THE DETERMINATION OF PROTEIN AND CERTAIN ESSENTIAL ELEMENTS IN EARTHWORM *EUDRILUS EUGENIAE* FED WITH DIFFERENT KINDS OF ORGANIC LITTERS

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การหาปริมาณโปรตีนและธาตุที่จำเป็นบางชนิดในไส้เดือนสายพันธุ์ยูดริลลัส ยูจีนิอี ที่เลี้ยงด้วยขยะอินทรีย์ต่างชนิด

นางสาวมัลลิกา ทองเขียว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE DETERMINATION OF PROTEIN AND CERTAIN ESSENTIAL ELEMENTS IN EARTHWORM *EUDRILUS EUGENIAE* FED WITH DIFFERENT KINDS OF ORGANIC LITTERS

Suranaree University of Technology has approved this thesis submitted in partial fulfillments of the requirement for a Master's Degree.

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การศึกษานี้มีวัตถุประสงค์ เพื่อหาปริมาณโปรตีนและธาตุที่จำเป็นบางชนิดในไส้เดือนที่เลี้ยงด้วยขยะอินทรีย์ต่างชนิดกัน เพื่อเป็นข้อมูลในการนำไส้เดือนไปใช้เป็นอาหารสัตว์ในอนาคต ในการทดลองได้ใช้ไส้เดือนสายพันธุ์ยูดริลลัส ยูจินีอี ซึ่งเป็นสายพันธุ์ที่สามารถขยายพันธุ์ได้ดีและโตเร็ว สามารถย่อยขยะอินทรีย์ได้ดี ได้เปรียบเทียบปริมาณโปรตีนและปริมาณธาตุที่จำเป็นบางชนิดในไส้เดือนที่เลี้ยงด้วยขยะอินทรีย์ต่างชนิดกัน ได้แก่ กากถั่วเหลือง ใบสะเดา กล้วยสุก และลังกระดาษ กับไส้เดือนที่ไม่ให้อาหารอื่นเพิ่มเติมซึ่งเป็นตัวควบคุม ผลการวิเคราะห์พบว่า ไส้เดือนมีโปรตีน 55.37-64.59 เปอร์เซ็นต์ ซึ่งใกล้เคียงกับปริมาณโปรตีนที่มีอยู่ในเนื้อผง ผลการวิเคราะห์ธาตุปริมาณหลักที่จำเป็นในหน่วยเปอร์เซ็นต์ มีดังนี้ แคลเซียม 0.52-0.65 คลอรีน 0.75-0.97 โพแทสเซียม 1.24-1.42 แมกนีเซียม 0.24-0.42 โซเดียม 0.44-0.50 ฟอสฟอรัส 0.24-0.27 กำมะถัน 0.21-0.27 และธาตุปริมาณน้อยที่จำเป็นในหน่วยมิลลิกรัมต่อกิโลกรัม คือ เหล็ก 2,116-3,231 ทองแดง 46.3-52.5 สังกะสี 180.2-192.7 แมงกานีส 35.4-94.7 และโคบอลต์ 1.16-4.96 ผลการศึกษาทำให้สรุปได้ว่าไส้เดือนอาจใช้เป็นแหล่งโปรตีนเสริมสำหรับอาหารสัตว์ได้เนื่องจากมีปริมาณโปรตีนค่อนข้างสูงและยังมีปริมาณธาตุที่จำเป็นต่อชีวิตในปริมาณที่ใกล้เคียงหรือมากกว่าที่มีอยู่ในอาหารสัตว์ชนิดอื่น

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ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

MALLIGA THONGKHEAW : THE DETERMINATION OF PROTEIN AND CERTAIN ESSENTIAL ELEMENTS IN EARTHWORM *EUDRILUS EUGENIAE* FED WITH DIFFERENT KINDS OF ORGANIC LITTERS. THESIS ADVISOR : ASST. PROF. TRITAPORN CHOOSRI, Ph.D. 91 PP.

EARTHWORM *EUDRILUS EUGENIAE*/PROTEIN/ESSENTIAL ELEMENTS/ ORGANIC LITTERS

The objective of this study was to determine the protein and certain essential elements contents in earthworms fed with different kinds of organic litters. The information would be useful for the utilization of earthworm as animal feed in the future. Earthworm *Eudrilus eugeniae* was used in the experiment because it has high rate of reproduction, grows extremely fast and is good in decomposing organic wastes. The protein and certain essential elements contents in earthworms fed with different kinds of organic wastes: soybean waste, neem leaves, ripe banana, and carton, were compared with the control group which was fed with nothing. The percentages of protein in earthworm were found to be in the range 55.37-64.59, comparable with meat powder meal. The results for the analyses of major essential elements contents of the earthworm in percentages were: Ca 0.52-0.65, Cl 0.75-0.97, K 1.24-1.42, Mg 0.24-0.42, Na 0.44-0.50, P 0.24-0.27, and S 0.21-0.27. The trace essential elements in mg/kg were: Fe 2,116-3,231, Cu 46.3-52.5, Zn 180.2-192.7, Mn 35.4-94.7, and Co 1.16-4.96.

The results showed that earthworms could be the protein source for animal feeds since the protein contents were moderately high, and the essential elements contents were quite comparable or better than the other feeds.

School of Chemistry Student's Signature_____

Academic Year 2010 Advisor's Signature_____

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Malliga Thongkheaw

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

AAS	atomic absorption spectroscopy
AES	atomic emission spectroscopy
^{o}C	degree Celsius
g	gram
h	hour
kg	kilogram
L	liter
mA	milliampere
min	minute
mg	milligram
ml	milliliter
mm	milimeter
N	normality
nm	nanometer
ppm	parts per million
% V/V	percent volume by volume
W	weight
µg	microgram
µl	microliter

CHAPTER I

INTRODUCTION

Earthworms appeared on earth 600 million years ago. They have since accompanied the build up and evolution of most soils and participated in the conservation of natural fertility. Earthworm is a common polyphagous annelid and plays an important role in the environment. According to their habitat types and ecological functions, earthworms can be divided into three major groups: the epigeic, the endogeic, and the anecic.

Epigeic earthworm species are essentially litter dwellers, they live in organic horizons in or near the litter surface and feed primarily on coarse particulate organic matter, ingesting large amount of uncomposed litter. They are essentially "litter transformers". They are typically small, uniformly pigmented species with high metabolic and reproductive rate, which represent adaptations to the highly variable environmental conditions at the soil surface. This group of epigeic species included *Lumbricus rubellus*, *Eisenia fetida*, *Eisenia Andrei*, *Dendrobaena rubida*, *Eudrilus eugeniae*, *Perionyx excavatus*, and *Eiseniella tatraedra* (Edwards and Bohlen, 1996).

Endogeic earthworm species live deeper in the soil profile and feed primarily on both soil and associated organic matter. They have little pigmentation, and they generally construct horizontal, deep-branching burrow systems that fill with cast material as they move through the organic-mineral layer of the soil. Species such as *Allolobophora caliginosa*, *Aporrectodea rosea*, *Aporrectodea caligenosa* and *Octolasion cyaneum* are included in this endogeic group (Edwards and Bohlen, 1996).

Anecic earthworm species live in more or less permanent vertical burrow systems that may extend several meters into the soil profile. The permanent burrows of the anecic earthworms create a microclimatic gradient, and the earthworm can be found at either shallow levels or deep in their burrows, depending on the prevailing soil environmental conditions. *Lumbricus terrestris*, *Aporrectodea trapezoides*, and *Allolobophora longa* are included in this ecological anecic group of earthworm (Edwards and Bohlen, 1996).

Earthworms have a major role in breaking down of organic matter and in releasing and recycling of the nutrients that it contains. They remove partially decomposed plant litter and crop residues from the soil surface, ingest it, fragment it and transport it to the subsurface layers. Their fecal material is in the form of casts and is deposited on the soil surface, in their burrows or in spaces below the soil surface, and having a major role in the development of soil horizons. Earthworms are often key organisms in the overall breakdown of the organic matter and transformation of major and minor mineral nutrients (Edwards and Bohlen, 1996).

Recently, interests have increased greatly in other roles of earthworms besides the agricultural benefits they provide. Two main research streams are in the using of earthworms in environmental management and the using of earthworms as protein supplement in animal feed.

Earthworms are able to process sewage sludges and solid paper-pulp mill sludge (Elvira et al., 1996), brewery wastes, processed potato wastes, paper industrical waste, supermarkets and restaurants wastes, animal (poultry, pigs, cattle, sheep, goats, horse, rabbits) wastes as well as horticultural residues from dead plants, yard wastes and wastes from mushroom industry (Edwards and Arancon, 2004).

For many years, earthworms have been farm-bred with a wide diversity of organic wastes, to be used as fish bait. The uses of earthworm as valuable soil additives and protein source in animal feed have been expanding rapidly. Many research results demonstrated the advantages of earthworm meal as diet supplement for the commercial feed which are quite expensive (Hilton, 1983; Stafford and Tacon, 1984; Nandeesha et al., 1988; Ibanez et al., 1993).

Among various species of earthworm, *Eudrilus eugeniae* (*E. eugeniae*) had attracted interest because of the high rate of reproduction and its capability to decompose rapidly large quantities of organic wastes (Dominguez et al., 2001; Gajalakshmi et al., 2001; Gajalakshmi et al., 2002).

The present study attempted to get more information on the nutritional value of the earthworm (*E. eugeniae*) bred in Thailand. Certain essential elements and the protein contents of earthworm fed with different kind of organic litters were investigated.

1.1 Background of the problem and significance of the study

The dominance of fish meal in feeds for cultivated fish has challenged fish nutritionists for many years. Successful replacements by other protein feedstuffs had been reported, mostly of animal origin: feather meal and meat meal (Tiews et al., 1976 quoted in Viola et al., 1982), single-cell protein (Atack et al., 1979 quoted in Viola et al. 1982), and algae meal (Sandbank and Hepher, 1978 quoted in Viola et al., 1982). However, most of these materials are as scarce and/or expensive as fish meal. Since the protein component of a fish diet is the single most expensive portion in the formulation, for this reason, it is desirable to use the most economical and readily available protein sources. However, at the present time, vegetable meals could be used only to limited extent in fish diet formulations, and fish meals should comprise approximately 25% of

the formulation (Hilton and Slinger, 1981 quoted in Hilton, 1983). So, nutritionists tried to decrease the amount of fish meal in the diet formulations and tried to replace it by other materials such as earthworms (Hilton, 1983; Stafford and Tacon, 1984; Nandeesha et al., 1988; Ibanez et al., 1993).

In many parts of the world, for example in Japan, earthworms had been used to treat waste materials such as waste of pulp industry and poultry manure (Yoshida and Hoshii, 1978). In addition to their role in waste management, earthworms also represent a valuable foodstuff suitable for animal feeding. It is easily expected that earthworms may be a good source of protein for domestic animals, if large amount of earthworms be produced by feeding them with waste materials and be supplied with reasonable price as feedstuffs.

Figure 1.1 *E. eugeniae* earthworm.

E. eugeniae or the African nightcrawler is an epigeic earthworm species. It is a large worm that grows extremely rapidly and is reasonably prolific and, under optimum conditions it would be ideal for animal feed protein production as well as for rapid organic waste conversion (Dominguez et al., 2001).

The African nightcrawler is an excellent vermicomposting worm. Vermi-composting is an excellent and natural form of recycling food and garden waste and

turning them into nutrient-rich compost or worm castings. They are playing a major role in solid waste management in Southeast Asian nations, for example, they are one of the main characters of vermicomposting in Phillippines. They are the perfect worms for home vermicomposting bins and for vermicomposting more fibrous materials such as leaves (Gajalakshmi, 2005), African nightcrawler worms are excellent as live fishing bait and also are used in exotic pet markets as food for birds, fishes, turtles and other reptilians.

In Thailand, researchers from Kasetsart University had interest and did research on the utilization of *E. eugeniae* earthworm in the treatment of wastes such as chicken manure (Kwansod et al., 2002) and dry sludge (DaDong, 2006). However, there are relatively few works on the nutritional value and very little information concerning *E. eugeniae* earthworm in Thailand. Therefore, it is interesting to do research on this subject.

In this study, the wastes materials: soybean wastes, neem leaves, banana, and carton would be used as earthworm feeds. Soybean waste is the wastes from the process of making soybean milk, it contains high amount of nitrogen and protein, and can be found in household wastes. Neem is a large, evergreen, hardy tree. It grows easily and survives even on dry, nutrient-lean soil. (Pundt, 2000 quoted in Gajalakshmi and Abbasi, 2004). In this work, neem leaves were used in earthworm breeding. There are a lot of neem trees at the earthworm farm in Uttaradit Province which was the supplier of earthworm for this experiment. Banana is food for human and animals but it is rancid and easily decomposed. Carton is a waste material from many processes and used carton can be found everywhere. It absorbs water and decomposes easily. Earthworm can consume this material as food (Sherman, 2003). Thus, it is interesting to use these materials as earthworm feeds in the experiment.

The results of this study may help to support the hypothesis that earthworm could be a source of essential elements and protein for animal feed. In addition, the results will provide more data concerning *E. eugeniae* earthworm in Thailand. The information will be transferred to the earthworm farm owner in Uttaradit Province, the supplier of the earthworms used in this study, who will use it as data base in the production of fish and frog formulations in the future.

1.2 Research objectives

1. To determine the protein and certain essential elements contents in *E. eugeniae* earthworm, fed with different kind of organic litters.

2. To compare the protein and certain essential elements contents among earthworms fed with different kind of organic litters; carton litter, neem leaves, banana litter and soybean waste.

1.3 Research hypotheses

After ingestion of organic litters, earthworm tissues may provide more valuable protein and certain essential elements, which may be important source of essential elements and protein for animal feed formulations.

1.4 Scope and limitation of the study

The study would be conducted on *E. eugeniae* earthworm supplied from the earthworm farm in Uttaradit Province, plus the earthworm bred at Suranaree University of Technology. These worms would be fed with different kind of organic litters which were soybean waste, neem leaves, banana litter, and carton litter.

1.5 Expected results

1. The results would support the assumption that the earthworm could be source of certain essential elements and protein for animal feed.

2. The results would provide the data on essential elements and protein content of the *E. eugeniae*, for the earthworm farm owner, who can use them in the formulation of fish and frog diets and provide the useful data for the general public.

CHAPTER II

LITERATURE REVIEWS

2.1 Earthworm as a source of protein

Lawrence and Millar (1945) were the first to suggest that earthworm contained sufficient protein to be considered as animal feed. They analyzed the total protein in common earthworms of a variety of colors and sizes dug from Wiltshire and the London area. The protein contents of the earthworm from this study were shown in Table 2.1.

Table 2.1 Protein and water contents of the earthworms from the study of Lawrence and Millar (1945).

	sample 1	sample 2	sample 3	sample 4	sample 5
Fresh worm (cleaned) (g)	4.66	8.0	5.0	15.0	26.0
Earthworm (desiccated) (g)	0.91	1.66	0.95	2.2	3.7
Water content in earthworm (%)	80.4	79.0	81.0	86.0	85.0
Protein, dry worm (%)	71.5	62.0	62.0	69.0	71.0
Protein, fresh worm (%)	13.95	12.9	11.7	9.9	10.0

Harwood and Sabine (1978) reported on chickens feeding with earthworm as a source of protein. They compared the use of earthworm meal as a protein supplement for chickens with that of meat meal, and found no significant difference in growth of chickens between the two diets. Similar results were reported by Taboga (1980) and Mekada et al. (1979) that when earthworms were fed to older birds, egg production was maintained. Jin-you et al. (1982) reported that chickens fed with earthworms put on

weight faster than those given other diets; they had more breast muscle and consumed less food. Fisher (1988) reported that chickens grew well, had a good weight gain per unit of food, and had excellent nitrogen retention when fed on diets with levels of worm meal from 72 to 215 g/kg.

Harwood and Sabine (1978) reported on the growth of pigs with earthworm protein supplements. The result showed that in feeding trials with both starter and grower pigs, young pigs fed on an earthworm protein supplement grew equally well and had similar food conversion ratios to those grown on commercial rations. Jin-you et al. (1982) reported that piglets grew better on earthworm protein supplements than on other protein supplements, and weaning was accelerated, estrus was also produced earlier in sows, disease resistance was increased and a decreased incidence of white diarrhea. Edwards and Niederer (1988) also reported good growth of pigs on earthworm protein.

Yoshida and Hoshii (1978) studied the nutritional value and elemental contents of dried earthworm as feedstuff for poultry. They concluded that earthworms had as high nutritional value as fish meal, except the calcium content was lower. Finally, their study suggested that if earthworms could be supplied in large quantity with reasonable price, they should be good source for energy, protein and phosphorus as poultry feed.

Tacon et al. (1983) had done two experimental feeding trials with rainbow trout. In the first trial, the nutritive values of three terrestrial lumbricid worms, *Eisenia foetida*, *Allolobophora longa*, and *Lumbricus terrestris* were compared with fish fed a commercial trout ration. In the second trial, the nutritive value of freeze-dried *E. foetida* meal was compared with that of herring meal at three dietary inclusion levels (replacing 0, 50, and 100% herring meal protein) within a semi-synthetic diet. Fish fed with frozen *A. longa* and *L. terrestris* grew as well as or better than fish fed on commercial trout pellet. But fish fed on frozen *E. foetida* displayed little or no growth over the

experimental test period. The overall conclusion was that certain terrestrial lumbricid worm species did have potential both as a complete feed and as a protein supplement feed for trout and other commercial fish feeds.

Akiyama et al. (1984) studied the outcome of using various meals as a supplement to fish meal diet for chum salmon fry. They found that fish fed with earthworm powder showed the best growth performance and feed efficiency as compared with fish fed with silkworm pupae powder, dried beef liver and krill meal.

Stafford and Tacon (1984) studied the nutritive value of the earthworm *Dendrodrilus subrubicundus* grown on domestic sewage, in trout diets. The earthworms were collected from the trickling filter beds of a domestic sewage works and freeze-dried. This study suggested that *D. subrubicundus* grown in a sewage substrate could replace herring meal in the diet of rainbow trout, providing 10% of the protein source, without losing in fish growth and feed utilization efficiency. At higher levels (50 and 100% protein replacements), fish growth and feed utilization efficiency were decreased and the concentrations of heavy metals had increased in the fish carcass and tissues.

Ibanez et al. (1993) studied on nutritional and toxicological evaluation of earthworm, *E. Fetida*, meal as a protein source for animal feed in rat. A group of rats was fed an experimental diet with inclusion of earthworm meal at 100 g protein/kg level and compared with a control group fed on diet containing casein to the same protein level. The results showed that the values of protein efficiency ratio and the net protein ratio were not significantly different for both diets. Moreover, they studied the long term toxicological evaluation of worm meal in feed, performed with an experimental diet containing 200 g earthworm protein/kg. They found no adverse effects on the rats health. The rats also showed good reproductive and lactation performance throughout the four generation periods. In addition, the study suggested that earthworm meal was similar to

casein as a protein source for rats and could be considered safe in diet formulations for livestock.

2.2 Researches on *E. eugeniae* earthworm

E. eugeniae is a large earthworm native to Africa but it has been bred extensively in the United States, Canada, Europe and Asia for the fish bait market (Dominguez et al., 2001). The classifications of earthworm (DaDong, 2006) are as follows:

Kingdom Animalia

Subkingdom Eumetazoa

Phylum Annelida

Class Oligochaeta

Order Haplotaxida

Suborder Lumbricina

Superfamily *Megascolecoidea*

Family *Eudrilidae*

Genus *Eudrilus*

Species *Eudrilus eugeniae*

The life cycle of the earthworm *E. eugeniae* is summarized in Figure 2.1

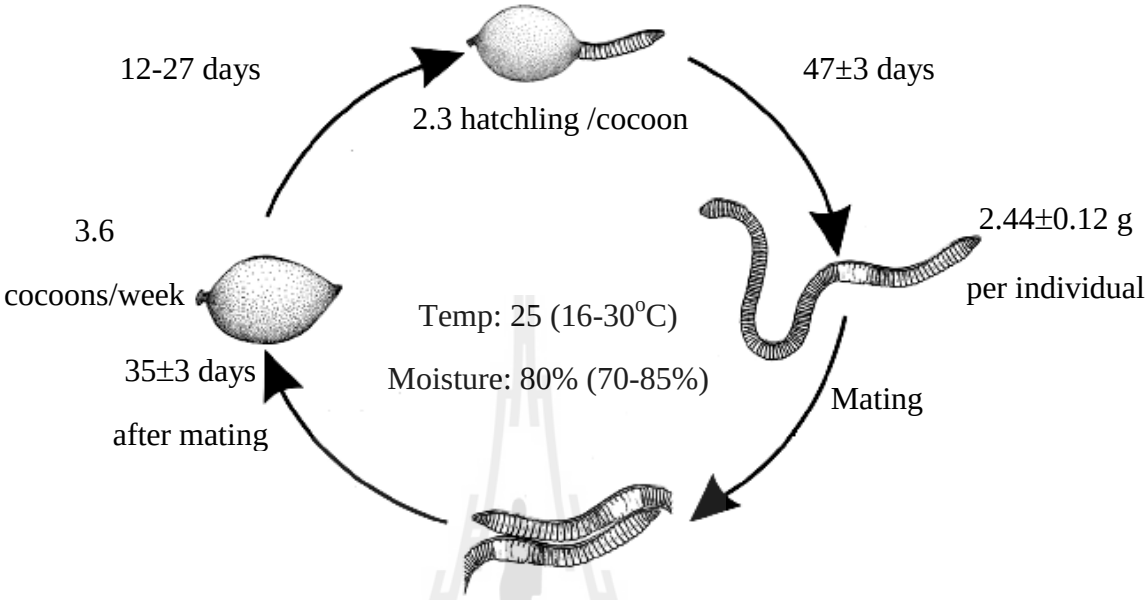

Figure 2.1 Life cycle of *E. eugeniae* (Dominguez et al., 2001; Edwards and Arancon, 2004).

Many researches demonstrated how to use *E. eugeniae* earthworm in the treatment of organic materials, since these species obtain nutrition from organic matter such as manure or activated sludge.

Dominguez et al. (2001) studied the biology and population dynamics of *E. eugeniae* in solids cattle waste. The study was performed by growing groups of 1, 2, 4, 8 or 16 small earthworms in 100 g of waste in small containers and incubated at 15, 20, 25 and 30°C. They reported that individuals of *E. eugeniae* grew well in separated cattle solids, and the maximum weight gain was 280 mg per week, compared to the highest weight gains reported for *E. fetida* of 60-80 mg per week (Graff 1974; Watanabe and Tsukamoto, 1976 quoted in Dominguez, 2001), 80-90 mg per week for *Eisenia Andrei*

(Elvira et al., 1996a quoted in Dominguez, 2001), 55-60 mg per week for *Lumbricus rubellus* (Elvira et al., 1996b quoted in Dominguez, 2001). The maximum rate of cocoon production by *E. eugeniae* was 3.6 cocoons per week at 25^{o}C which was the optimal temperature for growing the earthworm. The time from producing cocoons to sexual maturity was 47±3 days which was the shortest. The temperature for the greatest growth, maturation and biomass production was 25^{o}C, which was higher than the optimal temperature quoted for other species that inhabited temperate habitats. The study suggested that *E. eugeniae* was a fast-growing and productive earthworm in animal waste. It was ideally suited as a source of animal feed protein as well as for rapid organic waste conversion. It was more productive in terms of growth rate than other species and would seem to be a suitable candidate for vermicomposting systems, in regions where the optimal temperature of 25^{o}C was both feasible and economic.

Gajalakshmi et al. (2001) studied the potential of two epigeic (*E. eugeniae* Kinberg, and *Perionyx excavatus* Perrier) and two anecic (*Lampito mauritii* Kinberg and *Drawida willsi* Michaelson) earthworm species in terms of efficiency and sustainability of vermicomposting water hyacinth in different vermireactor. They found out that *E. eugeniae* was by far the most efficient producer of vermicasts and also was the species that showed the maximum net gain of weight.

Gajalakshmi et al. (2002) also studied the performance of the *E. eugeniae* in vermicomposting of different forms of water hyacinth such as fresh whole plants, dried whole plants and chopped pieces of fresh plants. Their experimental observations comfirmed that water hyacinth in any form could be sustainably vermicomposted with *E. eugeniae*.

Kurien and Ramasamy (2006) studied the vermicomposting of Taro with two epigeic earthworm species (*E. eugeniae* and *E. foetida*). The bioconversion potential of the two epigeic species was assessed in terms of efficiency and sustainability of vermicomposting of Taro. *E. eugeniae* was found to be more efficient producer of vermicasts than *E. foetida*.

Hilton (1983) studied the potential of freeze-dried worm meal as a replacement for fish meal in trout diet formulations. The feed formulated by using dried earthworm meal obtained from *E. eugeniae* species as a replacement for fish meal was used in the experiment. The results of the study showed that worm meal was a high protein feedstuff but low in a number of essential amino acids especially sulfur amino acids. Ash content was approximately 10%, with no high levels of minerals. They found that there was a significant linear regression of the final body weight of rainbow trout on level of worm meal in the formulation. Feeding response of trout was noticeably reduced by comparison with trout reared on high fish meal diets, indicating a lack of some unidentified essential component in the high worm meal diets. The results suggested that the freeze-dried worm meal was not a satisfactory replacement for fish meal in trout diet formulations.

Subsequently, Nandeesha et al. (1988) studied the influence of earthworm meal on the growth of common carp, Cyprinus carpio (*Linn.*). Three new fish feeds formulated by using dried earthworm meal obtained from *E. eugeniae* as a replacement for fish meal were evaluated. Fish meal was completely replaced by earthworm meal in one of the diets, while it was partially replaced in the other. In the third diet, earthworm meal was also partially replaced fish meal but was oil enriched by adding 5% sardine oil. Growth of fish fed on the test diets was compared to that on the fish meal-based control diet. The diet with 5% sardine oil provided the best growth of fish. The result of the

study also indicated the possibility of using earthworm meal at low levels in common carp diet. But it was necessary to further evaluate its influence on fish growth at different levels of incorporation.

From the literature reviews, it was clear that not many research works were done on the nutritional value of earthworm especially *E. eugeniae*. The present study was intended to provide more information on the nutritional value of *E. eugeniae* earthworm, including protein and contents of certain essential elements when fed with different organic litters. In this work, several techniques were used for the determination of the essential elements in the samples and Kjeldahl method was used for the determination of protein content.

2.3 Protein and Essential elements

2.3.1 Protein

Protein is the most important macronutrient needed for human and animals. Proteins provide the amino acids which are required to synthesize the protein in the body. Protein, in its many forms, is an essential and universal constituent of all living cells. Besides being plentiful, protein serves a variety of functions. They serve as structural components, as biocatalysts (in the form of enzymes), as antibodies, as lubricants, as messengers (in the form of hormones), and as carriers. Proteins are composed of amino acids which must be provided on food. After digestion, the amino acids which comprise the food proteins are absorbed and used to synthesize body proteins. (Berdanier, 1995). Table 2.2 shows the protein contents of some animal feeds (Marco et al., 2002).

Table 2.2 Protein contents of some animal feeds.

Animal feed	Protein (%)
Corn	5-10
Barley	10-15
Lupines	15-35
Sunflower powder	25-30
“Delicias” foodstuff	25-30
Full fat soya	35-40
Soya powder	35-50
Meat powder	50-55
Fish powder	70-75

2.3.2 Essential elements

Inorganic elements make up only 4% of the animal body tissues, but they are essential as structural elements and involve in many vital processes. Two main roles of these elements can be described as functional and structural. From the functional standpoint, they play a catalyzing role in enzymatic systems by binding their ions to substrates, thereby favoring various reactions, especially in the mediation of oxidation-reduction reactions, through reversible changes in the oxidation states of the metal ions. Structurally, they stand out for their roles as integrators of the body’s organic compounds, such as iron in hemoglobin and cobalt in vitamin B_{12} (Neves et al., 2009; Mcdowell, 1992).

All forms of living matter require inorganic elements, or minerals, for their normal life of processes. All animal tissues and all feeds contain inorganic elements in widely varying amounts and proportions.

Many inorganic elements are essential for animal well being. The four organically bound elements (C, H, O, and N) make up just over 96% of the animal's body weight. The principal cations and anions of Na, K, Ca, Mg, P, S, and Cl together account for 3.5-3.78% of the body weight and they are called major or macro essential elements. The amounts of major essential elements can be worked with conveniently in concentrations on a percentage (%) basis. The trace or micro essential elements are required in a minute amounts. They are usually expressed in milligrams per kilogram (mg/kg) unit. The trace essential elements are Cr, Co, Cu, Ni, Se, Mn, F, Mo, I, Fe, V, Br, and Zn. The element is considered essential if: (1) it is present in living tissues at a relatively constant concentration; (2) it provokes similar structural and physiological anomalies in several species when removed from the organisms; (3) these anomalies are prevented or cured by the supplement of the element (Gomes and Silva, 2007).

Twenty essential elements are known to be required by at least human and animals as shown in Table 2.3.

Table 2.3 Major and trace essential elements in some animal species.

Major elements or Macro essential elements	Trace or Micro essential elements	
Calcium (Ca)	Chromium (Cr)	Cobalt (Co)
Phosphorus (P)	Selenium (Se)	Zinc (Zn)
Potassium (K)	Copper (Cu)	Manganese (Mn)
Magnesium (Mg)	Fluorine (F)	Molybdenum (Mo)
Chlorine (Cl)	Iodine (I)	Iron (Fe)
Sodium (Na)	Nickel (Ni)	Vanadium (V)
Sulfur (S)	Bromine (Br)	

Quoted in Gomes and Silva, 2007.

2.4 Techniques used for the analyses

2.4.1 Atomic absorption spectroscopy (AAS)

AAS is a technique used for the determining of the concentration of a particular element in a sample. AAS has been successfully applied for the determination of over 70 elements with sensitivities that fall in the parts per million to parts per billion ranges (Skoog and Leary, 1992).

Flame atomic absorption spectroscopy is a very common technique for detecting metals and metalloids in samples. It is very reliable and simple to use. The technique is based on the fact that ground state metal atoms absorb light at specific wavelengths and are converted to atomic state by means of a flame. Light of appropriate wavelength is supplied to atomic metal and the amount of light absorbed can be measured and converting to the concentration of the metal atoms by using a standard curve.

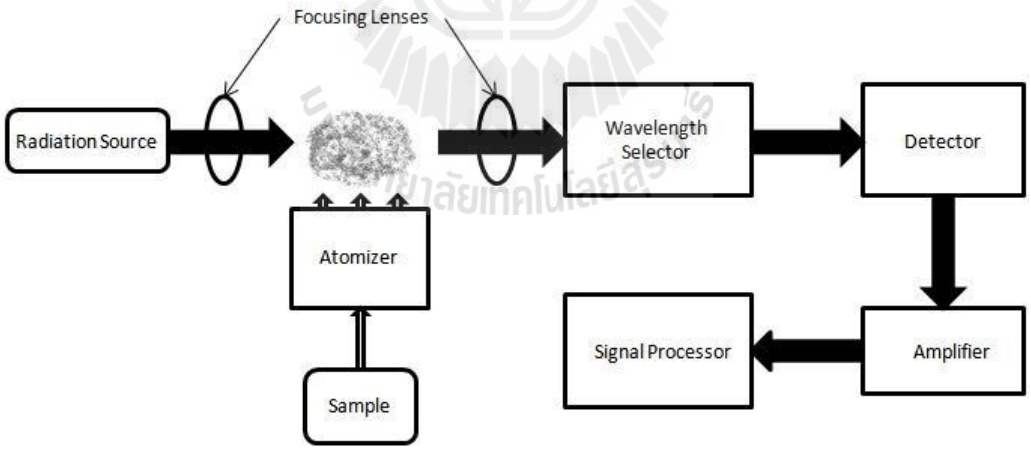

Figure 2.2 Components of the atomic absorption spectrophotometer (Atomic absorption spectroscopy, www, 2009).

2.4.2 Atomic emission spectroscopy (AES)

In this technique, flame is used as an atomizer and radiation source (also called flame emission spectroscopy). Its most important use has been in the determination of alkali and alkaline earth metals such as sodium, potassium, lithium and calcium, particularly in biological fluids and tissues (Skoog and Leary, 1992). The sample solution is converted into an aerosol in an atomizer. It then passes through an expansion chamber to allow a fall in the gas pressure and the larger droplets to settle out before passing to the burner, where the solvent evaporates instantly, and the atoms remain as a finely distributed gas. Atoms in the sample that are bound in the molecules should be decomposed at the flame temperature so rapidly that the same effect is achieved. In practice only small proportion of the sample (approximately 5%) is effectively atomized because the droplet size of the remaining 95% is so large that the water is never effectively stripped away (Holme and Peck, 1998). The components of atomic absorption spectrophotometer when using in an emission mode are shown in Figure 2.3.

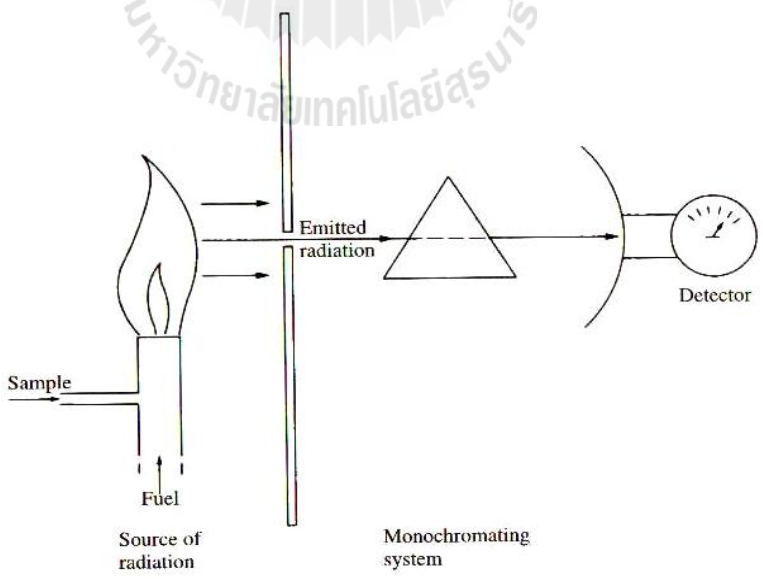

Figure 2.3 Components of the atomic absorption spectrophotometer when using in an emission mode (Holme and Peck, 1998).

2.4.3 CHNS analyzer

CHNS analyzer is a scientific instrument which can determine the elemental composition of a sample. The name is derived from the four elements measured by the device: carbon (C), hydrogen (H), nitrogen (N), and sulfur (S). A combustion process is used to break down substances into simple compounds which are then measured. By separating out inorganic carbon using a solvent, organic carbon in a sample can be measured using this device as well.

The CHNS analyzer can be used to analyze simultaneously the percentages of carbon, hydrogen, nitrogen, and sulfur in both solid and liquid samples. The technique used is dynamic flash combustion. The result of the combustion is the mixture of gases composed of N_yO_x products, CO_2, H_2O, and SO_2. The resulting four components are detected by thermal conductivity detector (TCD). The gas mixture passes through the reduction catalyst that eliminates any excess of oxygen and converts N_yO_x products into N_2. Gases which are carried forward by the helium flow are separated in a gas chromatography column located in the temperature-stable oven and detected by the highly sensitive TCD detector (Scientific Equipment Center, Prince of Songkla University, www, 2008).

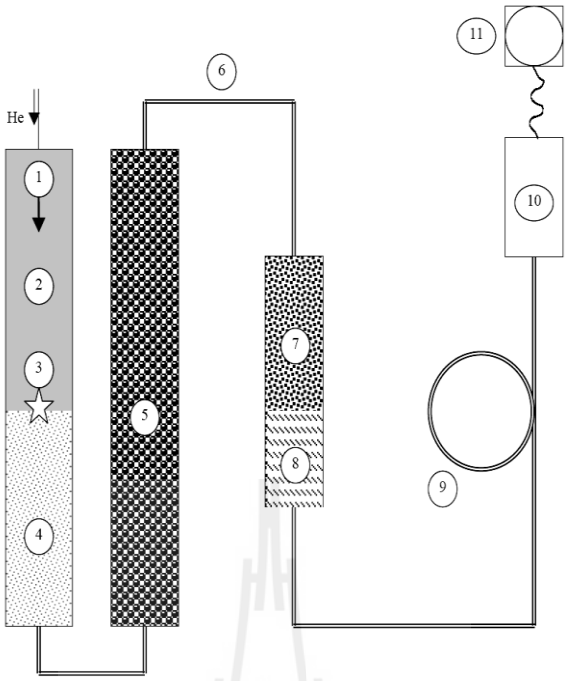

Figure 2.4 Components of a CHNS analyzer. (1) sample and oxygen in the combustion tube, (2) combustion at 900-950^{o}C, (3) combustion at 1800^{o}C, (4) oxidation and halogen trappings on Ag/Co, (5) reduction of SO_3 to SO_2 and excess oxygen by copper at 700^{o}C, (6) gases in helium include N_2, SO_2, CO_2 and H_2O, (7) trapping unmeasured H_2O, using anhydrone, (8) trapping unmeasured CO_2, by ascarite, (9) chromatography column, by using He as the carrier gas, (10) conductivity detector, (11) signal gathering and data processing (Résolution Oeno,www, 2002).

2.4.4 UV-Vis Spectroscopy

The technique involves the spectroscopy of photons in the UV-visible region. In this region of the electromagnetic spectrum, molecules undergo various electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited states to the ground state, while absorption measures transitions from the ground state to the excited states (Holme and Peck, 1998). The amount of radiation absorbed by a substance cannot be measured directly and it is usually determined by measuring the difference in intensity between the radiation falling on the sample (incident radiation, I_0) and the residual radiation which finally emerges from the sample (transmitted radiation, I).

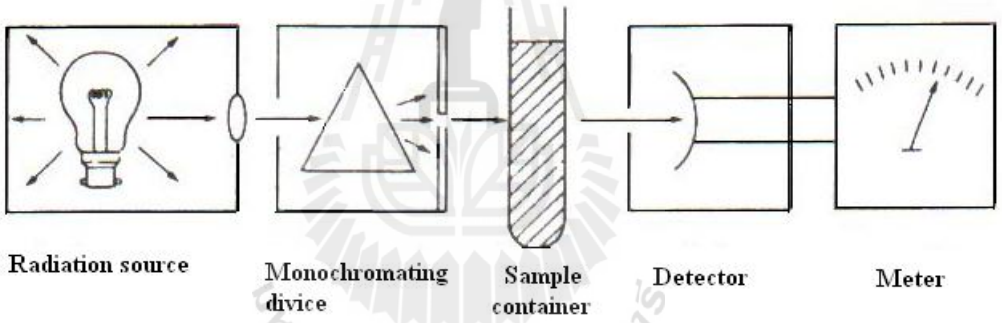

Figure 2.5 Components of a UV-Vis spectrophotometer (Holme and Peck, 1998).

2.4.5 Vanadomolybdophosphoric acid method

The vanadomolybdophosphoric acid method is a technique used to determine the concentration of phosphorus based on the reaction of ammonium molybdate with orthophosphate in acid solution to form molybdophosphoric acid. A typical reaction of the formation of molybdenum yellow in an acidic medium is as follows:

$$H_3PO_4 + 12(MoO_3) \longrightarrow H_3PMo_{12}O_{40} \text{.....} (2.1)$$

$$H_3PMo_{12}O_{40} + NH_4VO_3 \longrightarrow H_4PVMo_{11}O_{40} + NH_3 \text{.....} (2.2)$$

where MoO_3 represents the molybdate being present as Mo(VI) in an acidic medium, in the presence of oxoacids, such as vanadate and antimonite, ternary heteropoly acids can be formed (Motomizu and Hai, 2005).

In the presence of vanadium an intense yellow color of the complex $H_4PVMo_{11}O_{40}$ is formed, the intensity of which is proportional to the phosphate concentration. There exist more sensitive analytical methods for orthophosphate, one of which is basically involved the reduction of the molybdophosphoric acid to a blue complex, referred to as molybdenum blue. The ascorbic acid method is more commonly used, but the vanadomolybdate method allows analysis at a lower wavelength (400-470 nm), making it possible to easily obtain an absorption spectrum. For the ascorbic acid method, orthophosphate reacts in acid medium with ammonium molybdate and potassium antimonyl tartrate to form phosphomolybdic acid, which is reduced by ascorbic acid to form highly color molybdenum blue which absorbs at 880 nm.

2.4.6 Kjeldahl method

This method is used to estimate the protein content in the samples. The method is used to measure the nitrogen content of a compound and may be used to determine the protein content of the sample provided that the proportion of nitrogen in the protein is known. The nitrogen content of the proteins is usually accepted as 16% of the total weight (Holme and Peck, 1998). The Kjeldahl method consists of three steps, which have to be carefully carried out in sequences; (1) The sample is first digested in strong sulfuric acid in the presence of a catalyst, which helps in the conversion of the amine nitrogen to ammonium ions, (2) The ammonium ions are then converted into ammonia gas, then heated and distilled. The ammonia gas is led into a trapping standard solution where it dissolves and becomes an ammonium ion once again, and (3) Finally, the

amount of the ammonia that has been trapped is determined by titration with a standard solution, and a calculation is made.

The reactions are as follows:

(1) Digestion

$$\text{Organic sample (C,H,N,O)} \xrightarrow[H_2SO_4/\text{catalyst}]{} CO_2\,(g) + H_2O\,(l) + NH_4HSO_4 \quad \text{.....(2.3)}$$

$$NH_4HSO_4 + 2NaOH \xrightarrow[\text{pH 10-12}]{} NH_4OH + H_2O + Na_2SO_4 \quad \text{.....(2.4)}$$

(2) Distillation

$$NH_4OH + 2H_2O + Na_2SO_4 \longrightarrow [NH_3 + H_2O] \text{ as distillate} \quad \text{.....(2.5)}$$

$$NH_3 + H_3BO_3 \text{ (in excess)} \longrightarrow NH_4H_2BO_3 \quad \text{.....(2.6)}$$

(3) Titration

$$NH_4H_2BO_3 + HCl \longrightarrow NH_4Cl + H_3BO_3 \quad \text{.....(2.7)}$$

The calculations are based on the fact that 1 mole atom of nitrogen will result in the formation of 1 mol of ammonia, which will subsequently require 1 mol of acid.

1.0 mol of HCl ≅14 g nitrogen .....(2.8)

If A ml of acid solution containing B mol L^{-1} is required to neutralize the ammonia formed by a given amount of sample, the amount of nitrogen present in the sample is:

$$\frac{14}{1000} \times A \times B \text{ grams} \quad \text{.....(2.9)}$$

the amount of protein from which this nitrogen is derived:

$$\frac{14}{1000} \times A \times B \text{ grams} \times \frac{100}{16} \quad \text{.....(2.10)}$$

2.4.7 Volhard method

Volhard method is a technique used for the determination of chlorine (in the form of chloride ion) in the samples. Back titration with ammonium or potassium thiocyanate is being used to determine the concentration of chloride ions in a solution. Before the titration, an excess known volume of standard silver nitrate solution is added to the solution containing chloride ions, to form a precipitate of silver chloride. The term excess is used as the moles of silver nitrate added which are known to exceed the moles of chloride present in the sample so that all the chloride ions present will react.

$$Ag^{+}(aq) + Cl^{-}(aq) \longrightarrow AgCl(s) \quad \text{.....(2.11)}$$

Ferric ion indicator is then added and the solution is titrated with standard potassium or ammonium thiocyanate solution. The titrated solution remains pale yellow as the excess (unreacted) silver ions react with thiocyanate ions to form a silver thiocyanate precipitate.

$$Ag^{+}(aq) + SCN^{-}(aq) \longrightarrow AgSCN(s) \quad \text{……...(2.12)}$$

Once all the silver ions have reacted, the slightest excess of thiocyanate will react with Fe^{3+} to form a dark red complex.

$$Fe^{3+}(aq) + SCN^{-}(aq) \longrightarrow [FeSCN]^{2+}(aq) \quad \text{…….(2.13)}$$

The concentration of chloride ions is determined by subtracting the titration findings of the moles of silver ions that react with the thiocyanate from the total moles of silver nitrate added to the solution. This method is used when the pH of the sample solution is acidic (Burns and Muraca, 1960).

CHAPTER III

MATERIALS AND METHODS

3.1 Earthworm collection and breeding

Earthworm samples were collected from the earthworm farm in Uttaradit Province, but later were bred at Suranaree University of Technology. Earthworms were fed with different kinds of organic litters, which were soybean waste, neem leaves, ripe banana, carton, and fed with nothing as a control. The materials used for breeding; soil, cow manure, and rice straw (3:1:1) were mixed, then water was added to moist the material. The plastic drawers were used as breeding containers. There were 68 worms in each drawer. They were fed once with 1 kg of different organic litters in a 3-month period, then collected for the experiments.

3.1.1 Preparation of organic litters for earthworm breeding

Organic litters which were soybean waste, neem leaves, ripe banana, and small pieces of carton had been used in the experiment. Soybean waste was used as it was. Neem leaves were chopped to small pieces and fermented by soaking in water for 1 week until the substrate decayed. Ripe banana was chopped to small pieces and let stand for 1 day before used. Carton was soaking in water for 1 day to make it softer before used.

Soil Cow manure Rice straw

Figure 3.1 Materials used for earthworm breeding.

Plastic container Earthworm

Figure 3.2 Mixed materials in plastic container and earthworms before breeding.

Figure 3.3 Plastic containers for earthworm breeding.

3.2 Sample names

The samples being analyzed were named as follows:

EW-soy	=	Earthworm fed with soybean waste
EW-neem	=	Earthworm fed with neem leaves
EW-banana	=	Earthworm fed with banana
EW-carton	=	Earthworm fed with carton
EW-control	=	Earthworm with breeding material only
Litter-soy	=	Soybean waste
Litter-neem	=	Neem leaves
Litter-banana	=	Banana
Litter-carton	=	Carton
Litter-control	=	Breeding material

3.3 Cleaning of the glasswares and plasticwares

All glasswares used in the minerals analysis were washed, rinsed, and soaked in 10% (V/V) nitric acid for 24 h. Plasticwares were soaked in 1% (V/V) nitric acid for 24 h. Finally, all glasswares and plasticwares were rinsed thoroughly with deionized water and left at room temperature for drying before used.

3.4 Samples preparation for protein and essential elements analyses

3.4.1 Preparation of earthworm

Samples were prepared according to Dai et al. (2004).

Materials and equipments

1. Standard sieve 0.18 mm; Analysensieb, Retsch, USA.
2. Analytical balance; Model 205A, Precisa, Switzerland.

3. Oven; Model 400; Memmert, Germany.
4. Polypropylene plastic bottles and sealed plastic bags
5. Plastic trays for drying sample
6. Agate mortar and pestle
7. Beakers: 1500 ml
8. Spatula
9. Desiccator

Procedure

- Collected the adult earthworms from the containers and rinsed with distilled water. Placed one Whatman No. 42 filter paper in the beaker, added a few drops of distilled water to maintain moisture. Then put the cleaned earthworms on the filter paper.
- Kept the beaker with earthworms at room temperature for 2 days, changed filter paper daily to allow complete evacuation of the gut contents.
- Killed the earthworms by freezing in the freezer which was -4^{o}C for 1 day and then oven dried at 70^{o}C for 48 h to constant weight. After cooling to room temperature in a desiccator, weighed and recorded the dry weight of the earthworms.
- Crushed the earthworm samples using the agate mortar and sieved through a standard 0.18 mm sieve.
- Transferred the sieved samples into sealed plastic bags and kept in the desiccator until analysis.
- These samples would be used for the protein and essential elements analyses.

before breeding after breeding

Figure 3.4 Earthworms in the experiment.

Figure 3.5 Dried earthworms before analysis.

3.4.2 Preparation of organic litters

Organic litters used in the experiment; soybean waste, neem leaves, ripe banana (chopped), and small pieces of carton were oven dried at 80^{o}C before used.

The organic litters were consider dried, when their weights did not change more than 5% within 24 h. After drying, soybean waste and ripe banana were crushed using the agate mortar to small particles, neem leaves were crushed using the agate mortar and sieved through a standard 0.18 mm sieve, and carton was cut to small pieces. Put the litters in the sealed plastic bags and kept in desiccator until analysis.

3.5 Protein analyses

Kjeldahl method was used for the protein analyses of earthworm samples and the litters used for feeding.

Materials and equipment for Kjeldahl analysis

1. Digestion block and associate glasswares; Tecator 2006 Digestor, Perstorp Analytical Company, Sweden.
2. Distillation unit and associate glasswares; Tecator, Kjeltec system 1002 Distilling Unit, Perstorp Analytical Company, Sweden.
3. Exhaust acid apparatus; Tecator, Exhaust system 1013 Scrubber Unit, Perstorp Analytical Company, Sweden.
4. Analytical balance; Model 205A, Precisa, Switzerland.
5. Buret: 50 ml.
6. Stand and clamp.
7. Beakers: 100 ml, 250 ml.
8. Volumetric flasks: 25 ml, 100 ml, 1000 ml.
9. Erlenmeyer flask: 250 ml.

10. Glass beads.

11. Pipet.

Reagents

1. Potassium sulphate (K_2SO_4); Carlo Erba reagent, Italy.
2. Copper sulphate pentahydrate ($CuSO_4.5H_2O$); Ajax Finechem, New Zealand.
3. Methyl orange; Merck, Germany.
4. Methyl red; BHD, England.
5. Bromocresol green; Merck, Germany.
6. Catalyst tablets: Containing 5.00 g K_2SO_4 and 1.00 g $CuSO_4.5H_2O$.
7. Boric acid (H_3BO_3); Ajax Finechem, New Zealand.

   4% H_3BO_3 with mixed indicator.

   Dissolved 4.00 g H_3BO_3 in H_2O containing 0.70 ml 0.1% alcoholic solution of methyl red and 1.00 ml 0.1% alcoholic solution of bromocresol green, and transferred to 100 ml volumetric flask. Made up to volume with deionized water.
8. Sodium hydroxide (NaOH); Carlo Erba reagent, Italy.

   40% NaOH

   Dissolved 400.00 g NaOH in deionized water and transferred to 1000 ml volumetric flask. Made up to volume with deionized water.
9. Hydrochloric acid 37 % (HCl); Carlo Erba reagent, Italy.

   0.2000 N HCl

   Pipetted 16.70 ml conc. HCl into 1000 ml volumetric flask containning small volume of water then made up to 1000 ml with deionized water. The acid solution was standardized with standard sodium carbonate following the

AOAC official method 936.15. The concentration of the hydrochloric acid solution was found to be 0.1960 N.

10. Hydrogen peroxide 30% (H_2O_2); Analytical Univar Reagent, New Zealand.
11. Sulfuric acid 96 % (H_2SO_4); Carlo Erba reagent, Italy.

Procedure

- The analyses were being done in triplicates and the average mean values were reported.
- Accurately weighed ca 0.5000-1.0000 g well-ground samples (from 3.4.1 and 3.4.2) on weighing paper and transferred each sample to 250 ml digestion tubes.
- Placed tubes in a digester block which could accommodate six tubes at a time, added 4-5 glass beads, 15.00 ml conc. H_2SO_4, and slowly added 3.00 ml of 30% H_2O_2. Let the reaction subside and placed the digester block in the heating block.
- Digested at 410^{o}C until the mixture was clear. Removed the tubes from the digestion block, let them cool to room temperature, and carefully added 50-75 ml H_2O.
- Filled the digestion tubes with 50-75 ml 40% NaOH solution from an alkali tank of the steam distillation unit, then attached the digestion tube containing diluted sample solution to the distillation unit.
- Placed 250 ml receiving flask containing 25 ml of 4% H_3BO_3 with mixed indicator (absorbing solution) on receiving platform, with tube from the condenser extending below the surface of absorbing solution.

- Steam distilled one sample at a time until 100-125 ml were collected and the color of the indicator changed from pink to green. Removed the digestion tube and receiving flask from the unit.
- Titrated the absorbing solution with standard 0.1960 N HCl solution to pink color end point and recorded the volume of the acid used. Titrated the reagent blank similarly.

$$\%N = \frac{(V_A - V_B) \times N \times 1.4007}{W} \quad \text{.....(3.1)}$$

$$\%\ \text{Protein} = \frac{(V_A - V_B) \times N \times 1.4007 \times 6.25}{W} \quad \text{.....(3.2)}$$

Where

V_A = volume of standard hydrochloric acid used in the sample titration (ml)

V_B = volume of standard hydrochloric acid used in the blank titration (ml)

N = concentration of standard hydrochloric acid (0.1960 N)

1.4007 = milliequivalent weight of nitrogen × 100 (%)

6.25 = factor to change %N to %protein for meat products (meat product usually contains 16% nitrogen)

W = weight of sample used (g)

Figure 3.6 Digester and digestion tubes for digestion step.

Figure 3.7 Distillation Unit.

3.6 Elemental analyses

In this work, atomic absorption and atomic emission spectroscopy (AAS and AES) techniques, Volhard method, and CHNS analyzer were being used to determine the concentrations of the essential elements in earthworms and in litters used in feeding. The analyses were being done in triplicates and the average mean values were reported.

3.6.1 Elemental analyses by atomic absorption spectroscopy technique

Materials and equipments

1. Atomic absorption spectrophotometer; Model Spectra 250 plus, Varian, Australia.
2. Micro pipet; Model Pipet Man, Gilson Medical Electronics, France.
3. Analytical balance; Model 205A, Precisa, Switzerland.
4. Volumetric flasks.
5. Filter paper; Whatman No. 42.
6. Aluminium foil.
7. Parafilm.

Reagents

1. Nitric acid 65% (HNO_3); Carlo Erba reagent, Italy.
2. Hydrochloric acid 37% (HCl); Carlo Erba reagent, Italy.
3. Deionized water.
4. Standard solutions for AAS:
   - Ca, Cu, Fe, and Zn; Merck, Germany.

     Ca: 999±2 ppm, Cu: 998±2 ppm, Fe: 999±2 ppm, Zn: 999±2 ppm.
   - K, Mg, Mn, and Na; Carlo Erba reagent, New Zealand.

     K: 1000 ppm, Mg: 1000 ppm, Mn: 1000 ppm, Na: 1000 ppm.

- Co; BDH Laboratory Supplies, England.

  Co: 998±5 ppm.

Preparation of standard solutions

99.9 ppm Ca

Pipetted 5.00 ml 999 ppm Ca into 50 ml volumetric flask, then made up to 50 ml with deionized water.

99.8 ppm Co

Pipetted 5.00 ml 998 ppm Co into 50 ml volumetric flask, then made up to 50 ml with deionized water.

99.8 ppm Cu

Pipetted 5.00 ml 998 ppm Cu into 50 ml volumetric flask, then made up to 50 ml with deionized water.

99.9 ppm Fe

Pipetted 5.00 ml 999 ppm Fe into 50 ml volumetric flask, then made up to 50 ml with deionized water.

10 ppm K

Pipetted 2.50 ml 1000 ppm K into 250 ml volumetric flask, then made up to 250 ml with deionized water.

10 ppm Mg

Pipetted 0.50 ml 1000 ppm Mg into 50 ml volumetric flask, then made up to 50 ml with deionized water.

10 ppm Mn

Pipetted 2.50 ml 1000 ppm Mn into 250 ml volumetric flask, then made up to 250 ml with deionized water.

10 ppm Na

Pipetted 0.50 ml 1000 ppm Na into 50 ml volumetric flask, then made up to 50 ml with deionized water.

9.99 ppm Zn

Pipetted 1.00 ml 999 ppm Zn into 100 ml volumetric flask, then made up to 100 ml with deionized water.

Preparation of sample solution

- Weighed ca 0.5000 g (W) well-ground samples (from 3.4.1 and 3.4.2) into the digestion tubes.
- Added 30 ml of mixed acid, 2:1 ratio of conc. HNO_3: conc. HCl.
- Placed the tubes on the digester block and set the temperature at 50^{o}C for 2 h.
- Increased the temperature of the digester block progressively to 150^{o}C, and kept for 2 h, then increased the temperature to 200^{o}C and kept for 1 h.
- Cooled the solutions to room temperature.
- Made up the volume of the solutions to 250 ml (V_1) with deionized water, kept in polypropylene bottles, and stored in a refrigerator until analysis.
- Sample solutions from this step would be called the sample stock solution and would be used for the analyses of Ca, Co, Cu, Fe, K, Mg, Mn, Na, and Zn.

3.6.1.1 Determination of calcium

Procedure

- Measured the amount of Ca in the sample stock solution using AAS technique with standard addition method.
- Pipetted 0.20 ml (V_3) of the sample stock solution into four 10 ml (V_2) volumetric flasks.
- Added exactly 0, 300, 500, and 700 μl of standard 99.9 ppm Ca into each of the four volumetric flasks to obtain 0, 3, 5, and 7 ppm of standard Ca solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Ca from standard addition curve.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	422.7 nm
Lamp current	15 mA
Slit width	0.70 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

Calculation

$$\text{Calcium (μg/g)} = \frac{C_{Ca} \times V_1 \times V_2}{W \times V_3} \qquad \text{……..……..(3.3)}$$

Where

C_{Ca} = concentration of Ca from standard addition curve (µg/ml)

V_1 = total volume of sample solution from 3.6.1 (250 ml)

V_2 = total volume of sample solution used in AAS measurement (10 ml)

V_3 = volume of the sample stock solution being diluted for the measurement (0.20 ml)

W = weight of sample (g)

3.6.1.2 Determination of cobalt

Procedure

- Measured the amount of Co in the sample stock solution using AAS technique with standard addition method.
- Pipetted 5.00 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 200, 300, and 400 µl of standard 99.8 ppm Co into each of the four volumetric flasks to obtain 0, 2, 3, and 4 ppm of standard Co solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Co from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Co analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	240.7 nm
Lamp current	30 mA
Slit width	0.20 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.3 Determination of copper

Procedure

- Measured the amount of Cu in the sample stock solution using AAS technique with standard addition method.
- Pipetted 5.00 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 300, 500, and 700 μl of standard 99.8 ppm Cu into each of the four volumetric flasks to obtain 0, 3, 5, and 7 ppm of standard Cu solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Cu from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Cu analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	324.8 nm
Lamp current	20 mA
Slit width	0.70 nm

Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.4 Determination of iron

Procedure

- Measured the amount of Fe in the sample stock solution using AAS technique with standard addition method.
- Pipetted 0.50 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 400, 600, and 800 μl of standard 99.9 ppm Fe into each of the four volumetric flasks to obtain 0, 4, 6, and 8 ppm of standard Fe solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Fe from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Fe analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	248.3 nm
Lamp current	30 mA
Slit width	0.20 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.5 Determination of potassium

Procedure

- Measured the amount of K in the sample stock solution using AAS technique with standard addition method.
- Pipetted 0.2 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 1.00, 2.00, and 3.00 ml of standard 10 ppm K into each of the four volumetric flasks to obtain 0, 1, 2, and 3 ppm of standard K solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of K from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for K analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	766.5 nm
Lamp current	12 mA
Slit width	0.70 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.6 Determination of magnesium

Procedure

- Measured the amount of Mg in the sample stock solution using AAS technique with standard addition method.
- Pipetted 0.1 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 300, 500, and 700 µl of standard 10 ppm Mg into each of the four volumetric flasks to obtain 0, 0.3, 0.5, and 0.7 ppm of standard Mg solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Mg from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Mg analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	285.2 nm
Lamp current	6 mA
Slit width	0.70 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.7 Determination of manganese

Procedure

- Measured the amount of Mn in the sample stock solution using AAS technique with standard addition method.
- Pipetted 5.00 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 1.00, 2.00, and 3.00 ml of standard 10 ppm Mn into each of the four volumetric flasks to obtain 0, 1, 2, and 3 ppm of standard Mn solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Mn from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Mn analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	279.5 nm
Lamp current	20 mA
Slit width	0.20 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.8 Determination of zinc

Procedure

- Measured the amount of Zn in the sample stock solution using AAS technique with standard addition method.
- Pipetted 5.00 ml (V_3) of the sample stock solution into four 10 ml volumetric flasks.
- Added exactly 0, 0.50, 1.00, and 1.50 ml of standard 9.99 ppm Zn into each of the four volumetric flasks to obtain 0, 0.5, 1, and 1.5 ppm of standard Zn solutions respectively.
- Made up to volume with deionized water.
- Measured the absorbance of the four solutions by AAS technique.
- Calculated the concentration of Zn from standard addition curve using the same calculation as Ca analysis, but changed V_3 to the volume of the sample stock solution being used for Zn analysis.
- Instrumental parameters for atomic absorption measurements were as follows:

Wavelength	213.9 nm
Lamp current	20 mA
Slit width	0.70 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

3.6.1.9 Determination of sodium

Procedure

- Measured the amount of Na in the sample stock solution using AES technique with external standard method. This could be done using the atomic absorption spectrophotometer in the emission mode.
- Constructed the calibration curve using five concentrations of standard Na.
- Pipetted 0.50, 1.00, 1.50, 2.00, and 2.50 ml of the standard 10 ppm Na into five 10 ml volumetric flasks to obtain 0.5, 1, 1.5, 2, and 2.5 ppm of standard Na solutions respectively. Made up to volume with deionized water.
- Measured the concentrations of the five standard solutions by AES technique.
- Instrumental parameters for atomic emission measurements were as follows:

Wavelength	589.0 nm
Slit width	0.20 nm
Air flow	10.0 l/min
Acetylene flow	3.00 l/min

- Constructed a calibration curve of Na.
- Pipetted 2.00 ml of the sample stock solution into 10 ml volumetric flask then made up to volume with deionized water.
- Measured the concentration of the sample solution by AES technique.
- Calculated the concentration of Na using the emission data and the calibration curve.

Calculation

$$\text{Sodium } (\mu g/g) = \frac{C_{Na} \times D \times V}{W} \quad \text{.....(3.4)}$$

Where

C_{Na} = concentration of Na from external standard curve (µg/ml)

D = Dilution factor

V = total volume of sample solution from 3.6.1 (250 ml)

W = weight of sample (g)

3.6.2 Determination of chloride by Volhard method

Materials and equipment

1. Buret: 50 ml.
2. Erlenmeyer flask: 250 ml.
3. Stand and clamp.
4. Beaker: 100 ml.
5. Glass stirring rod.
6. Hot plate.

Reagents

1. Nitric acid 65% (HNO_3); Carlo Erba reagent, Italy.
2. Silver nitrate ($AgNO_3$); BDH, England.

   0.1000 N $AgNO_3$

   Dissolved 1.6900 g $AgNO_3$ in water and transferred to 100 ml volumetric flask. Made up to volume with deionized water. Standardized the $AgNO_3$

solution with KCl solution according to AOAC official method 941.18. The concentration of $AgNO_3$ was found to be 0.0979 N.

3. Ammonium thiocyanate (NH_4SCN); Merck, Germany.

   0.1000 N NH_4SCN

   Dissolved 7.6120 g NH_4SCN in water and transferred to 1000 ml volumetric flask. Made up to volume with deionized water. Standardized the NH_4SCN solution with 0.0979 N $AgNO_3$ according to AOAC official method 942.26. The concentration of NH_4SCN was found to be 0.0938 N.

4. Ferric ammonium sulphate ($FeNH_4(SO_4)_2.12H_2O$); Aldrich chemicals, USA.

   Ferric indicator.

   Dissolved $FeNH_4(SO_4)_2.12H_2O$ in water to obtain saturated solution.

Procedure

- The analyses of chloride were being done in triplicates and the average mean values were reported.
- Weighed ca 0.5000 g (W) of the sample (from 3.4.1 and 3.4.2) into 250 ml Erlenmeyer flasks. Added 10.00 ml of standard $AgNO_3$ solution to precipitate all Cl^- as AgCl, and then added 20 ml conc. HNO_3.
- Boiled a solution on hot plate until all solids except AgCl dissolved which took about 15 min, cooled, then added 50 ml H_2O, and 5.00 ml ferric indicator.
- Titrated a solution with standard NH_4SCN solution to get permanent light brown color at the end point.
- Recorded the volume of NH_4SCN used to calculate the amount of chloride which was equivalent to chlorine concentration.

Calculation

$$\text{Chloride (\%)} = \frac{M(aV_1 - bV_2) \times 100}{1000 \times W} \quad \text{.....(3.4)}$$

= Chlorine (%)

Where

M = molecular weight of the chloride salt being titrated (53.5 for NH_4Cl)

a = normality of ammonium thiocyanate solution (0.0938 N)

b = normality of silver nitrate solution (0.0979 N)

V_1 = volume of NH_4SCN used in the titration (ml)

V_2 = volume of $AgNO_3$ solution added (ml)

W = weight of sample (g)

before titration

during titration

end point

after end point

Figure 3.8 Color changes in Volhard titration.

3.6.3 Determination of phosphorus by vanadomolybdophosphoric acid method

Materials and equipments

1. UV-Vis spectrophotometer; Varian model Cary1E, Australia.
2. Cuvett.
3. Micro pipet; Model Pipet Man, Gilson Medical Electronics, France.
4. Volumetric flasks: 10, 50, and 100 ml.
5. Beaker: 100 ml.

Reagents

1. Standard solution 1000 ppm P: SPEX Certiprep, METUCHEN, USA.

   100 ppm P

   Pipetted 5.0 ml 1000 ppm P into 50 ml volumetric flask, then made up to volume with deionized water.
2. Nitric acid 65% (HNO_3); Carlo Erba reagent, Italy.
3. Hydrochloric acid 37% (HCl); Carlo Erba reagent, Italy.
4. Ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$): Merck, Germany.
5. Ammonium metavanadate (NH_4VO_3): Merck, Germany.
6. Molybdovanadate reagent:

   Prepared solution A by dissolving 25.00 g ammonium molybdate in 300 ml distilled water. Prepared solution B by dissolving and heating to boil 2.50 g ammonium metavanadate in 300 ml distilled water. Cooled the solution and then added 330 ml conc. HCl. Cooled solution B to room temperature. Poured solution A into solution B, mixed, and then diluted to 1 L with distilled water to get molybdovanadate reagent.

Procedure

Preparation of standard solutions of phosphorus

- Pipetted 100, 250, 500, 750, and 1000 µl of the standard 100 ppm P into five 10 ml volumetric flasks, to obtain the standard solutions of 1.00, 2.50, 5.00, 7.50, and 10.00 ppm P respectively.
- Added 1 ml of molybdovanadate reagent into each of the volumetric flask, diluted to volume and let them stand for 10-30 min to allow the complete complexation reaction.
- Measured the absorbance of the five standard solutions at 400 nm using the UV-Vis spectrophotometer and constructed a calibration curve.

Preparation of sample stock solution

- Weighed the samples (from 3.4.1 and 3.4.2) ca 2.0000 g using weighing paper and transferred to the crucibles, calcined in the furnace at 600^{o}C for 4 h to obtain sample ash.
- Cooled the ashed samples to room temperature.
- Weighed the sample ash ca 0.2000 g using weighing paper and transferred to the digestion tube.
- Added 12.00 ml of mixed acid, 2:1 ratio of conc. HNO_3: conc. HCl.
- Placed the digestion tubes on the digester block and set the temperature at 150^{o}C for 1 h.
- Cooled the solution to room temperature.
- Made up the volume of the solution to 100 ml with deionized water, kept in polypropylene bottles and stored in a refrigerator. The solutions would be used for the analysis of P.

Preparation of sample solution for analysis

- Pipetted 3.00 ml of the sample stock solution into 10 ml volumetric flask.
- Added 1.00 ml of molybdovanadate reagent, diluted to volume with deionized water and let it stand for 10-30 min.
- Measured the absorbance of the sample solution at 400 nm using the UV-Vis spectrophotometer.
- Calculated the concentration of P using the absorbance data and the calibration curve.
- All the analyses were being done in triplicates and the average mean values were reported.

Figure 3.9 Color changes after adding molybdovanadate reagent into solutions of various concentrations of P.

3.6.4 Determination of sulfur by CHNS analyzer

In this work, the CHNS analyzer was used for the determination of S in the samples. The analyses were being done in triplicates and the average mean values were reported.

Materials and equipment

1. CHNS analyzer; CHNS-932, LECO Corporation ST. JOSEPH MI USA.
2. Analytical balance; Sartorius, Frankfurt, Germany.
3. Standard reference material 245, (National Institute of Standards and Technology, USA).
4. Tin capsules.
5. Forceps.
6. Spatula.

Procedure

- Weighed ca 2.000 mg well-ground of each sample (from 3.4.1 and 3.4.2) into separate tin capsules then furled the capsules tightly.
- Prepared the standard reference material and blank (empty tin capsule) using the same procedure as the sample.
- Put the standard, blank, and samples into loading chamber, then put the chamber on the block in CHNS analyzer.
- Operated the instrument according to the instruction manual.
- The results obtained were reported as the percentages of C, N, H, and S.

3.7 The study on the effect of organic wastes on the biomass and the population of earthworms

The influences of organic litters supplemented to the breeding material on the biomass and the population of earthworms were investigated in separated experiment. Earthworms were fed with different kinds of organic litters, which were soybean waste, neem leaves, ripe banana, carton, and fed with nothing (breeding material only) as a control. The breeding material and organic litters were prepared according to 3.1 and 3.1.1 respectively. The sample names were designed according to 3.2 for the earthworm samples. The experimental period was four months.

Procedure

- Prepared the breeding materials for earthworm and put equally in five containers.
- Fresh specimens of 150 adult earthworms were collected from soil and divided equally into five groups.
- Weighed each group of the fresh worms using a two decimal balance, recorded the weights of the earthworms.
- Put the earthworms into the containers and let them stay there for one week, maintained the moisture.
- Put 500.00 g each of the organic litters into each container except for EW-control which the earthworms would stay in breeding material only.
- The earthworms were fed weekly with the same amount of organic litters through the experimental period.
- After four months, the earthworm were collected from soil, rinsed with distilled water and counted the numbers of earthworm in each group.
- Weighed each group of the earthworm by using a balance and recorded the weight and numbers of earthworms in each group.

- Calculated the percentages of the weight gained of the earthworms by using equation:

$$\% \text{ Weight gained} = \frac{\text{Weight of earthworm at start}}{\text{Weight of earthworm after four months}} \times 100$$

CHAPTER IV

RESULTS AND DISCUSSION

The major aim of this study was to determine and compare the amount of protein and certain essential elements contents of *E. eugeniae* earthworm which had been fed with different kind of organic litters. The results and the discussion on the protein and essential elements contents of the samples were presented in this chapter. In this study, one-way ANOVA was used to compare the amount of protein and certain essential elements contents of earthworm fed with different kind of organic litters.

4.1 Protein analyses

Proteins are high molecular weight compounds which are present in almost all foods, although often in very small amount. In the present study, Kjeldahl method was used to determine the amount of N in the sample. Since most proteins contain approximately 16% N, the percentages of the nitrogen found could be changed to the percentages of the protein by multiplying with 6.25 (Holme and Peck, 1998).

Table 4.1 shows the protein and nitrogen contents of the earthworm samples. The direct correlation between the contents of the nitrogen and protein were demonstrated. The protein contents based on dry weight, were in the ranges of 55.37% (EW-control) to 64.59% (EW-soy), while the protein contents in organic litters were from 1.03% (Litter-carton) to 29.60% (Litter-soy) as shown in Table 4.2.

Table 4.1 Protein and nitrogen contents of the earthworms fed with different kinds of organic litters.

Sample	% Nitrogen	% Protein
EW-soy	10.33	64.59
EW-neem	8.97	56.06
EW-banana	9.39	58.70
EW-carton	9.54	59.63
EW-control	8.86	55.37

Table 4.2 Protein and nitrogen contents of the organic litters used as feeds.

Sample	% Nitrogen	% Protein
Litter - soy	4.74	29.60
Litter - neem	1.78	11.14
Litter - banana	0.51	3.20
Litter - carton	0.16	1.03
Litter-control	0.75	4.66

Table 4.1 shows the protein contents of the earthworms fed with different kind of organic litters. The graphic presentation of the data from Tables 4.1 and 4.2 are shown in Figure 4.1. Even though earthworm can use a wide variety of organic materials for food, protein and carbohydrate rich litters seem to be preferred to litters with the lower protein content. EW-soy had the highest amount of protein (64.59%) as the Litter-soy had the highest amount of protein (29.60%). Considered the protein contents in other organic litters used as feeds in Table 4.2: the protein content in Litter-neem (11.14%) was higher than the protein contents of Litter-banana (3.20%) and Litter-carton (1.03%). But the protein contents in the earthworms fed with these litters were not in the same order as the order of organic feeds. The EW-carton had higher protein content (59.63%) than EW-banana (58.70%) and EW-neem (56.06%). The EW-neem had the lowest amount of protein, even though the protein content in Litter-neem was not the lowest. In this case, palatability might be the explanation since neem leaves had bitter taste. It is known that the neem leaves contained some biopesticide against various insects and most of its parts contain azadirachtin, which has most insecticidal activity among other limonoids, or tetranortriterpenoids present in neem trees. Its properties such as toxicity, repellence, feeding deterrence, and insect growth regulator activity contribute mainly toward insecticidal activity (Gopal et al., 2007; Singh et al., 2010). These properties of neem leaves might affect the palatability to the earthworm. But since the earthworms were all in the same breeding materials (bovine manure + soil + straw), they also consumed these materials as food. The Litter-control or the breeding material itself contained 4.66% protein. This was the reason why the protein contents of EW-neem and EW-carton were not that low. It was also clear that the protein contents of the earthworms fed with organic litters as supplement to the breeding materials contained higher amount of protein than the

earthworm fed with only breeding materials. The results were confirmed using one-way ANOVA test, (n=3), P≤0.05. This study might conclude that any organic litters could contribute to higher protein production for earthworms.

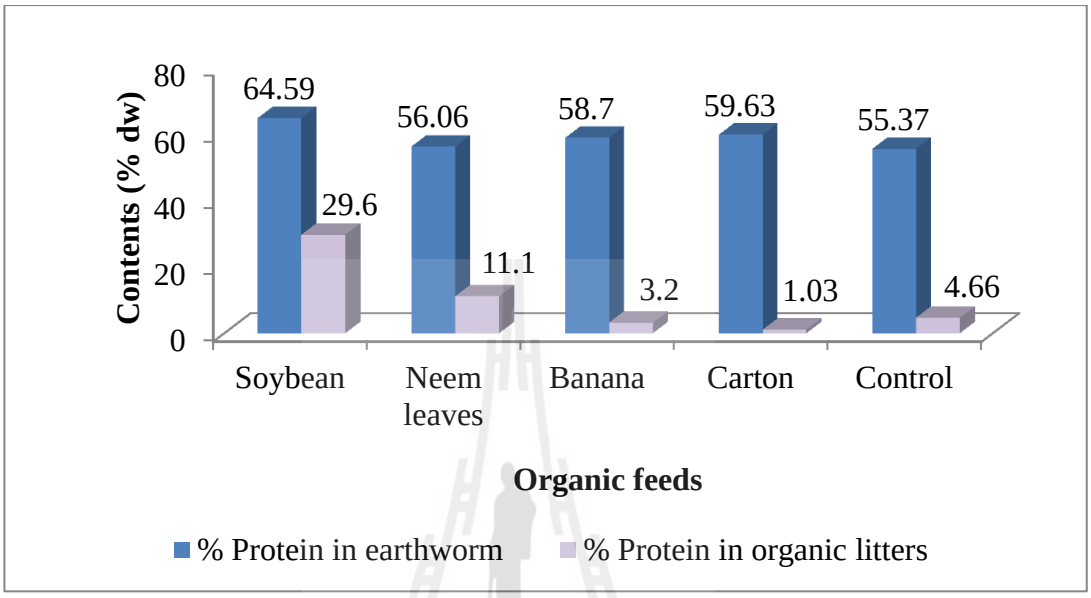

Figure 4.1 Graph showing the protein contents in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

Table 4.3 shows the protein contents of the earthworm samples from other studies compared with the results from this study. Kjeldahl method was used in the analysis in all studies. The percentages of the proteins in other studies were from 50.43% to 66.04% which were in the same ranges of the present study. The findings in Table 4.3 suggested that the protein contents were not varied that much. Different kinds of feed and earthworm species might contribute to the variation in the percentages of the protein found.

The average amount of protein found in earthworms fed with different organic litters was 59.06% which was moderately high. The amount was higher than the protein content in meat powder (50-55%) and comparable to the protein content in fish powder (70-75%) as shown in the Table 2.2 in Chapter II.

Table 4.3 Protein contents in earthworm samples from other studies.

Researcher/year	Species	Feed type	% Protein
Yosida and Hoshii (1978)	*E. foetida*	Poultry manure	56.44
Hartenstein et al. (1980)	*E. foetida*	Horse manure and activated sludge	65.2
Hilton (1983)	*E. eugeniae*	-	60.4
Tacon et al. (1983)	*A. longa*	-	50.43
	L. terrestris	-	56.1
	E. foetida	-	58.78
Stafford and Tacon (1984)	*D. subrubicundus*	Domestic sewage	65.13
Nandeesha et al. (1988)	*E. eugeniae*	-	66.04
Ibanez et al. (1993)	*E. foetida*	Bovine manure and wheat straw	57.5
Results obtained in this study	*E. eugeniae*	Organic litter + breeding material	
		Litter-soy	64.56
		Litter-neem	56.06
		Litter-banana	58.68
		Litter-carton	59.63
		Breeding material (Bovine manure + rice straw + soil)	55.37

4.2 Elemental analyses

In this study, the concentrations of Ca, Co, Cu, Fe, K, Mg, Mn, and Zn in the earthworms and organic litters were measured by atomic absorption spectroscopy technique and Na was determined by using the technique of emission spectroscopy. Volhard method was used to determine the amount of chloride. Vanadomolybdophosphoric acid method was used for the analysis of phosphorus, and sulfur was analyzed by CHNS analyzer.

4.2.1 Major essential elements

It is well established that many inorganic elements are required for vital processes in human and animals, and are considered as essential elements. As previously mentioned in Chapter II, several researchers suggested that earthworms could ingest organic wastes and had high efficiency in vermicomposting. So, it was interesting to find out the essential elements in earthworm after ingested organic litters and to compare the amount of essential elements in the earthworms when ingesting various organic litters.

The results of the analyses of the seven major essential elements in the earthworms fed with different kinds of organic litters and in organic litters used as feeds are displayed in Table 4.4 and Table 4.5, respectively. The concentrations of major essential elements in earthworms found were: Ca 0.52-0.65%, Cl 0.75-0.97%, K 1.24-1.42%, Mg 0.24-0.42%, Na 0.44-0.50%, P 0.24-0.27%, and S 0.21-0.27%. The concentrations of major essential elements in organic litters were: 0.26-3.57% for Ca, 0.15-0.82% for Cl, 0.09-2.14% for K, 0.42-0.74% for Mg, ND-0.048% for Na, 0.004-0.136% for P, and ND-0.095% for S.

Table 4.4 Major essential elements contents of the earthworms fed with different kinds of organic litters.

Sample	Concentration (%)						
	Ca	Cl	K	Mg	Na	P	S
EW-soy	0.53	0.97	1.33	0.24	0.45	0.27	0.27
EW-neem	0.63	0.83	1.37	0.42	0.48	0.26	0.25
EW-banana	0.52	0.82	1.42	0.29	0.5	0.26	0.24
EW-carton	0.65	0.8	1.24	0.36	0.44	0.24	0.21
EW-control	0.6	0.75	1.39	0.3	0.46	0.27	0.22

Table 4.5 Major essential elements contents of the organic litters used as feeds.

Sample	Concentration (%)						
	Ca	Cl	K	Mg	Na	P	S
Litter-soy	0.53	0.17	1.54	0.68	0.014	0.09	0.095
Litter-neem	3.57	0.82	1.59	0.67	ND	0.056	0.035
Litter-banana	0.26	0.23	2.14	0.45	ND	0.022	ND
Litter-carton	1.57	0.15	0.09	0.42	0.008	0.004	0.026
Litter-control	1.37	0.40	0.98	0.74	0.048	0.136	0.036

Note: ND = not detected

Figure 4.2-Figure 4.8 show the graphic presentations of the concentrations of Ca, Cl, K, Mg, Na, P, and S in the earthworms fed with different kinds of organic litters compared with the concentrations of Ca, Cl, K, Mg, Na, P, and S in organic litters, respectively.

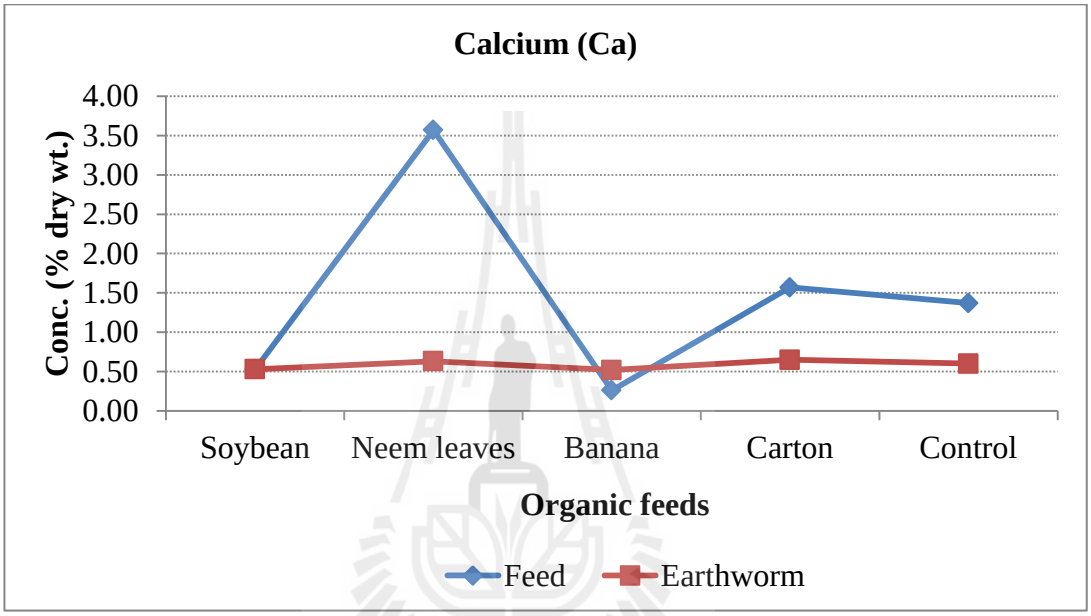

Figure 4.2 Graph showing the concentrations of Ca in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

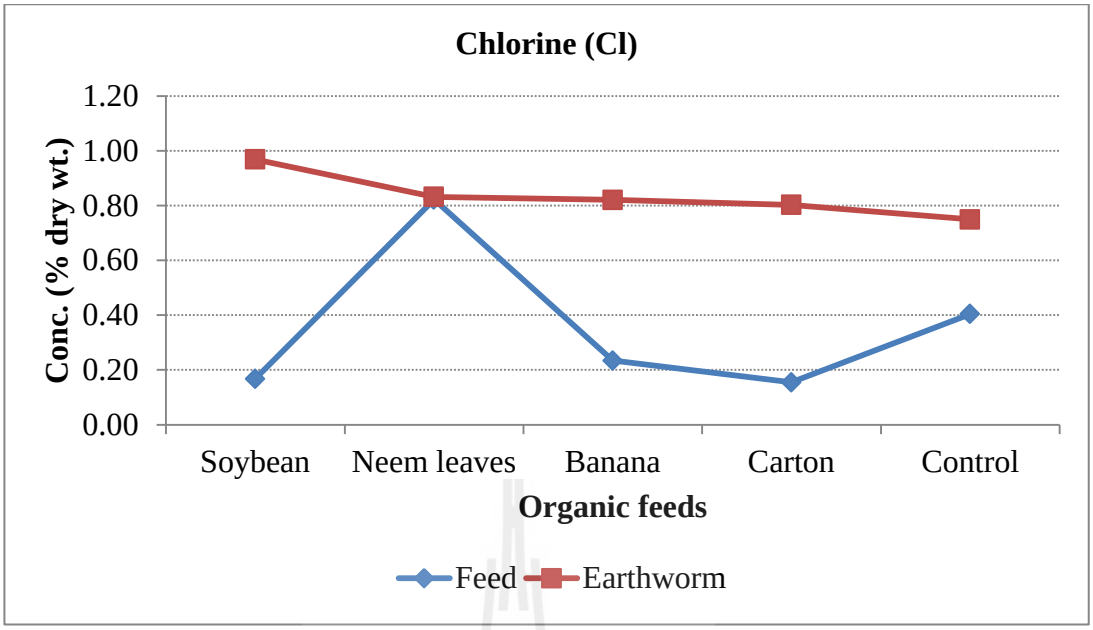

Figure 4.3 Graph showing the concentrations of Cl in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

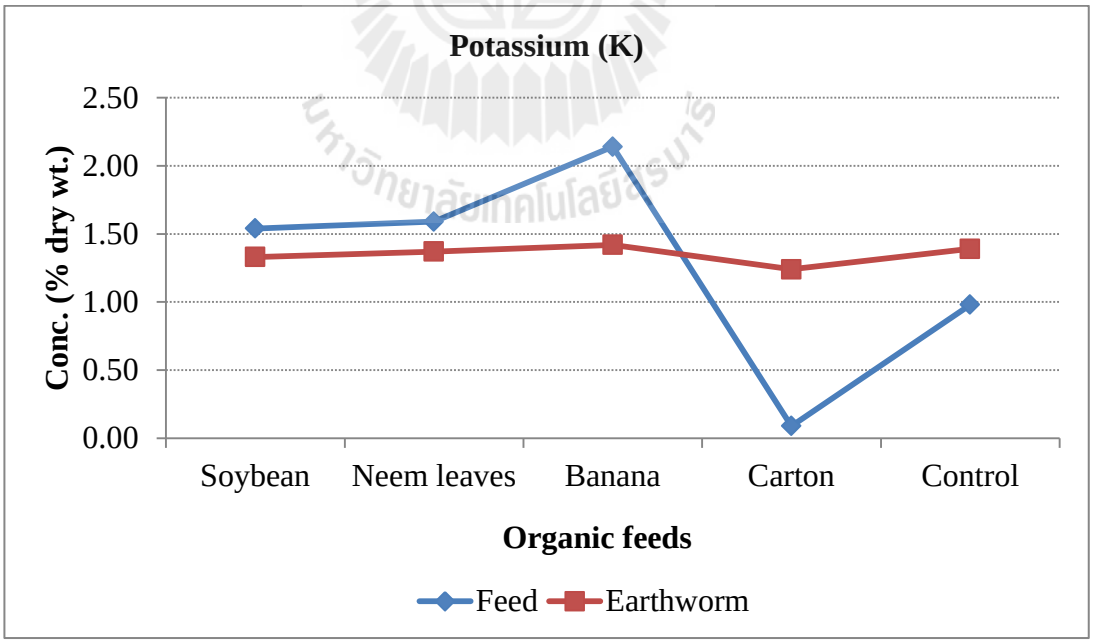

Figure 4.4 Graph showing the concentrations of K in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

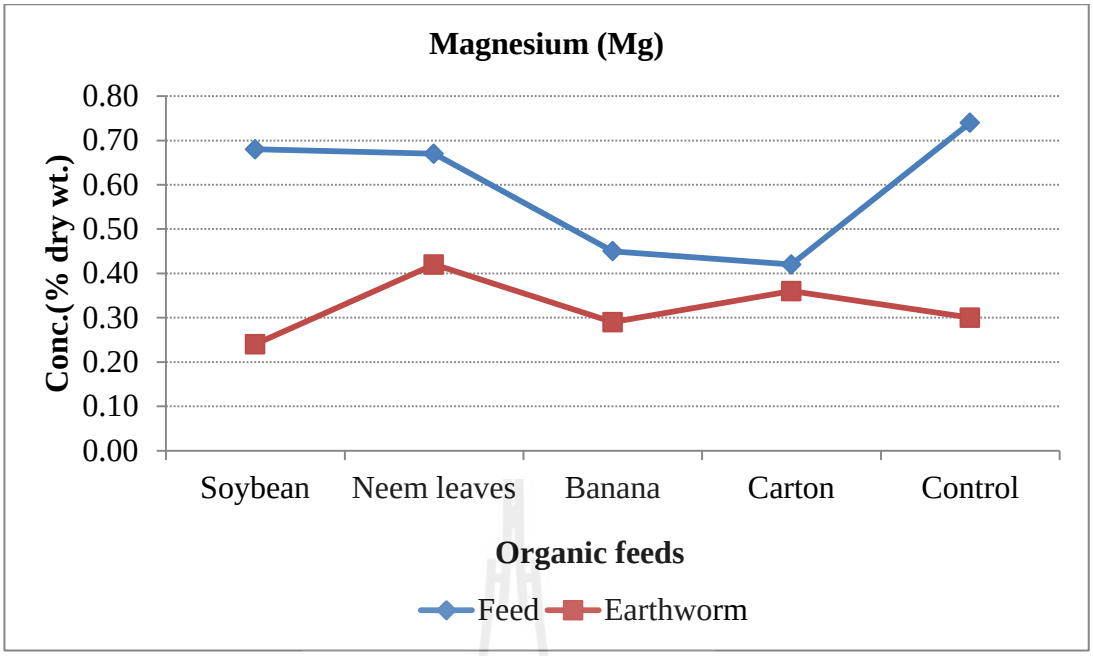

Figure 4.5 Graph showing the concentrations of Mg in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

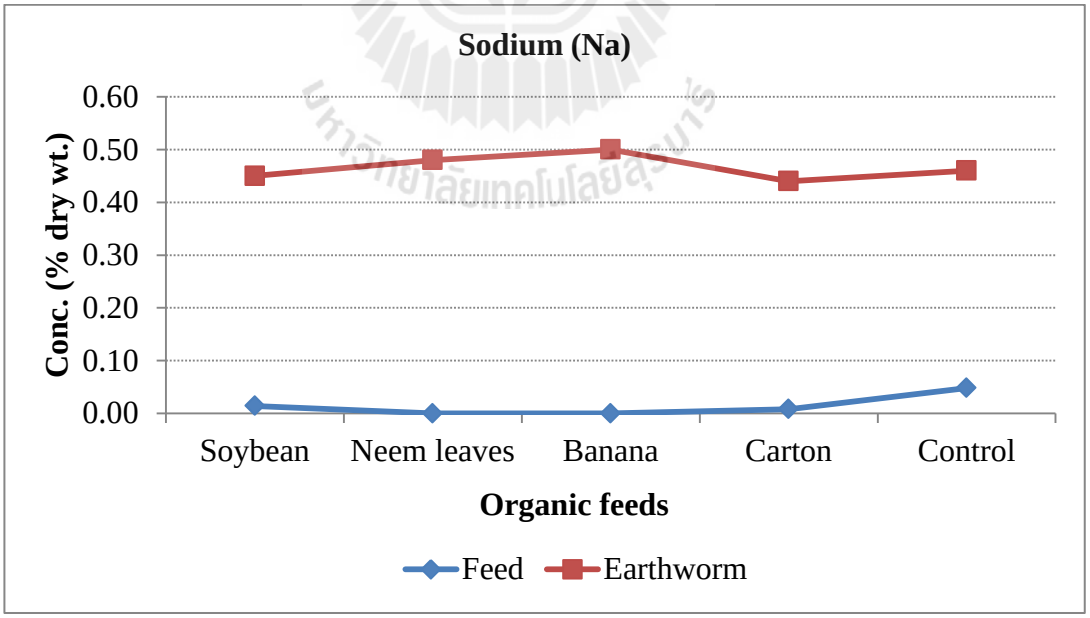

Figure 4.6 Graph showing the concentrations of Na in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

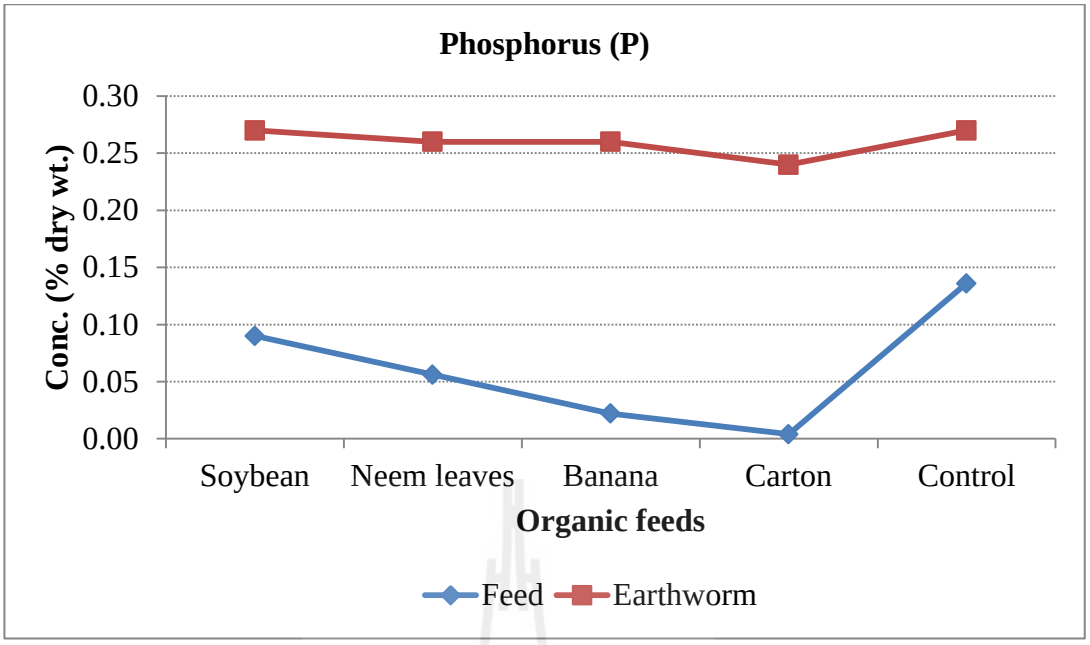

Figure 4.7 Graph showing the concentrations of P in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

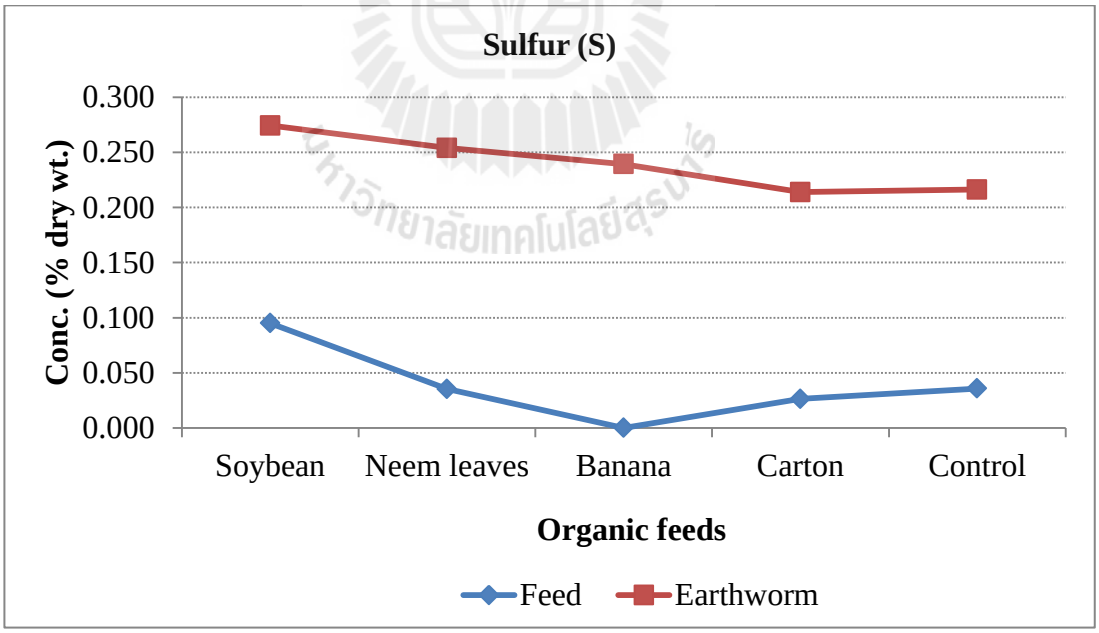

Figure 4.8 Graph showing the concentrations of S in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

The results from the Table 4.4 show that the concentrations of each element were about the same in each earthworm groups. For example, Cl concentrations in earthworms fed with four different organic feeds were 0.97% in EW-soy, 0.83% in EW-neem, 0.82% in EW-banana, and 0.80% in EW-carton. Chloride is the major anion of the body involved in osmotic pressure, making more than 60% of the total anionic equivalents in the extracellular fluid. It seemed that the amount of Cl in organic litters did not affect the amount of Cl in earthworms. Litter-carton contained only 0.15% Cl (the lowest amount) while Litter-neem contained the highest amount of Cl (0.82%). Figure 4.3 shows the concentrations of Cl in the earthworms fed with different kinds of organic litters and in organic litters used as feeds. Another example were the concentrations of K found in the earthworms. Potassium is the third most abundant elements in the animal body. It plays many vital roles in life processes. The concentrations of K in each earthworm groups were 1.33% in EW-soy, 1.37% in EW-neem, 1.42% in EW-banana, and 1.24% in EW-carton. It was clear that the concentrations of both Cl and K in each earthworm groups were relatively constant and not depended upon the concentrations of the elements found in the organic feeds. Figure 4.4 shows the concentrations of K in the earthworms fed with different organic litters and in the organic litters used as feeds.

The concentrations of each element were relatively constant as shown in Table 4.4 because they were essential elements. The earthworms tried to keep those concentrations constant for their proper functions in life processes. Even though the organic feeds and the breeding media contained very small amount of some essential elements for example, Litter-carton contained only 0.09% K or the breeding materials contained only 0.048% Na, the earthworms could get supply of the elements they needed through the accumulation processes. The amounts of major essential elements

found in earthworms were depended on many factors such as the chemical form of the element, which would determine its biological availability to the earthworms, the absolute amount of the element in organic feed which affected the elemental uptake of the earthworms.

When compared the concentrations of these major essential elements found in earthworms of this study with the concentrations of major essential elements in some important feeds (Table 4.10), it was clear that earthworms could supply quite moderate amount of these essential elements.

Table 4.6 compares the contents of major essential elements found in the earthworms from other studies with the results from the present study. The available data showed that the element with the highest concentration among major essential elements was K. The difference in other element concentrations might be due to the difference of earthworm species. Different kinds of feeds might play some role especially when considered on the palatability.

Table 4.6 Major essential elements contents of the earthworms from other studies compared with the results from this study.

Researcher/ year	Species/ food type	Major element content : concentration in percentage						
		Ca	Cl	K	Mg	Na	P	S
Yosida and Hoshii, 1978	*E. foetida*/ Poultry manure	0.48	-	0.89	0.21	0.69	0.87	-
Hartenstein et al. (1980)	*E. foetida*/ Horse manure and activated sludge	0.69	-	1.66	0.28	0.79	0.32	-
Hilton, 1983	*E. eugeniae*/ Not specified	1.49	-	-	0.16	-	0.89	-
Stafford and Tacon, 1984	*D. subrubicundus*/ Domestic sewage	0.18	-	0.83	0.06	0.45	-	-
Results obtained in this study	*E. eugeniae*/ Organic litter + breeding material							
	Litter-soy	0.53	0.97	1.33	0.24	0.45	0.27	0.27
	Litter-neem	0.63	0.83	1.37	0.42	0.48	0.26	0.25
	Litter-banana	0.52	0.82	1.42	0.29	0.50	0.26	0.24
	Litter-carton	0.65	0.80	1.24	0.36	0.44	0.24	0.21
	Breeding material	0.60	0.75	1.39	0.30	0.46	0.27	0.22

4.2.2 Trace essential elements

Trace essential elements are required by the living organism in a minute amounts usually less than 100 mg/kg (McDowell, 1992). Although the living organism contained and required very small amount of these elements, deficiency of some elements can lead to the malfunction of life processes.

Table 4.7 shows the concentrations of certain trace essential elements of earthworms fed with different kinds of organic litters, while Table 4.8 shows the concentrations found in organic litters used as feeds respectively.

Table 4.7 Trace essential element contents of the earthworms fed with different kinds of organic litters.

Sample	Concentration (mg/kg)				
	Co	Cu	Fe	Mn	Zn
EW-soy	4.96	50.3	2,517	35.4	180.9
EW-neem	1.16	50.7	2,497	85.4	187.4
EW-banana	2.91	46.3	2,116	54.9	180.2
EW-carton	1.28	46.4	3,231	43.1	180.7
EW-control	2.48	52.5	3,129	94.7	192.7

Table 4.8 Trace essential element contents of the organic litters used as feeds.

Sample	Concentration (mg/kg)				
	Co	Cu	Fe	Mn	Zn
Litter-soy	35.9	53.4	3,573	42.8	38.6
Litter-neem	8.57	39.2	4,252	64.5	24.6
Litter-banana	26.7	42.6	801	132	17.9
Litter-carton	7.61	50.8	5,439	54.8	36.2
Litter-control	14.1	67.9	33,983	677	253

Figures 4.9-4.13 show the graphic presentations of the concentrations of Co, Cu, Fe, Mn, and Zn in the earthworms fed with different kinds of organic litters compared with the concentrations of Co, Cu, Fe, Mn, and Zn in organic litters, respectively.

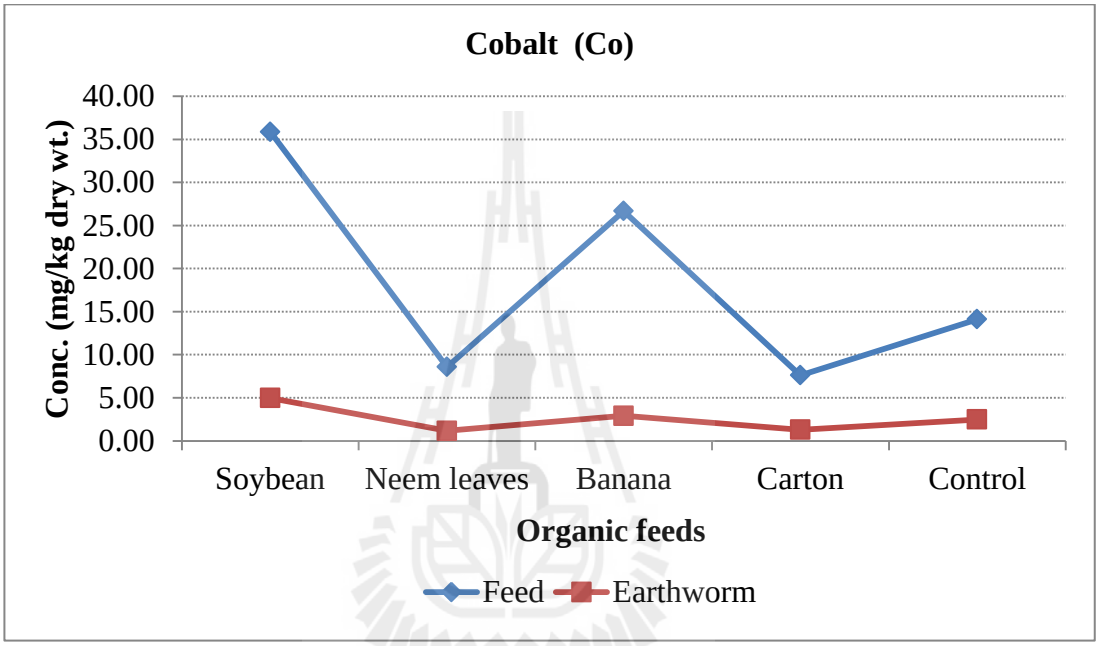

Figure 4.9 Graph showing the concentrations of Co in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

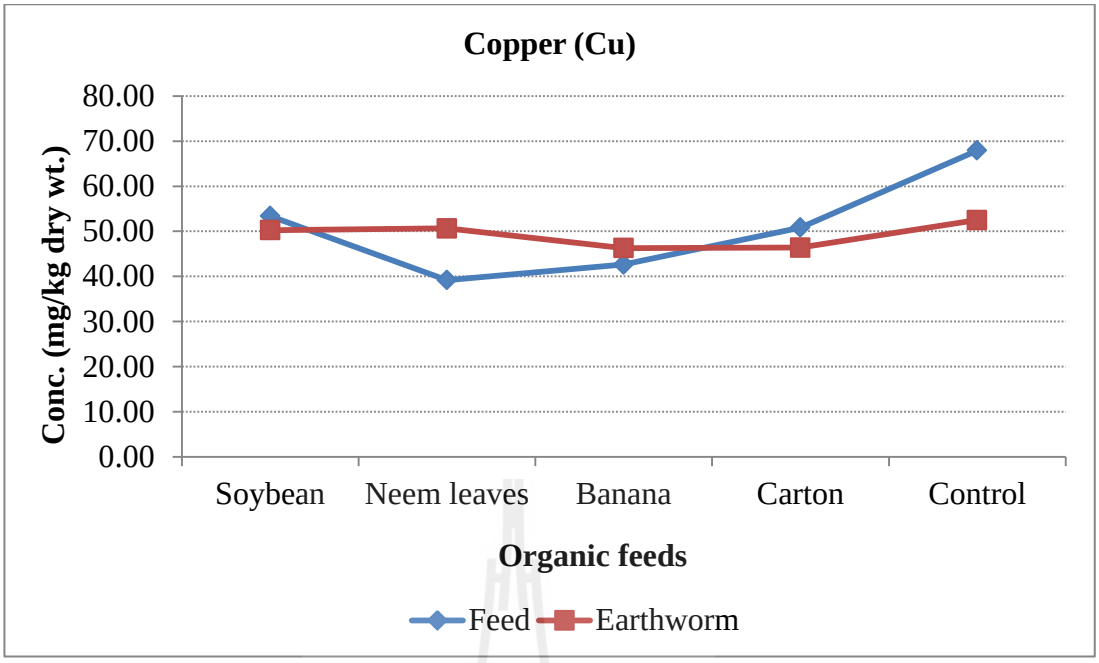

Figure 4.10 Graph showing the concentrations of Cu in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

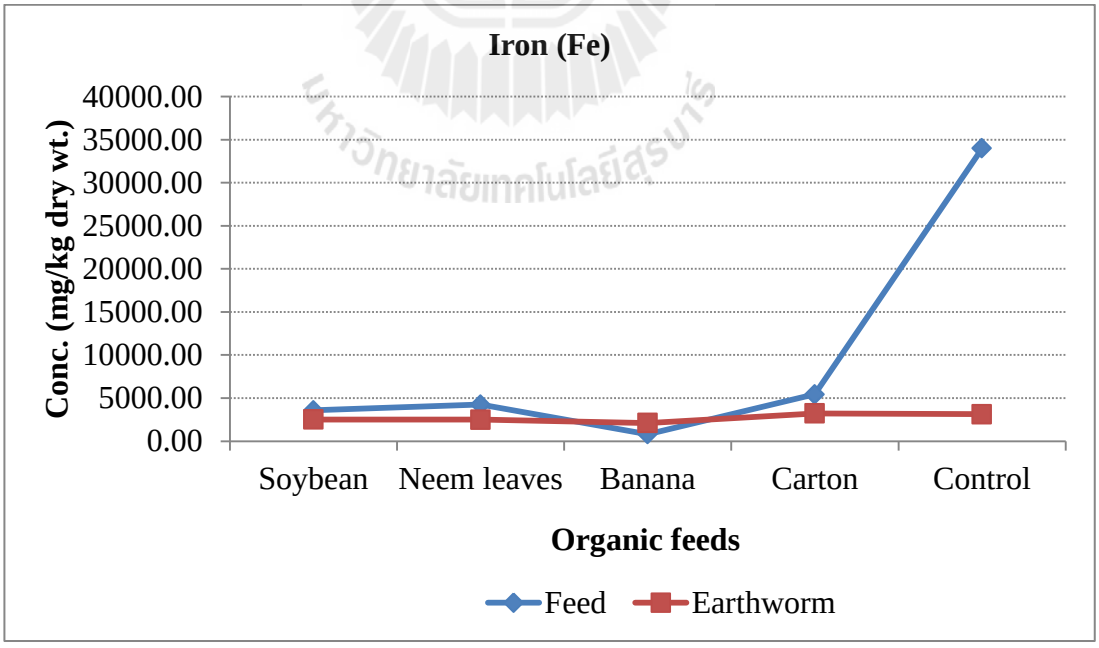

Figure 4.11 Graph showing the concentrations of Fe in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

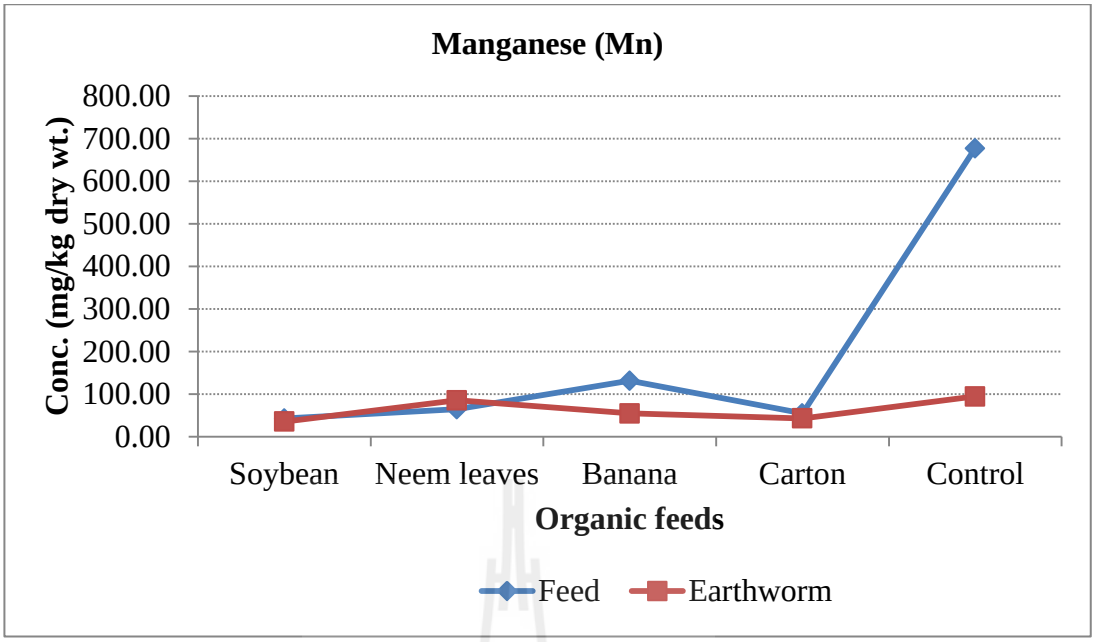

Figure 4.12 Graph showing the concentrations of Mn in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

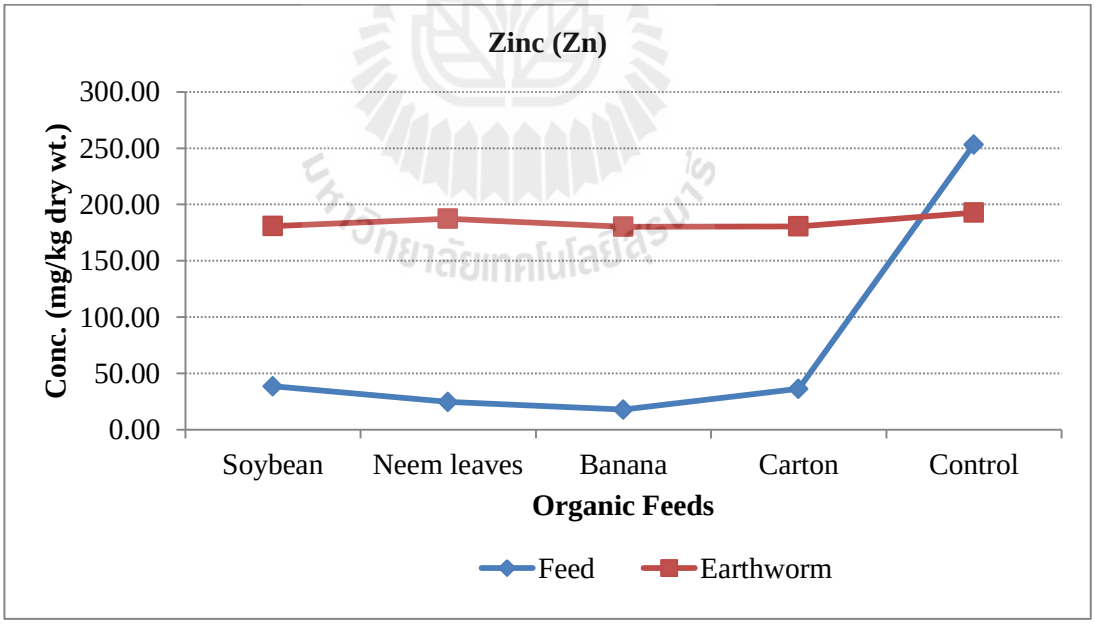

Figure 4.13 Graph showing the concentrations of Zn in the earthworms fed with different kinds of organic litters and in organic litters used as feeds.

The findings showed the same trends as what had been found for the major essential elements. The concentrations of each element in each group of earthworms were relatively constant except for Co which were quite varied. Considered Cu as an example. Copper is a component of many enzymes involved in electron transport during aerobic respiration, also shows relatively constant concentrations in different earthworm groups, being 50.3 mg/kg in EW-soy, 50.7 mg/kg in EW-neem, 46.3 mg/kg in EW-banana, 46.4 mg/kg in EW-carton, and 52.5 mg/kg in breeding materials (Figure 4.10). Another example, zinc, which is a part of many metalloenzymes. It plays an important role in protein, carbohydrate, and lipid metabolism. The concentrations of Zn were quite constant in earthworms fed with different organic litters: 180.9 mg/kg in EW-soy, 187.4 mg/kg in EW-neem, 180.2 mg/kg in EW-banana, 180.7 mg/kg in EW-carton, and 192.7 mg/kg in EW-control or the breeding materials. Again, the concentrations of Zn found were not depended on the amount of Zn presented in organic feeds (Figure 4.13). All except Fe have been shown in other studies to accumulate in earthworm tissues to levels above those of the surrounding media (Gish and Christensen, 1973; Van Hook, 1974; Ireland, 1975 quoted in Stafford and Tacon, 1984). Concerning the amount of trace essential elements found in earthworms, besides the absolute concentrations of the elements in organic litters and the chemical form of the elements which determines their biological availability to the earthworms, and an antagonistic reaction may have arisen. For example, the antagonistic reactions of Fe and other elements in organic feeds. High intakes of Zn, Mn, Cu, Co, Cd, have been reported to interfere with Fe uptake and metabolism (Underwood, 1977 quoted in Stafford and Tacon, 1984). Antagonism and interaction between trace essential elements will also vary from feed to feed as levels of the individual elements vary. The results from the study provided the conclusion that the

concentrations of the trace elements found in earthworms fed with different organic litters might get some effect from the feeds due to the antagonistic reaction and interactions between elements in different feeds.

Table 4.9 compares the trace essential elements from other studies with the results from this study. In all studies, Fe concentration was the highest while Zn concentrations were about the same. Again, earthworm species and different kinds of feeds resulted in various concentrations of the trace essential elements.

Table 4.9 Trace essential element contents of earthworms from other studies compared with the results from this study.

Researcher/year	Species/ food type	Trace element contents : concentration in mg/kg				
		Co	Cu	Fe	Mn	Zn
Hartenstein et al., 1980	*E. foetida*/ Horse manure and activated sludge	-	62.4	1,415	71.1	168
Hilton, 1983	*E. eugeniae*/ Not specified	-	7.83	1,300	-	122.5
Stafford and Tacon, 1984	*D. subrubicundus*/ *Domestic sewage*	0.33	28.72	356.9	18.32	197.9
Results obtained in this study	*E. eugeniae*/ Organic litter + breeding material					
	Litter-soy	4.96	50.3	2,517	35.4	180.9
	Litter-neem	1.16	50.7	2,497	85.4	187.4
	Litter-banana	2.91	46.3	2,116	54.9	180.2
	Litter-carton	1.28	46.4	3,231	43.1	180.7
	Breeding material	2.48	52.5	3,129	94.7	192.7

Table 4.10 shows the comparison of certain essential element contents obtained from the analysis of various feeds. The concentrations of K, Mg, Co, Cu, Fe, Mn, and Zn in the earthworms were comparable to other feeds. Sodium, S, and Cl were in the ranges of the other feeds. In contrast, the concentrations of Ca and P were quite lower than the other feeds. Calcium was quite low because earthworm is an invertebrate. The results suggested that earthworm could be sources of certain essential elements for animal feed production.

Table 4.10 Composition of certain essential elements composition of important feeds.

Essential elements	Feed name/ Concentrations						
	Chicken[a]	Poultry[a]	Crab[a]	Fish[a]	Shrimp[a]	Meat[a]	Earthworm[b]
Ca*	6.45	3.76	15.77	0.43	10.8	9.44	0.58
Cl*	-	0.58	1.63	5.38	1.15	1.27	0.83
Mg*	0.10	0.19	1.02	0.06	0.60	0.29	0.32
P*	2.33	1.96	1.72	1.18	2.05	4.74	0.25
K*	0.26	0.42	0.49	3.22	0.92	0.61	1.35
Na*	0.38	0.87	0.95	4.67	1.74	1.37	0.46
S*	-	0.56	0.27	0.25	-	0.50	0.23
Co**	6.22	0.24	-	0.14	-	0.14	2.56
Cu**	2.00	15.0	35.0	92.0	-	10.0	49.2
Fe**	96.0	473	4719	445	116	470	2698
Mn**	2.00	12.0	144	27.0	33.0	10.0	62.7
Zn**	49.0	129	-	87.0	32.0	85.0	184.4

* Reported in term of percentage (%)

** Reported in term of milligrams per kilogram (mg/kg)

[a] Quoted in McDowell, (1992)

[b] Mean value of the results obtained in this study

4.3 The study on the effect of organic wastes on the biomass and population of earthworm

The influence of organic litters on the biomass and the population of earthworms were investigated. Table 4.11 shows the results of the study after feeding the earthworms with four different kinds of organic litters for four months. The highest %weight gained was the EW-banana group while the lowest %weight gained was the EW-control group. The explanation might be from the palatability and the ability to decompose easily of the banana. It should be noted that this was different from the results of the determination of protein contents of the earthworms as shown in Table 4.1. In those experiments, only the adult earthworms were used in the analyses and the EW-soy had the highest protein content while the control group, again, contained the lowest amount of protein.

Table 4.11 Biomass and population of the earthworms after feeding for four months.

Sample	No. EW at start	Weight of EW at the start (g)	No. EW four months period	Weight of EW after four months (g)	% wt. gained
EW-soy	30	36.28	131*	67.40	185.77
EW-neem	30	40.09	157*	78.35	195.44
EW-banana	30	33.48	186*	87.21	260.48
EW-carton	30	38.97	165*	80.79	207.31
EW-control	30	34.97	120*	58.92	168.49

* Number of earthworms included adult, juvenile, and larva worms.

Considered the other groups of earthworms, the EW-carton and EW-neem groups got higher %weight gained than the EW-soy group. Carton is a good material for earthworm bedding so it might be suited for the reproduction of the earthworms (Sherman, 2003). Even though neem leaves had been known as the nematode killer, they had no deleterious effect on the earthworms (Gajalakshmi and Abbasi, 2004) which was noticed from the survival of earthworms through the experimental period. The EW-soy group gained lower %weight gained when compared with EW-carton and EW-neem groups because soybean waste was slowly decomposed and produced bad odor. Some of earthworms died during feeding with soybean waste. The %weight gained of the EW-soy was still higher than the EW-control group.

The results suggested that the organic wastes supplemented to the breeding material provided higher biomass and population of the earthworms than feeding them with breeding material only. Organic wastes which seemed to be priceless such as neem leaves or used carton can be supplemented as food in vermiculture to produce higher biomass and population of earthworms.

CHAPTER V

CONCLUSIONS

The analyses of protein and certain essential elements contents of earthworm *E. eugeniae* species fed with different kinds of organic litters: soybean waste, neem leaves, ripe banana, carton, and fed with nothing as a control were carried out in this thesis.

Kjeldahl method was used for the determination of protein. The concentrations of Ca, Co, Cu, Fe, K, Mg, Mn, and Zn were measured by atomic absorption spectroscopy and Na was analyzed by atomic emission spectroscopy. Volhard method was used to determine chloride, vanadomolybdophosphoric acid method was used for the determination of phosphorus, and sulfur was analyzed by CHNS analyzer.

The results showed that the earthworms contained moderately high amount of protein (59.06% dw) which was higher than the meat powder meal (50-55%), so it would be an ideal source for protein supplement in animal feeds in the future. The earthworms bred in breeding material supplemented with organic litters produced higher protein concentration than the earthworms bred in breeding material only. Many research papers confirmed that the earthworm meals could be partially replaced the fish meal for culture fishes diet, with no detrimental effect (Akiyama et al., 1984; Stafford and Tacon, 1984; Nandeesha et al., 1988; Ibanez et al., 1993). This might be very useful since the protein component of a diet was the single most expensive portion in the formulation. When considered the essential elements, earthworms could be good source of essential elements. They contained moderately amount of many essential elements

especially K and Fe. The amounts of essential elements were relatively constant and depended very little on the amounts of essential elements in organic feeds. The amount of essential elements in earthworms depended on many factors such as the regulations of elements in the body, the absolute concentration of the element in organic feeds, the chemical form of the element which would determine its biological availability to the earthworm, and the natural accumulation processes. So this was meant that any organic wastes could be used in vermiculture.

It was also found in this study that any organic litters supplemented to breeding material could produce higher biomass and population of the earthworms than feeding with breeding material only. Banana seemed to be the best in providing the highest % weight gained of the earthworm.

There should be more researches on relation between food ingested and the protein and element contents in earthworms. The approach should be multidisciplinary to produce a complete assessment of the earthworm behavior.

REFERENCES

REFERENCES

Akiyama, T., Mural, T., Hirasawa, Y., and Nose, T. (1984). Supplementation of various meals to fish meal diet for chum salmon fry. **Aquaculture.** 37: 217-222.

Atack, T. H., Jauncey, K., and Matty, A. J. (1979). The utilisation of some single-cell proteins by fingerling mirror carp (Cyprinus carpio). **Aquaculture.** 18: 337-348. Quoted in Viola, S., Mokady, V., Rappaport, U., and Arieli, Y. (1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. **Aquaculture.** 26: 223-226.

Berdanier, C. D. (1995). **Advanced nutrition: macronutrients.** Boca Raton: CRC Press.

BWCNfarms. (2008). **African Nightcrawlers.** [Online]. Available: http://www.bwcn farms.com/index.php?pr=Worms.

Burns, E. A. and Muraca, R. F. (1960). Determination of small amounts of chloride by Volhard titration: Evaluation of operator determinate end-point error. **Anal. Chim. Acta.** 23: 136-144.

DaDong, S. (2006). Anatomy and treatment of dry sludge from Kasetsart University Dairy Center by *Eudrilus eugeniae*. **Thesis**: Master of Science (Zoology). Kasetsart University, Bangkok, Thailand.

Dai, J., Becquer, T., Rouiller, J. H., Reversat, G., Bernhard-Reversat, F., Nahmani, J., and Lavelle, P. (2004). Heavy metal accumulation by two earthworm species and its relationship to total and DTPA-extractable metals in soils. **Soil Biol. Biochem.** 36: 91-98.

Dominguez, J., Edwards, C. A., and Ashby, J. (2001). The biology and population dynamics of *Eudrilus eugeniae* (Kinberg) (Oligochaeta) in cattle waste solids. **Pedobiologia.** 45: 341-353.

Edwards, C. A. and Arancon, N. Q. (2004). The use of earthworms in the breakdown of organic wastes to produce vermicomposts and animal feed protein, Quoted in Edwards, C. A. (2004). **Earthworm Ecology.** New York: CRC Press. pp. 345.

Edwards, C. A. and Bohlen, P. J. (1996). **Biology and Ecology of Earthworms.** New York: Chapman and Hall. pp. 113.

Edwards, C. V. and Niederer, A. (1988). The production and processing of earthworm protein, Quoted in Edwards, C. A., and Bohlen, P. J. (1996). **Biology and Ecology of Earthworms.** New York: Chapman and Hall. pp. 262.

Elvira, C., Domínguez, J., and Briones, M. J. I. (1996a). Comparison between *Eisenia fetida* and *Eisenia andrei.* Interactions and life cycles. **Pedobiologia.** 40: 377-384. Quoted in Dominguez, J., Edwards, C. A., and Ashby, J. (2001). The biology and population dynamics of *Eudrilus eugeniae* (Kinberg) (Oligochaeta) in cattle waste solids. **Pedobiologia.** 45: 341-353.

Elvira, C., Domínguez, J., and Mato, S. (1996b). The growth and reproduction of *Lumbricus rubellus* and *Dendrobaena rubida* in cow manure. Mixed cultures with *Eisenia andrei.* **Appl. Soil Ecol.** 5: 97-103. Quoted in Dominguez, J., Edwards, C. A., and Ashby, J. (2001). The biology and population dynamics of *Eudrilus eugeniae* (Kinberg) (Oligochaeta) in cattle waste solids. **Pedobiologia.** 45: 341-353.

Elvira, C., Goicoechea, M., Sampedro, L., Mato, S., and Nogales, R. (1996). Bioconversion of solid paper-pulp mill sludge by earthworms. **Bioresource Technol.** 57: 173-177.

Fisher, C. (1988). The nutritional value of the earthworm meal for poultry, Quoted in Edwards, C. A. and Bohlen, P. J. (1996). **Biology and Ecology of Earthworms.** New York: Chapman and Hall. pp. 261.

Gajalakshmi, S. and Abbasi, S. A. (2004). Neem leaves as a source of fertilizer-cum-pesticide vermicompost. **Bioresource Technol.** 92: 291-296.

Gajalakshmi, S., Ramasamy, E. V., and Abbasi, S. A. (2001). Potential of two epigeic and two anecic earthworm species in vermicomposting of water hyacinth. **Bioresource Technol.** 76: 177-181.

Gajalakshmi, S., Ramasamy, E. V., and Abbasi, S. A. (2002). Vermicomposting of different forms of water hyacinth by the earthworm *Eudilus eugeniae*, Kingberg. **Bioresource Technol.** 82: 165-169.

Gajalakshmi, S., Ramasamy, E. V., and Abbasi, S. A. (2005). Composting-vermi-composting of leaf litter ensuing from the trees of mango (Mangifera indica). **Bioresource Technol.** 96: 1057-1061.

Gish, C. O. and Christensen, R. E. (1973). Cadmium, nickel, lead and zinc in earthworms from roadside soil. **Environ. Sci. Technol.** 7: 1060-1062.

Gomes, C. S. F. and Silva, J. B. P. (2007). Minerals and clay minerals in medical geology. **Appl. Clay Sci.** 36: 4-21.

Gopal, M., Gupta, A., Arunachalam, V., and Magu, S. P. (2007). Impact of azadirachtin, an insecticidal allelochemical from neem on soil microflora, enzyme and respiratory activities. **Bioresource Technol.** 98: 3154-3158.

Graff, O. (1974). Gewinnung von Biomasse aus Abfallstoffen durch Kultur des Kompostregenwurms *Eisenia fetida* (Savigny, 1862). **Landbauforschung Völkenrode.** 2: 137-142. Quoted in Dominguez, J., Edwards, C. A., and Ashby, J. (2001). The biology and population dynamics of *Eudrilus eugeniae* (Kinberg) (Oligochaeta) in cattle waste solids. **Pedobiologia.** 45: 341-353.

Hartenstein, R., Leaf, A. L., Neuhauser, E. F., and Bickelhaupt, D. H. (1980). Composition of the earthworm *Eisenia foetida* (savigny) and assimilation of 15 elements from sludge during growth. **Comp. Biochem. Physiol.** 66C: 187-192.

Harwood, M. and Sabine, J. R. (1978). The nutritive of worm meal, Quoted in Edwards, C. A., and Bohlen, P. J. (1996). **Biology and Ecology of Earthworms.** New York: Chapman and Hall., pp. 261.

Hilton, J. W. and Slinger, S. J. (1981). Nutrition and feeding of rainbow trout. **Can. Spec. Publ. Fish Aquat. Sci.** 55: 15. Quoted in Hilton, J. W. (1983). Potential of freeze dried worm meal as a replacement for fish meal in trout diet formulations. **Aquaculture.** 32: 277-283.

Hilton, J. W. (1983). Potential of freeze dried worm meal as a replacement for fish meal in trout diet formulations. **Aquaculture.** 32: 277-283.

Holme, D. J. and Peck, H. (1998). **Analytical Biochemistry.** (3rd ed). London. Prentice Hall., pp. 36-381.

Ibanez, I. A., Herrera, C. A., Velasquez, L. A., and Hebel, P. (1993). Nutritional and toxicological evaluation on rats of earthworm *(Eisenia fetida)* meal as protein source for animal feed. **Anim. Feed Sci. Tech.** 42: 165-172.

Ireland, M. P. (1975). Metal content of Dendrobaena rubid (Oligochaeta) in a base metal mining area. **Oikos.** 26: 74-79. Quoted in Stafford, E. A. and Tacon, A. G. J. (1984). Nutritive Value of the Earthworm, *Dendrodrilus subrubicundus,* Grown on Domestic Sewage, in Trout Diets. **Agri. Wastes.** 9: 249-266.

Jin-you, X., Xian-Kuan, Z., and Zhi-ren, P. (1982). Experimental research on substitution of earthworm for fish meal in feeding broilers. **J. South China Norm. Coll.** 1: 88-94.

Kurien, J. and Ramasamy, E. V. (2006). Vermicomposting of taro *(Colocasia esculenta)* with two epigeic earthworm species. **Bioresource Technol.** 97: 1324-1328.

Kwansod, T., Chantsavang, S., and Bunchasuk, C. (2002). Treatment of chicken manure using *Lumbricus rubellus* and *Eudrilus eugeniae.* **Proceedings.** The 41th Kasetsart University Annual Conference: Animals, Veterinary Medicine: 3-7.

Lawrence, R. D. and Millar, H. R. (1945). Protein content of earthworm. **Nature. Lond.** 155(39): 157.

Marco´, A., Rubio, R., Compano´, R., and Casals, I. (2002). Comparison of the Kjeldahl method and a combustion method for total nitrogen determination in animal feed. **Talanta.** 57: 1019-1026.

Mekada, H., Hayashi, N., Yokota, H., and Olcomura, J. (1979). Performance of growing and laying chicken fed diets containing earthworms. **J. Poult. Sci.** 16: 293-297.

Motomizu, S. and Hai, L. Z. (2005). Trace and ultratrace analysis methods for the determination of phosphorus by flow-injection techniques. **Talanta.** 66: 332-340.

Nandeesha, M. C., Srikanth, G. K., Basavaraja, N., Keshavanath, P., Varghese T. J., Kubra Bano, Ray, A. K., and Radha D. Kale. (2008). Influence of Earthworm Meal on the Growth and Flesh Quality of Common Carp. **Biol. Wastes.** 26: 189-198.

Neves, R. C. F., Moraes, P. M., Saleh, M. A. D., Loureiro, V. R., Silva, F. A., Barros, M. M., Padilha, C. C. F., Jorge, S. M. A., and Padilha, P. M. (2009). FAAS determination of metal nutrients in fish feed after ultrasound extraction. **Food Chem.** 113: 679-683.

Pundt, L. (2000). Neem based insecticides. **Hame and Garden News.** July/August, p. 6. Quoted in Gajalakshmi, S. and Abbasi, S. A. (2004). Neem leaves as a source of fertilizer-cum-pesticide vermicompost. **Bioresource Technol.** 92: 291-296.

Résolution Oeno. (2002). Quantification of total nitrogen according to the Dumas method. [Online]. Available: http://news.reseau-concept.net/images/oiv_uk /Client/MA-E-AS323-02-AZOTDU.pdf.

Sandbank, E. and Hepher, B. (1978). The utilization of microalgae as a feed for fish. **Arch. Hydrobiol. Beiheft.** 11: 108-120. Quoted in Viola, S., Mokady, V., Rappaport, U., and Arieli, Y. (1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. **Aquaculture.** 26: 223-226.

Science-equipment. (2008). **CHNS/O Analyzer.** [Online]. Available: http://share.psu .ac.th/blog/science-equipment/5319.

Sherman, R. (2003). **Raising earthworm successfully.** [Online]. Available: http://www.bae.ncsu.edu/topic/vermicomposting/pubs/earthworms.pdf.

Singh, B., Sharma, D. K., Kumar, R., and Gupta, A. (2010). Development of a new controlled pesticide delivery system based on neem leaf powder. **J. Hazard. Mater.** 177: 290-299.

Skoog, D. A. and Leary, J. J. (1992). **Principles of Instrumental Analysis.** (4th ed). Saunders College Publishing: Florida. pp. 196-230.

Stafford, E. A. and Tacon, A. G. J. (1984). Nutritive value of the earthworm, *Dendrodrilus subrubicundus*, grown on domestic sewage, in trout diets. **Agri. Wastes.** 9: 249-266.

Taboga, L. J. (1980). The nutritional of earthworms for chickens. **Br. Poult. Sci.** 21: 405-410.

Tacon, A. G. J., Stafford, E. A., and Edwards, C. A. (1983). A preliminary investigation of the nutritive value of three terrestrial worms for rainbow trout. **Aquaculture.** 35: 187-199.

Tiews, K., Gropp, J., and Koops, H. (1976). On the development of optimal rainbow trout pellet feeds. **Arch. Fishereiwiss. Beiheft.** 1: 27. Quoted in Viola, S., Mokady, V., Rappaport, U., and Arieli, Y. (1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. **Aquaculture.** 26: 223-226.

Underwood, E. J. (1977). Trace elements in human and animal nutrition. Academic Press, London and New York, pp. 545. Quoted in Stafford, E. A. and Tacon, A. G. J. (1984). Nutritive value of the earthworm, *Dendrodrilus subrubicundus*, grown on domestic sewage, in trout diets. **Agri. Wastes.** 9: 249-266.

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Van Hook, R. I. (1974). Cadmium, lead and zinc distributions between earthworms and soils: Potential for biological accumulation. **Bull. Envr. Contam. Toxicol.** 12: 509-11. Quoted in Stafford, E. A. and Tacon, A. G. J. (1984). Nutritive value of the earthworm, *Dendrodrilus subrubicundus*, grown on domestic sewage, in trout diets. **Agri. Wastes.** 9: 249-266.

Viola, S., Mokady, V., Rappaport, U., and Arieli, Y. (1982). Partial and complete replacement of fishmeal by soybean meal in feeds for intensive culture of carp. **Aquaculture.** 26: 223-226.

Watanabe, H. and Tsukamoto, J. (1976). Seasonal changes in size class and stage structure of the lumbricid *Eisenia fetida* population in a field compost and its practical application as the decomposer of organic waste matter. **Revue d'Ecologie et Biologie du Sol.** 13: 141-146. Quoted in Dominguez, J., Edwards, C. A., and Ashby, J. (2001). The biology and population dynamics of *Eudrilus eugeniae* (Kinberg) (Oligochaeta) in cattle waste solids. **Pedobiologia.** 45: 341-353.

Wikipedia. (2011). **Atomic absorption spectroscopy.** [Online]. Available: http://en.wikipedia.org/wiki/Atomic_absorption_spectroscopy.

Yoshida, M. and Hoshii, H. (1978). Nutritive value of earthworms for poultry feed. **Jpn. J. Poult. Sci.** 15: 308-310.