

Identification of earthworms using *cox1* gene and assessing the toxicological responses of an endogenic earthworm *Metaphire nanaoensis* exposed to heavy metal lead (pb) from paddy fields of Assam, Northeast India

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Abstract

Earthworms being the allies of farmers increasing the porosity and fertility of agricultural soil. The current work was to investigate the diversity of earthworm along with the effect of Lead (Pb) toxicity on earthworms in the paddy fields of Assam. Being the biodiversity hotspot, there is a keen interest of the researchers to explore the cryptic, novel earthworm species in the paddy fields. The current exploration reveals eight different species of earthworm with accession number *Eudrilus eugeniae* (OP999656); *Metaphire nanaoensis* (OQ073567), *Polypheretima elongata* (OR053673), *Perionyx depressus* (OQ073568), *Amyntas morrissi* (OR518282), *Amyntas phaselus* (OR518347), *Metaphire formosae* (OR518417) and *Eudrilus eugeniae* PBP (OR053675) belonging to two dominant family Megascolecidae and Eudrilidae. The Shannon diversity index (H') was found to be 2.045. These open up into a new heterogeneity and more integrated approaches is required to explore the unknown which is yet to discovered in the near future. Acute toxicity was evaluated using filter paper test with an endogenic earthworm *Metaphire nanaoensis* exposed to heavy metal Lead (Pb) with LC_{50} found to be 0.5462 mg/ml. Morphological changes like swelling of body, mucous secretion was observed when exposed at highest concentration of 0.1mg/ml of Pb. The growth was seen to be minimum at 100 ppm on 25th day of Pb exposure which were significantly less ($p < 0.05$) in soil toxicity test. This would help in assessing the toxic potential of Pb and environmental impact with such heavy metal.

Keywords: Earthworm diversity, paddy fields, acute toxicity, Assam Northeast India

Introduction

The natural productivity of agricultural systems is often influenced by the diversity of life on earth. Earthworm populations in agro-ecosystems are extremely susceptible to the physio-chemical characteristics of soil, which may have impact on the resources necessary for earthworm survival (Singh *et al.*, 2016) [27]. By regulating the concentration of organic matter, decomposition and nutritional output, it also alters the soil's porosity (De Wandeler *et al.*, 2016) [8]. Periodically, it seems intensive agriculture methods are seriously harmful to both human and soil health (Bertrand *et al.*, 2015) [5]. The extensive application of chemical-based fertilizers and pesticides endangers the ecotoxicological health of earthworms in many developing nations like India. Earthworms being the ecological engineers originated during the Precambrian epoch of the phylum Annelida, class Oligochaeta (Darwin 1890). The biological profile and interspecific dynamics of the soil ecosystem are impacted by the earthworm's functional interactions with these flora and fauna (Ray 2018) [20]. The species of earthworms are classified as epigenic, endogenic, and anecic based on their morpho-ecological habitat (Yadav and Mullah *et al.*, 2017) [34]. The species that live in the top soil, which is rich in decomposing organic materials, are the epigenic species. Endogenic species are underground and prefer to eat soil that is rich in organic content. They typically dig horizontal burrows. Anecic creates vertical, permanent burrows that go deep into the ground.

Dealing with earthworm morphological types it presents a taxonomic challenge that can be resolved through the application of DNA barcodes. The sequences of two morphologically similar species might be compared using the cytochrome-c oxidase I (COX-1) gene (Huang *et al.*,

2007; Shekhovtsov *et al.*, 2017) [12, 26]. Additionally, COX1 DNA barcode eventually led to the formation of a reference library that could be used to place unidentified species in their appropriate matching (Porco *et al.*, 2018) [19]. Different soil habitats, such as alluvial soil and agricultural soil, need to be studied in order to investigate and characterize more earthworm fauna. A recent work reported that out of eight species, five species of *Metaphire posthuma* (Vaillant, 1868), *Lampito maruitii* (Kinberg, 1866), *Perionyx pulvinnatus* (Stephenson, 1916), *Perionyx excavates* (Perrier, 1872) *Amyntas diffringens* (Baird, 1869) and *Perionyx pulvinnatus* (Stephenson, 1916) all of which belong to the dominant Megascolecidae family in Assam (Saikia *et al.*, 2021) [22].

Assam Northeast, India is one of the major ecological hotspots and is known as a hub for the ongoing evolution of numerous unique gene pools. It serves as the meeting place for the exceptional biological resources of the Indo-Burman and Himalayan biodiversity regions. The Eastern Himalaya Region in India has been noted for having a great diversity of earthworm species, with 128 species documented and 72 indigenous species (Tiwari *et al.*, 2019) [31]. The soil macro fauna has drawn a lot of interest because of their advantageous effects, especially in the agricultural sectors. Earthworms referred as cultivators of land, serve as a useful role in the agricultural ecosystem. They improve soil quality for plant growth by lowering soil acidity and alkalinity (Deepthi *et al.*, 2016) and are known for increasing the soil porosity and structural stability.

Due to industrialization, heavy metal pollution in soil has become a major concern as it has detrimental effect on both lower and higher organisms (Ray *et al.*, 2019) [21]. Heavy metal has negative consequences in the earthworms

resulting in alteration of enzymatic activities, DNA damage, damage of internal organs etc. Heavy metals are toxic to metal-sensitive enzymes resulting in acute or chronic conditions in soil-dwelling organisms (Ali *et al.*, 2019)^[1]. Earthworms are highly susceptible to soil contamination due to their porous surface of skin and direct contact with soil through their gastrointestinal tract. Reports shows that earthworms are usually used for eco-toxicological investigations (Yadav *et al.*, 2023). Prolong exposure heavy metals can have detrimental effects on earthworm survival, morpho-physiological changes (Uwizeyimana *et al.*, 2017)^[32]. Lead (Pb) contamination in agricultural soils can originate from historical use of lead-based pesticides, atmospheric deposition from industrial emissions etc. (Yeboah *et al.*, 2019)^[35]. Although a number of approaches have been used to document and address this issue, it is still a troubling one. Baruah *et al.*, (2021)^[2] and Bora *et al.*, (2024)^[3] observed high concentration of Pb in the semi-urban paddy fields of Assam. Pb contamination in the river ecosystem thus posing a significant threat to the agri-ecosystem was studied by Bhuyan *et al.*, in (2023). Due to some challenging topographical conditions, information regarding the diverseness of different dominant earthworm species in paddy fields of Assam, Northeast India has been lacking. We still have limited understanding of earthworm diversity, community organization and distribution patterns over biodiverse agricultural ecosystem in Assam. Being the significant biological indicators of soil pollution, an attempt has been made to study the ecotoxicology of earthworm exposed to relevant concentration of Pb and to explore the variety of earthworm communities in the paddy fields of Assam. The study discovers different species and argues that the northeastern region has many newly discovered or undiscovered species and supports different ecosystems of both flora and animals. This work would further highlight the toxic effect of heavy metal in soil-dwelling organisms.

Materials and Methods

Experimental Sites

The current study was carried out in the Kamrup district of Assam located in (N26°4.8172', E91°33.5767'). Kamrup district being the hotspot for the urban industrialization as well as a major semi-rural area for the livelihood. Samples from different paddy fields were collected and transferred to experimental sites. The fertile soil is attributed to the great Brahmaputra that flows across the state.

Collection and Analysis of Samples

Earthworms were collected and obtained by hand-sorting method along with the soil samples from the paddy fields of Kamrup district (Julka 1988)^[14]. At the sampling site, a quadrant is randomly designated, and the edges are cut by removing the soil depth 12 to 18 cm. An average of five to ten earthworms were statistically collected from the specific site. The earthworms were picked up and were sorted out. The morphological characteristics such as color of earthworms, width and length of the earthworms were also noted at the time of collection and transferred to experimental sites (Julka 1976). The different physio-chemical characteristics of soil like pH, total organic carbon, total nitrogen content and phosphorous content was measured from the collected locations (Baruah *et al.*, 2021; Bray and Kurtz 1945)^[2, 6].

Analysis of Morphological Characteristics of Collected Earthworms

The morphological characteristics like body color and size, segments, clitellum, prostomium were recorded under microscope in the laboratory. The earthworms were first given a 5–10% ethyl alcohol anesthesia. They were then placed down dorsally in a dissecting tray and a transverse incision was made. The anatomical characters such as beginning of the intestine, gizzard, crop and calciferous glands were noted. The earthworm taxonomical characteristics were determined using keys of (Stephenson 1923; Julka 1988; Gates 1972)^[10, 14].

Molecular Identification of the Earthworms

Molecular analysis was carried by CTAB method with slight modification. The pellet thus obtained by discarding the supernatant was air dried, and cleaned with ethanol. The pellet obtained was dissolved in 30-50 µl of T.E. buffer.

Using standard universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') the amplification of cytochrome oxidase I (COX1) gene of earthworms were amplified (Folmer *et al.*, 1994; Dey *et al.*, 2018). The amplification reaction was set for 20 µl, at 94°C for 2 min, followed by 36 cycles at 94°C. The annealing was set at 54°C for 30 sec which was followed by the extension and final extension at 72°C for 10 min. The product obtained was sent for sequencing for identification of different earthworm species. The sequenced obtained was submitted in NCBI. Phylogenetic tree was constructed by MEGAX 11 software using bootstrap method.

Acute Toxicity Test

The adult earthworms (at least three months old with matured clitellum) with an individual weight of 200 to 500 mg and length of 9-14cm were subjected to Acute toxicity test using filter paper following to OECD (1984) guidelines for testing of chemicals no. 207. The experiments were conducted using petri plates of 8cm in length and 3cm in diameter. A relevant concentration for Lead in the salt form Pb (NO₃)₂ (0.02, 0.05, 0.07, 0.09, 0.10) mg/ml was taken for the experiment. 15 replicates (n=15) were taken for each replicate consisting of one earthworm per petri plates and control (c) without heavy metal. The test was performed based on slight modification following OECD protocol (Yue *et al.*, 2021; Duo *et al.*, 2022)^[9, 36].

Soil Toxicity Test

In soil toxicity test, tested air-dried natural soil of 1 kg was placed in earthen pots size of 13*15 cm. Heavy metal lead (Pb) at relevant concentrations (70,90,100 ppm) were mixed with dry soil following OECD guidelines (Yue *et al.*, 2021; Duo *et al.*, 2022; Hullot *et al.*, 2021)^[9, 11, 36] and incubated for 15 days with some modification as shown in Fig 13. After incubation, the prepared inoculated pots with heavy metals were used for 30 more experimental days. (E+ HM) with both earthworms and heavy metal and for control treatment (C) with only earthworm and without heavy metal (HM) was used for the experimental type. About 10 adult endogenic earthworms (at least three months old with matured clitellum) with an individual weight of 200 to 500 mg and length of 9-14cm were subjected to the experimental pots. The water was sprinkled at regular intervals after every 2 days. The mortality, avg. wt. gain/ loss and rate of

earthworms in the pre-incubation treatment was monitored for every 5 days.

Statistical Analysis

All the data are expressed as mean±S.E in triplicate by using software XLSAT 2023 version and excel. The value less than p value (p<0.05) is significant.

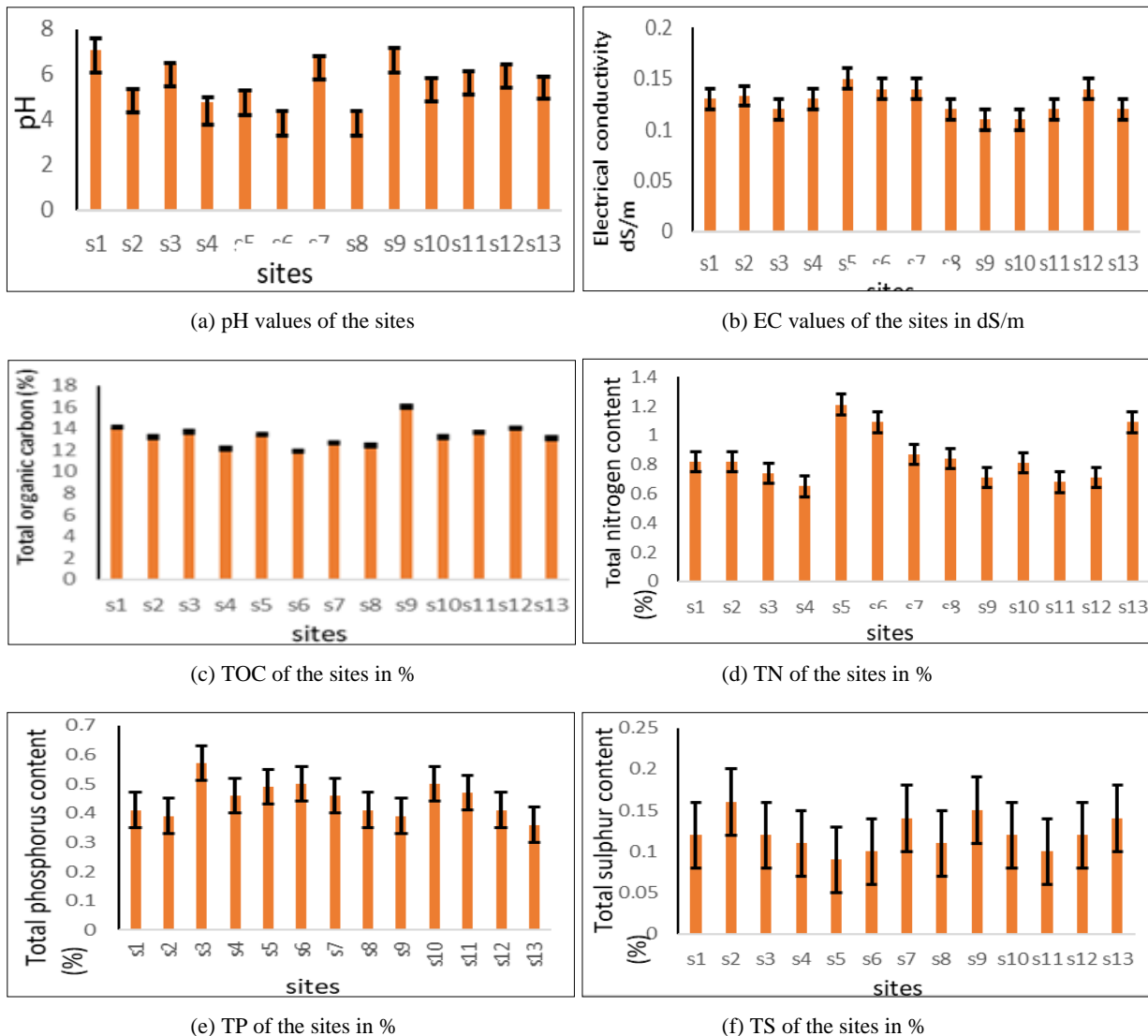
Results

Experimental Sites and Analysis of soil Parameters

Earthworms along with soil samples were collected from

thirteen (13) paddy fields in triplicates from Kamrup district of Assam. A brief description of the sites along with different soil physio-chemical parameters shown in Table 1. The pH of the collected soil samples ranged from slightly neutral to acidic in nature (7.1±0.05-4.3±0.08). The lowest was found to be in the Dhemai gaon (s6); Tegharia (s8) area. The slightly neutral pH was obtained in the Dharapur (s9) area (7.1±0.05). The type of the soil was found to be mostly alluvial type. The soil texture was found to be fine loamy to course loamy with soil color from light brown to dark brownish.

Table 1: Table showing different Physico-chemical characteristics of soil



Collection and Morphological Analysis of Earthworms

A total of hundred (100) earthworms were collected from the paddy fields. In the present study, records based on the external characteristics such as body length, body color, clitellum, prostomium, segments and previous records (Julka 1987,1988; ZSI) [13, 14] were evaluated. The most abundant earthworms' genera that were found in the paddy fields of Assam were observed as *Eudrilus sp.*, *Metaphire sp.*, *Polypheretima sp.*, *Perionyx sp.* and *Amyntas sp.* Zoological survey of India provides an insight detail about the fauna of earthworms and its distribution.

Molecular Identification of Earthworms

The earthworms were subjected to molecular identification using COX-1 gene. A list of identified earthworms with the accession number are shown in Table 2. *Eudrilus eugeniae*, *Metaphire nanaensis*, *Metaphire formosae*, *Perionyx depressus*, *Amyntas morrissi*, *Polypheretima elongata* and *Amyntas phaselus* were reported in the paddy fields of Assam. The percentage distribution of the identified earthworm species is shown in Fig. 1.

Table 2: List of identified earthworm species with their accession number

Names of the earthworm	Accession number
<i>Eudrilus eugeniae</i>	OP999656
<i>Perionyx depressus</i>	OQ073568
<i>Amyntas morrisi</i>	OR518282
<i>Metaphire nanaoensis</i>	OQ073567
<i>Polypheretima elongata</i>	OR053673
<i>Amyntas phaselus</i>	OR518347
<i>Metaphire formosae</i>	OR518417
<i>Eudrilus eugeniae PBP</i>	OR053675

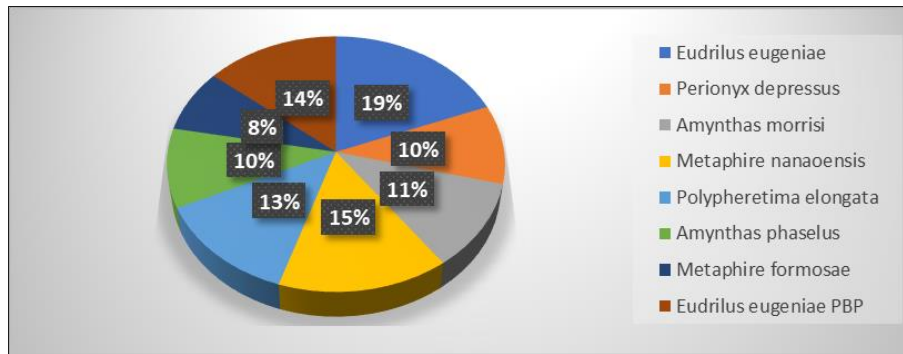


Fig 1: Showing the percentage distribution of different earthworm species

A brief detailed account of diagnostic features of Earthworms are listed below:

Eudrilus Eugeniae (Kingberg 1867)

Eudrilus eugeniae is primarily a detritivore, feeding on decaying organic matter such as dead plants, kitchen waste, and other compostable materials. It prefers warm and humid environments with organic-rich soils, often thriving in compost piles, decomposing plant material, and other similar habitats. Kingberg (1867) reports gives us both taxonomical and anatomical evaluation of the species *Eudrilus eugeniae*.

Polypheretima elongata (Perrier, 1872)

Polypheretima elongata, originally described by Perrier in 1872, a species of earthworm belonging to the family Megascolecidae. It contributes significantly to the health of the soil and ecosystem through activities including soil aeration and nutrient cycling. A kind of burrowing species known as *Polypheretima elongata* is frequently found in soil, especially in areas with tropical and subtropical climates. It contributes to improved soil fertility and structure through burrowing and the breakdown of organic materials.

Perionyx Depressus (Stephenson 1914)

In *Perionyx depressus*, the clitellum is circular, intersegmental furrows, and setae are found from XIII to XVIII. Due to their bluish coloring and similar outward appearances, it is sometimes difficult to distinguish this species from *Polypheretima elongata*. It has a major impact on the circulation of the soil, recirculating of nutrients, and the decomposition of organic matter.

Amyntas morrisi (Beddard, 1892) [4]

The *Amyntas morrisi* has segments that range in length from 91 to 120, and it is cylindrical throughout. Setae are uniformly distributed along segmental equators and within male pores. These "Asian jumping worms" are most likely

native to East or Southeast Asia. *Amyntas* earthworms are renowned for their exuberant behavior and capacity to move in a writhing, snake-like fashion, frequently referred to as "jumping" when disturbed. They prefer moist environments and are often found in leaf litter, compost, and soil. *Amyntas* earthworms are voracious eaters and have the potential to significantly alter soil structure.

Metaphire Nanaoensis (Julka 1987) [13]

Metaphire earthworms *sp.* are often found in various soil habitats, including forest floors, gardens, and agricultural fields. In *Metaphire nanaoensis* the clitellum is from 14–16, annular, smooth, 8–12 mm in length, no dorsal pore, no setae. Diverticulum stalk is narrow, equal to spermathecal stalk in length, and slightly twisted. *Metaphire* earthworms are detritivores, primarily feeding on decaying matter. Their feeding activities are essential for enhancing the soil's organic matter breakdown and enrichment.

Metaphire formosae

Metaphire formosae under the genus *Metaphire* are found in different soil condition similar to *M. nanaoensis*. The external morphology reveals male pores around 132-135. Clitellum at 14–16, annular, smooth, 10–11 mm in length, no dorsal pore, no setae. There are no genital papillae near the spermathecal orifice. 14 has a single female ventral pore. 18 pairs of tiny, latero-ventral male pores. In 10–13, There are ellipsoidal ampulla that are 3–5 mm long and have a 2 mm stalk. Small diverticulum that extends to the basal 1/3 of the ampulla, with an oval and smooth seminal duct.

Amyntas Phaselus (Hatai, 1930)

Amyntas phaselus belongs to the genus *Amyntas*. They are voracious eaters and have the power to dramatically change the composition of the soil. The species *Amyntas phaselus* have segments ranging from 80 to 130. The specimen neither awarded special classification as a syntype nor a neotype. The majority of individuals have ventral spermathecal pores as in *Amyntas morrisi*. Setae are

uniformly scattered along segmental equators and between male pores. They are indigenous to East or Southeast Asia.

The species of *Amynthas* frequently inhabit in dirt, compost because they favour damp habitats.



Fig 2: (a) *Eudrilus eugeniae* (b) *Polypheretima elongata* (c) *Perionyx depressus* (d) *Amynthas morrissi* (e) *Metaphire nanaoensis* (f) *Metaphire formosae* (g) *Amynthas phaselus* (h) *Eudrilus eugeniae PBP*

Table 3: Showing the internal and external characteristics of the different earthworm species

Characteristics	<i>Eudrilus eugeniae</i>	<i>Polypheretima elongata</i>	<i>Perionyx depressus</i>	<i>Amynthas morrissi</i>	<i>Metaphire nanaoensis</i>	<i>Metaphire formosae</i>	<i>Amynthas phaselus</i>	<i>Eudrilus eugeniae PBP</i>
Body length (mm)	90-185 mm	75 to 180 mm	76-97 mm	94 to 104 mm	81 to 160 mm	90-182 mm	98 to 110 mm	95-175mm
Body color	Reddish-brown	Light grey to reddish body	Purple to brown	Dark brown	Reddish-brown	Brownish-yellow	Light to dark brown	Reddish-brown
Clitellum segments	13-15	14-16	13-17	14-16	14-16	14-16	14-17	13-15
Clitellum shape	Saddle	Annular	Circular	Circular	Annular	Annular	Circular	Saddle
Gizzard (in segment)	5	8	8	8-10	8	8	8-10	5-7
No. of segments	120-240	130-296	180-216	119-156	81-160	90-178	120-150	120-252
Prostomium	epilobous	Prolobous	epilobous	epilobous	Prolobous or epilobous	Prolobous	epilobous	epilobous
Female pore (in segment)	14	14	13	14	14	14	13-14	14
Male pore (in segment)	17	18	18	18	18	18	18	17
Intestinal caeca (in segment)	25-26	Lacking	26-27	27	27	27	27	25-26
Ampulla shape	Oval	Large and round	ovoid	thumb	Ovoid to almond	ellipsoidal	thumb	oval

Acute toxicity test

The earthworms' responses varied according to the heavy metal content in the filter paper test. *Metaphire nanaoensis* being an endogenic earthworm abundantly found in the

paddy fields was subjected to acute test toxicity to evaluate the toxic potential of lethal condition of heavy metal (Pb). After 1-4 hrs exposure at low concentration of Pb (0.02, 0.05) mg/ml it didn't show any changes except coiling of

body. After 24 hours, earthworms exposed to high concentrations of Pb (0.07, 0.09) mg/ml showed changes in body colour, sluggish locomotion, and mucous secretion shown in Fig 4. After 48 hours, it was observed that about half of the earthworm didn't respond to external stimuli. The mortality was found to be 80% at high concentration of

0.1 mg/ml as represented in Fig 3. The lethal concentration (LC₅₀) was found to be 0.5462 mg/ml indicating that 50% of the test organisms were found to be dead during toxicity test. Control sample (c) without heavy metal didn't show any morphological changes within 48hrs with no mortality rate.

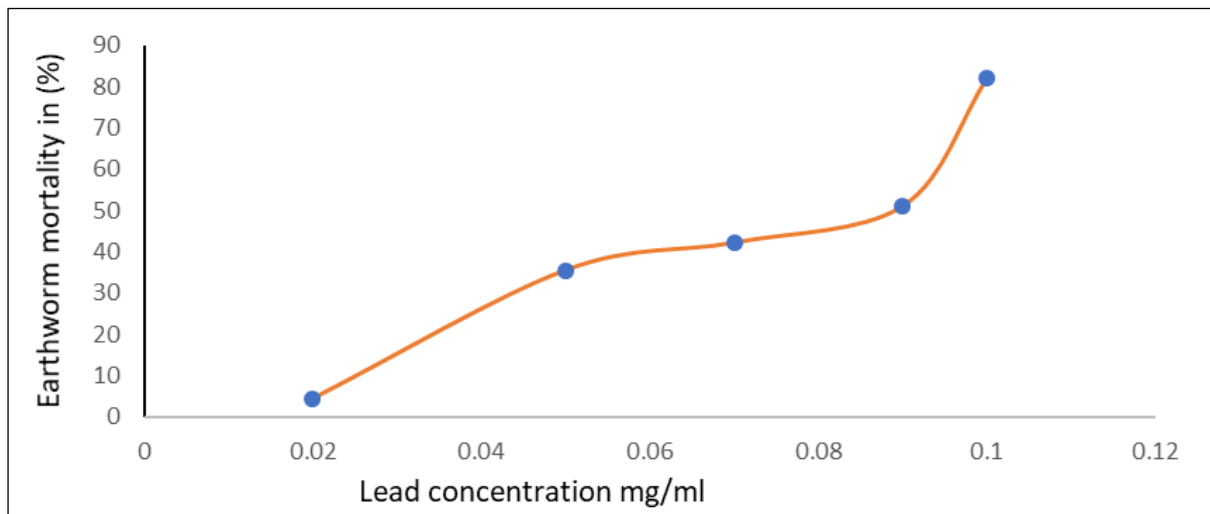


Fig 3: Showing the earthworm mortality in (%) of acute toxicity test with relevant concentration of *Metaphire nanaoensis*

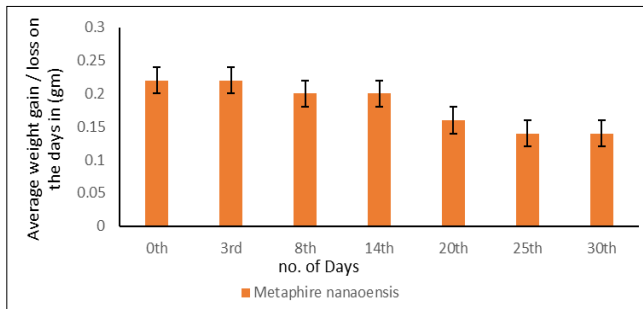


Fig 4: showing the toxic effect of lead in *Metaphire nanaoensis* (a) Coiling and mucous secretion of the body; (b) Discoloration of the body; (c) Swelling and coiling of the body; (d) mucous secretion from the body; (e) slow locomotion with mucous secretion; (f) distortion of the body

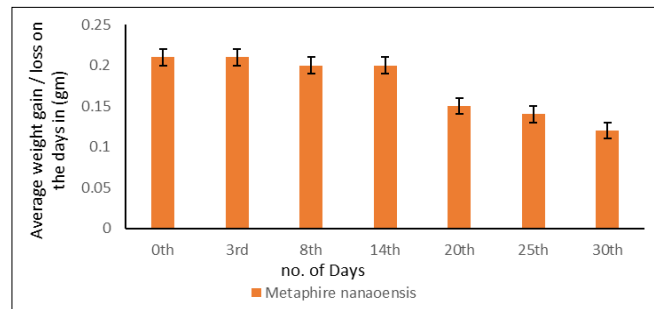
Soil toxicity test

The *Metaphire nanaoensis* was exposed to different relevant concentration of Pb along with the control (C) for a period of 30 days and the observation was evaluated. In (Fig 5) it shows the average weight gain/loss of different earthworms in relation to the heavy metal exposure. The result shows a decrease in all heavy metal exposure. The growth was seen

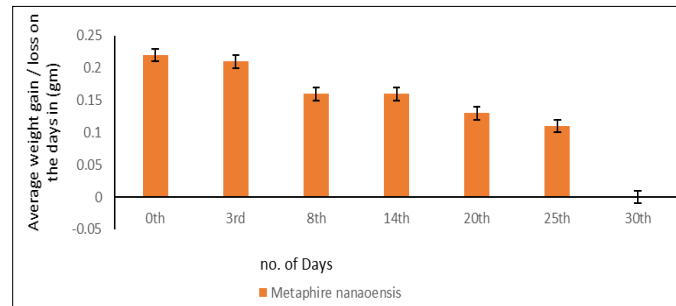
to be minimum at 100 ppm on 25th day of Pb exposure which were significantly less (p<0.05). The highest survival percentage (66.6%) was observed at 70 ppm of Pb on 30th. Heavy metal lead (Pb) at 70 ppm showed least toxicity in earthworms. Heavy metal Pb affected negatively to the cocoon formation and reproduction. The size of the earthworm slightly decreased with decrease in body weight.



(a) Concentration at 70 ppm



(b) Concentration at 90 ppm



(c) Concentration at 100 ppm

(a) Fig 5: Change in average weight gain/loss of earthworms in concentration of heavy metal Pb at (a) 70ppm (b) 90 ppm (c) 100ppm

Discussion

Assam's biodiversity is blessed with diverse ecosystems, including grasslands, forests and riverine habitats which support a wide range of flora and fauna. As most of the previous reports investigated the biodiversity of earthworm in the sewage, grasslands and forests, the primary focus on paddy fields has not been evaluated so far in Assam. Therefore, an attempt has been made to carry out the DNA barcoding approach survey of earthworms and to study the toxicity effect of *Metaphire nanaoensis* exposed to Pb in paddy fields of Kamrup district, Assam. In current content, a total of hundred (100) earthworms were collected belonging to different families and genera on the basis of morphoanatomical parameters of Julka (1988) [14] and COI barcode. Earthworm taxonomists can easily add new taxa to the database with the help of DNA barcoding, which furthers the database's ability to explore different taxa. In the present study, morphological identification was followed by molecular identification where the sequences obtained was submitted in NCBI which showed high similarities with different species of earthworm: *Eudrilus eugeniae* (Acc. no. – OP999656), *Metaphire nanaoensis* (Acc. No. – OQ073567), *Polypheretima elongata* (Acc. No. – OR053673), *Perionyx depressus* (Acc. No. – OQ073568), *Amyntas morrissi* (Acc. No. - OR518282), *Amyntas phaselus* (Acc. No. -OR518347), *Metaphire formosae* (Acc. No. -OR518417) and *Eudrilus eugeniae PBP* (Acc. No. - OR053675) represented in Table 3. The identified specimens of earthworm species are shown in Fig. 2. The Shannon diversity index (H') was found to be 2.045 which indicates moderate diversity among them in a particular area. It gives a better description of an ecosystem's diversity. A comparative analysis has been made of both external and internal characteristics of different earthworm species in Table 3.

In relation to the ecological aspect, *Eudrilus eugeniae* and *Perionyx depressus* are epigenic in nature whereas *Metaphire nanaoensis*, *Polypheretima elongata*, *Metaphire*

formosae, *Amyntas phaselus* and *Amyntas morrissi* are endogenic resulting in more endogenic than anecic and epigenic earthworms in paddy fields. It might be possible that due to the disturbance caused by ploughing, the amount of anecic species in different paddy fields is usually low. In the current work a total of five different species (*Metaphire nanaoensis*, *Metaphire formosae*, *Perionyx depressus*, *Amyntas morrissi*, *Amyntas phaselus*) was found belonging to same dominant family Megascolecidae in the paddy fields of Assam. The maximum number of earthworms (*Metaphire sp.*, *Eudrilus eugeniae*) were found in Sonapur (s7) and Panikhaiti (s1) area. In the Sonapur (s7) and Tegheria (s8) area the concentration of earthworms (*Metaphire nanaoensis* and *Polypheretima elongata*) was found to high in comparison with other areas of paddy fields. The most common earthworms (*Eudrilus eugeniae*, *Eudrilus eugeniae PBP*) was found to be in all the selected areas of the paddy fields except in some area like Dhemai gaon (s6). 12 different species of earthworms was identified by Lalthanzara *et al.*, (2020) [18] in Arunachal Pradesh. Kumar *et al.*, (2021) [17] pointed out earthworm populations on farmed land were often lower than those in ecosystems that had not been altered.

In agro-ecosystems, earthworm populations are usually susceptible to the physical, biological and chemical soil properties. The pH along with other physio-chemical characteristics plays an important factor about the occurrence of earthworms in paddy fields. In the current content, the soil collected from different paddy fields of Kamrup district, Assam is mainly alluvial type with a pH ranging from slightly neutral to acidic in nature (7.1 ± 0.05 - 4.3 ± 0.08). According to Kumar *et al.*, (2020) [16] the quantity of earthworms is greatly influenced by soil physiochemical parameters in various ecosystems. In the current context, the highest total organic carbon (TOC) was (16.01%) in (s9), total phosphorous content ranged from (0.39-0.49) %, total nitrogen content (0.65-1.21) % and total sulphur content (0.09-0.16) % as shown in Table 1 which

validates from previous work of Singh *et al.*, in (2020) [28]. In Northeast India's lower Brahmaputra valley, where the pH ranged from (3.2 to 7.55) was examined by Baruah *et al.*, in (2020).

In the present investigation, two endogenic earthworm species (*Metaphire nanaoensis* and *Metaphire formosae*) reported belonging to family Megascolecidae in the paddy fields which correlates with (Singh *et al.*, 2016; Singh *et al.*, 2020) [27, 28]. It was found that the prostomium of *Metaphire nanaoensis* is probolous or epilobous whereas in *Metaphire formosae* it is probolous. The occurrence of *Metaphire birmanica* was initially noted in Manipur and in Northeast India (Singh *et al.*, 2020) [28]. There are about ten different species of *Metaphire* that have so far been discovered in diverse parts of India (Thakur and Yadav 2018) [30]. Singh *et al.*, in (2020) [28] observed a high percentage of *Metaphire posthuma* in both cultivated and uncultivated areas. However, study on the indigenous native species of earthworm in the paddy fields has not been evaluated so far.

In the current context, *Eudrilus eugeniae* belonging to family Eudrilidae was found to be more in the decomposed rice straw compared to submerged region of paddy fields. Along with *Eudrilus eugeniae*, *Amyntas morrissi*, *Amyntas phaselus* and *Polypheretima elongata* were found to be more in banong area of paddy fields which can be compared with previous records of Saikia *et al.*, (2021) [22]. *Perionyx depressus* were also found near the edges of the submerged area of paddy fields. Saikia *et al.*, in (2020) reported two new species of the same genus, *Perionyx excavates* (Perrier) and *Perionyx annandalei* in Assam. Because of paddy cultivation requires extensive ploughing and water, earthworm species were found in greater quantity around the edges of the paddy fields compared to the submerged region of fields. This results correlates with the previous investigation of Van Schaik *et al.*, (2016) [33]. Singh *et al.*, (2020) [28] pointed out that compared to non-cultivated fields, the abundance of *Polypheretima elongata* is substantially lower in cultivated fields. The distribution of earthworm species inside the soil is significantly influenced by the soil structure and its pore size, and even little alterations in physio chemical factors can have adverse effect on the community structure of earthworms (Schon *et al.*, 2017) [23]. Therefore, certain integrated approaches must be taken to explore the indigenous native species in Assam Northeast, India. This would be helpful for taxonomists in addressing issues with comparing earthworms that were previously thought of being synonymy, cryptic, novel or undescribed species that are under risk due to exploitation and ecosystem modification. Additionally, when dealing with severely endemic species, combining morphological description, DNA barcoding, and phylogeny is worthwhile. Earthworms are extremely sensitive to chemical stimuli and have many sensory cells with thin cuticles. In the filter paper contact test, heavy metal Lead (Pb) sticks to the paper's surface, travels through the skin of earthworms to reach the coelomic fluid, and then spreads throughout the body, causing toxic symptoms (Jeyaprakasam *et al.*, 2021) [15]. The present work reveals that when exposed to long duration and with increased concentration, the toxicity of Pb also increases in the body of the earthworm. They exhibit toxic symptoms such swelling, curling, fragmentation, and mucous secretion in endogenic earthworm *Metaphire nanaoensis*. Current work revealed changes in body color,

sluggish locomotion after 24 hrs of exposure at high concentration of lead (0.07,0.09) mg/ml. The mortality rate was seen to be increased with increased in concentration and about 80% was found to be dead after 48 hrs of exposure at 0.1 mg/ml concentration. The 48hrs-LC₅₀ of Pb was found to be 0.5462mg/ml. A similar finding was reported by Yue *et al.*, (2021) [36] where the toxicity of selenium was evaluated in earthworms *Eisenia fetida*. The 48hrs-LC₅₀ in the filter contact test was found to be 1.52µg/cm². Jeyaprakasam *et al.*, (2021) [15] observed the acute toxicity of earthworms with malathion pesticides. Duo *et al.*, (2022) [9] studied the toxicity of Graphene oxide in earthworms and studied the histopathological changes after exposure. Another work investigated the toxicity effect of two chemical pesticides chlorpyrifos and cypermethrin. The mortality was found to be 70% of chlorpyrifos at a concentration of 0.1960 µg/cm² (Tiwari *et al.*, 2019) [31] indicating a potential health risk accumulated in soil. In the present soil toxicity test the growth of *Metaphire nanaoensis* was found to be minimum at 100 ppm on 25th day of Pb exposure which were significantly less (p<0.05). The average weight of all the earthworms were seen to be decreased in all the concentrations with no cocoon production. The reproductive rate didn't show any increased in number which correlates with previous report which showed decreased in growth of the earthworm after Pb exposure (Žaltauskaitė *et al.*, 2020) [37]. Similar work reported a decrease in growth with high mortality rate in *Eisenia fetida* after exposure with Pb (Sujetovienė *et al.*, 2019) [25]. The toxic effect of heavy metal Selenium (Se) exposure to earthworms showed higher accumulation of Se in the artificial soil rather than other substrates (Yue *et al.*, 2021) [36]. It has been studied that earthworm mortality is directly proportional to pollutants found in the soil. In the current content the mortality was seen to be maximum at 100ppm. The present findings attributes to the notion that with increasing concentration of Pb resulted in morphological changes in the soil-dwelling organisms thus effecting the food chain. This study would help in assessing the toxicity effect of heavy metal in the agricultural fields. Further research is necessary to understand the ecological risk associated with heavy metal toxicity in soil dwelling earthworms along with terrestrial ecosystems.

Conclusion

Assam, Northeast India is not only a region of rising sun for ethnic and cultural diversity, but also a huge biodiversity center. Paddy cultivation plays a major role for the livelihood for the people of Northeast, India. Therefore, the main aim has been focused on the paddy fields and to explore the richness and species diversity of ecological engineers, earthworms associated with it. In the present work, eight different earthworm species have been identified belonging to two dominant families Megascolecidae and Eudrilidae. It was observed that endogenic earthworm occupies a dominant status in relation to epigenic and anecic species in cultivated paddy fields. Therefore, the toxicity of an endogenic earthworm *Metaphire nanaoensis* was evaluated to determine the LC₅₀ exposed to heavy metal (Pb). The soil toxicity test showed maximum growth at 100 ppm on 25th day of Pb exposure. Morphological changes were observed after 24 hrs of Pb exposure in filter paper test with LC₅₀ found to be 0.5462 mg/ml. This would help in evaluating the lethal condition of

the soil-dwelling organism in contaminated soil. Therefore, more attention is needed to evaluate the toxic risk associated and to mitigate with heavy metal Pb from the environment. To increase the diversity of earthworms in the agricultural areas, farmers need to know the importance and significance of earthworms in soil enhancement which would also help in generating higher-quality crops, which is a positive step towards sustainable farming and development.

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