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Water Logging Affect Tissue Protein, Lipid Peroxidation and Enzyme Activities in the Epigeic Earthworm *Eudrilus eugeniae* (Kinberg)

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ABSTRACT
Oxygen deprivation caused due to water logging during flood could affect physiology of earthworms with significant increase in oxidative stress. This study reports the effects of short-term water logging of 12h and 24h duration with different water column heights and consequent depletion in oxygen level in water on survivability, tissue protein, lipid peroxidation (LPX) levels and activities of the enzymes, lactate dehydrogenase (LDH) and catalase (CAT) of the earthworm, *Eudrilus eugeniae*. The survivabilities were 30% and 15% under 5cms (T1) and 10cms (T2) water columns after 24h. With depletion of DO in the water, tissue protein of the earthworm indicated significant increase up to 12h but there after declined sharply up to 24h. Significant increase in LPX level, LDH and CAT activities were observed after 12h and 24h.The biochemical parameters of the animal showed significant variation between treatments and was found to be negatively correlated with DO of water column. It was concluded that hypoxia due to short-term waterlogging could seriously impair the metabolism and survival of earthworms.

Key words: Hypoxia, earthworm; survivability; oxidative stress; biomarker; antioxidant enzyme activity

INTRODUCTION

The life of soil organisms is considerably influenced by variations in environmental factors including moisture and temperature (Singh et al. 2016, Mishra et al. 2019, 2020). It has been reported that soil moisture considerably impacts physiology of organisms due to its role as a suitable solvent for uptake of nutrients by microbes and plants. An optimal soil moisture is also desirable for cutaneous respiration of certain groups of soil fauna including the earthworms (Wall et al. 2015, Blankinship et al. 2011, Eisenhauer et al. 2012). It is also a driving factor for the community composition and ecological functions of the soil biota. Climate change could bring in alterations in the soil moisture level due to flooding and drought which are likely to significantly impact soil biota (Thakur et al. 2018, Singh et al. 2019). Below ground ecosystems are likely to be influenced by excess of soil moisture with adverse impact on respiration, nutrient availability and decomposition rates (Mishra et al. 2020).

Epigeic earthworms live in and consume litter on the soil. In the top soil, microbial and faunal activities are dependent on moisture and temperature, which could lead to highly variable oxygen concentrations. In the top soil, oxygen deficiency could easily be compensated from the atmosphere. Therefore, epigeic soil fauna including earthworms have not evolved long-term adaptations to survive longer in hypoxic or anoxic conditions.

Terrestrial invertebrates cope with periods of hypoxia or anoxia caused by fluctuating soil moisture levels by altering their behavior, cellular and physiological functioning, and level of activity (Harrison et al. 2006, Hoback and Stanley 2001, Marx et al. 2009, 2012). Field studies have indicated that flooding causes significant reduction in earthworm abundance, biomass and diversity (Plum 2005, Plum and Filser 2005, Ivask et al. 2012, Kiss et al. 2021). It has also been reported that flooded soil can reach anoxic levels within as little as 24 h (Ponnamperuma 1984, Kiss 2019). Therefore, understanding how epigeic earthworms respond to decreasing oxygen concentrations in terms of oxidative stress is likely to throw light on their survival and adaptive mechanism in long- and short-term waterlogging.

Zorn et al. (2008) observed flooding induced avoidance behavior and significant loss of biomass

in three earthworms *Allolobophora chlorotica, Aporrectodea caliginosa* and *Lumbricus rubellus* in laboratory-controlled conditions. No information is available on how earthworms biochemically respond to the hydrological stress. While earthworms of all ecotypes are likely adapted to some degree of oxygen stress, the biochemical response of epigeic earthworms like *Eudrilus eugeniae* to hypoxic conditions due to waterlogging is not known. Therefore, this study was undertaken to evaluate the effects of oxygen deprivation caused due to short term waterlogging in a laboratory-controlled condition, on this earthworm in terms of survivability, tissue protein, lipid peroxidation (LPX) levels and activities of the enzymes, catalase (CAT), lactate dehydrogenase (LDH).

MATERIALS AND METHODS

Sampling of soil, earthworms and experimental design

Soil was collected from the top 0–10 cm horizon of an agricultural field of no prior history of agrochemical application, dried, sieved through a 5 mm mesh and stored at room temperature (28.6 ± 1.36ºC) for further use. The soil was sandy loam type. The characteristics of soil samples were analyzed (Gómez et al. 2014) prior to the experiment and has been presented in Table 1. The water holding capacity of the soil was measured by Boycos hydrometer and was found to be 36%. Distilled water was used to bring the soil to the required soil moisture content. 3kg soil was transferred to a total of nine experimental pots.

Clitellated earthworms (*E. eugeniae*) were sampled from the vermiculture facility of Odisha University of Agriculture and Technology and carried to the laboratory. Ten earthworms were transferred in to each experimental pot to acclimatize them with the soil conditions for 4 days. Moisture level was maintained at the desired level by intermittent addition of distilled water and monitored with the help of a digital moisture meter (OMEGA-HSM50). In total, three treatments were used in triplicate, C- control pot without water logging, T1- pot with 5cm water above soil surface and T2- pot with 10cms water above soil surface. In the sets for waterlogging, wire mesh (0.5mm) were placed just above the water surface to prevent escape of the worms and ensure that the animals remain inside soil and water for the experimental period of 24h.

Table1. Chemical characteristics of the experimental soil

Parameter	Value
pH	7.8±0.09
OC (%)	4.2±0.12
Nitrogen (g/kg)	6.8±1.1
Phosphorous (g/kg)	2.9±1.3
Potassium (g/kg)	6.2±1.5

DO of water columns and survivability of earthworms

The DO of water columns in waterlogging sets was measured using a portable DO meter (YSI 55; YSI Inc., Ohio, USA) at different time intervals. The percent survivability of the animals was calculated by counting the number of live worms in different experimental pots after 12h and 24h and calculating the percentage with respect to the number of animals released at the beginning of the experiment.

Survivability % = Number of live worms / Number of worms released

Biochemical assay

Biochemical analysis of the animal tissue was done after 12h and 24h. Randomly sampled worms from each experimental pot were sacrificed and weighed quickly to avoid post mortal tissue degradation. Whole body tissues were homogenized with phosphate buffer (0.05M, pH 7.4) after removing the gut contents. Aliquots were collected after 15-min centrifugation at 10,000 rpm in a cooling microfuge (REMI C-24BL). Aliquots of supernatant were stored at -20°C in a deep freezer (Celfrost) until further use. The method prescribed by Lowry et al. (1951) was adopted for estimation of tissue protein at 700 nm taking bovine serum albumin as standard. Protein was expressed as mg/g of tissue. LPX level was measured as per Ohkawa et al. (1979) at 532 nm through a spectrophotometer by measuring malondialdehyde (MDA) and expressed in nmoles/ mg protein. LDH activity was measured spectrophotometrically at 340 nm in a medium

containing phosphate buffer (pH 7.4), 7.5 mM NADH taking 30 mM sodium pyruvate as substrate (Cabaud et al. 1958). CAT activity was measured according to the method prescribed by Cohen et al. (1970) at 242 nm taking phosphate buffer (pH 7.4) and hydrogen peroxide using UV-VIS spectrophotometer (Systronics). The absorbance for this enzyme was taken at a 30-s time interval. Enzyme activities were expressed in U/mg protein.

Statistical analysis

Two way ANOVA of the data was conducted followed by Dunnett's multiple comparison test (alpha= 0.05, 0.1, 0.001 level) to determine the variation in biochemical parameters between C, T1and T2 over time. Correlation coefficient (r) was determined between DO of water column of different heights and biochemical parameters of the earthworm at 0.05 level of significance using correlation matrix and principal component analysis (PCA). Student's t-test was conducted to observe the significance of difference in the DO of water columns over the experimental periods. All the statistical analyses and graphs were done through XL-STAT and Graph Pad Prism 8 software package (Graph Pad Software, Inc., La Jolla, CA).

RESULTS AND DISCUSSION

The earthworms in C remained inside the soil over the experimental period of 24h. However, in T1 and T2, the animals wriggled out of soil and floated in water after 7-8 h of water logging and tried to escape. However, due to the wire mesh barrier above the water surface, the animals were forced to remain in water till they survived. The survivability of the earthworms was 30% in T1 and 15% in T2 after 24h. No survivability was recorded after 28h and 32h of flooding in T1 and T2, respectively.

The DO decreased by 39% and 22% in T1 and T2, respectively, after 12 h of water logging. T1 indicated the maximum (93%) depletion in DO after 24 h (Fig. 1). Student's t-test indicated significant difference ($p<0.05$) in DO of water columns over the experimental period.

The protein content in earthworms in T1 and T2 were 143.3±6.03, 221.09±5 mg/g tissue after 12h of flooding in comparison to C where it was 146.6±5.3 mg/g tissue. After an initial increase in protein levels in both T1 and T2 at 12h, it declined to 124.8±5.7mg/g tissue and 119.8±6.2 mg/g tissue respectively at 24h (Fig. 2a). The variation in protein contents

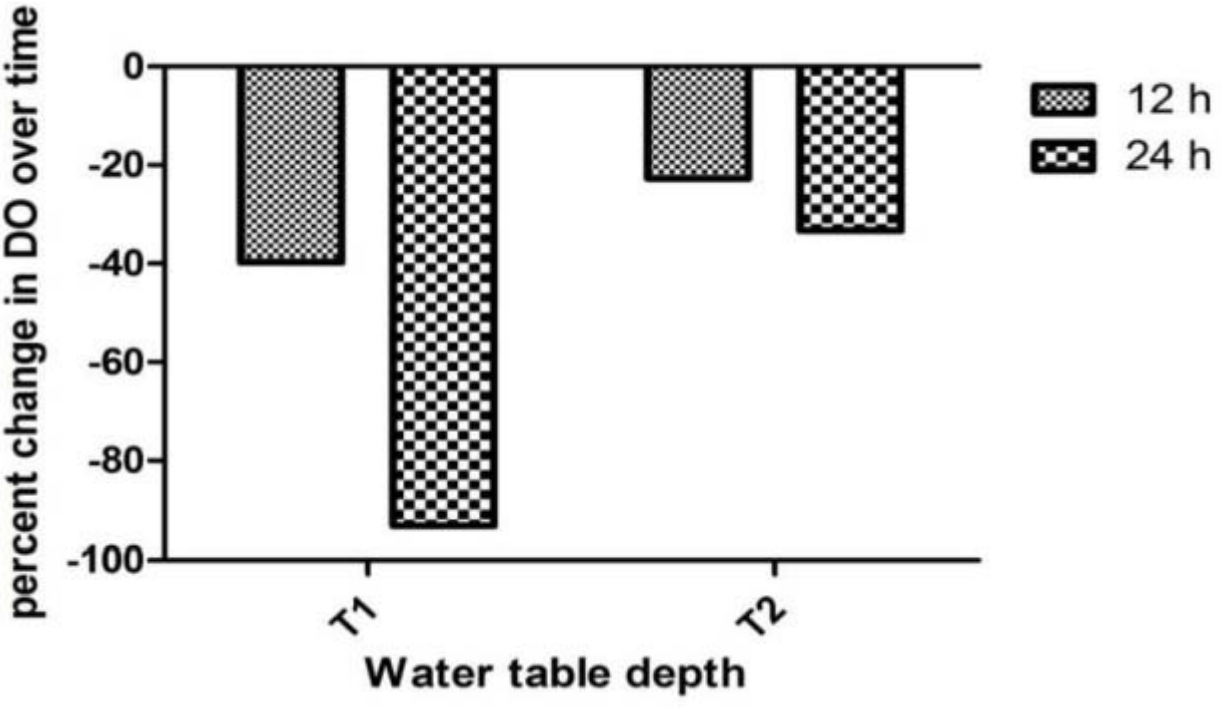

Figure 1. Change in DO (%) of water columns after 12h and 24h

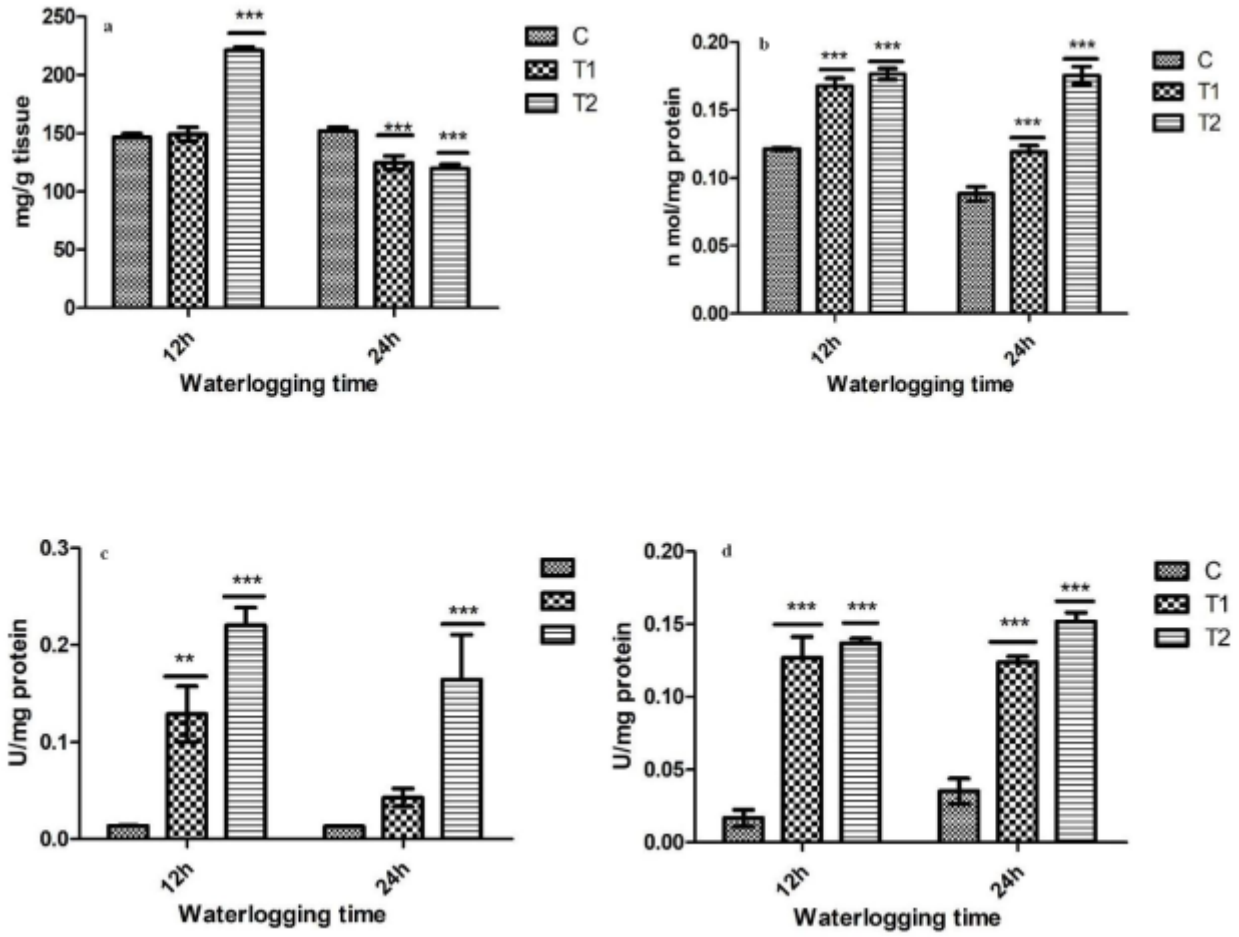

Figure 2. Biochemical alterations in *E. eugeniae* in response to water depths and flooding time. a) Protein, b) LPX, c) LDH, d) CAT. Variations were statistically significant at * (P<0.05), ** (p<0.01), *** (p<0.001)

between C, T1 and T2 was highly significant (p<0.001).

A significant increase in LPX level of the earthworm was noticed in T1 and T2 at 12h and 24h of water logging (p<0.001). The LPX level in C was 0.12±0.009 nmol/mg protein. It increased in T1 to 0.16±0.01, 0.11±0.08 nmol/mg protein at 12h and 24h of water logging (Fig. 2b). The maximum LPX level (0.175±0.004 nmol/mg protein) was recorded in T2 at 24h.

The LDH activity of *E. eugeniae* in C was 0.013±0.005 and 0.013±0.004 U/mg protein at 12h and 24h, respectively. The enzyme activity increased in both T1 and T2 over time. The activities were 0.12±0.02 and 0.22±0.03 U/mg protein in T1 and T2 after 12h. In T2, the enzyme activity increased to 0.16±0.08 U/mg protein at 24h (Fig 2c). The variation between C, T1 and T2 at 24h was statistically significant (p<0.05).

CAT activity of the earthworm increased under water logging conditions. The activity was 0.12±0.02U/mg protein and 0.13±0.006 U/mg protein in T1 and T2, respectively, at 12h. The highest enzyme activity was in T2 (0.15±0.01 U/mg protein) at 24h (Fig 2d). The variation in enzyme activity between C, T1 and T2 was highly significant (p<0.001).

The DO of water column indicated significant negative correlation with CAT activity of the earthworms (r= - 0.82, p<0.05) which has been depicted in correlation matrix colour map (Fig. 3a). Different colours in correlation matrix map presented the correlation coefficient values. Five variables were reduced to a few principal components to study the principal component analysis (PCA). Two components were extracted and those two components accounted for 90% of the total variance. The Eigen value against the component number was

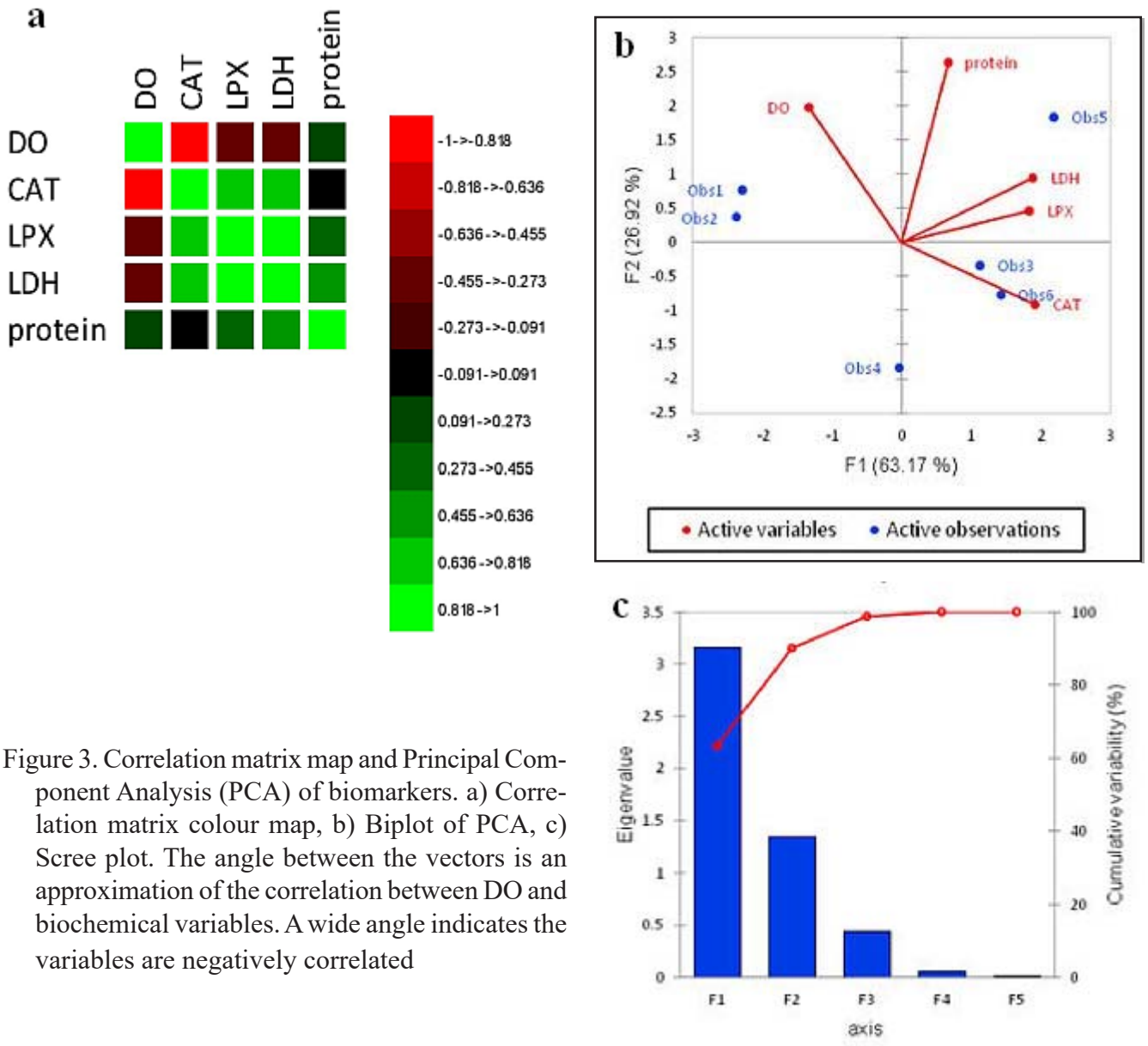

Figure 3. Correlation matrix map and Principal Component Analysis (PCA) of biomarkers. a) Correlation matrix colour map, b) Biplot of PCA, c) Scree plot. The angle between the vectors is an approximation of the correlation between DO and biochemical variables. A wide angle indicates the variables are negatively correlated

taken to create the scree plot. From the third component on, we observed that the line was almost flat indicating that each successive component accounted for smaller amounts of the total variance. Eigen values greater than 1 were taken into account. In the present study wide angles between the parameters indicated negative correlation (Fig. 3 b, c).

Metabolism in majority of the living organisms depended on availability of oxygen. However, certain organisms are adapted to survive in an oxygen deprived environment (Turrens 2003). Due to various reasons, organisms might face conditions ranging from mild deficiency to complete absence of oxygen in their habitats. Information is available on physiological and biochemical alterations in animals in response to oxygen deficient environment (Storey and Wu, 2013). For ensuring survival in a hypoxic environment, organisms might opt for transient metabolic depression with significant reduction in ATP generation (Turrens 2003, Hermes Lima et al. 2015).

Deficiency of O_2 in sea water significantly influences marine invertebrates with reduced respiration and growth (Pörtner and Grieshaber 1993, Pörtner et al. 1998, Pörtner et al. 2005, Steckbauer et al. 2015). O_2 deficiency coupled with an increased CO_2 concentration in water impact transmembrane ion exchange with consequent reduction in metabolic processes in marine invertebrates (Reipschläger et al. 1997). It has been earlier reported that protein

synthesis and ATP production in mammalian tumor cells are suppressed with depletion in O_2 supply (Sorensen et al. 2015). Preedy et al. (1985) had observed that in rat, protein synthesis is inhibited in the tissues when the animal was exposed to hypoxic conditions relative to control. Reports are rare on how hypoxia impacts tissue protein in invertebrtes. The results obtained in the present study indicating consistent decrease in protein level of *E eugeniae* after 24h in O_2 deficient conditions is more or less similar to these earlier reports. The initial increment in the protein level at 12h in the earthworm was possibly due to accumulation of stress protein in the body tissues.

Studies have shown increased lipid peroxidation in the tissues of aquatic invertebrates under hypoxic conditions (Luschak et al. 2005, Oliviera et al. 2006, Giraud-Billoud et al. 2011, 2013, Perez-Jimenez et al. 2012, Woo et al. 2013). Pannunzio et al. (1998) had observed increased lipid peroxidation in foot muscle of the marine gastropod *Littorina littorea* under anoxia (Welker et al. 2013). Significantly high lipid peroxidation was also observed in the hepatopancreas of the crab *Chasmagnathus granulata* due to O_2 deficiency (Oliviera et al. 2006). The explanation was that the residual O_2 present in the tissues during anoxia triggered ROS formation. The enhanced levels of LPX of *E eugeniae* in hypoxic stress in the present study corroborate these earlier reports.

LDH is a crucial enzyme of anaerobic metabolism as it is responsible for the regeneration of NAD^+ which sustains glycolysis in the absence of oxygen. LDH in the O_2 deficient conditions converts pyruvate to lactate. Marx et al. (2009) reported that in soil microarthropods, lactate content in tissues of collembolan species increased significantly during anoxia. Hodkinson and Bird (2004) had earlier reported similar results for arctic microarthropods. Glycolysis with modest production of lactate could facilitate post-hypoxic recovery, as discussed by Wegener (1993). Marti et al. (1994) had observed significant increase in the LDH activity in cultured mammalian smooth muscle cells in an oxygen deficient environment. Identical results were obtained in the cerebro-cortical neurons of rat where O_2 deficiency of 3 days enhanced LDH activity significantly relative to control (Malthankar-Phatak et al. 2008). The enhanced levels of LDH activity in the body tissue of the earthworm in the present study under flooding stress can be linked to the requirement of higher lactate production in the earthworm tissues in hypoxic conditions.

It has been reported that anoxia or hypoxia exposure in several other organisms induces increased activity or gene expression of antioxidant enzymes. Leopard frogs (*Rana pipiens*) under 30h anoxia showed increased activities of CAT in muscle and heart (Hermes-Lima and Storey, 1993). The gold fish (*Carassius auratus*) under anoxia showed increased CAT activity in liver (Lushchak et al. 2001). The crab (*C granulata)* under anoxia showed increased CAT activities in both anterior and posterior gills (Oliviera et al. 2005). Common carps (*Cyprinus carpio*) under hypoxia for 5h presented increased CAT activity in brain tissue (Lushchak et al. 2005). Exposure of the amphipod *Monoporeia affinis* to hypoxia induced a rise in the activities of this enzyme (Gorokhova et al. 2010, 2013). *Balanus amphitrite* barnacles under anoxia and hypoxia indicated a sharp increase in CAT activity in larvae and adults (Desai and Prakash 2009). In the pupae of the Caribbean fruit fly *Anastrepha suspense*, CAT activity remained unchanged in response to1h anoxia (Lopez-Martinez and Hahn 2012). All these results are more or less identical to the results obtained on the CAT activity of *E eugeniae* in O_2 deficient environment.

The results of the present study thus indicated that epigeic earthworms like *E eugeniae* are not adapted adequately to survive for long period of time in a hypoxic environment created by waterlogging. These earthworms are likely to remain within soil for few hours, utilizing available oxygen, but as the soil gets super saturated with excess moisture, there is rapid depletion of oxygen and the animals come out of soil in to the water for respiration. As the dissolved oxygen in the water depletes, the earthworms suffer from significant metabolic depression with enhanced oxidative stress. The earthworms could survive as long as they are able to counter the stress and in the absence of an escape route, they die. The present study also indicated that CAT activity in *E eugeniae* could be considered as the potential marker to evaluate oxidative stress due to water logging

induced hypoxia. The results from this laboratory study can also be correlated to the findings of Kiss et al (2021) who reported that earthworm abundance in the crop and pasture soils and total earthworm biomass was significantly lower in the frequently flooded areas than in the rarely flooded areas.

CONCLUSION

Earthworms with cutaneous respiration are sensitive to conditions which could result in short supply of oxygen. Earthworms with limited migratory ability cannot escape flooding and are therefore likely to be exposed to an oxygen deficient environment. Epigeic earthworms have not developed effective adaptation to survive for a longer period under oxygen deficient conditions and therefore are susceptible to physiological stress which could inhibit their normal ecological functions and even survival. A longterm investigation on how different species of earthworms respond to environmental stressors such as flooding in diverse terrestrial ecosystems will help in understanding the degree of their adaptation and the mechanism behind it.

Authors' Contributions: All the authors contributed equally

Conflicts of interest: Authors declare no conflict of interest

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