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SELECTION OF EARTHWORM SPECIES FOR VERMICOMPOSTING WITH THE AID OF ATOMIC FORCE MICROSCOPIC STUDIES

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Abstract: Earthworm was being specialized to live in decaying organic wastes and can degrade into fine particulate materials, which were rich in available nutrients to revive productivity and fertility of soil. The coelomic cavity filled with coelomic fluid; generally secreted by the earthworm is for maintaining the moisture, respiration and burrowing. The Coelomic fluid contain different enzymes including proteases, amylases, phosphateases, some of hormones like oxin, cytokinins and essential nutrients like K, Ca, Mg, Cl, Cu, P and Na for plant growth and increases disease resistance. The coelom communicates with the outer environment directly by dorsal pores. Atomic Force Microscopy (AFM) is a powerful diagnostic tool, have been proven itself to be a tool of choice to image super soft biological samples for the exploration of the mechanical properties of living cells. AFM measurements gave access to the roughness, pore size, pore density and pore distribution of a membrane. For the present investigation the skin of selected species of earthworms were exposed under Atomic Force Microscope to study the pore size and the roughness of the skin. Highly significant results in roughness were found out from the earthworm species such as *Octochetona pattoni* (489.148 nm), *Priodocheta pellucida* (195.370 nm). The significant pore sizes were found in *Notoscolex palniensis* (26.750 nm), *Drawida parva* (42.467 nm), *Hoplochetella stuarti* (3.423 nm) and *Octochetona surensis* (604.000 nm) where the expulsion of coelomic fluid may be higher than the rest of the species. These species can be suggested for better vermicompost production.
Keywords: Dorsal pore size, Roughness, Atomic Force Microscopy (AFM), Selected species of earthworm.

Introduction

Earthworms are able to protect themselves against invading microorganisms through their immune system. (Cooper, *et al*., 1999). Soil pathogens activity decrease by coelomic fluid and earthworm compost bears its natural characteristics of black color without any odour.

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Organic earthworm cast increases the soil nutrition and microbial population that directly promotes the plant growth and increase the yield.

The coelomic cavity is a fluid filled cavity within the mesoderm of an earthworm (Hideshi *et al.*, 2004; Weidong *et al.*, 2003)., generally secreted by the earthworm for maintaining the moisture and to help in its physiological activities including respiration and burrowing (Hideshi *et al.*, 2004; Weidong *et al.*, 2003), containing free, wandering coelomocytes (Tucˇková and Bilej 1996). The coelom communicates with the outer environment directly by dorsal pores and paired nephridial tubules through which metabolites are excreted. The dorsal pores represent also one of the important routes for the elimination of bacteria and "exhausted" coelomocytes. Under stress conditions, the coelomic fluid with its suspended cells can be rapidly expelled by increased intra-coelomic pressure (Tucˇková and Bilej 1996). Earthworms release the coelomic fluid along with mucus through the pores present on the dorsal surface of their body called dorsal pores (Kathireswari *et al.*, 2014). Coelomic fluid contain different enzymes including proteases, amylases, phosphateases, some of hormones like oxin, cytokinins and essential nutrients like K, Ca, Mg, Cl, Cu, P and Na for plant growth and increases disease resistance (Packialakshmi and Mahalakshmi, 2014, and expresses naturally a strong proteolytic activity (Roch *et al.*, 1998).

AFM - Biological application:

AFM is a powerful diagnostic tool, especially in force mode. The AFM had been developed from an exotic instrument into relatively widespread surface-imaging and probing instrument. Today this method was used to obtain the high- resolution static and dynamic images in investigation of the physical and mechanical properties of the thin film and biological molecules.

The ability of this microscope to achieve high resolution (sub nano meter) in liquids and to probe the mechanical properties of the sample at a nano metric scale make this instrument increasingly interesting for the study of biological specimens(Hansma, 1996). Every conceivable type of biological material had been explored using the AFM (Bustamante, *et al.*, 1994**,** Bustamante and Keller., 1995, Lal and John., 1994, Louder and Parkinson., 1995, Parrat, *et al.*, 1994**,** Shao, *et al.*, 1996)**.** One advantage of the AFM is the ease with which data can be acquired over a wide range of length scales.

The AFM measurements give access to the roughness, pore size, pore density and pore size distribution of a membrane (Khulbe and Matsuura, 2000). They can also provide information on the surface electrical properties of a membrane, its fouling potential towards a specific colloid (Hilal, *et al*., 2003) and its filtration performance as a function of its roughness (Kwak *et al*., 1997).

AFM based nano-indentation makes it possible to identify specific pores of earthworm. In the present study, the pore sizes (nm) of fourteen species of earthworms were measured in mechanical response. This investigation is mainly focussed on the earthworm skin microstructure (pore), their size (nm) and earthworm skin roughness [Img.Rms (Rq)].

Materials and Methods

This AFM work was conducted in the Department of physics, UGC-DAE consortium, Devi Ahilya Vishwavidyalaya University, Indore, Madhyapradesh. North India.

The skin pieces of the size approximately 0.5 cm × 0.5 cm in area of fourteen species of earthworms viz.,.*Lemnoscolex scutarius, Hoplochetella stuarti, Eudrilus Eugenia, Octochetona surensis, Lampito mauritii, Drawida parva, Glyphidrilus tuberosus, Notoscolex palniensis, Celeriella Ditheca, Celeriella quadripapillata, Celeriella punctata, Perionyx sansibaricus, Octochetona pattoni, Priodocheta pellucid* were cut from dorsal skin and fixed over a magnetic holder by using double side adhesive tape. Nanoscope E of AFM from Digital instruments, USA for imaging in contact mode, Triangular cantilevers of Si3N4,˜100 micron, wide leg, with a force constant of 0.58N/M was used in the present investigation. All the Contact mode-AFM images were made in air at room temperature at different locations of each skin sample. Scan made on areas of 5 micron ×1 micron were selected randomly on the earthworm skin surface for analysis. The pore sizes and roughness parameters of the sample (Earthworm skin) were determined by AFM. To obtain the pore size, profiles were selected to traverse micron scan surface areas of the Contact mode-AFM images. The diameters of pores (i.e. low valleys) were measured by a pair of cursors along the reference line. The horizontal distance between each pair of cursors was taken as the diameter of the pore. The AFM software program allows quantitative determination of pores by use of the images in conjunction with digitally stored line profiles. The average pore diameters were determined for a minimum of 5 pores on each sample. In addition, the AFM analysis software program allowed computation of various statistics related to the surface roughness and pore density on a predetermined scanned earthworm skin area. The evaluation of the roughness parameters of each membrane sample was based on various micron scan areas (i.e. 1 m × 1m) (m = micron).

Statistical Analysis

Students ‘t’ test (SPSS version 17.0) was used to evaluate the earthworm body pore size measurement and skin roughness measurements.

Results and Discussion

Skin samples with AFM:

Skin samples of earthworms collected from Theni District, Tamilnadu, South India and identified as *Lemnoscolex scutarius, Hoplochetella stuarti, Eudrilus Eugenia, Eudrilus Eugenia, Octochetona surensis, Lampito mauritii, Drawida parva, Glyphidrilus tuberosus, Notoscolex palniensis, Celeriella Ditheca, Celeriella quadripapillata, Celeriella punctata, Perionyx sansibaricus, Octochetona pattoni, Priodocheta pellucida* were focussed under the atomic force microscope in contact mode and AFM nanoscope images were recorded without damage of the skin part and also perfect images were pictured (Fig.1-8).

AFM had been proven itself to be a tool of choice to image super soft biological samples. The exploration of the mechanical properties of living cells is a key requirement today and also a real technical challenge, and had been successfully tested on polymers. This was applied to living and super soft samples (Alexandre Berquand, 2011).

Mean Pore size of the skin & Pore size Distribution:

In the present study earthworm skin pore size were analysed and measured along with the skin roughness (Table 1) by the AFM nanoscope instrument.

The technique had been applied, since then, extensively for studying various types of membranes and materials (Khulbe, and Matsuura, 2000, Bowen, 1999), including microfiltration membranes (Dietz *et al.,* 1992, Chahboun, *et al.,*1992), ultra filtration membranes(Singh *et al*., 1998, Khayet *et al*.,2002, Bowen, *et al.,* 1996), nano filtration membranes (Bowen, *et al.,* 1997, Mohammad, *et al.,* 2003), reverse osmosis membranes (Hirose, *et al*.,1996, Stamatialis, 1999) and gas separation membranes (Khulbe, *et al*., 1996, Tan and Matsuura, 1999) giving useful information about surface morphology, pore size, nodule size, pore density, porosity and roughness.

The maximum pore size of the skin of earthworm species (*Celeriella Ditheca)* ranges from 93.45 nm to 93.090 nm and minimum pore size (*Octochetona pattoni)* ranges from 0.404 nm to 0.4095 nm (Table 1). Khayet *et al.,* (2004) reported that the membranes having pores with size (i.e. diameter) below 0.5524m lend themselves to the Knudsen region. All pores having sizes between 552.4m and the maximum pore sizes are in viscous flow region, and pores in the intermediate range 0.5524m and 552.4m are considered in the transition region.

Earthworm skin surface roughness:

The skin roughness of different parts of the same sample were measured and images (Fig. 9-11) were captured and the mean ± S.D values were reported (Table 1). The roughness of the skin in their descending order is as follows: *Lampito mauritii* 83.70 Img.Rms (Rq), *Celeriella Ditheca* 39.04 Img.Rms (Rq), *Octochetona pattoni* 33.02 Img.Rms (Rq), *Lemnoscolex scutarius* 31.15 Img.Rms (Rq), *Hoplochetella stuarti* 9.35 Img.Rms (Rq). Hirose *et al*., (1996) explained that, the membrane roughness was higher for membranes having larger pore sizes and nodule sizes as well as higher surface porosity. In fact, the increase of the membrane permeation flux with the membrane roughness was being observed previously by Hirose *et al*., for polyamide composite reverse osmosis (RO) membranes, Stamatialis *et al*., (1999) for dense and integrally skinned cellulose acetate RO membranes.

Relationship between Pore size and roughness:

Generally, the result of roughness is greater in the earthworm skin with an increase in pore size. In fact, when the surface consists of deep depressions that characterize pores correspond to high roughness parameters were expected. In this study, earthworm skin having larger pore sizes have more coelomic fluid outfall. The same relationship between roughness parameter and pore size was also observed by other researchers (Bowen, *et al*., 1996). The AFM was well adapted to these types of studies, this system had been successfully applied to probe biotin–streptavidin (Florin, *et al*., 1994), avi-din–biotin [Moy, *et al.,* 1994], cell-adhesion proteoglycans (Dammer, *et al*., 1995), antigen–antibody (Dammer, *et al.,* 1996), and complementary DNA strands (Lee, *et al*., 1994) interactions.

Lower pore density, larger nodule sizes and higher roughness parameters were being observed by TM-AFM (Tapping Mode Atomic Force Microscopy) for the membranes prepared with higher concentrations of water in the PVDF (polyvinylidene fluoride) casting solution. The measured pure water flux increased with the increase of the roughness parameters of the prepared PVDF (polyvinylidene fluoride) membranes. This must be explained since the membrane roughness was higher for membranes having larger pore sizes and nodule sizes as well as higher surface porosity; Khayet *et al.,* (2004). But, In our result differ from this result earthworm species skin pore sizes and roughness were compared in between, but maximum pore sizes are not in relation with the higher roughness, maximum pore size ranges from (*Celeriella Ditheca)* 93.45 ± 132.006 nm and the higher roughness in the species was *Lampito mauritii* 83.70 ± 3.627. so, same species could not have maximum roughness and greater pore size. The maximum pore size *Celeriella Ditheca* (93.45 ± 132.006

nm) having the normal range of the roughness 39.04 ± 0.60175 Img.Rms (Rq). The higher ranges of roughness have the *Lampito mauritii* (83.70 ± 3.627 Img.Rms (Rq)) at the same time normal range of pore sizes 0.489 ± 0.388 nm.

Statistical consequence of Earthworm skin Pore size and roughness:

The ‘t’ values for skin roughness of all the species of earthworms were found to be significant. But, highly significant in species such as *Octochetona pattoni* (489.148) and *Priodocheta pellucida* (195.370). It was concluded, that these two species may be tolerant to unfavourable conditions of the environment such as stress and drought. This may be in accordance to the previous work of Mari ivask and Annely, 2008, that the roughness of the skin plays an important role in tolerance to external environmental condition. The specific and ecological composition of earthworm community serves as a better indicator of the intensity of agricultural activity in the field. The exclusive occurrence of species like *Aporrectodea caliginosa, Aporrectodea rosea, Lumbricus rubellus,* which were tolerant to disturbance, was the result of intensive tillage and agricultural practice or the influence of strong limiting ecological factor. A community including more sensitive species such as *Lumbricus terrestris* and *Aporrectodea longa,* or the most sensitive species *Allolobophora chlorotica* and *Lumbricus castaneus*, indicates more favourable agricultural or ecological conditions.

The statistical analysis of one sample‘t’ test was applied for earthworm pore size and roughness. The significant pore size was observed in species such as *Notoscolex palniensis* (26.750 nm)*, Drawida parva (*42.467 nm*)* and *Hoplochetella stuarti* (3.423 nm) and it was found to be highly significant in *Octochetona surensis* (604.000 nm).

Earthworm coelomic fluid was particularly interesting from a toxicological perspective, because it was responsible for pollutant disposition and tissue distribution to the whole organism. Its cells (coelomocytes) were involved in the internal defense system (Cooper *et al*., 2002 and Engelmann *et al*., 2004) and any impairment of coelomocyte functioning could compromise the health of the entire organism. Therefore, this physiological fluid is very interesting for the development of novel nondestructive pollution biomarkers.

Soil with vermicompost and coelomic fluid mixed sample had been shown the faster. Germination and flowering rate and quality yield when compared with control (soil). This is in concordance with the work of Packialakshmi and Mahalakshmi, 2014 that Vermicompost with coelomic fluid treated soil gave the good growth and good yield because the vermicompost is increasing microbial count and nutrient level and the coelomic fluid has the immune mechanism to inhibit the soil pathogens.

Lumbricus terrestris could be exposed to pollutants present not only in the soil surface but also in the soil deeper layer. It had a large size which permits handling and collection of enough amounts of coelomic fluid without compromising the survival of the animal (Calisi *et al*., 2014). This is in concordance with the present work, that bigger size of the earthworm body will expel large amount of coelomic fluid. The AFM result gave the skin pore size measurements of fourteen species of earthworm and the significant values were found in *Octochetona surensis* (604.00nm), *Notoscolex palniensis* (26.750nm), *Drawida parva* (42.467 nm) and *Hoplochetella stuarti* (3.423 nm). It can be concluded that the quantity of expulsion of coelomic fluid depends on the body pore size of earthworm species identified and measured in the present investigation which may be higher than the rest of the species and these species can be suggested for better vermicompost production.

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Tables and Figures

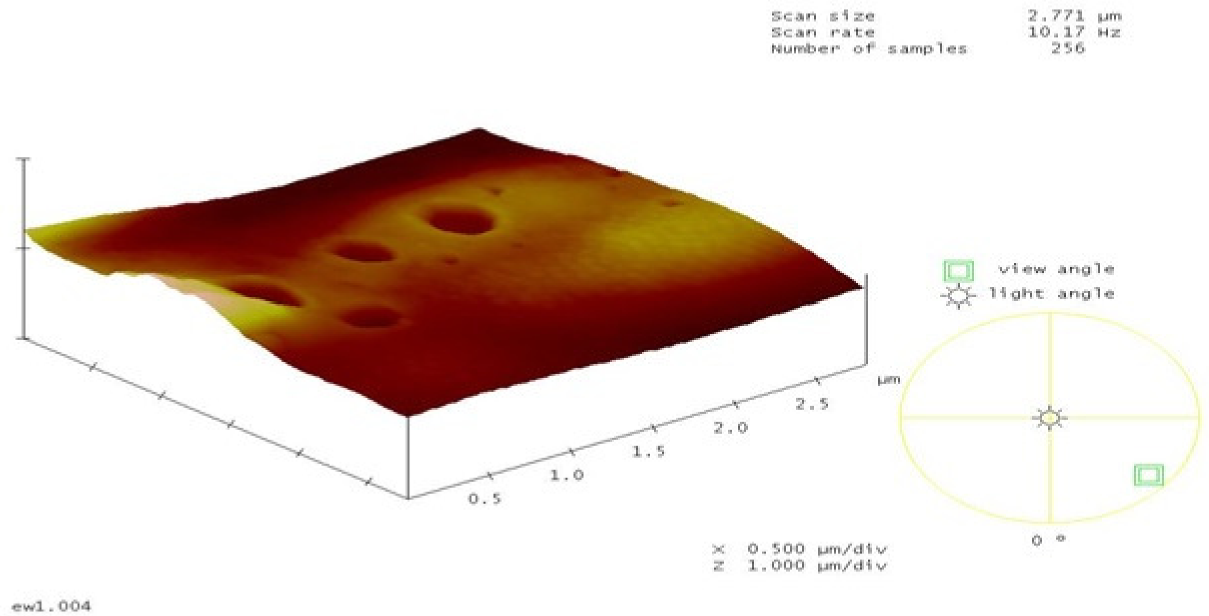
Table 1: AFM measurements of earthworm body pore & Skin Roughness

Sl.No	Earthworm Species	Pore Size		Skin Roughness [Img.Rms (Rq)] (S.D ± S.E)
		Maximum(in nm)	Minimum(in nm)	
1.	*Lemnoscolex scutarius*	0.245 ±0.245	0.225 ±0.177	31.1140 ± 1.56129
2.	*Hoplochetella stuarti*	0.324±0.000	0.178 ±0.207	9.3510 ± 0.56710
3.	*Eudrilus eugenia*	0.186±0.146	0.191 ±0.059	9.5445 ± 0.47305
4.	*Octochetona surensis*	0.334±0.056	0.271 ± 0.055	27.5045 ± 1.39229
5.	*Lampito mauritii*	0.489±0.388	0.439 ±0.346	83.7045 ± 3.62675
6.	*Drawida parva*	0.133±0.0085	0.186 ± 0.002	20.1850 ± 0.64347
7.	*Glyphidrilus tuberosus*	0.478±0.121	0.307±0.008	18.0665 ± 1.33431
8.	*Notoscolex palniensis*	0.593±0.322	0.477±0.266	12.5690 ± 0.66609
9.	*Celeriella Ditheca*	93.45±132.006	93.09 ±131.19	39.0355 ± 0.60175
10.	*Celeriella quadripapillata*	0.21±0.007	0.334±0.321	11.5410 ± 0.66327
11.	*Celeriella punctata*	0.957±1.104	1.122 ±1.225	10.2990 ± 0.34083

12.	*Perionyx sansibaricus*	0.518±0.538	0.447 ±0.162	16.5950 ± 0.57276
13.	*Octochetona pattoni*	0.404±0.389	0.4095±0.430	33.0175 ± 0.09546
14.	*Priodocheta pellucida*	71.87±22.52	91.68 ± 62.21	26.3750 ± 0.19092

AFM images of the body pores of Earthworm species

Figure 1: <u>3D Image of Earthworm (*Lemnoscolexscutarius*) body pore size measurement</u>



<u>Figure 2: Glyphidrilus tuberosus - 3D Image of one pore magnification of Earthworm skin</u>

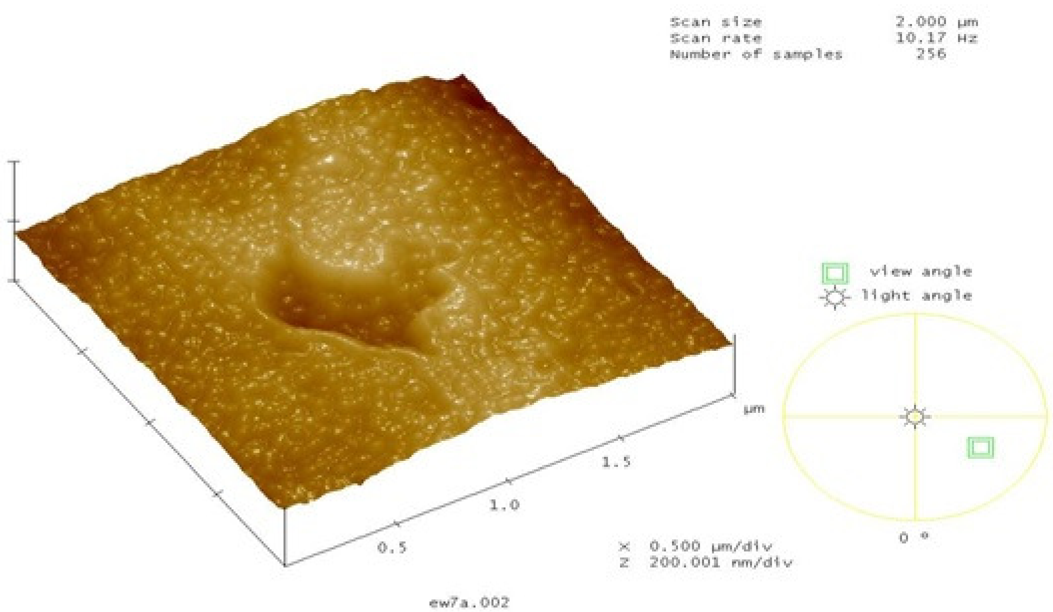


Figure 3: 3D Image of Earthworm (*Lampito mauritii*) body pore size measurement

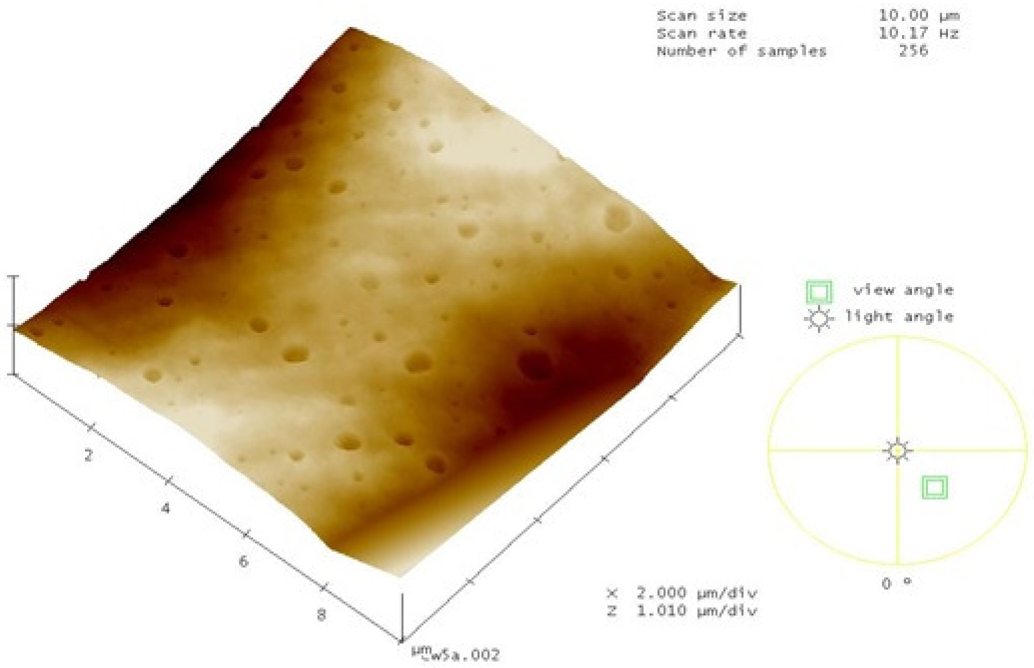


Figure 4, 5 & 6: Image of Earthworm (*Perionyx sansibaricus*) body pore size measurement

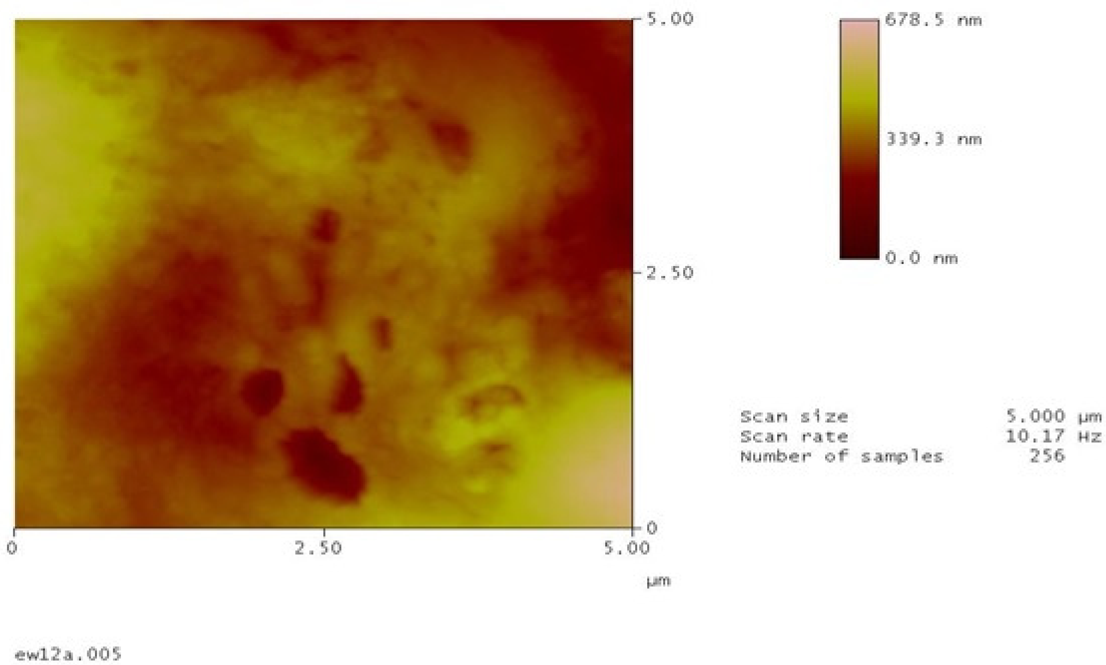


Figure 4

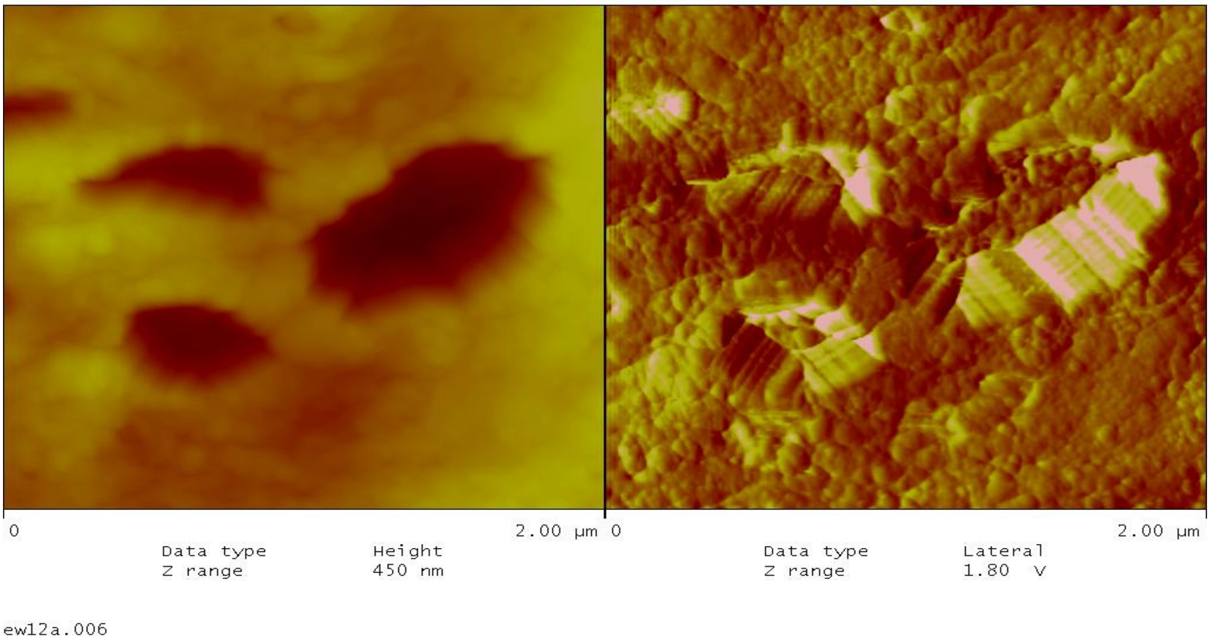


Figure 5

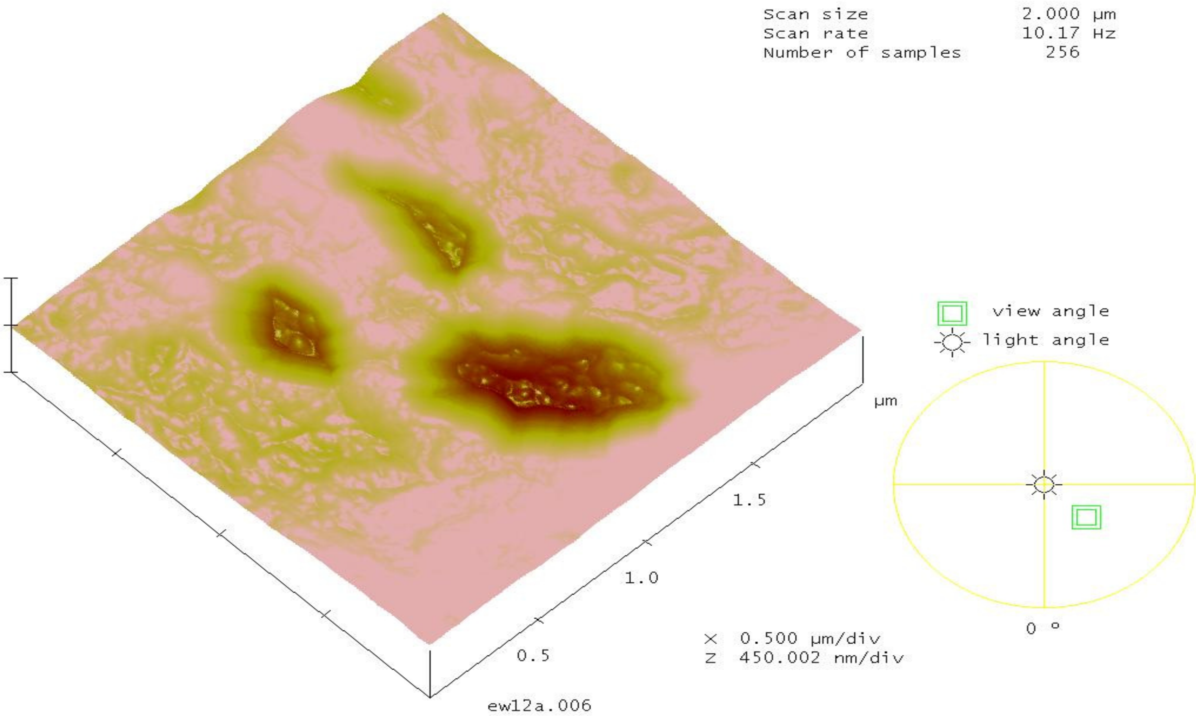


Figure 7: 3D Image of Earthworm (*Priodocheta pellucida*) body pore size measurement

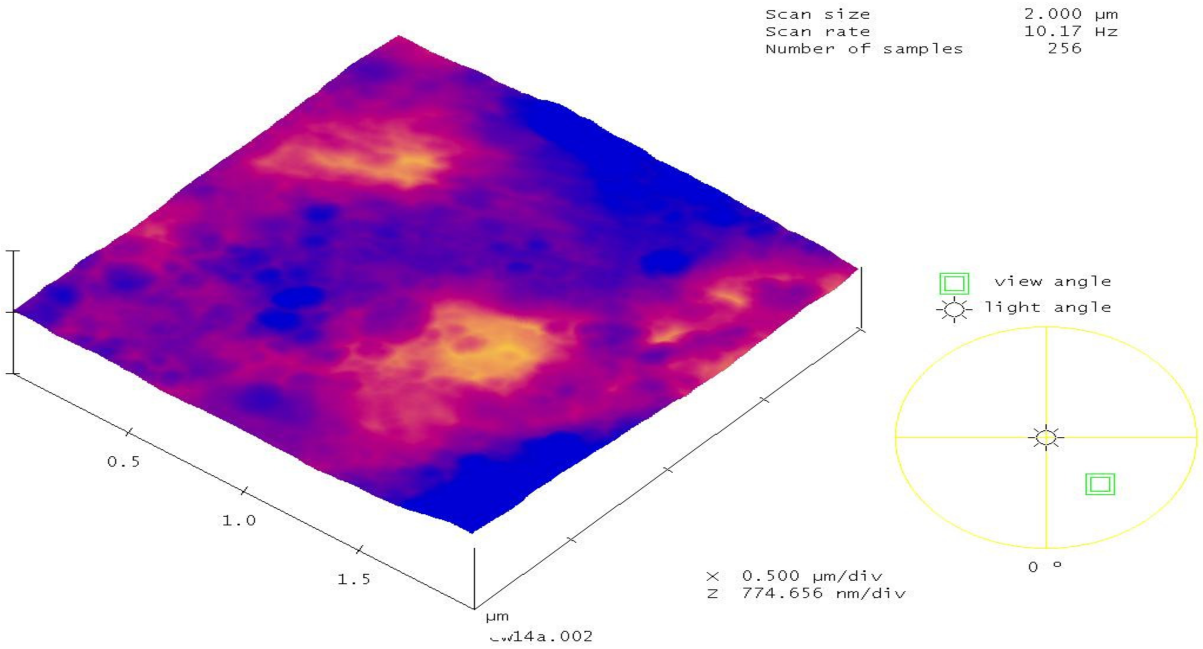


Figure 8: 3D Image of Earthworm (*Notoscolex palniensis*) body pore size measurement

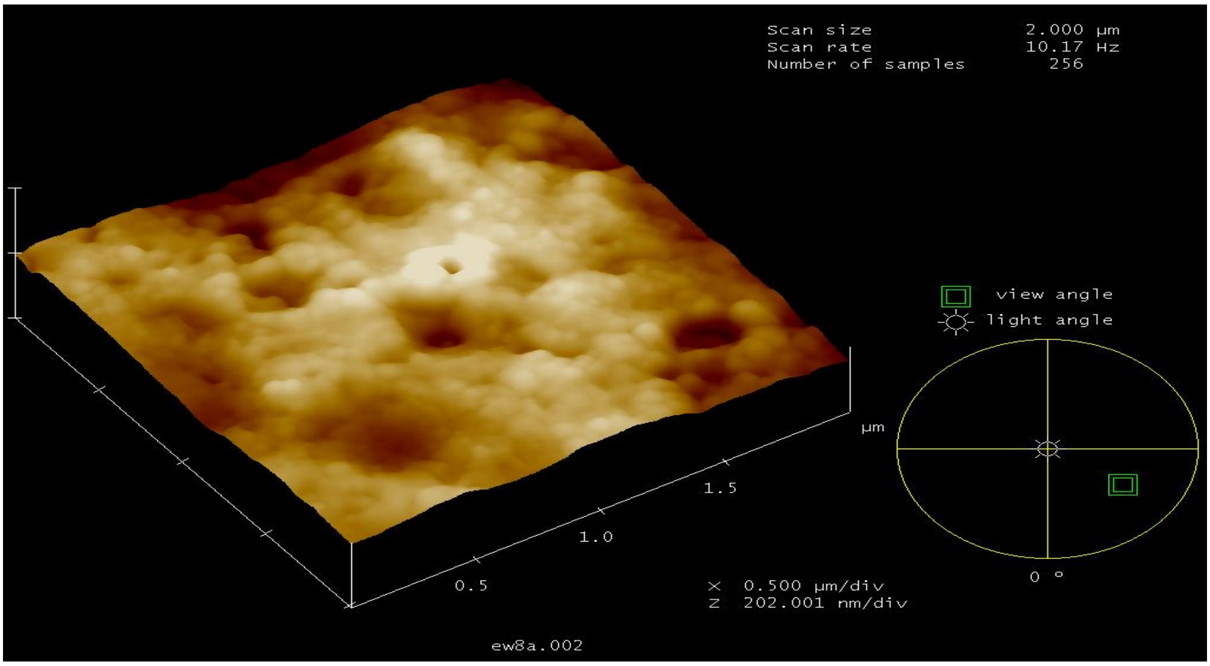


Figure 9, 10 & 11 : <u>AFM Image of Earthworm (*Hoplochetella stuarti*) skin roughness measurement</u>