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Research Article

ELEMENTAL ANALYSIS ON THE NEW SPECIES OF EARTHWORM FROM ORISSA

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ABSTRACT: Earthworms are natural tillers of the soil and are known as the farmer's friends. The earthworm physiology is capable of combating pollution and pathogens and is therefore worth exploring. Although effect of pollution on earthworm immune system is being studied, elemental analysis of earthworm is not studied, how pollution affects the elements in earthworms, is not known. New species of earthworm was recorded from the state of Orissa, India. In this study we have tried to understand the elemental composition in a new species of earthworm, *Perionyx shyamasreetus (P shyamasreetus)* isolated and reported earlier, from the soil of Institute of Physics (IOP) campus, Bhubaneswar, Orissa India. We have performed fluorescence spectrophometry, UV-Vis studies, The Fourier-transform infrared (FTIR) and Proton induced X-ray Emission (PIXE) studies on earthworm from the wild to understand the elements that exists in it. We observed that the elements including Sulphur (S), Chlorine (Cl),Potassium (K), Calcium (Ca), Titanium (Ti),Vanadium (V),Iron (Fe), Zinc (Zn), Copper (Cu), Chromium (Cr)are present from the PIXE studies on the tissues of earthworm as trace elements and Fe as minor element while Carbon (C), Hydrogen (H), Nitrogen (N) and S as major element. FTIR spectroscopy and UV-Vis fluorescence studies reveal the presence of amino acids such as tryptophan, tyrosine and cysteine. The future scope of the study lies in understanding the elemental composition of earthworm in environmentally toxic soils including radioactivity prone areas of Orissa.

Keywords: Earthworm, Perionyx, PIXE.

INTRODUCTION

Earthworms reveal existence in habitats ranging from organic heaps, manure, compost, litter, humus, kitchen drainage etc. to forest land, grassland, agricultural land, plant nursery etc feeding on a variety of food of both plant and animal origin and dead organic matter. The importance of earthworms in increasing soil fertility has been reported since the time of Darwin (Darwin 1881). Earthworms are the natural tillers of the soil and also are known as farmer's friends. The earthworm immune system is robust (Ghosh 2018) and contains molecules that can digest the polyphenols of plants (Liebeke *et al.* 2015). Earthworms belong to Phylum Annelida, Class Oligochaeta, some of them being terrestrial while others are freshwater forms. Their segmented body reveals absence of parapodia, but possesses setae, and movement is by peristaltic contraction of muscles (Ruppert *et al*. 2003).

The different existing families of earthworms include Acanthodrilidae, Glossoscolecidae, Lumbricidae, Megascolecidae. Genus Perionyx is under family Megascolecidae (Baird 1869, Julka 1988) that reveal most distribution in the Indian subcontinent.A newly discovered species named as *Perionyx shyamasreetus* (*P shyamasreetus*, Fig. 1) has been recorded from Orissa, is placed under genus Perionyx, with distinctly unique

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morphological features and have been recorded in Zoological Survey of India (ZSI, Kolkata, Ghosh *et al.* 2018). In order to understand the biology and elemental composition of the newly discovered species, *P shyamasreetus,* earthworms from the wild and its natural habitat, in Bhubaneswar, Orissa different spectrophotometric analysis, and multi-elemental approaches were used. We have seen no study has been done on earthworms on these lines. We have purposely selected earthworms in wild to understand their composition in their natural habitat in the wild.

We report here for the first time, the chemicals composition of *P shyamasreetus* (Ghosh *et al.* 2018b) detected by Fluorescence, UV-Vis spectroscopy, FITR and PIXE approaches. Again, the CHNS (carbon, hydrogen, nitrogen, and sulfur) analysis was carried out to quantify the major elemental constituents present in the earthworm sample.

The PIXE is a non-destructive multi-elemental analysis technique plays a major role in detection of minor and trace elements present in this life sample. The solid sample in the forms like pellet, thin film, powder, fiber, fluid, semi-solid etc. can be analyzed (Johansson and JL 1988). Liquid and gaseous samples analyzed through this technique, is limited to atomic number (Z)>12.The existence of different elements in the earthworm is indicative of their composition in the wild, which control various important biological activities inside the living body.

MATERIALS AND METHODS

Sample collection and identification

This Earthworm specimen is collected from root of bushes near IOP campus Bhubaneswar, Orissa (Fig. 1). After making the collection, earthworms were sorted out and cleaned and processed as discussed in detailed in Ghosh *et al.* 2018. They were placed in a tray with a small quantity of water and were slowly killed by anaesthetising with alcohol allowing them to expand before death. Just after death, the earthworms were kept in 70% alcohol for permanent preservation. For good dissection material were kept in 4% formalin for 12 hours just after narcotisation. Specimens should be kept straight because straight and hard specimen helps to dissect properly. Structure of Prostate gland, Peneal setae, stomach, spermatheca etc internal organs helps to identify the specimen. Setal arrangement (perichaetine, lumbricine and quinqancial), superficial structure and position of gonopores, number of segments etc. of fresh and straight specimens were identified (Ghosh *et al*. 2018).

Dissection

The specimens prepared as described in details in (Ghosh *et al*. 2018) were observed under 10X of Leica microscope with zoom magnification from 8X to 35X with high resolution images. Photograph of main identifying organs like prostate, spermathecal glands, superficial characters like male and female pores, setal arrangement, prostomium, clitella region etc. were studied under 10X microscope (Ghosh *et al*. 2018).

Cryopreservation

The earthworms were reduced to a fine powder by impact pulverization at liquid nitrogen temperature. The earthworms cleaned as in (Ghosh *et al*. 2018) were incubated in liquid nitrogen and instantly the body become brittle and were grinded in a mortar and pestle in toa powder form and stored in -80°C refrigerator till further use.

Preparation of sample for Fluorescence, CHNS analysis and UV-Vis spectroscopy

Distilled water was used as solvent to form the slurry of the earthworm powder prepared for the spectroscopic studies.

Preparation of sample for FTIR

To prevail the free radicals and functional-group of earthworm using FTIR spectrophotometry (Thermo scientific) were recorded in the region 1000 cm^{-1} – 4000 cm^{-1}analysis was carried out. The earthworm powder as described above was used for the FTIR study (Adina *et al.* 2010).

Preparation of sample for CHNS study

The solution of the earthworm powder was prepared using distilled water as solvent for the CHNS analysis.

Preparation of sample for PIXE analysis

The solid pellet from *P shyamasreetus* was prepared for the PIXE experiment. Earthworms cleaned and narcotized with 70% alcohol was immersed in liquid nitrogen causing them to become hard and frozen. They were then powdered in mortal and pestle, dried as above. Powder form of earthworm and graphite was mixed together to form a pallet of weight ratio 1:1 and behaves as a conducting system. This pellet was placed in the vacuum chamber and exposed to 3 MeV-proton beam by using 3 MV Horizontal Tandem Pelletron Accelerator (9SDH-2, National Electrostatic Corporation, USA) facility at the Ion Beam Laboratory, Institute of Physics, Bhubaneswar, Odisha, India (Maxwell *et al.*1995, Campbell *et al.* 2000, Hopman *et al*. 2002).

RESULTS AND DISCUSSION

Identification of the species

The species was identified as *Perionyx shyamasreetus* (*P shyamasreetus*, Fig. 1, Table 1, Ghosh *et al*., 2018).

Its diagnostic features included morphology of medium and elongated, brownish in colour, ventral region with pink in colour, and size of a total length of about is 3.2 cm and breadth of 3 mm with anal pore: 0.3 mm, number of body rings of total 157, with male pores opening between the rings 18, almost eye shaped, small to medium sized with female pore obscure and clitellar region (Ghosh *et al*. 2018), pinkish white in colour (Fig. 1).

Physicochemical characterization of the species

Physiochemical analyses of 05 number of same samples were carried out by different experimental techniques. Fluorescence analysis of the *P shyamasreetus solution* was carried out using 260 nm excitation produces an emission at 340 nm (Fig. 2A). In proteins, the important amino acids such as phenylalanine ($C_9H_{11}NO_2$), tyrosine ($C_9H_{21}NO_3$) and tryptophan ($C_{11}H_{12}N_2O_2$) are fluorescent in nature. The fluorescent band obtained at 340 nm as shown in Fig.2 confirm the presence of above fluorescent-protein in the earthworm.

The UV-Vis absorption band at 280nm shown in Fig. 2(B) indicates the proteins. This absorbance (A_{280}) of protein is mainly due to the presence of amino acids viz. tryptophan ($C_{11}H_{12}N_2O_2$), tyrosine ($C_9H_{21}NO_3$) and cysteine ($C_3H_7NO_2S$) in the earthworm material.

The bands in FTIR spectrum shown in Fig. 3 identify the free radicals and functional-group presence in the earthworm sample. The bands at 1405 cm^{-1} confirm hydrocarbons (-CH2-), 1627 cm^{-1} confirms alkenes (-C=C-), 2076 cm^{-1} confirms alkynes (-C ºC-) and 2500 cm^{-1} confirms the free-SH, present in the earthworm. The UV-Vis absorption band of cysteine ($C_3H_7NO_2S$) observed at 280nm support the FTIR bands at 2500 cm^{-1}confirm the presence of sulphur in a complex form inside the body of the earthworm.

Table 1. Taxonomic Position of *Perionyx sp.*

Phylum	**Annelida Lamarck, 1809**
Class	Clitellata Michaelsen, 1919
Order	Opisthopora Michaelsen, 1930
Family	Megascolecidae Rosa, 1891
Genus	*Perionyx* Perrier, 1872

The CHNS analysis was performed to understand the major elemental composition of the earthworm. The major elemental concentration is presented in the Table 2. It was observed that the weight percentage of Carbon was 5.929, Nitrogen was 16.545, Hydrogen was 6.749, while no Sulphur was found in this technique. This is because of the concentration of S is below the detection limit (nil). The existence of elemental S is found in trace amount and quantifies later using PIXE technique.

The PIXE spectrum of the *P shyamasreetus* is shown in Fig. 4. The elemental concentrations of each individual element were quantified applying GUPIX software. The Standard Reference Material (SRM) – 1577c consists of Bovine Liver tissue of National Institute of Standards and Technology (NIST), USA was used for the quantification purpose. The element Fe is found out as a minor constituent in the *P shyamasreetus* sample. The

Table 2. Multi-elemental analysis of earthworm (*P shyamasreetus*): CHNS, PIXE studies.

Major element(%)		**Minor elements(mg/Kg)**		**Trace elements(ppm)**		
CHNS		**PIXE**		**PIXE**		
Elements	**Concentration**	**Elements**	**Concentration**	**Elements**	**Concentration**	**σ**
				S	133 ± 34	0.084
				Cl	35 ± 4	0.241
H	6.749		2758.304	K	144 ± 7	0.123
C	5.929		± 55.174	Ca	741 ± 73	0.035
N	16.545	Fe		Ti	64 ± 57	0.137
S	NIL		s = 0.0096	V	274 ± 41	0.035
				Cr	20 ± 11	0.151
				Cu	18 ± 3	0.019
				Zn	2± 2	0.278

†Concentration, 1% = 10,000 ppm = 10 mg/g, 1 mg/kg = 1 ppm

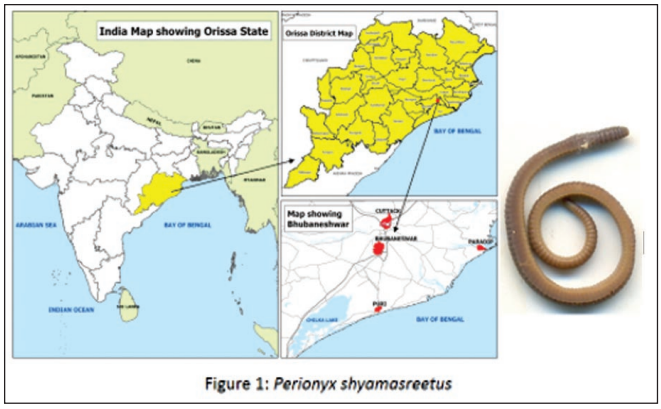

Fig. 1. Location of isolation of *P shyamasreetus.*

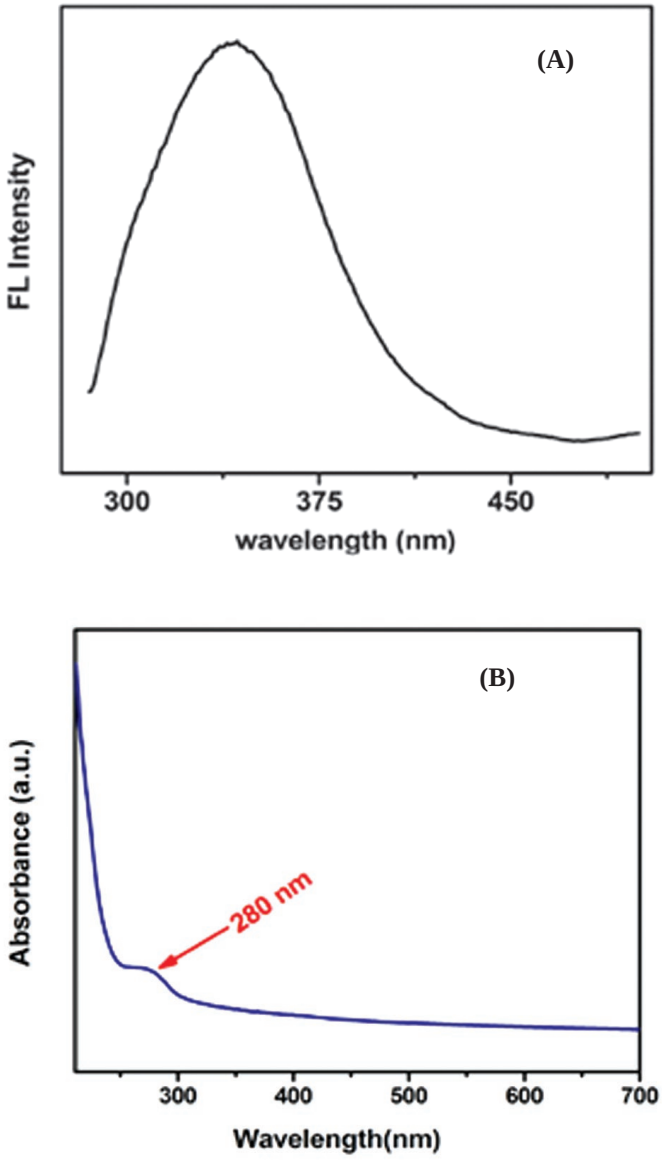

Fig. 2. (A) Fluorescence spectrum and (B) UV-Vis spectrum of Earthworm: *P shyamasreetus*.

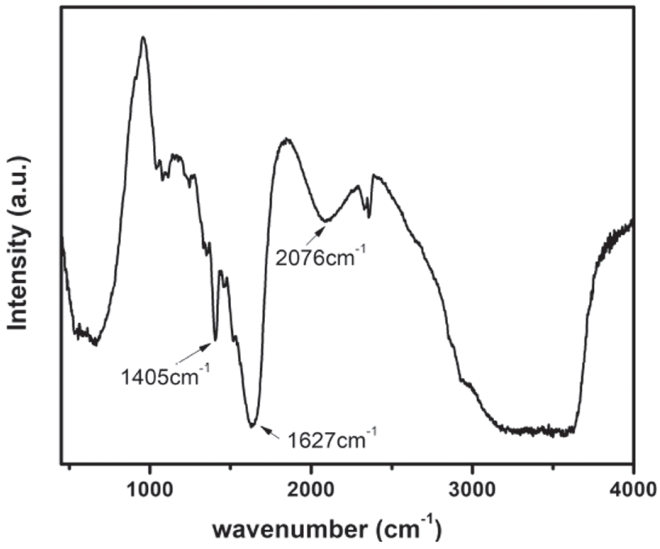

Fig. 3. FTIR spectrum of Earthworm: *P shyamasreetus.*

concentration of iron calculated to be 2758.304 ± 55.174 mg/Kg. The high value of Fe concentration may be due to the high-concentration of Fe in the soil.

A number of trace elements including S, Cl, K, Ca, Ti, V, Zn were observed. Among the above minor elements, concentration of elemental S is found out to be 133 ± 34 ppm. This is why the elemental sulphur is not detected by the CHNS technique. The sulphur is an important and essential element in the biology of any living tissue and play a major role as an electron acceptor for respiration process.

In a study from earthworm collected from forest soil in urban area in USA revealed that earthworms *Amynthas agrestis* (*Am agretis*) and *Metaphire hilgendorfi* (*M hilgendorfi*) may be bioaccumulating trace elements including Cd, Cr, Co, Cu, Mn, Ni, Pb, V, and Zn from the soil (Richardson 2019). However, no study has been conducted on Perionyx genus. We hypothesize that the trace elements from the earthworm tissues have been accumulated also from the soil from where they were inhabiting and residing. Although there have characterization of Trace elements by Frieden (1985) in humans included (i) Essential trace elements: Boron (B), Cobalt (Co), Copper (Cu), Iodine (I), Iron (Fe), Manganese(Mn), Molybdenum (Mo), and Zinc(Zn), (ii) Probable essential trace elements: Chromium (Cr), Fluorine (F), Nickel (Ni), Selenium (Se), and Vanadium (V) and (iii)Physically primitive trace elements including Bromine (Br), Lithium (Li), Silicon (Si), Tin (Sn), and Titanium (Ti) and their role in human health is proven (Frieden 1985, Prashanth *et al*. 2015), the role of trace elements in indication of earthworm is not at all known.

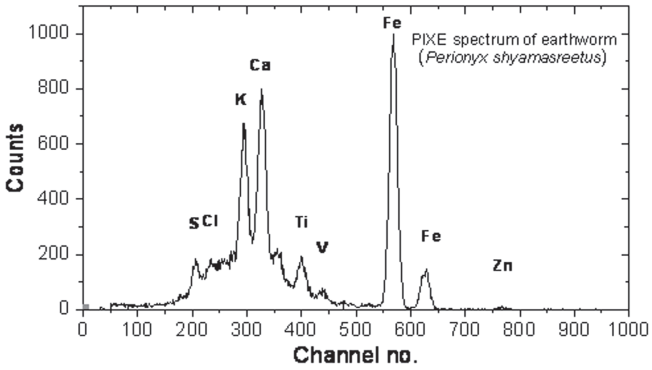

Fig.4. PIXE spectrum of Earthworm: *P shyamasreetus*.

This study finds importance as is the first study of elemental composition reported from this genus and this new species, isolated from wild normal soil. This forms the baseline for all the future studying other species of earthworm from Indian soil. Since bioaccumulation is a phenomena reported in animals in the ecosystem, and earthworms have been reported to bioaccumulate radioactive contaminants from soil habitats (Ghosh 2019) that may adversely affect their health, it is of our interest to study, the elemental composition of earthworm tissue and casts when isolated from radioactivity predominant belts of Orissa, in our ongoing study.

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