase on day 14 (+6.27 %), while suppressed activity levels were evident on day 21 (-15.34 %) and 28 (-11.99 %). Zn^{2+} exposures carried out at 24^{o}C, exhibited a significant (p<0.05) exacerbation in activity on day 14 (+248.66 %) and 28 (+577.37 %) respectively, while an insignificant increase was evident on day 7 (+70.44 %) and 21(+8.55 %). However, Zn^{2+} exposures made at 28^{o}C exhibited a marginal increase in AChE activity on day 14 (+45.14 %) followed by a suppression in activity on day 7 (-34.74 %), 21 (-40.80 %) and 28 (-63.34 %) respectively.

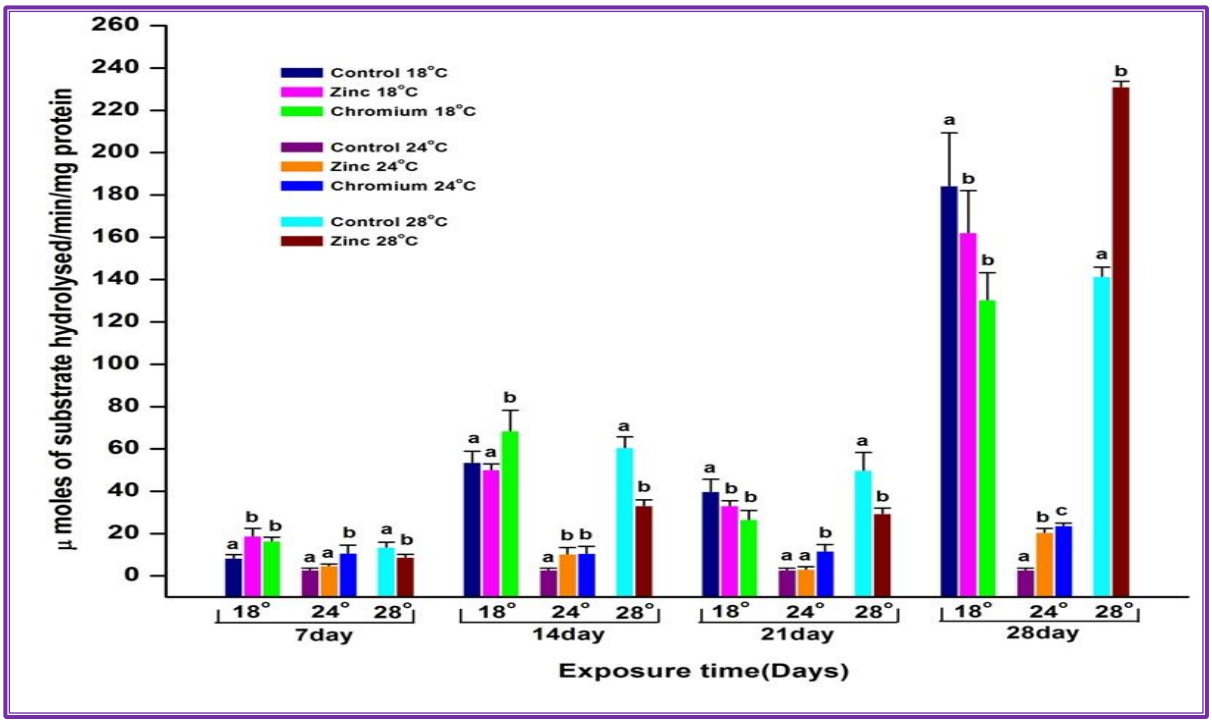

Figure 1: Impact of temperature stress (18ºC, 24ºC, 28ºC) on the modulation of heavy metal [Zn^{+2} (350 ppm) and Cr^{+6} (8 ppm)] induced toxicity in earthworm species '*Eudrilus eugeniae'.* Each Bar with SE (n=6) represents the activity levels of 'Acetyl cholinesterase' at different time intervals (7, 14, 21, 28 d) upon toxic exposure. Alphabets (a, b, c) represent statistical significance (p<0.05) between groups (One-way ANOVA, Duncan post hoc test. SPSS 20.0).

Chromium exposures carried out at 18ºC caused a significant (p<0.05) augmentation in AChE activity on day 7, 14 and 21 and the observed % change found to be +95.75, +27.71 and +32.99 respectively; while a considerable suppression in its activity was evident on day 28 (-29.17 %). Further, Cr^{6+}exposures at 24ºC exhibited a significant (p<0.05) exacerbation in AChE activity on all exposure days and the observed % change found to be +277.31, +257.0, +290.13, and +680.0 on day 7, 14, 21 and 28 respectively.

Lampito mauritii

Data presented in fig.2 depict the alterations occurred in the activity levels of AChE as a functional consequence of exposure to temperature stress and heavy metals. In species *L.mauritii*, Zn^{2+} exposures carried at 18ºC caused a significant (p<0.05) suppression in AChE activity on all exposure days and the observed % change found to be -22.43, -19.41, -38.11,-39.24 on day 7, 14 , 21 and 28 respectively. In contrast Zn^{2+} exposures carried out at 24ºC caused a significant (p<0.05) exacerbation in the activity on day 7 (+161.57 %), 21 (+207.02 %), 28 (+444.05 %) and a suppression in activity was evident on day 14 (-51.64 %). Whereas, Zn^{2+} exposures made at 28ºC exhibited a suppression in AChE activity on day 7 (-58.42 %), 14 (-73.25 %) and 28 (-20.14 %) while an exacerbation was observed on day 21 (+313.45 %).

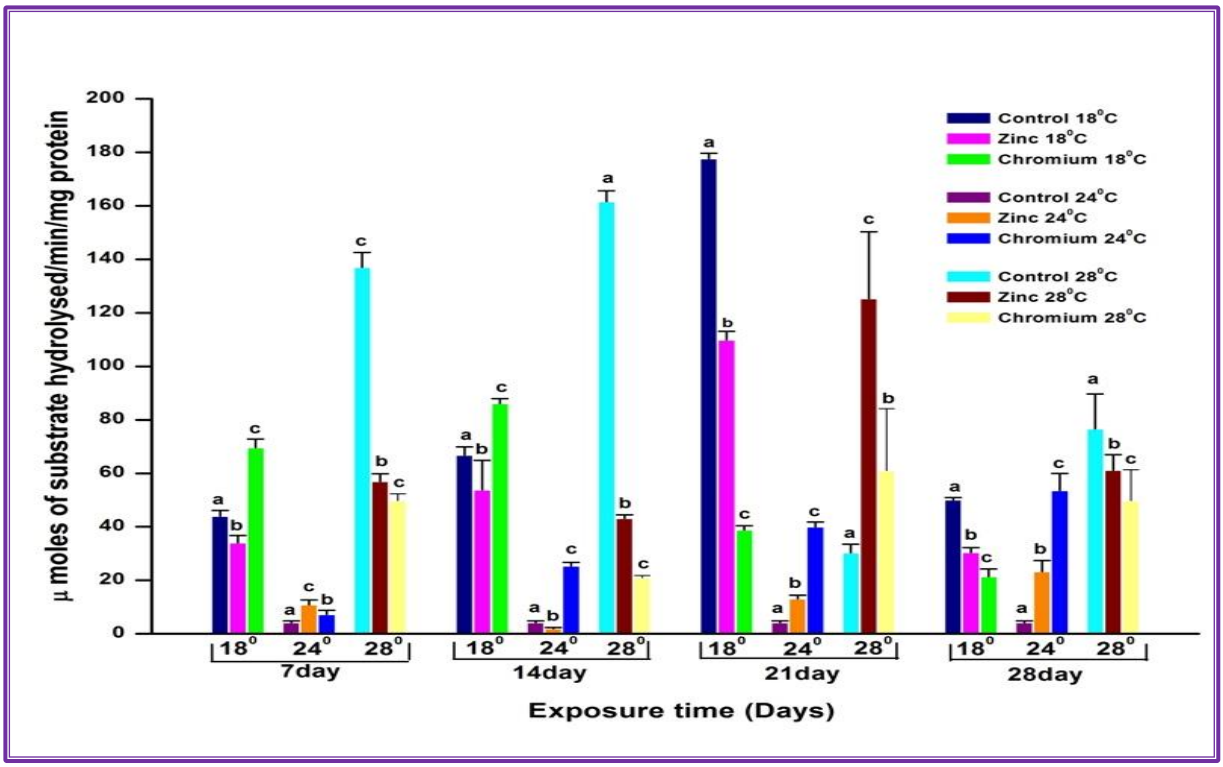

Figure: 2 Impact of temperature stress (18°C, 24°C, 28°C) on the modulation of heavy metal [Zn^{+2} (350 ppm) and Cr^{+6} (8 ppm)] induced toxicity in earthworm species '*Lampito mauritii*'. Each Bar with SE (n=6) represents changes in the activity levels of 'Acetyl cholinesterase' at different time intervals (7, 14, 21, 28 d) upon toxic exposure. Alphabets (a, b, c) represent statistical significance (p<0.05) between groups (One-way ANOVA, Duncan post hoc test. SPSS 20.0).

Cr^{6+} exposures made at 18°C exhibited a significant (p<0.05) augmentation in AChE activity on day 7 (+58.35 %) and 14 (+28.75 %) while a suppression in activity was found on day 21 (-78.13 %) and 28 (-57.27 %). Cr^{6+} exposures carried at 24°C caused an increase in AChE activity on day 7 (+72.55 %) while exacerbation was evident on day 14 (+497.65 %), 21 (+837.70 %) and 28 (+1149.41 %) respectively. Contrarily, significant (p<0.05) suppression in AChE activity was evident on day 7, 14 and 28 and the observed % change found to be -63.70, -87.0, -35.01 respectively, while an augmentation in AChE activity was evident on day 21 (+100.98 %).

Discussion

Acetylcholinesterase serves as a biomarker in earthworms, indicating neurotoxic effects of pollutants by measuring enzyme activity, which suggests chemical toxicity and impacts on neurocholinergic transmission (Singh *et al*., 2022). As a structural protein, AChE has been demonstrated to be involved in processes that are primarily unrelated to its enzymatic capacity to hydrolyze acetylcholine, such as axonal outgrowth (Bigbee *et al*. 2000), synaptogenesis (Sternfeld *et al*., 1998), cell adhesion (Bigbee & Sharma 2004), neuronal migration and hemopoietic stress responses (Grisaru *et al*., 2006), and apoptosis (Park, Kim & Yoo, 2004). AChE activity in earthworms plays a crucial role in their adaptation to environmental stressors like plant polyphenols, heavy metals, and microplastics by aiding in detoxification processes and maintaining homeostasis (Gudeta *et al*., 2023).

In this study a significant augmentation in AChE activity, was seen upon exposure to both Zn^{2+} and Cr^{6+} toxicity. In *Eudrilus eugeniae*, being an epigeic exhibited more sensitivity by inhibiting AChE while aneic, *Lampito mauritii*, showed delayed inhibition of enzyme activity. Likewise, Brown *et al*. (2004) also reported species- specificity in three marine invertebrate species *Patella vulgata*, *Mytilus edulis* and *Carcinus maenas* upon exposure to copper at identical exposure conditions and reported variations in responses of haemolymphatic AChE activity as indicated a significant augmentation in

Patella vulgata, and a significant suppression in *Carcinus maenas* and no effect in *Mitilus edulis.* Pertaining to this study, at specific temperatures the variability observed in responses suggests a great species-specificity and interaction occurred to modulate the toxic effect. Thereby the observations of this study corroborate with the findings of other investigations reported (Brown *et al*. 2004). Further, Narváez, Sabat & Sanchez-Hernandez (2022) highlights the detrimental impact of combined stressors (20 °C temperature, 25% moisture, and pesticide contamination) on earthworm populations and reported that earthworms exposed to the adverse environmental conditions, experienced a 50% mortality rate and a 49% loss in body weight. Additionally, Baetz, Schmidt & Tuerk (2022) demonstrated enzyme's sensitivity to various pollutants especially to detect neurotoxic effects from organothiophosphates in aquatic environments. Hence many authors suggest using AChE as a potential biomarker. As organisms are exposed to a variety of chemical and physical stresses in field conditions (Hagger *et al*. 2006), the use of multiple biomarkers can integrate overall physiological status with specific, molecular effects, providing a "diagnosis of stress". Therefore, understanding interactions between different enzyme systems is crucial for interpreting the toxic responses. In *Eudrilus eugeniae*, an augmentation in the level of AChE was observed upon exposure to both metals (Zn^{2+} and Cr^{6+}) while Zn^{2+} co-exposures carried at 18^{o}C and 28^{o}C found to inhibit the activity on day 21 and 28 highlighting the modulatory effect of temperature in augmenting the toxic potential of Zn^{2+}. In contrast, Cr^{6+} co-exposures carried at 18^{o}C exhibited an inhibition in AChE activity on day 28 highlighting combined impact of dual stressors in altering the function of cholinergic system.

In *Lampito mauritii*, a considerable augmentation in AChE activity was evident upon exposure to both metals, while a significant (p<0.05) inhibition in enzyme activity levels was evident in Zn^{2+} exposure at 18^{o}C and 28^{o}C explaining the influence of temperature in augmenting the toxicity. Likewise, Cr^{6+} co-exposures carried out at 18^{o}C found to inhibit AChE activity on day 21 and 28 and similar, inhibition was evident in exposures made at 28^{o}C on day 7 and 28 with exception on day 21 wherein increased AChE activity levels were observed. Likewise, Dzul-Caamal, Vega-López and Osten (2024) has reported higher AChE inhibition in glyphosate-polluted soils, indicating potential indirect effects on earthworm physiology.

Limitations

Earthworms have a simple, decentralized nervous system (a ventral nerve cord and ganglia), which limits the study of complexity of their responses. However, the future scope of acetylcholine esterase studies in earthworms has wide scope. It enhances the knowledge on basic neurobiological mechanisms that might be conserved across species and could provide a non-invasive method to assess soil health and the presence of toxic substances.

Conclusion

The study highlights the combined impact of dual stressors in altering the function of cholinergic system and emphasis on the need for multidimensional approach towards toxicity testing by involving abiotic factors like temperature and its role in assessing toxicity. Further, the toxic responses toward any pollutant is species specific, use of multiple species belonging to different ecological habitats is highly recommended in ecotoxicological monitoring and assessment of polluted sites.

Conflict of Interest

The author(s) declares no conflict of interest.

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