Use of Fruit Beetles, Waxworms Larvae and Tiger Worms in Waste Conditioning for Composting

By

Waleed Alwaneen

MSc, King Saud University, Riyadh, Saudi Arabia
MPhil, University of Sheffield, England

Thesis submitted in part fulfillment of the requirement for the degree of Doctor of Philosophy

Department of Molecular Biology and Biotechnology
The University of Sheffield, UK

بِسْمِ اللهِ الرَّحْمٰنِ الرَّحِيْمِ

Dedication

This thesis is dedicated to my parents, to my wonderful wife Mona and to my children, Reema, Yazeed, Omar and Leena.

Acknowledgments

ABSTRACT

1) Numbers of all three bacteria were higher in the cow faeces treated with fruit beetles than in the control, while the number of all three bacteria was lower in the cow faeces treated with Waxworms than in the control. Numbers were lower in cow faeces treated with Tiger worms than in the control. Liquefaction appears the main reason why bacterial numbers were higher in cow faeces treated with Fruit Beetle larvae.

2) Tiger worm treatment generally improved plant growth parameters, especially in the compost-faeces ratio (75% compost, 25% cow faeces), followed by a ratio of 50% compost, 50% cow faeces, Plant growth in Waxworm treated faeces was weak compared with plant growth in compost treated by FB, WW and control, notably in the compost (25% compost, 75% cow faeces) and 100% cow faeces where there was no growth at all. Fresh plant weight, fresh leaf weight and fresh root weight were significantly higher in the compost treated with Tiger worms in (75% compost, 25% cow faeces); no evidence was seen for any significant differences in the dry root weight measurement between TW, WW, FB and control in all composts. Tiger worms produced the best product, especially at the compost ratio of 75% compost, 25% cow faeces followed by 50% compost, 50% cow faeces.

3) Waxworms feed on wax that covered waxed paper, causing holes in the paper as a result of eating parts of paper therefore. This event decreased significantly waxed paper weight during the experiment. While there was no change or weight loss of waxed paper with Fruit Beetle larvae and most of the larvae died within 7 days. The waste weight of Waxworms after feeding on recycled paper was increased over the 28 day incubation period, notably at day 21. The Fruit Beetle larvae waste weight decreased during the experimental period, and a sharp decline occurred from day 21 to day 28. The results show significant differences between WW, FB waste weight after feeding on recycled

paper in days 14, 21 and 28. The numbers of *E.coli*, *salmonella Spp* and total bacteria in WW and FB waste after recycling paper feeding were very low or zero in most of days over the entire incubation period.

4) Fruit beetle haemolymph and whole-body disks produced only small inhibition zones (2-4 mm) against *E.coli*, while against *Salmonella,* FB haemolymph gave a zone of 2mm; Waxworm haemolymphs produced only a small zone (2mm) against, *E.coli*. Waxworm whole-body extract gave no zones against both *E.coli* and *Salmonella* cultures. All of the larvae (FB, WW) haemolymphs and whole-body extracts failed to produce inhibition zones against all bacteria.

List of Contents

List of Figures

XIII

List of Tables

Chapter One: Introduction

1. Introduction

1.1. Effects of adding cow manure to agricultural soil

Like other organisms, cows produce undigested residue of plant matter which has passed through their gut. This dung material is rich in minerals and organic matter including undigested cellulose and lignin, originating from the cell walls of the plants and methane. In many countries dung is used as manure; it also may be collected and used to produce biogas methane (Adegunloye *et al.,* 2007)

Farm manure comprises the dung and urine of domestic animals, along with bedding material, as well as straw, peat, leaves, sawdust, shavings and other vegetable waste. Names for such material include manure, barn manure, stable manure, farmyard manure and barnyard manure (Westover, 1926). Manure of various forms is one of the earliest materials to have been used for soil improvement. As well as providing small amounts of nutrients for plants, manure also supplies humus, greatly increasing the soil's water-holding capacity, whilst improving its physical character and making it a more suitable environment for the essential growth of bacteria (Westover, 1926).

Pennington *et al.* (2011) reported that manure produced by dairy cattle has a characteristic nutrient composition, the specifics of which are reliant on numerous factors. Farming operations have an impact on many of these, and include storage methods, the manure application system, housing and bedding systems, the diet fed to the cattle, and the environmental temperature. The quantities of micronutrients, nitrogen (N), potassium (K) and phosphorus (P) are affected by such factors, as is the manure's net value (Pennington *et al.*, 2011).

1.2. Risks of using cow manure in agriculture

Manure can be used as an inhibitor of plant diseases. Several microbes isolated from cow dung (e.g. *Aspergillus niger, Trichoderma harzianum, Bacillus cereus* and

Bacillus subtilis) were characterized to have potential to be used as inhibitors agents against seedling blight inducing pathogens (Muhammad and Amusa, 2003).

Many people do not realize the hidden dangers when they fertilize their gardens with composted cow manure, which may harbor diseases such as Listeriosis, Salmonellosis, Cryptosporidiosis and even mad cow disease. These diseases are faecal to oral in transmission and may be passed to humans if garden vegetables are not properly washed. Several bacterial strains which cause diseases associated with vegetable crops and fresh fruits are likely to directly contaminate food products in the field (Kirk, 2011). Many field crops are prone to contamination with remains of faeces of farm animals or other domestic animals, which are in contact with manure contaminated soil or dust. This may likely contribute significantly to pre-harvest contaminations. Comparatively little research has been reported on the outbreak of human bacterial diseases, mainly intestinal pathogens, such as *Campylobacter*, *Salmonella sp* and *Escherichia coli*, in the developing countries compared with the developed countries (Philips, 1999; Locking *et al*., 2005). Nevertheless, contamination associated with *E.coli* O157 is an occurring zoonosis in several countries as well as the United Kingdom and the United States (Tauxe, 1997; Rangel *et al*., 2005). Scotland has the highest percentage of human bacterial infections within the United Kingdom, with an average of 4.1 cases per 100,000 persons in 2004 (Locking *et al*., 2005).

By the end of May 2011, the BBC News in Europe and The ABC news from Australia reported that cucumbers, which it is believed were imported from Spain, were contaminated with *E.coli* and left people ill with haemolytic-uremic syndrome (HUS). There have also been around 300 of infection reported in several European countries in 2011 (Centre for Disease Control and Prevention, 2011). The aggressive form of *E.coli* is known to cause kidney failure and affect the central nervous system.

Austria's Agency for Health and Food Safety has included some other items (e.g. tomatoes and eggplants) in the food ban list. Meanwhile, one European Union spokesman declared that two greenhouses in Spain were identified as sources of the disease outbreak and their activities ceased. Investigations were still carried out to see whether the contamination had occurred in the identified place or elsewhere (BBC News, 2011).

Pathogens that affect the nervous system can cause convulsions, confusion and loss of balance, according to the Centre for Disease Control and Prevention (2011) a particular condition, *Salmonellosis,* is caused by the *Salmonella* bacteria, of which there are many species, only a few of which affect humans and cattle. Tests on the faecal matter of animals suggest that around 75 percent of dairy cattle carry *Salmonella*, with the survival rate of the bacterium dependent on both temperature and ammonia concentration. The parasite *Cryptosporidium* is spread by livestock and humans, the main source of infection being from water supplies tainted by parasite-infected manure. Such organisms divide and multiply once inside a host, and are able to survive for a long period in manure (Kirk, 2011). The progressive and fatal neurological disease known as 'Mad Cow Disease' (*Bovine spongiform encephalopathy)* was previously thought to be spread solely by rendering and feeding of infected flesh to other cud-chewing mammals. Kirk (2011) has shown, however, that faecal-oral prion (infectious protein) transmission is possible between healthy animals in the presence of manure or soil where prion concentrations are high.

1.3. Management of cow manure

As a fertilizer, compost is a superior choice to fresh manure. When compared to composted manure, the fresh, non-composted equivalent will generally display a higher N content, although the composted variant contributes more in the way of

organic matter content. Fresh manure has a higher concentration of soluble N compounds, such that over-application can cause leaching losses and salt build-up (Rossen and Bierman, 2005). Additionally, the large numbers of viable seeds often present in such manure can lead to issues with weeds. Fresh manure can contain pathogens such as *E.coli*, which can cause illness in people who eat produce grown on such media unless relevant precautions are taken. Rossen and Bierman (2005) have suggested that in order to overcome the contamination of crops, three particular precautions should be taken:

a) In the case of fields for crops intended for human consumption, ensure that raw manure is applied and incorporated at least three months prior to harvesting.

b) In the particular case of root and leaf crops which contact the soil, allow a four month period between application and harvesting.

c) In the case of trees where fallen fruit will be collected, refrain from surface application of manure.

Management of manure before usage is important. Dung pre-treatment, using physical or biological methods to control the pathogens content, has become a common practice. Treatment processes, such as manure digestion, controlled manure storage and use of certain insects, are potentially applicable (Callaghan *et al.*, 2002). Each treatment practice carried out may cause a decrease in the numbers of pathogens from the initial load found in the manure. The manure, both in solid and liquid form, can be treated with anaerobic digestion for the purpose of increasing the decomposition rate (Callaghan *et al.*, 2002). Several studies have been conducted to investigate the effect of temperature of incubation of the manure as well as the dairy manure water content on survival of faecal bacteria, *E.coli*, faecal streptococci and faecal coliforms. Wang *et al.* (2004) investigated the effect of many temperature

5

ranges (e.g. 4°C, 27°C and 41°C) as well as various levels of manure moisture content (e.g. 30%, 55% and 83%) on survival of faecal bacteria. This research suggested that manure management practices should maintain manure at high temperature (41°C), because it would decrease the *E.coli* and faecal coliform counts in the treated manure.

Kirk (2011) has outlined three manure storage protocols which are designed to reduce the pathogen content; composting, lagoon storage (slurry) and deep stacking. The decomposition rate of the manure is known to vary, dependent on the specific method.

1) Deep stacking involves removal of manure from livestock housing facilities, with the resultant material being placed in large piles. These piles do not usually undergo a turning procedure, but heating does occur within them. The lack of homogeneity in the stack can however result in areas which are unheated in which pathogens are able to survive. The outer crust of the stack is one particular region where pathogen survival is more likely, and reports suggest that *Salmonella* bacteria can survive in this area for up to 200 days.

2) Stacked manure can be subjected to composting, in which periodic turning for at least three days improves heat distribution, making sure that all parts of the pile reach at least $55 - 65^{\circ}$C. Such a procedure avoids the possibility of areas existing, particularly within the crust, which are not subject to the heating process. Protocol suggests that after being turned several times, the pile must be left for at least a month in the undisturbed condition.

3) 'Slurry' is the term used to denote a mixture of liquid and dry matter that can be lagoon stored for varying amounts of time before being applied to either crops or pastures. Initial pathogen load usually decreases over the storage period, with the level of *Salmonella* being substantially lowered if storage persists for at least a month before

spreading occurs. Lower temperatures correlate with a smaller reduction in *Salmonella* load; however the first two weeks of lagoon storage usually see bacterial quantity reduced by up to 90%.

1.4. Effect of insects to improve the manure

On the Davis Ranch in Southern Oklahoma, dung beetles have been released to manage cattle dung on a grazed area of 2500 acres (1000 hectares). *Onthophagus gazella* was one of the first insects to appear when the landowner ceased use of insecticides in 1975. This tropical species, used for bio-control of pests such as hornflies, was introduced by the U.S. Department of Agriculture during the mid 1970's. The insects are able to bury dung into the soil through spreading and riddling, with a wet manure burial rate of a ton per acre per day in mature plots, along with a surface removal capacity of 90% (Richardson and Richardson, 2000). Due to their effect on soil aeration, removal of non-point source pollution, ability to increase organic matter, improvement of water infiltration and pest control properties, such insects represent a valuable soil-restoration tool (Richardson and Richardson, 2000).

Dung beetles have been described by Fabre (1911) as scavengers of meadows contaminated by herds. Such 'scavenging' facilitates the decomposition of pasture dung, improving the cycling of nutrients, and is thus extremely beneficial. Despite the more diminutive stature of British species when compared to their larger, dung-rolling African counterparts, they are still capable of rapidly degrading dung when present in sufficient numbers (Fabre, 1911). Dung pats, with their large populations of invertebrates, represent an excellent feeding environment for vertebrates as well as other predatory beetles such as Staphylinids, Elaterids and Calerids.

An attempt at *Esherichia coli* and *Salmonella enterica* reduction by using black soldier fly in larval stage in chicken manure has been studied by Erickson *et al*. (2004).

The obtained results showed that the larvae accelerated inactivation of *E. coli* and *Salmonella* in chicken manure, either on autoclaved or non-autoclaved manure. During this process, the larvae become contaminated with the pathogen and may transmit it to the fresh manure, thereby leading to cross contamination (Erickson *et al.*, 2004).

In their study on bacterial counts in cattle dung, Lussenhop *et al.* (1980) concluded that Sarcophagid maggots and *Aphodius* beetles boosted bacterial counts and decreased hyphal density in cattle dung. These effects can be referred to insect mixing of the substrate, giving bacteria a competitive advantage over fungi.

Bertone *et al.* (2004) reported that pastures can be improved through the incorporation of manure into the soil via beetle activity. In the laboratory setting, two beetles, *O. gazella* and *O. taurus*, were assessed for their ability to improve soil quality, with a coastal-plain sandy-loam, common to East Carolina, used as the basis for the study. Treatments chosen for the study included untreated bovine dung, dung with added *O. gazella*, dung with added *O. taurus*, and a control with no dung addition. Inclusion of beetles was seen to improve P, K and Mg levels, as well as the amount of cations in the soil beneath the dung pat.

OVERALL AIM

The overall aim of the work described in this Thesis was to investigate the potential role of a variety of insect larvae in the conditioning of cow manure, and waxed carton-paper waste.

Chapter Two: Isolation of Bacteria and Changes in Bacterial Populations following Treatment of Cow Faeces with the Waxworm *Galleria mellonella,* the Fruit beetle *Pachnoda marginata* and the Tiger worm *Eisenia fetida*

2.1. Introduction

An obvious problem with cow faeces when used as fertilizer relates to their potential microbial content, which might include pathogenic bacteria and parasitic worms, a problem which is clearly not found in relation to inorganic fertilizers. The average feedlot steer produces 1.62 kg of faeces (dry matter) per day, which results in more than 18 million metric tons of faeces (dry matter) per year in the United States alone. Enteric microbes of cattle affect animal health and food safety and can be used as an indicator of faecal pollution of drinking and recreational surface waters (Shanks *et al.*, 2011). Pathogenic bacteria like *Escherichia coli* O157: H7 which are present in the bovine gastrointestinal tract have been linked to disease outbreaks due to the consumption of contaminated beef, milk, and drinking water. Pathogens are potentially released into the environment when bovine faecal waste is sprayed on farmland, or is accidentally discharged into the environment following severe storms, or onsite failure of waste management practices. Pathogens associated with such releases include *E. coli* O157:H7, *Campylobacter jejuni*, *Salmonella spp.*, *Leptospira interrogans*, and *Cryptosporidium parvum* (Gannon, 2004).

Faecal microbes play a critical role in animal health and productivity but also in food safety. Shanks *et al.* (2011) using parallel pyrosequencing of a hypervariable region of the rRNA coding region profiled the faecal microbial communities of cattle from six different feedlots. A total of 633,877 high-quality sequences were obtained from the faecal samples of 30 adult beef cattle (5 individuals per operation). Overall, the composition of the bacterial community correlated significantly with faecal starch concentrations, largely reflected in changes in the *Bacteroidetes*, *Proteobacteria*, and *Firmicute s*populations (Shanks *et al.*, 2011).

Comparatively few studies of the survival of enteric bacteria in cow pats on pasture have been reported (Sinton *et al*., 2007). Coliforms and faecal coliforms often survive for up to 18 weeks in cattle faeces in hot, dry summers and release of faecal coliforms and *Escherichia coli* from cattle faeces has been recorded for up to 30 days and 100 days. *Campylobacter* spp. have been detected in cattle faeces after incubation for 1 to 3 weeks at 5°C and after incubation for 1 week at 30°C, while *Salmonella* spp. have been found for 12 to 28 weeks after incubation at 5°C and for 4 weeks at 30°C. More recent survival studies have focused primarily on *E. coli* O157 and related strains because of the importance of cattle wastes as a major source of these pathogens. Most of these studies either have involved manure and slurries or have been laboratory based and have produced extinction times ranging from 24 h to 100 days, with faster inactivation generally associated with higher temperatures (Sinton *et al*., 2007). Few field-based studies of survival in cow pats have been conducted, although a 4- to 5-$\log_{10}$ decrease in *E. coli* O157:H7 within 50 days has been recorded in inoculated cow pats placed on grassland (Sinton *et al*., 2007).

The aim of the work described in this Chapter was to investigate the use of insect larvae (Fruit Beetle (FBL), Waxworms (WW) and Tiger worms (TW)) to improve the breakdown of agricultural wastes and reduce their pathogen loads, hopefully leading to an improved material for use as a fertilizer or potting compost.

2.2. Materials and Methods

2.2.1. Methods for bacterial isolation and identification

1) A selective medium of HiCrome *E. coli* Agar was used. It is based on Tryptone Bile Agar and is used to detect *Escherichia coli* in foods, where recovery of *E. coli* has been shown to be faster, more reliable and accurate. Most *E. coli* strains can be recognized from other coliforms by the presence of the enzyme glucuronidase which is

highly specific for *E.coli.* The chromogenic agent X-glucuronide used in this medium helps to detect glucuronidase activity. *Escherichia coli* cells absorb x-glucuronide and the intracellular glucuronidase splits the bond between the chromophore and the glucuronide. The released chromophore gives the blue coloration of the colonies.

The medium is made as follows:

- ➤ Powder (36.6g) is suspended in 1000 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes.
- ➤ It was the cooled to 50°C and poured into sterile Petri dishes.

2) XLT-4 (Xylose Lactose Tergitol-4) Agar is a highly selective plating medium used for isolation and identification of Salmonellae from clinical, environmental and food samples (Miller *et al.,* 1990). The amino nitrogen, essential nutrients and vitamins are obtained from peptones and the yeast extract present in this formulation, ensuring optimal growth of Salmonellae. The selective agent Tergitol-4 (also known as Niaproof 4 or sodium tetradecylsulpate) is an anionic surfactant. This largely hinders the growth of unwanted background flora. Due to the inclusion of phenol red, background colour of the plate is red, which results from pH changes due to fermentation and decarboxylation reactions. The fermentation of xylose, lactose and sucrose as well as the decarboxylation of lysine facilitates the differentiation on the medium. Due to the ability of *Salmonella* to reduce thiosulphate to hydrogen sulphide, Salmonellae appear as black or red colonies.

- ➤ Powder (59 g) of XLT-4 Agar Base was suspended in 1000 ml of distilled water
- ➤ XLT-4 Selective Supplement (4.6ml) was added and the medium was boiled (avoiding overheating and autoclaving).

➢ Finally the medium was cooled to approximately 50°C and poured into sterile Petri dishes (Miller *et al.,* 1990).

3) Nutrient Agar is a basic culture medium used to subculture organisms for maintenance purposes or to check the purity of subcultures from isolation plates prior to biochemical or serological tests. In semi-solid form, agar slopes, the medium is used to maintain control organisms (Lapage *et al.,* 1970). It contains 1.5% agar to permit the addition of up to 10% blood or other biological fluid, as required. The medium, without additions, may be used for the cultivation of organisms which are not exacting in their nutritional requirements.

➢ Nutrient Agar (23g, pH 6.8) was suspended in 1000 ml of distilled water and boiled; it was then autoclaved at 121°C for 15 minutes.

4) Potato extract serves as a source of carbon, nitrogen, minerals, vitamins and other essential growth nutrients, while dextrose acts as source of carbohydrate. Agar is added as the solidifying agent. The accompanying bacterial flora is suppressed by the pH value of 3.5. The growth of yeasts and moulds is promoted on this medium and fungi develop typical morphologies. Rose Bengal (8.4 mg/l) can be added to further suppress the development and restrict the size and spreading of mould colonies (Beever *et al.,* 1970).

➢ Potato dextrose agar was suspended 39 g of powder in 1000 ml of distilled water.

➢ The mixture was brought to the boil to dissolve completely and sterilize by autoclaving at 121°C for 15 minutes.

2.2.2. Larvae, Worms and Cow Faeces: Collection and Incubation

Larvae and worms were purchased online from Ricks LiveFood and Original Organics Ltd. Fresh cow faeces were collected from Butterthwaite Cattle Farm,

Ecclesfield, Sheffield (Figure 1.1), and distributed in 12 plastic boxes (30×20×15 cm) with a lid perforated to allow for gas exchange (Figure 1.2), three control boxes and 9 treatment boxes were set up. 100 larvae of Waxworms (Figure 1.3 B) were then added. One hundred grams of cow faeces was added to each control box (3 replicates). Fruit beetle larvae (Figure 1.3 C) (ten larvae to 100 g cow faeces) were then added to each box (3 replicates), and Tiger worms (Figure 1.3 A) were then added, 40 worms to 100 g cow faeces in each box (3 replicates). Approximately were used, as suggested by Li *et al.*, (2011). All samples were incubated at 25°C throughout the incubation period.

2.2.3. Bacterial Isolation from Cow Faeces

Bacteria were isolated every seven days from both control cow faeces and cow faeces to which larvae were added; from zero time to 28 days in order to determine the populations of *E.coli, Salmonella Sp* and total bacteria count. Samples (1g) were diluted in 9 ml of sterilized water then a serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) was performed and 0.1 ml of each diluted sample was spread onto *E.coli* agar (Figure 1.4), XLT4, LB (Figure 1.5) and Plate Count Agar (Figure 1.6) and incubated for between 18 and 24 h at 37^{0}C.

2.2.4. Fungal Isolation on PDA medium

To isolate and count fungi in cow faeces, samples (0.1 g) of fresh cow faeces were inoculated onto PDA (Potato Dextrose Agar) and then incubated at 25°C for 3 to 5 days (Fig 1.7). Identification of fungi was achieved by extraction of genomic DNA and using 18S rRNA primers. Samples were 0.5-1 ml of an overnight Potato Dextrose Broth culture.

2.2.5. Genomic DNA extraction for bacteria and fungi

Genomic DNA was extracted from each strain of bacteria and fungi, then PCR with appropriate primers was conducted to produce sufficient quantities of the 16S

rRNA or 18S rRNA gene. Genomic DNA was isolated by using the Key prep-Bacterial DNA Extraction Kit (ANACHEM, lab store, UK) by the following procedures described for bacteria; and the Norgen Fungi Genomic DNA Isolation Kit (GENEFLOW LIMITED, Labstore, UK) by following procedures described for fungi. The genomic DNA was separated by gel electrophoresis using 1% agarose gel to check for purity.

(A) Procedures (Key prep- Bacterial DNA Extraction Kit)

1-Centrifugation: 3 ml of pure bacterial culture grown overnight in LB medium was collected by centrifugation at 6000×g for 2 minutes and supernatant was decanted completely.

2- Re-suspension of pellet: 100 μl Buffer R1 was added, along with pipetting the pellet up and down to re-suspended it.

3- Lysozyme treatment: For gram-negative bacteria, 10 μl of lysozme (50mg/ml) was added to the suspension while for Gram-positive bacteria 20μl of lysozyme was used. The suspensions was mixed completely and incubated at 37°C for 20 minutes.

4- Centrifugation: For 3 minutes, the sample was centrifuged at1000×g, discarding the supernatant.

5- Protein denaturation: 180 μl of Buffer-R2 and 20 μl of Proteinase-K was added to the pellet and mixed completely and incubated in a shaking water bath at 65°C for 20 minutes.

6- Homogenization: 410 μl of Buffer-BG was added to the sample, mixed thoroughly, and incubated at 65 °C for 10 minutes.

7- Addition of ethanol: Then 200 μl of absolute ethanol was added to the samples followed by mixing.

8- Loading to column: The sample was transferred into a column and centrifuged at 10,000×g for 1 minute; the supernatant was then discarded.

9- Column washing: The column was washed gently using 750 μl of wash buffer and then centrifuged at 10,000×g for 1 minute; flow through was discarded (wash buffer was diluted by absolute ethanol before use). The column was again centrifuged at 10,000×g for 1 minute to remove residual ethanol.

10- DNA elution: Finally, DNA was collected using a clean microcentrifuge tube by adding 100 μl of preheated Elution Buffer directly into the column membrane and leaving to stand for 2 minutes at room temperature, and then centrifuging at 10,000×g for 1 minute to elute the DNA. DNA obtained was stored at -20°C. DNA ready for gel electrophoresis.

(B) Procedures (Norgen- Fungi Genomic DNA Isolation Kit)

1- Centrifugation: Pure fungal culture (50 mg wet weight, grown for 3 days in PDA medium) was collected by centrifugation at 14,000×g for 1 minute to pellet the cells. The supernatant was decanted carefully.

2- Lysate preparation: the pellet was re-suspended by adding 500 μl of lysis solution and the cells were re-suspended by gentle vortexing (optional RNase treatment). 10 KUnits of RNase was added to the suspension and mixed completely.

3- The samples were transferred into bead tubes and vortexed horizontally for 5 minutes on a flat bed vortex pad with tape and then the samples were incubated at 65°C for 10 minutes.

4- The samples were transferred into a DNase-free microcentrifuge tubes and centrifuged at 14,000 xg for 2 minutes, and then supernatant transferred to a new microcentrifuge tube (note the volume)

5- Addition of ethanol: equal volume of absolute ethanol was added into the samples and vortexed immediately, and then 300 μl of binding solution was added into the samples and vortex.

6- Binding nucleic acids to column: 650 μl of the lysate with ethanol was transferred into a column and then centrifuged at 6,000xg for 1 minute; the supernatant was then discarded.

7- Column washing: 500 μl of wash solution was washed through the column and centrifuged at 6,000×g for 1 minute, and the flow through discarded (wash buffer was diluted by absolute ethanol before use). The column was centrifuged at 6,000×g for 1 minute again to remove any residual ethanol. The samples were centrifuged at 14,000xg for 2 minutes and the collection tube was discarded.

8- DNA elution: an elution tube was used to collect DNA by adding 100 μl of Elution Buffer directly into the column membrane, followed by centrifuging at 6,000×g for 2 minutes to elute DNA. DNA was stored at -20°C for a few days or -70°C for long term storage. DNA was then analysed by gel electrophoresis and PCR reaction.

2.2.6. DNA quantification

DNA can be quantified using several methods. The most common approach is to use a spectrophotometer (Haque *et al*., 2003). 98.0 μl of elution buffer (EB) was added to 2.0 μl of the genomic DNA sample, which was mixed thoroughly and added to special UV cuvettes (UVette, Eppendorf) and the optical density OD was then measured at 260 nm using a spectrophotometer (Unicam, Hexios).

2.2.7. Agarose gel electrophoresis

Using 1% Agarose-gel, DNA fragments were separated. These gels were prepared in the following manner. Because of the small size of the BioRad Subcell GT electrophoretic tank with just a 6 or 14 well comb, 0.4 g of molecular biology grade

agarose will be dissolved in 0.8 ml of 50× TAE buffer and 40 ml distilled water followed by heating for 3 minutes in a microwave oven on medium power until the agarose gel was dissolved. This solution was then cooled and 2.5 μl of ethidium bromide was added to visualise the DNA before setting the solution in a gel tray and finally pouring the gel in the gel rack. After pouring the gel, the combs were inserted at one side of the gel and kept at room temperature until the gel set. The gel was then submerged in TAE buffer 1× the samples added into the wells, each 10 μl was mixed with 2 μl loading dye. 6 μl of Hyper Ladder was used to determine the size of fragments. The gel was then run for 40 min, operated at 80V. Finally, DNA was visualized taking digital images using an UVitec ''Uvidoc'' attached to a digital camera. If any smearing was observed, 1.0 μl of RNAse was added to the whole extracted gDNA, followed by incubation at 4°C overnight.

2.2.8. Polymerase chain reaction (PCR) amplification of 16S and 18S rRNA genes

After the successful extraction of genomic DNA from the unknown microorganisms, 16S and 18S rRNA genes were amplified using the polymerase chain reaction (PCR) Table 1.1. shows the amount of each component used for amplification of 16S and 18S rRNA genes. Bacterial and fungal universal primers (Forward and Reverse) were used for DNA amplification (Table 1.2). To anneal to a certain region of the proposed DNA to amplify, primers were designed specifically. Primers were purchased from Eurofins (mwg/operone). The following thermal cycling order was used for the amplification of the 16S gene:

➤ initial denaturing at 94°C for 3 min to separate double stranded DNA into two single strands, followed by 30 cycles of DNA denaturation at 94°C for 1 min, and then primer annealing at 60°C for 1 min.

➤ The temperature was then decreased to allow primers to anneal.

➢ Strand elongation was conducted at 72°C for 5 min to allow the Taq polymerase to replicate the remaining single strand of DNA.

➢ Final elongation was done at 75°C for 5 min (Table 1. 3)

Table 1.1: Components of the PCR reaction.

Component	Quantity
Sterile Milli-Q water	35.0-39.0µl
10xTaq buffer	5.0 µl
MgCl2 solution (50mM)	2.5 µl
Forward Primer (10 ppmole.I-1)	0.5 µl
Reverse Primer (10 ppmole.I-1)	0.5 µl
dNTPs mix (2.5 mM each)	1.0 µl
Genomic DNA (10-100 ng)	1.0-5.0 µl
Ampli Taq polymerase (5U/µl)	0.5 µl

Table 1.2: Oligonucleotide primers from Eurofins (mwg/operone) Germany.

Primer name	Sequence(5`-3`)	Target gene	Reference
16SUN1.FOR	CCGAATTCGTCGACAACAG AGGATCCTGGCTCAG (34)	Bacterial 16S rRNA	Weisburg et al., 1991
16SUN1. REV	CCCGGGATCCAAGCTTACG GCTACCTTGTTACGACTT (37)	Bacterial 16S rRNA	Weisburg et al., 1991
Nu-SSU-0817.FOR	TTAGCATGGAATAATRRAA TAGGA(24)	Fungal 18S rRNA	Borneman and Hartin, 2000
Nu-SSU-1196-39.REV	TCTGGACCTGGTGAGTTTC C(20)	Fungal 18S rRNA	Borneman and Hartin, 2000

Table 1.3: PCR amplification procedure for 16S rRNA.

Steps	Temperature and time	Number of cycle
Initialization	94°C for 3 min	1
Denaturating	94°C for 1 min	
Annealing	60°C for 1 min	30
Elongation	72°C for 5 min	
Final elongation	75°C for 5 min	1
Hold		

Similarly, the thermal cycling order for amplification of 18S rRNA gene used in this experiment was as follows:

> initial denature at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 0 sec, primer annealing at 56°C for 10 sec.

> strand elongation at 72°C for 30 sec.

> Final elongation at 75°C for 2 min. The thermal cycling conditions for amplification of 18S rRNA gene are presented in table 1.4.

Table 1.4: PCR amplification procedure for 18S rRNA.

Steps	Temperature and time	Number of cycle
Initialization	94°C for 2 min	1
Denaturating	94°C for 0 sec	
Annealing	56°C for 10 sec	35
Elongation	72°C for 30 sec	
Final elongation	75°C for 2 min	1
Hold		

After finishing the amplification processes of both 16S and 18S rRNA genes, the PCR reaction was examined in electrophoresis on a 1% agarose gels. 10 µl of PCR product was mixed with 2 µl of Blue/Orange 6× loading dye and was run on a 1% agarose gel. In addition, 6 µl of 1 Kb Hyper ladder are loaded on the gel to confirm the correct sized product (Figure 1.8).

2.2.9. Phylogenetic identification of unknown bacteria and fungi

All samples were sent to Sheffield University Medical School Core Genetics Unit for sequencing. Finch TV software will be used to correct the sequence then comparing it with BLAST to look for the closest matches available from the website of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). In Finch TV software the unknown nucleotide represents as N, and it could be either A, or T, or G, or C, according to the different colours appear (Mishra *et al*., 2010) (Figure 1.9).

2.2.10. Statistical analyses

All observations were presented as means ±SE (standard error). Bacterial population numbers were converted to log CFU/g before statistical analysis. Statistical analyses were performed on experimental datasets using the t-test between two groups and comparing significance of means between treatment and control (P=0.05). Results were analysed using Sigma Plot 12.0$^{©}$ software.
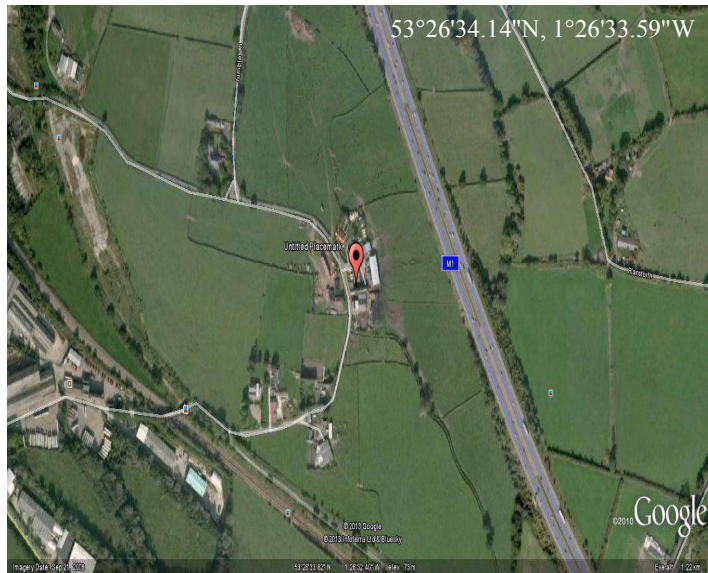
Figure 1.1: Aerial view and coordinates of Butterthwaite cattle Farm site in Ecclesfield from Google earth program.
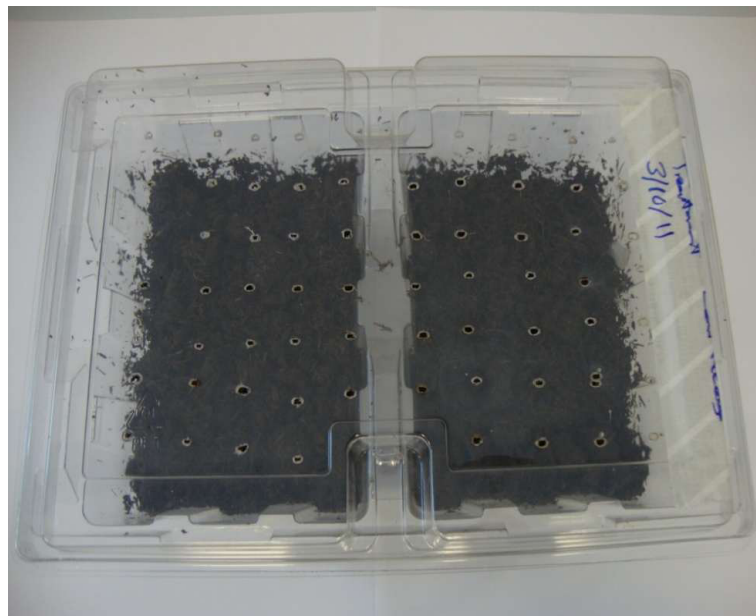
Figure 1.2: Plastic box with a perforated lid, containing compost and larvae (Author's image).
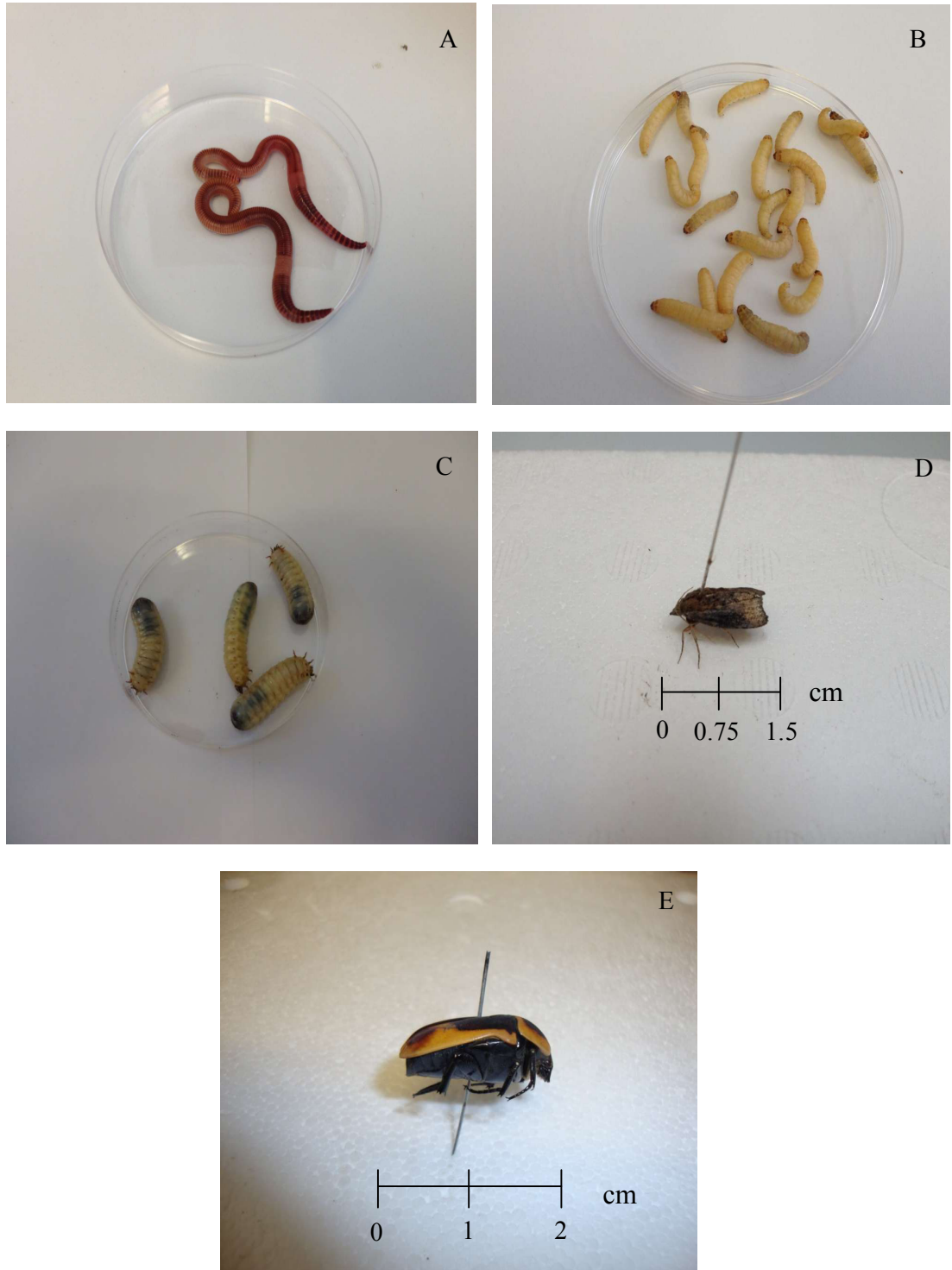
Figure 1.3: A) Tiger worms *Eisenia fetida*, B) Waxworms larvae *Galleria mellonella*, C) Fruit beetles larvae *Pachnoda marginata*, D) Waxworm adult *Galleria mellonella*, E) Fruit beetle adult *Pachnoda marginata* (Author's images).
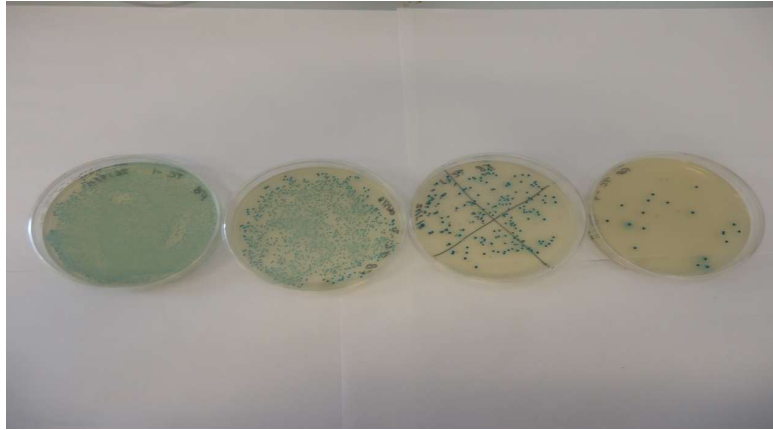
Figure 1.4: HiCrome *E. coli* agar showing blue colonies in different dilution.
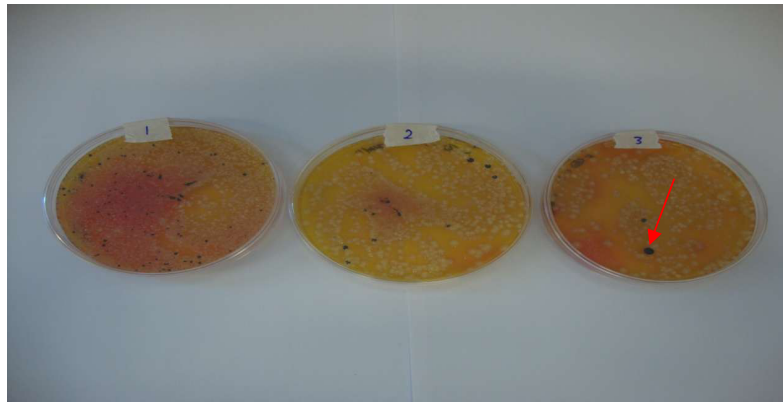
Figure 1.5: *Salmonella Spp* (black colonies) grown on XLT-4 selective *Salmonella*
agar medium at different dilutions.
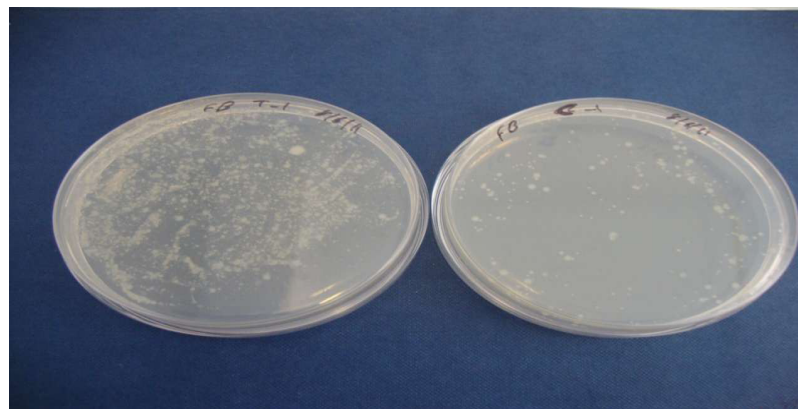
Figure 1.6: Colonies of bacteria grown on plate count agar medium.
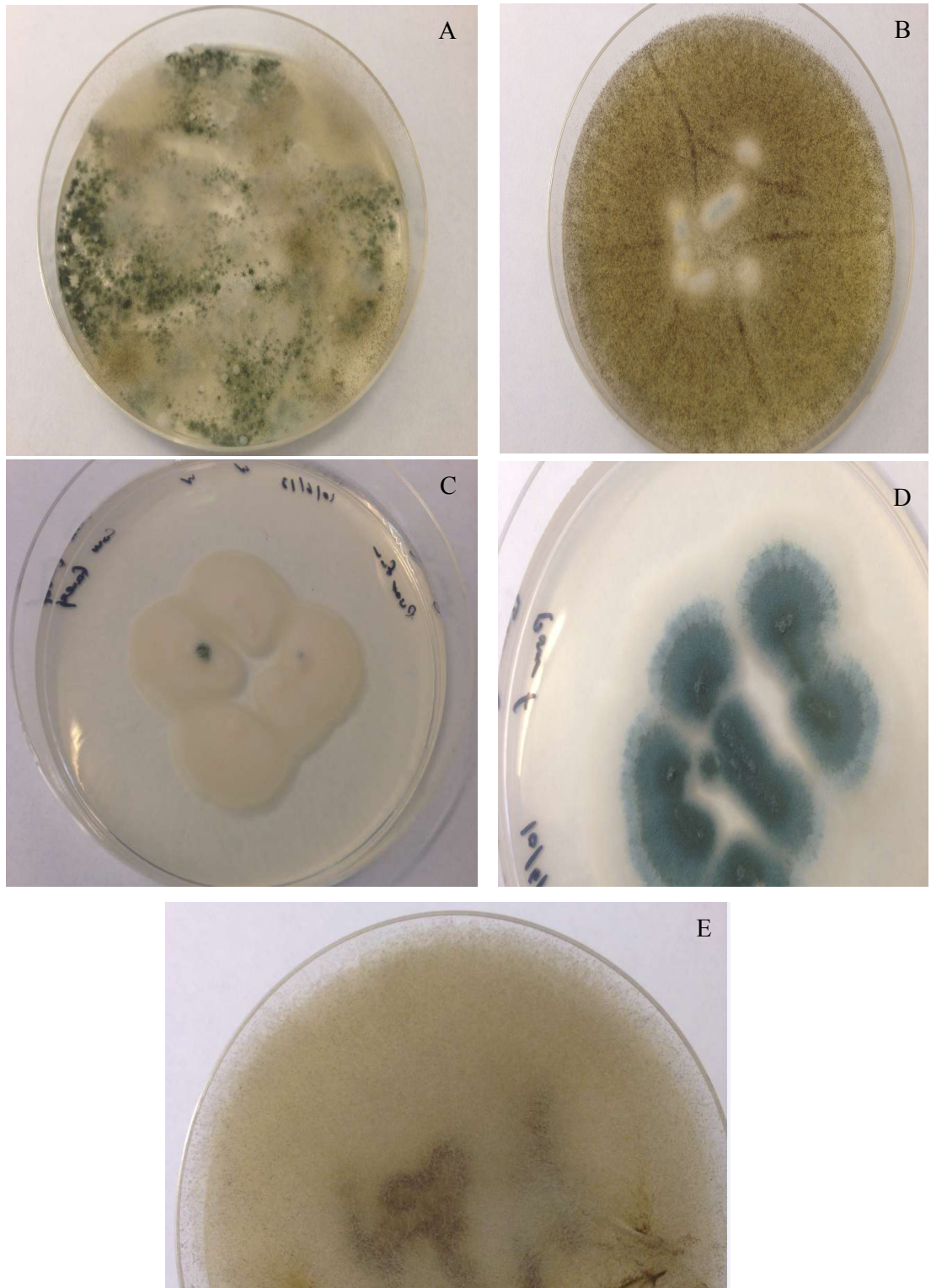
Figure 1.7: (A, B) Fungi isolated from cow faeces using PDA medium: (C,D) *Penicillium roqueforti*; (E) *Mucor circinelloide.*
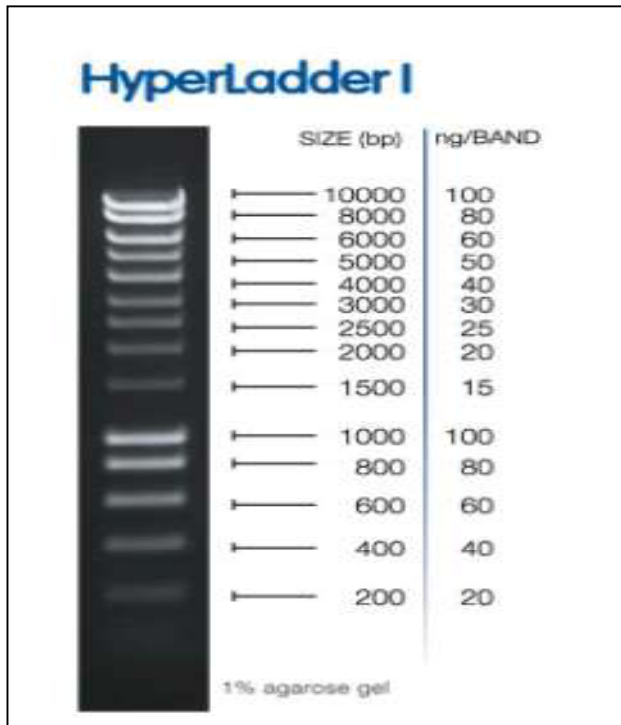
Figure 1.8: Standard hyperladder I with 14 lanes indicates higher intensity bands, 1000 and 10,000 and each lane (5μl) provides 720ng of DNA (BIOLINE supplier).
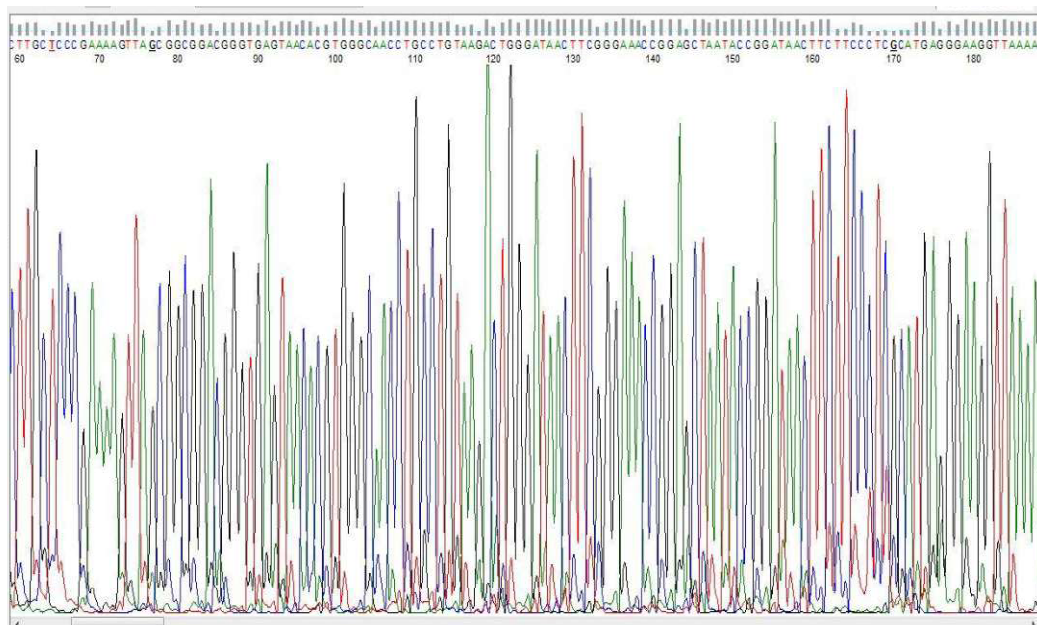
Figure 1.9: FinchTV software which manually adjusts errors of consensus sequences prior to before BLAST in the NCIMB database.

2.3. Results and Discussion

2.3.1. The effect of fruit beetle larvae, Waxworms and Tiger worms on bacterial populations in cow faeces

Numbers of *E.coli* in the control samples declined steadily over the 28 day incubation period (Fig.1.10 A). A decrease in the number of this bacterium also occurred in soils with added fruit beetle larvae (FBL), but from day 14 the number of *E.coli* was higher in the treated compared to control samples. The number of *Salmonella* fell sharply in the cow faeces at day 7 and then decreased more slowly over the incubation period. From day 7, more *Salmonella* were found in the faeces treated with FBL than in the control (Fig.1.10 B). The total heterotrophic bacterial count is shown in fig.1.10 C. Numbers of bacteria declined in the untreated, control faeces, but remained higher in the FBL treated cow faeces over the entire incubation period. The overall trend in these results was that the number of bacteria was higher in the cow faeces treated with fruit beetles than in the control.

The number of *E.coli* in the samples treated with Waxworms was less than the control over the 28 day incubation period (Fig.1.11 A), but in days 7 and 14, the number of *E.coli* was less in the treated compared to control samples. The number of *Salmonella* fell sharply in the cow faeces at day 7 and the decreased more slowly over the incubation period. From day 7, less *Salmonella* were found in the faeces treated with Waxworms than in the control (Fig 1.11 B). The total heterotrophic bacterial count is shown in Fig1.11 C. Numbers of bacteria declined sharply in the cow faeces treated with Waxworms and control at day 7, and then decreased more slowly over the incubation period; however, throughout the incubation period, the number of bacteria in the control was higher than in the treatment. The overall trend in these results was that the number of bacteria was lower in the cow faeces treated with Waxworms than in the control.

The number of *E. coli* fell sharply in the cow faeces treated with Tiger worms was less than the control over the 28 day incubation period. In days 7, 14 and 21 the number of *E.coli* was very low in the treated compared to control samples. (Fig.1.12 A). Numbers of *Salmonella* fell sharply in the cow faeces at day 7 and the decreased more slowly over the incubation period (Fig 1.12 B). After day 7, less *Salmonella* were found in the faeces treated with Tiger worms than in the control. The total bacterial count is shown in (Fig1.12 C). Numbers of bacteria declined sharply in the cow faeces treated with Tiger worms and the control at day 7, and then decreased more slowly over the incubation period, but at day 14, the number of bacteria was close between treatment and control; however, throughout the incubation period the number of bacteria in the control was higher than in the treatment. The overall trend in these results was that the numbers of bacteria were higher in the cow faeces treated with Tiger worms than in the control.

It is not immediately apparent why the number of bacteria in cow faeces treated with fruit beetle larvae should be higher than the control, when the opposite is true of the Waxworm and Tiger worm treatments. It was noticeable, however, that the fruit beetle larvae were extremely active and moved around in the faeces much more so than did the larvae of the other two beetles. The activity and overall metabolism of the fruit beetle larvae led to the faeces being liquefied, an effect not seen with the other larvae. This liquefaction effect will be discussed in more detail below, as it may have important biotechnological implications. We assume the liquefaction was the main reason why bacterial numbers were higher in cow faeces treated with fruit beetle larvae, as this would have encouraged aeration and the tendency for bacterial numbers to be higher in liquid compared to solid faeces.
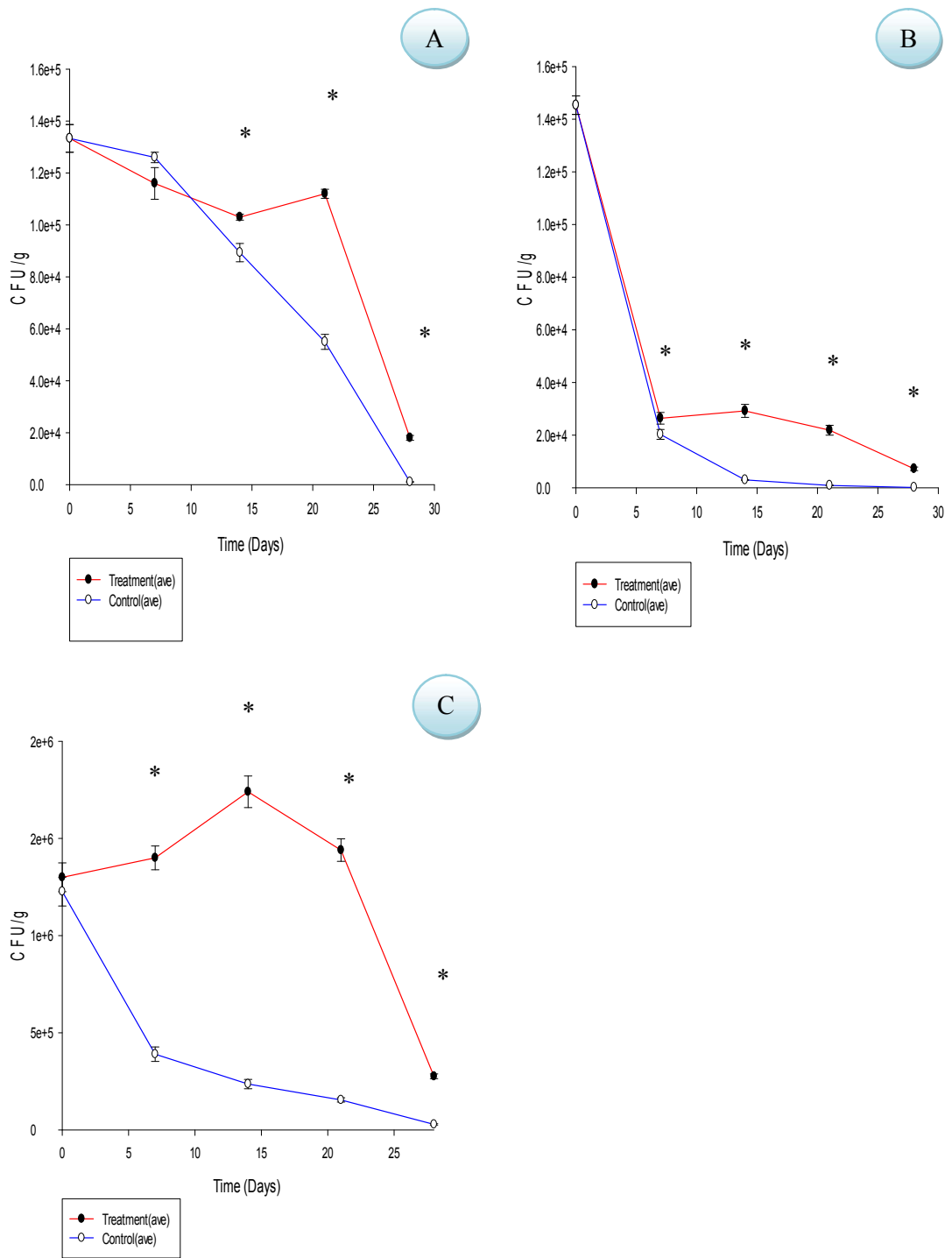
Figure 1.10: Bacterial average numbers isolated from cow faeces treated with Fruit beetle, bacteria grown on; A) HiCrome *E. coli* medium B) *Salmonella Sp* medium and C) plate count medium. (*Significantly different from control).
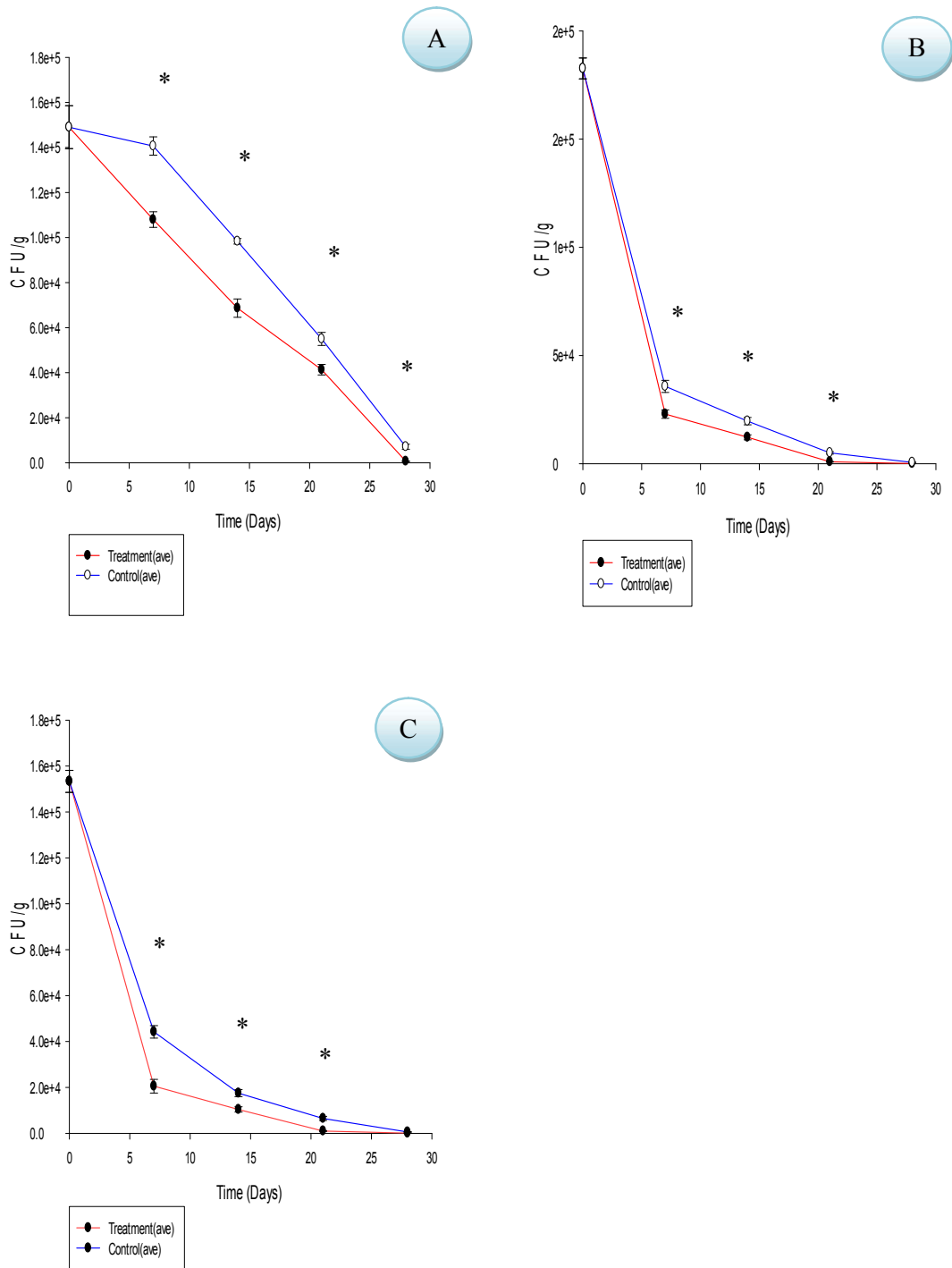
Figure 1.11: The effect of Waxworm larvae on numbers (Average) of A) *E. coli*, B) *Salmonella Sp* and C) bacterial total account in cow faeces (*Significantly different from control).
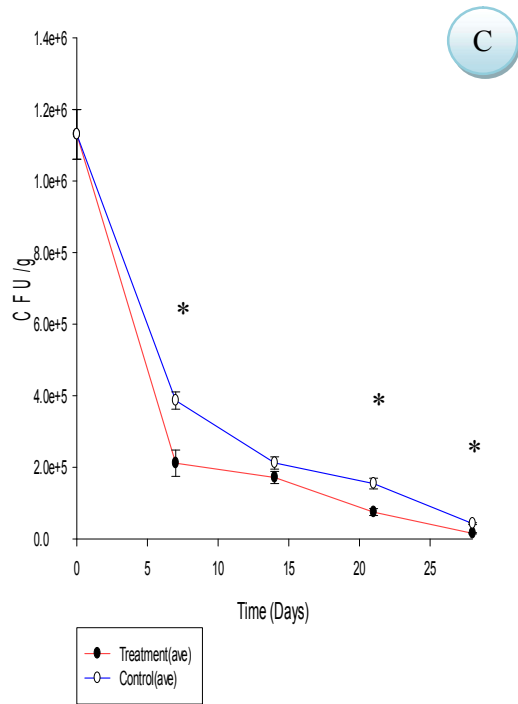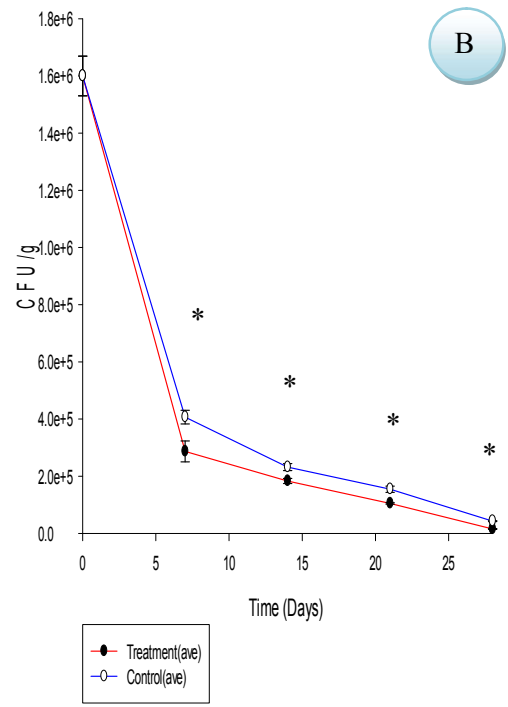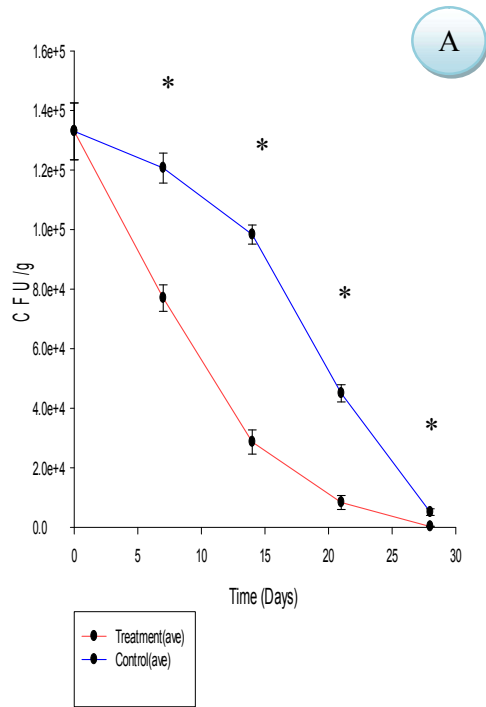
Figure 1.12: Bacterial numbers (Average) isolated from cow faeces, bacteria grown on; A) *E. coli* selective media B) *Salmonella Sp* selective media and C) plate count medium, treated with Tiger worms (*Significantly different from control).
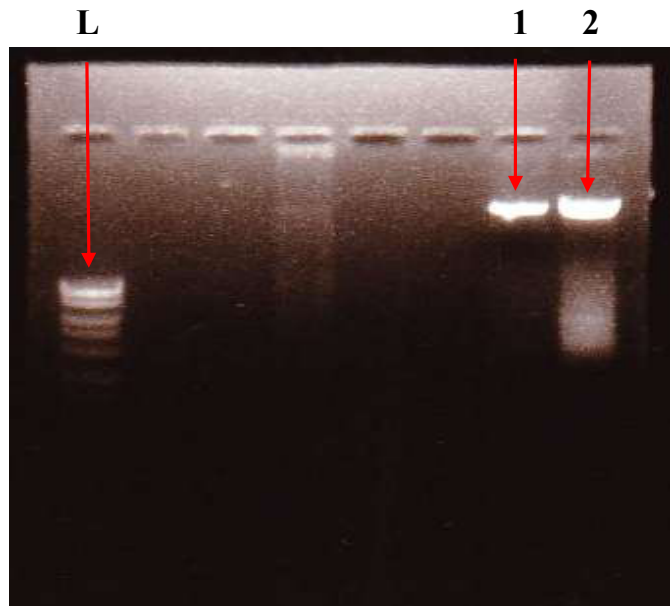
Figures 1.13: Extraction of the genomic DNA of isolated bacterial species; (lane L) hyper ladder, (Lane 1) *Salmonell*a and (Lane 2) *E. coli*.
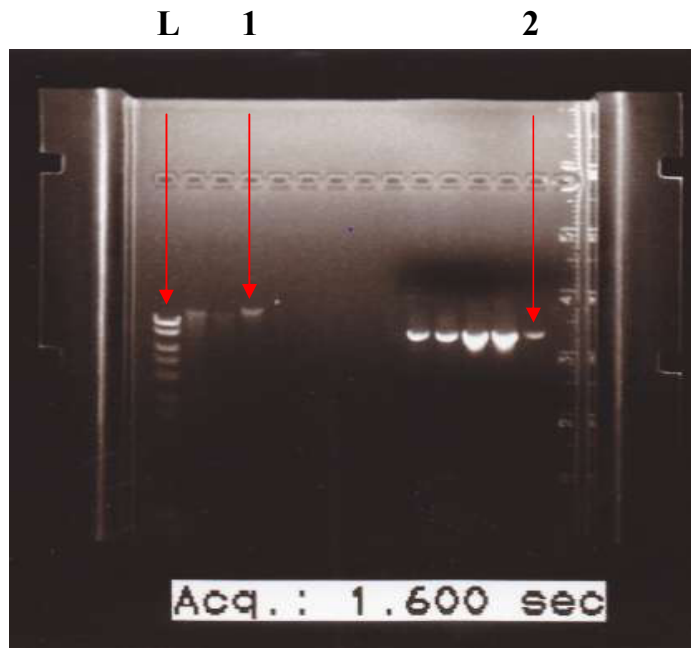
Figure 1.14: PCR- 16Sr RNA, amplification products of *E. coli* and *S. enerica* analyzed by electrophoresis in agarose gel lanes represent; (lane L): hyper ladder; (lane1): *S. enerica.* (lane 2): *E.coli.*
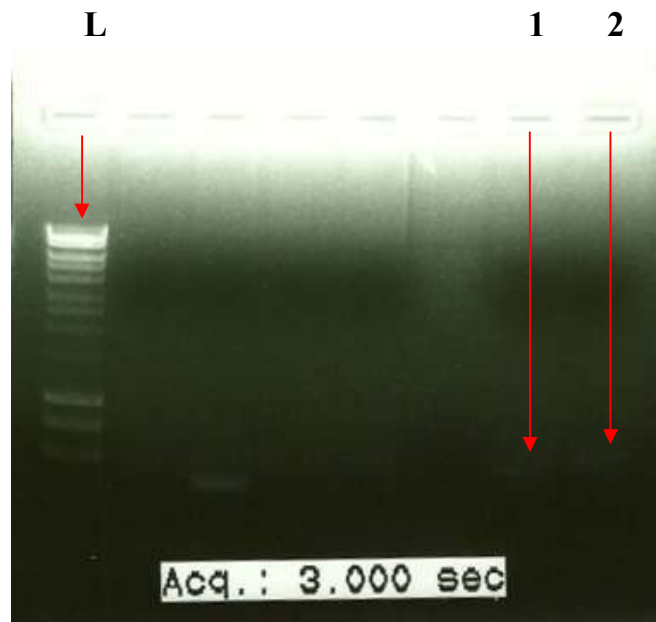
Figure 1.15: PCR- 18SrRNA, amplification products of fungal species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder; (lane1) *Penicillium roqueforti* (lane 2) *Mucor circinelloides*.

2.3.2. Microbial isolation and molecular identification

Bacteria were isolated from cow faeces using *E.coli* agar, XLT4, nutrient agar (Fig 1.4, 1.5. 1.6). Results of g DNA and PCR-16SrRNA analysis are respectively shown in Fig. 1.14. Two bacteria isolates were identified using 16SrRNA analysis, which are *E.coli* (99% similarity) and *Salmonella enterica* (97% similarity). The results of the isolation and molecular identification of the bacteria from the untreated cow faeces showed that two bacteria *Salmonella enterica* and *E.coli* were isolated. This clearly denotes the existence in cow faces of pathogenic bacteria and shows why it is necessary to compost faeces before applying them to agricultural land or suing them in potting composts. Were this not done, then these pathogenic bacteria might infect crops such as lettuce and then go on to infect humans.

Several species of fungi were isolated from cow faeces using PDA media (Fig 1.7). The results of g DNA and PCR-18SrRNA analysis are respectively shown in Fig.1.15. Two fungal isolateS were identified using 18SrRNA analysis, namely *Penicillium roqueforti* and *Mucor circinelloides* (100% similarity).The two species of fungi isolated are not, at first sight, regarded as human pathogens although all fungi can cause problems with immunocompromised patients. *Mucor circinelloides,* however, has recently been directly linked to pathogenicity as it causes invasive maxio-facial zygomycosis (Kuan *et al.*, 2009).

Chapter Three: Modification of Cow Faeces by Fruit Beetle Larvae, Waxworms and Tiger worms in relation to Nutrient Release and Potential Use of Modified Faeces as a Compost Additive

3.1. Introduction

Nutrients like nitrogen, phosphorus and potassium which are eaten by cattle in feed are eventually excreted in dung and urine and end up in the slurry store or farmyard manure heap (Atiyeh *et al.*, 2004). Such manures contain appreciable amounts of plant nutrients and can be used to reduce expenditure on inorganic fertilisers. Unfortunately, they also present a risk of pollution through nutrient losses to water or air; as a result, management needs to be aimed at using the plant nutrients in manures specifically for crop production in order to both save on fertiliser and minimises losses as pollution.

The farmer needs information about the concentration of nutrients in manure to be able to use them effectively, a result which is best achieved by the analysis of a representative sample or alternatively by reference to typical, or standard analyses for all different types of manure which are published in the advisory literature. Typically cattle farm yard manure contains, dry matter, nitrogen, phosphate and Potash (25, 6, 3.5, 8) (Pain, 2007).

The nitrogen content which is available to the next crop will be lower and will depend on the timing of application and whether or not it has been stored or is fresh.

For cattle slurry, the main source of variation is the amount of dry matter present. The more water added, (e.g. from rain during storage), the lower will be the concentration of nutrients (Beauchamp, 1983).

Some of the manure nutrients are present as inorganic (or plant available) compounds which can be taken up by crops immediately, in the same way as inorganic fertilizer. The remainder are present as organic compounds that cannot be directly taken up by plants. Over time, organic compounds are broken down in soil to release inorganic nutrients (Atiyeh *et al.*, 2004). Nitrogen is present in both inorganic and

organic compounds and is readily lost from the soil. Nitrates from manure spread in autumn or winter, for example, can be lost through leaching to groundwater and drainage, especially from sandy or shallow soils. This is why there is a recommended closed period for spreading at this time of the year in the UK; where possible, spreading should be delayed until late winter/spring when nitrates are rapidly taken up by actively growing crops (Atiyeh *et al*. 2004). The nitrogen present in manures is also rapidly lost as ammonia gas (NH_3) and as a result it is often desirable to plough in cow manures immediately after spreading to retain 90% of the available N in slurry (Webb *et al.,* 2010). An alternative is to apply the slurry via an injector or band spreading machine. Slurry dry matter has an effect on ammonia loss, and losses from dilute slurries are lower because they soak into the soil more rapidly than thicker slurries or farm yard manure. Unlike with phosphate and potash, the appropriate availability of manure nitrogen depends upon the time of spreading, soil type and slurry dry matter content (Pain, 2007).

As we have seen, cow faeces are potentially an important fertilizer resource. However, in many parts of the developed world animal wastes like these also represent a problem. This is often because the areas of agriculture devoted to intensive arable crop production are often distant from the farming areas where cattle are reared, either for beef or dairy products. In former times, when most farmers practised mixed agriculture, animal wastes were used as part of a regular fertilizer regime often based on the famous Norfolk Rotation (Gregory and Nortcliff, 2013). Today however, most arable crops are grown intensively using artificial fertilizers and animal wastes are often regarded as a problem. This has led to farmers applying wastes to their land not so much in regard to their fertilizer potential (which provides an obvious bonus) but merely as a means of disposing of a waste product which would otherwise present a

problem in relation to sewage disposal. This last problem has become increasingly relevant as environmental authorities have tightened up on the accidental release to local watercourses of run-off from feed lots and dairy farms (Dickey and Bodman, 1992).

Despite these problems, cow faeces, and other animal wastes, are when used correctly, a valuable fertilizer source (Beauchamp, 1983). However, another major problem associated with them is their variability. While inorganic fertilizers can be precisely applied to crops, organic fertilizers are far more varied and often the farmer simply does not know what is being applied to the land in terms of nutrients (Atiyeh *et al*., 2004). The aim of the following research was to determine if the larvae used here have an effect on the nutrient content of cow faeces and to relate any observed changes to the use of these organic fertilizers in crop production.

Materials and Methods:

3.2.1. Cow Faeces, Larvae and Worms: Collection and Incubation

Fresh cow faeces were collected from Butterthwaite Cattle Farm, Ecclesfield, Sheffield. The larvae and worms were bought online from Ricks LiveFood and Original Organics Ltd., and distributed into 12 plastic boxes (30×20×15 cm) with a lid perforated to allow for gas exchange, 3 boxes control and 9 boxes treatment. 100g of cow faeces in each box were then added, 100 larvae of Waxworms (3 boxes), ten Fruit beetle (3 boxes) and 40 Tiger worms (3 boxes). All samples were incubated at 25°C throughout the 28 day incubation period.

3.2.2. Determination of Nitrate: the oxidation of ammonium to nitrate in cow faeces samples:

Cow faeces samples were incubated in plastic boxes with small holes in lids to allow for gas exchange for 28 days at 25°C, which were used at various time intervals

(e.g. 0, 7, 14, 21 and 28 days) of the experiment. Cow faeces (1 g) were shaken for 15 min with 20 ml of water and the samples were filtered through Whatman No.1 filter paper. Nitrate was determined by using the method of Sims and Jackson (1971). Chromotropic acid (7 ml) was mixed with 3 ml of filtrate and then incubated at 40^0C in a water bath for 45 minutes; the yellow colour formed was measured at 410 nm and the concentration of nitrate was determined by reference to a standard curve of nitrate concentration.

Reagents:

<u>Chromotropic acid</u>

1- Stock solution:

Chromotropic acid ($C_{10}H_6O_8S_2Na_2$) (0.92 g) was dissolved in 500 ml of sulphuric acid (H_2SO_4). The solution can be stored at 4°C for several months.

2- Working solution:

Stock solution (100 ml) was added to 10 ml of concentrated HCL and to 1000 ml of concentrated H_2SO_4. The solution can be stored at 4°C for several weeks.

3.2.3. Determination of sulphate: Determination of the oxidation of sulphur in cow faeces samples:

Cow faeces samples were incubated in plastic boxes with small holes in lids to allow for gas exchange. The boxes were set up in triplicate and were incubated for 28 days at 25°C. The experiments were conducted at various time intervals as follows: (0, 7, 14, 21 and 28 days). Cow faeces samples (1 g) are shaken with 20 ml of distilled water for 15 minutes and then samples were filtered through filter paper (Whatman No.1). In order to determine sulphate concentration of the cow faeces samples, by using the method of (Hesse, 1971). Filtrate (5 ml) was treated with 1 g of barium chloride and 2 ml of gum acacia and distilled water used to make up 25 ml. The OD

was then measured at 470 nm using a spectrophotometer. The sulphate concentration was then determined by reference to a standard curve (10-100 ug sulphate ml^{-1}) prepared from a standard solution of Na_2SO_4.

3.2.4. Determination of Ammonium: Determination of the hydrolysis of urea to ammonium in cow faeces samples:

The cow faeces samples were incubated in plastic boxes with small holes in lids to allow gas exchange. The boxes were set up in triplicate and were incubated for 28 days at 25°C. The experiments were conducted at various time intervals as follows (0, 7, 14, 21 and 28 days) from the experiment start. Cow faeces (1 g) in 10 ml KCL was shaken for 30 min and then filtered through filter paper (Whatman No.1). Filtrate (2ml) was then added to 1 ml of EDTA (6% w/v), 7 ml of distilled water, 5 ml of phenolate reagent and 3 ml of sodium hypochlorite solution (10%v/v). The reaction mixture was mixed thoroughly and incubated at 25^0 for 20 min in the dark. The volume was then made up to 50 ml with distilled water and mixed and the concentration of the indophenol-blue ammonium complex was measured at 630 nm using a spectrophotometer (Wainwright and Pugh, 1973); the concentration of ammonium intensity was then determined by reference to standard curve (10-100 ug NH_4-N ml^{-1}) prepared from a standard solution of ammonium sulphate.

Reagents:

Ethylenediamineteraacetic acid (EDTA):

EDTA (60 g) was dissolved in 900 ml of distilled water then diluted to 1 litre.

Phenol solution:

Phenol (62.5 g) was dissolved in ethanol (25 ml) and acetone (18.5 ml) added to make up 100 ml. The phenol solution was stored in the dark at 4^0C.

Phenolate reagent:

Phenol solution (20 ml) was mixed with 20 ml of sodium hydroxide (27% NaOH w/v) and diluted to 100 ml.

3.2.5. Determination of phosphate

Cow faeces samples were placed in plastic boxes in triplicate and incubated at 25^0C for 7, 14, 21 and 28 days. After incubation, 5 g samples of cow faeces were placed into screw capped glass bottles containing 50 ml of 0.5 N NaHCO3 and all the bottles were shaken for 30 minutes at 70 rpm, using an orbital shaker; the contents were then filtered through Whatman No.1 filter paper. Phosphorus ions were determined as described by Al-Falih and Wainwright (1995) as follows: Filtrate (3 ml) was mixed with 7 ml of working solution then the mixture incubated at 37^0C for 1 hour. The blue colour was measured at 820 nm using a spectrophotometer and the concentration of P were determined by reference to a calibration curve (0-8 μg PO_4-P ml^{-1}).

Reagents:

Working solution:

1- Ascorbic acid 10%

2- Ammonium molybdate 0.42 in 1N H_2SO_4

1 vol. of ascorbic acid mixed with 6 vol. of ammonium molybdate.

3.2.6. Statistical analysis

All data were presented as means ± SE (Standard Error). Results were analysed using a t-test between two groups and compared significant difference of means between treatment and control using Sigma Plot $12.0^©$ software.

3.2. Results and discussion:

3.3.1. Effect of fruit beetle larvae and Waxworms on concentrations of ions in cow faeces

In the case of sulphate and ammonium concentrations, addition of fruit beetle larvae to cow faeces generally led to increases over the incubation period (Figs.2.1 B,C), while the trend was decreased in the case of phosphate and nitrate. However, concentrations were higher at day 14 in the case of phosphate (Figs.2.1 A, D). In the case of phosphate, sulphate and ammonium concentrations, addition of Waxworms to cow faeces generally led to increases over the incubation period. Concentrations were higher at day 21 in case of phosphate and day 7 in case of ammonium (Fig.2.2 A, C), while the trend was decreased in the case of nitrate (Fig.2.2 D). For ammonium and phosphate concentrations, addition of Tiger worms to cow faeces led to increases over the incubation period (Figs.2.3 A, B), while the trend was decreased in the case of sulphate and nitrate. There were significant differences between treatments and control for all nutrients. (Figs.2.3 B, D).

The use of manures in agriculture in relation to plant nutrients has been discussed at length by Edmeades (2003). This author pointed out that organic manures, like cow faeces, can provide most of the essential nutrients and micronutrients required by crop plants and in terms of their fertilizer potential are potentially equivalent to inorganic fertilizers. They also provide the added bonus of improving soil structure and soil biological activity. The downsides of their use relate to enhanced transport and handling costs and cost related to the need for them to be composted to reduce potential pathogen loads. Most conclude that manures will never replace inorganic fertilizers and return to the dominant position they had a century ago (Edmeades, 2003).

The findings presented here show that with the exception of nitrate, the concentration of all plant available ions increased in cow faeces in the presence of all three types of worm. This is a potentially positive result as it would lead to enhanced fertiliser potential of the faeces when used as mulches, when spread to the field as a fertilizer, or where used as a compost additive to improve the growth of potted plants. The reduction in nitrate concentration can also be seen as positive since, provided there is sufficient nitrogen present as other forms, this reduction in nitrate need not be detrimental. In the case of field reductions in nitrate, this reduced concentration can be viewed as being positive since this ion is rapidly denitrified under anaerobic conditions, and, when it reaches potable water can cause medical problems, notably blue baby disease and gastric cancer (Knobeloch *et al.,* 2000). It is also very mobile in the soil due to the fact that it is not fixed by ion exchange mechanisms as a result it is rapidly lost from the soil in wet periods (Evanylo *et al*., 2008).
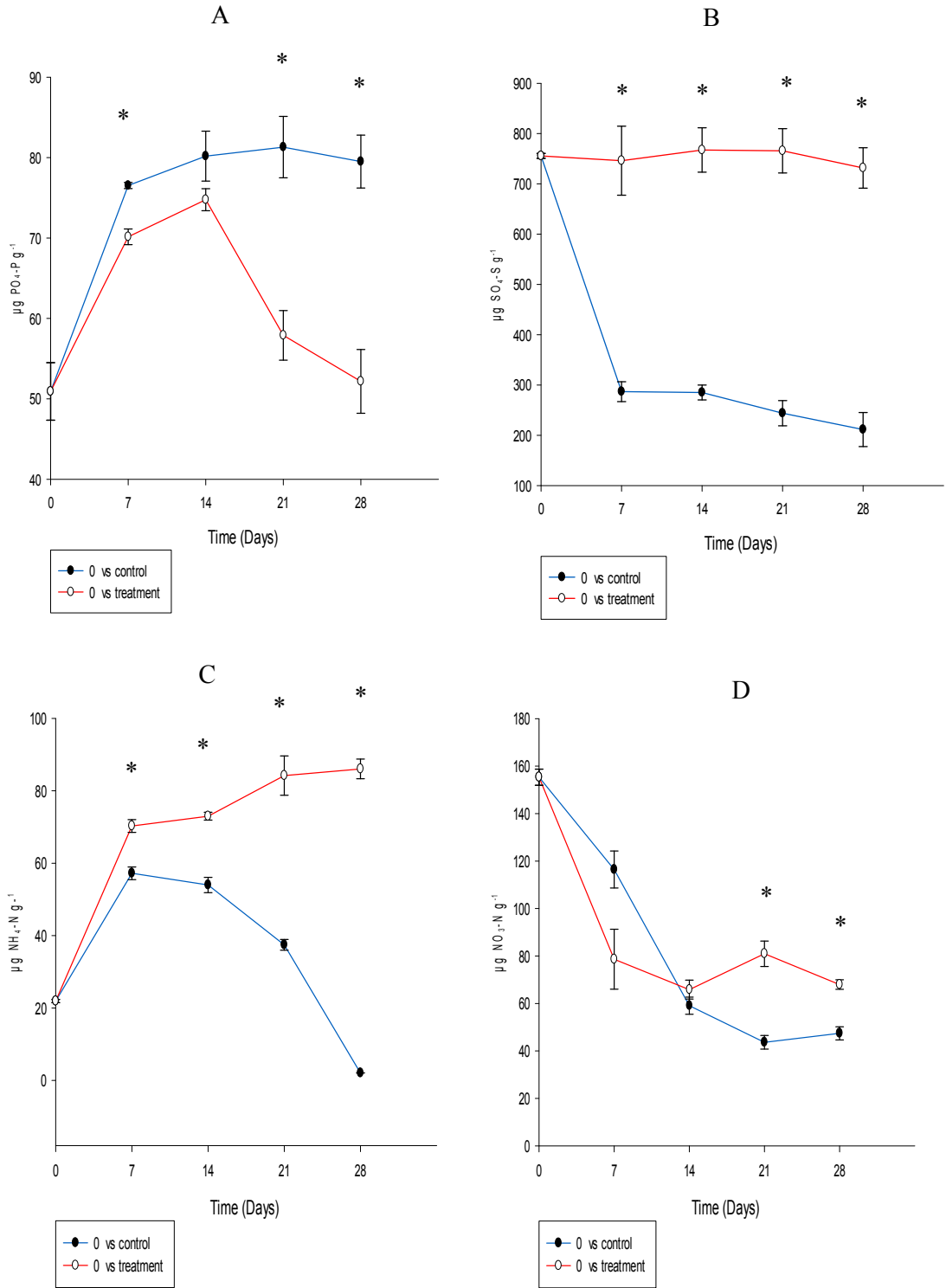
Figure 2.1: The effect of FB larvae activity on indigenous; A) phosphate, B) sulphate, C) ammonium and D) nitrate, concentration in cow faeces (* Significantly different from control, p=0.05).
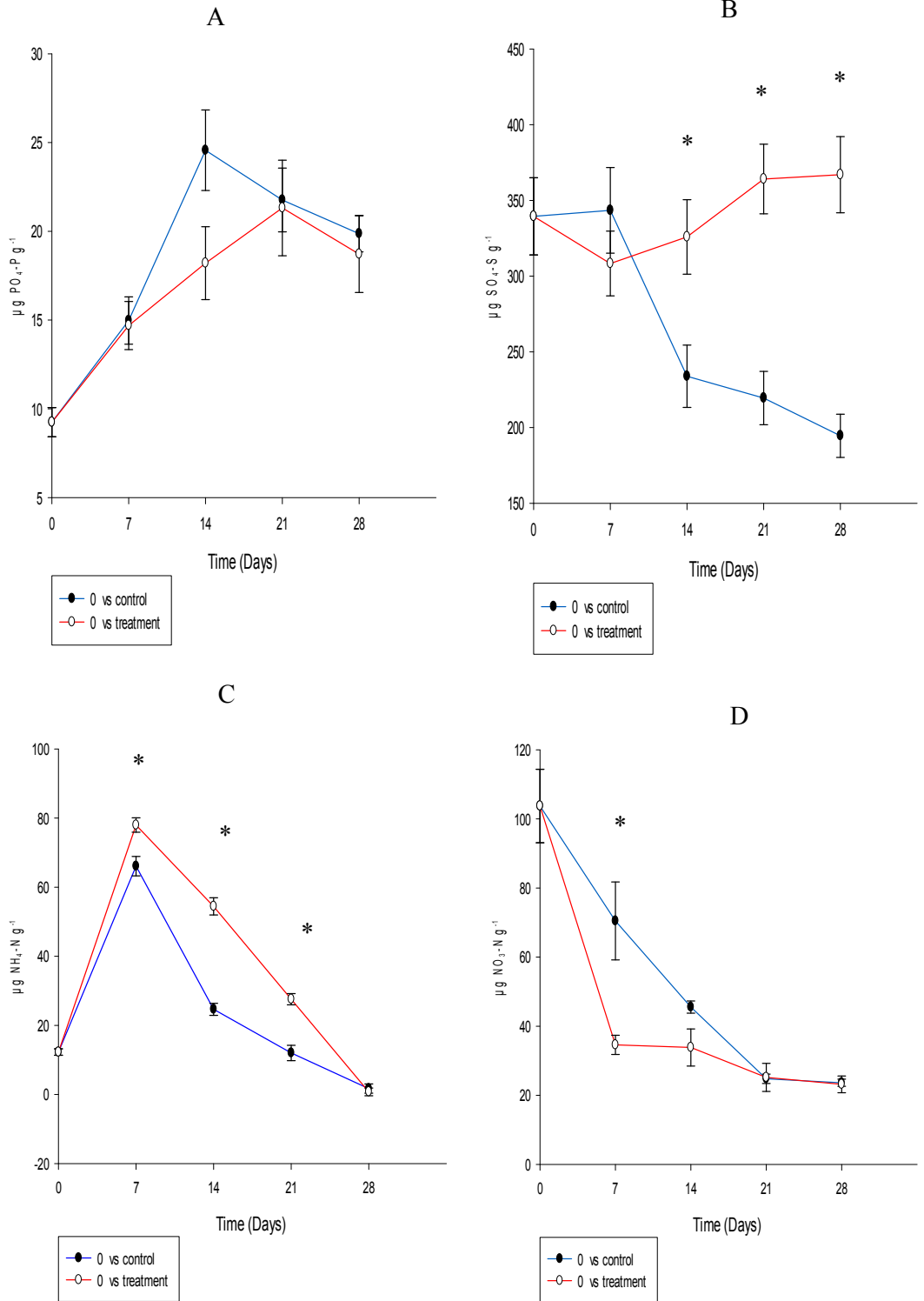
Figure 2.2: The effect of Waxworm activity on indigenous; A) phosphate, B) sulphate, C) ammonium and D) nitrate, concentration in cow faeces (* Significantly different from control, p=0.05).

45
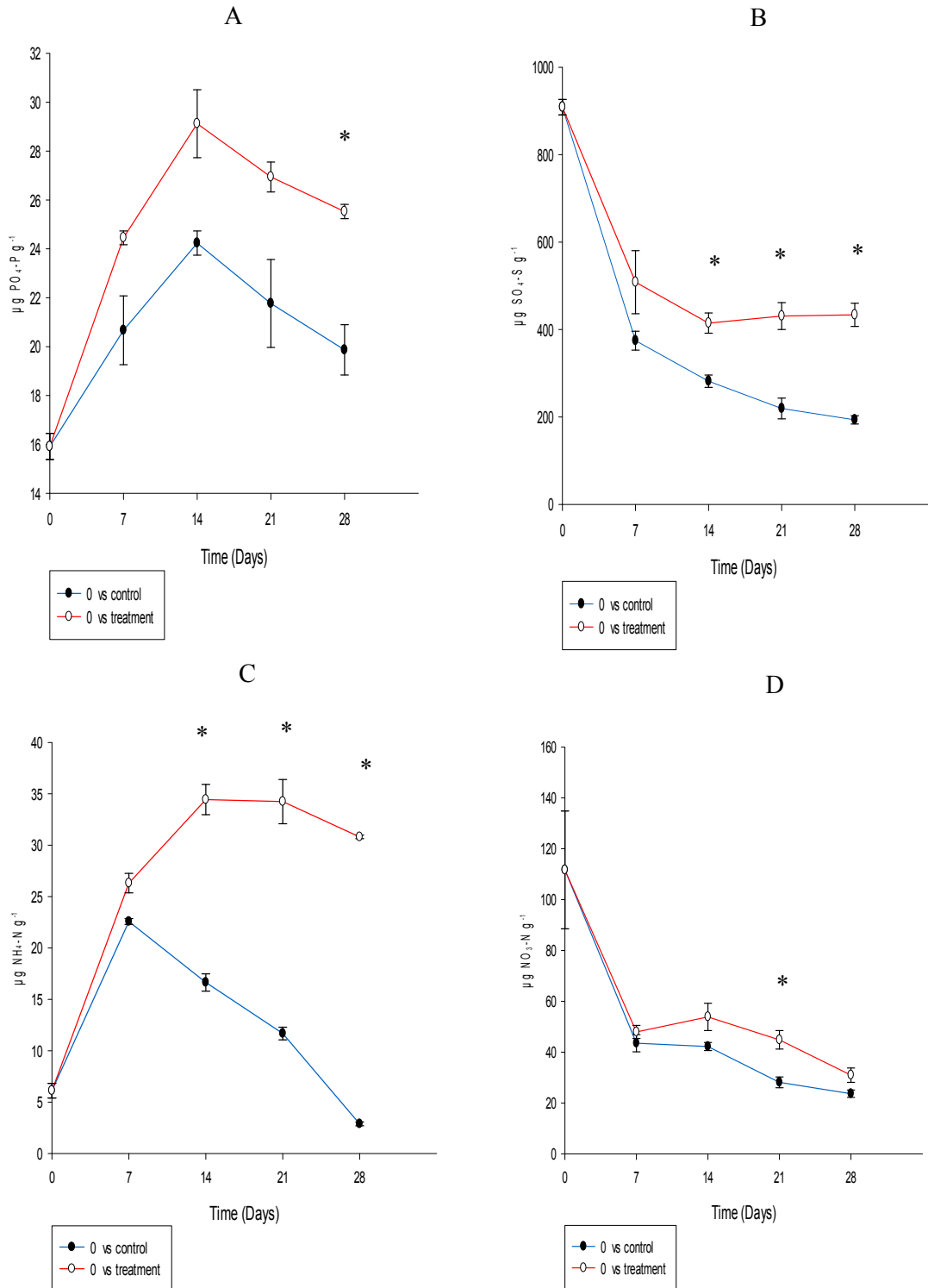
Figure 2.3: The effect of Tiger worms activity on indigenous; A) phosphate, B) sulphate, C) ammonium and D) nitrate, concentration in cow faeces (* Significantly different from control, p=0.05).

Chapter Four: The effects of Cow Faeces treated by Fruit Beetle Larvae, Waxworms and Tiger worms on Plant growth in relation to its use as potting compost

4.1. Introduction

In addition to being used in large scale agriculture (Edmeades, 2003), cow manure has the potential to be used as potting compost, either directly or more probably as an additive to peat and soil based potting composts. Animal wastes, like cow manure can also be profitably used as fertilizers by allotment owners.

The high levels of micronutrients contained in bovine manure, such as potassium, phosphorus and perhaps most importantly, nitrogen, make it an ideal fertilizer as well as growing medium when mixed with a mineral-rich compound (Atiyeh *et al*., 2004). Potted plants require adequate room to grow, a warm ambient temperature, proper lighting, regular watering and nutrients for maximum growth. Composted manure should always be used as opposed to fresh manure, as fresh manure will burn the root system.

The safest manure for potting compost use comes from herbivores, while manures from carnivores such as dogs and cats possess an added risk of containing human pathogens. Even herbivore manures, however, are extremely strong and need to be aged before being used as potting composts or compost additives (Atiyeh *et al*., 2004). Cow manure contains the three main elements of garden soil fertiliser (i.e. N.P, K) but it may contain pathogens like *Salmonella* (Atiyeh *et al*., 2004). By allowing the manure to age, the risk of planting weeds or transmitting diseases through garden crops or contact with the soil is greatly reduced. Manure contains abundant amounts of nitrogen, which help improve the soil and boost plant foliage production. Nitrogen stimulates beneficial bacteria in the soil and speeds up the decomposition of organic materials (Atiyeh *et al*., 2004). But fresh un-aged manure can cause rapid immobilization of nutrients. Tender young plants are also susceptible to too much

nitrogen in the soil and can be damaged by it, while plants that absorb too much nitrogen are attractive to insect and other pests (Atiyeh *et al*., 2004).

When used in allotments, cow manure is best spread as fresh manure in the autumn after plants are harvested; it is then worked into the soil the following spring before planting. Another approach is to compost the manure in a pile which heats sufficiently to kill any pathogens or viable undigested weed seeds. Aged cow manure can be used to enrich established garden plots by lightly digging the fertiliser in to the side of plants. Irrigation or rainfall will cause the nutrients to seep into the soil around the plant, feeding the roots (Atiyeh *et al*., 2004).

The aim of the work reported in this Chapter was to determine if cow faeces, when modified by the insect larvae, used here, can provide an improved potting compost, especially when added to peat-based composts, for use in house plant culture.

4.2. Materials and Methods:

4.2.1. Seeds, worms, larvae and cow faeces-collection and incubation

Seeds (pea shoots) and cell inserts trays were bought from BandQ. Larvae and worms were purchased online from Ricks Livefood and Original Organics Ltd. Fresh cow faeces were collected from Butterthwaite cattle Farm, Ecclesfield, Sheffield, UK, and distributed in 12 plastic boxes (30×20×15 cm) with a lid perforated to allow for gas exchange; 3 boxes of controls (100g in each box) and 9 boxes of treatment were also set up; Waxworm larvae were then added, One hundred larvae to 100 g of cow faeces in each box (3 replicates). Fruit beetle larvae were then added, ten larvae to 100 g cow faeces were added to each box (3 replicates). Tiger worms were then added 40 worms to 100 g cow faeces in each box (3 replicates). All samples were incubated at $25^{o}C$ throughout the 28 day incubation period.

49

4.2.2. Compost transfer to the cell insert trays and seedings

A peat based compost was used here. After completion of the incubation period, the manure was moved to cell insert trays (each cell size was L6 cm, D6 cm, W6 cm) with different concentrations from 100% compost (temperate grassland soil), 75% TGS mixed with 25% cow faeces, 50% TGS mixed with 50% cow faeces, 25% TGS mixed with 75% cow faeces and finally 100% cow faeces. Each treatment (FBL, WW, TW and control) was set up in triplicate (Fig.3.1). Seeds were then placed in the composts (10 seeds to each). The treated samples were then left at laboratory temperature (circa 23°C) and the trays were left near a window to allow for adequate light for plant growth for a period of three weeks (Fig.3.2). At the end of the three weeks growth period the plants were harvested and the following characteristics were determined, plant height, total plant wet weight, fresh leaf weight, fresh root weight, total plant dry weight and root dry weight; the measurements used to assess plant growth were then made.

4.2.3. Statistical analyses

All observations are presented as means ±SE (Standard Error). Bacterial populations were converted to log CFU/g before statistical analysis. Statistical analyses were performed on experimental datasets using the t-test between two groups and a one way ANOVA at $P<0.050$ probability level compared significance means for four kinds of compost. Results were analysed using Sigma Plot 12.0© software.

4.3. Results and Discussion

This study shows that the Tiger worms generally led to improvements in the plant growth parameters studied here, especially in the compost-faeces ratio (75% compost, 25% cow faeces), followed by a ratio of 50% compost, 50% cow faeces, which showed significant differences for most measurements. Also the results of the statistical

analysis showed significant differences between the Tiger worms and other larvae (FB, WW) as well as the control. In contrast, plant growth in Waxworm treated faeces were weak compared with plants growth in compost treated by FB, WW and control. This was especially in the compost (25% compost 75% cow faeces) and 100% cow faeces where there was no growth at all (Fig.3.2 B). The results show that the fresh plant weight, fresh leaf weight and fresh root weight were significantly higher in the compost treated by Tiger worms in 75% compost 25% cow faeces (Fig.3.4-3.5-3.6). The results did not show evidence of any significant differences in the dry root weight between TW, WW, FB and control In all composts (Fig 3.8). The control gave greatest in cases in plant height and plant dry weight, showing significant differences in composts (25% compost, 75% cow faeces) and 100% cow faeces (Fig.3.3-3.7). Tiger worms were shown to produce the best product when compared to that produced by WW, FB and the control, especially at the compost ratio of 75% compost, 25% cow faeces, followed by 50% compost, 50% cow faeces.

Atiyeh *et al.,* (2004) have showed that the use of worms to treat animal wastes for use in the commercial culture of tomatoes (i.e. vermicomposting) is efficient for converting solid wastes to useful products. The incorporation of composts and vermicomposts into potting and container media was soon to be a potential use for these materials. They conducted a greenhouse trial where they determined the effects of a vermicompost produced from raw dairy manure (RDM) along with some other composting materials. All potting mixtures produced significantly higher biomass than the control. Other workers have also concluded that cow manure can be useful in potting compost (Chen *et al.,* 1988), while Aryantha *et al.,* (2000) concluded that such manures can be useful in reducing the incidence of plant pathogenic fungi, such as *Phytopthora* species.

In conclusion, the results of this study show that pretreatrnent of cow faeces with the worms used here can improve the fertilizer potential of these materials such that they can become a potentially valuable additive to peat-based potting composts for use in households or horticulture. An important observation was made in relation to the odour of the cow faeces which was noticeably reduced following worm treatment. Such a reduction in odour has obvious benefits especially when the faeces are to be used as additives to household potting composts. The wide availability and cheapness of cow manure points to it being profitably used as a potting compost additive, and together with the fact that high protein-fat worms are produced as a by–product, this means that the production process could be economically viable. Although peat based composts are the norm in the UK in the USA, and other countries, household potting composts are usually mineral soil-based (Atiyeh *et al.*, 2004). Obviously, the investigations here would have to be repeated for such soil-based composts if any product based on this research were to be marketed outside the UK.

100% compost (TGS)	75% compost (TGS), 25% cow faeces	50% compost (TGS), 50% cow faeces	25% compost (TGS), 75% cow faeces	100% cow faeces
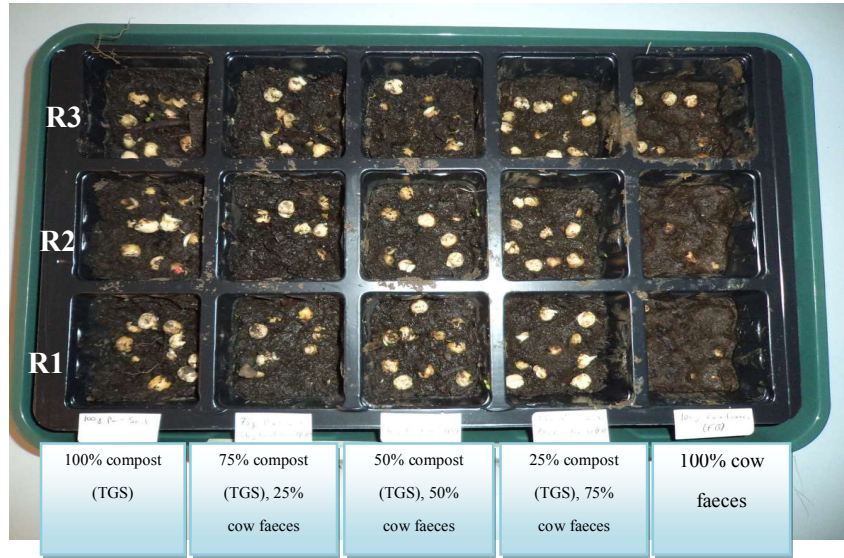
Figure 3.1: The compost ratios and replication in cell insert trays.

Figure 3.2: The effect of cow faeces treated by A) Fruit beetle, B) Waxworms, C) Control, D) Tiger worms, on pea shoots growth in different compost ratios.
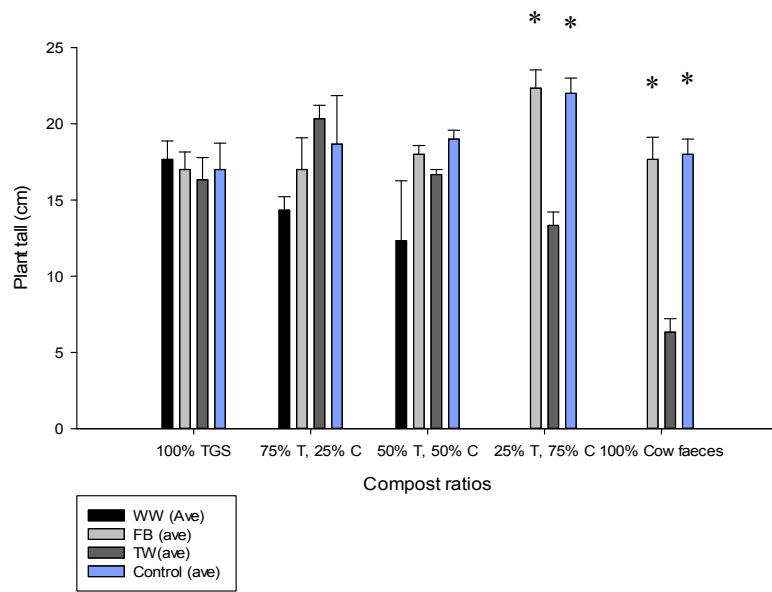
Figure 3.3: Effect of cow faeces treated with FB, Waxworms, Tiger worms and non-treated cow faeces on height of pea shoots grown in different compost ratios (Means of triplicate (±)SE log plant height (*Significant difference between group).
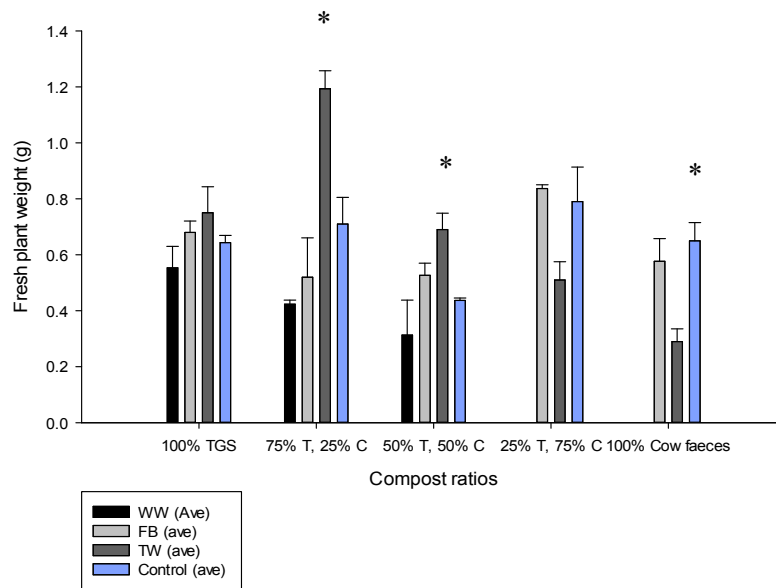
Figure 3.4: Effect of cow faeces treated with FB, Waxworms, Tiger worms and non-treated cow faeces on fresh plant weight of pea shoots grown in different compost ratios (Means of triplicate (±) SE log fresh plant weight (*Significant difference between groups).
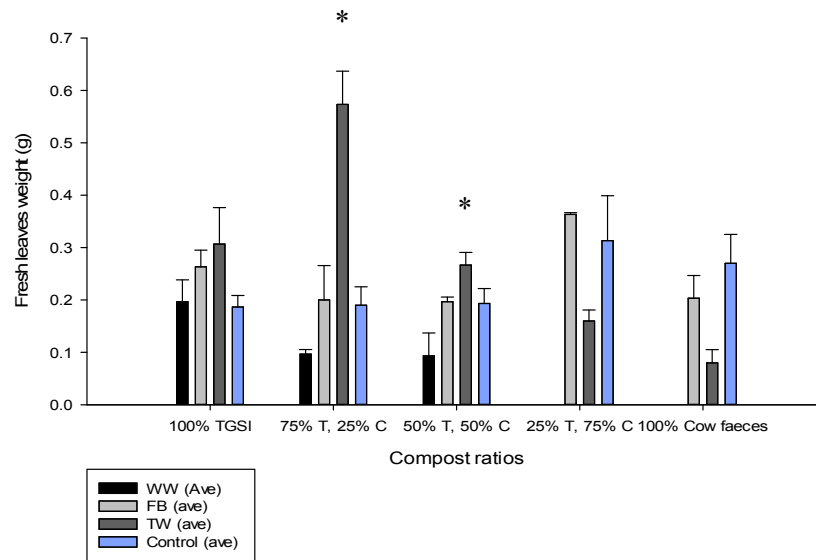
Figure 3.5: Effect of cow faeces treated by FB, Waxworms, Tiger worms and non-treated cow faeces on fresh leaves weight of peas grown in different compost ratios (Means of triplicate (±)SE log fresh leaves weight (*Significant difference between group).
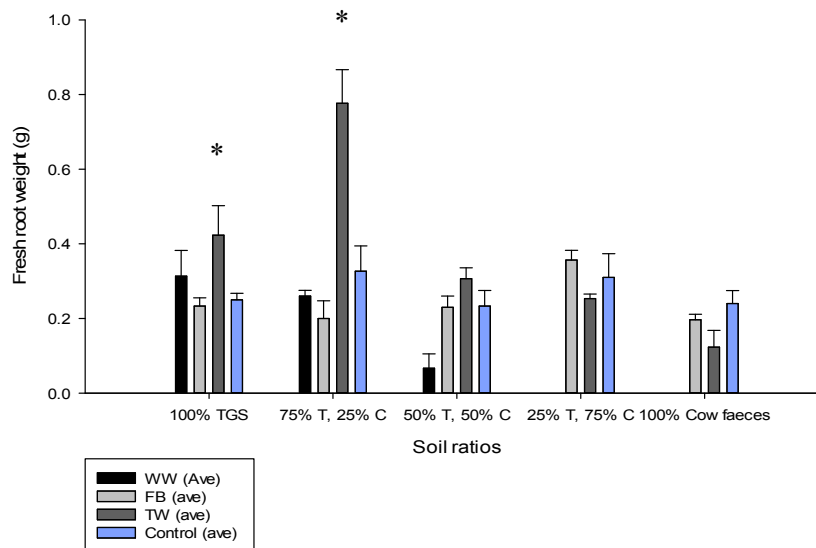
Figure 3.6: Effect of cow faeces treated with FB, Waxworms, Tiger worms and non-treated cow faeces on fresh root weight of peas grown in different compost ratios (Means of triplicate (±)SE log fresh roots weight (*Significant difference between groups).
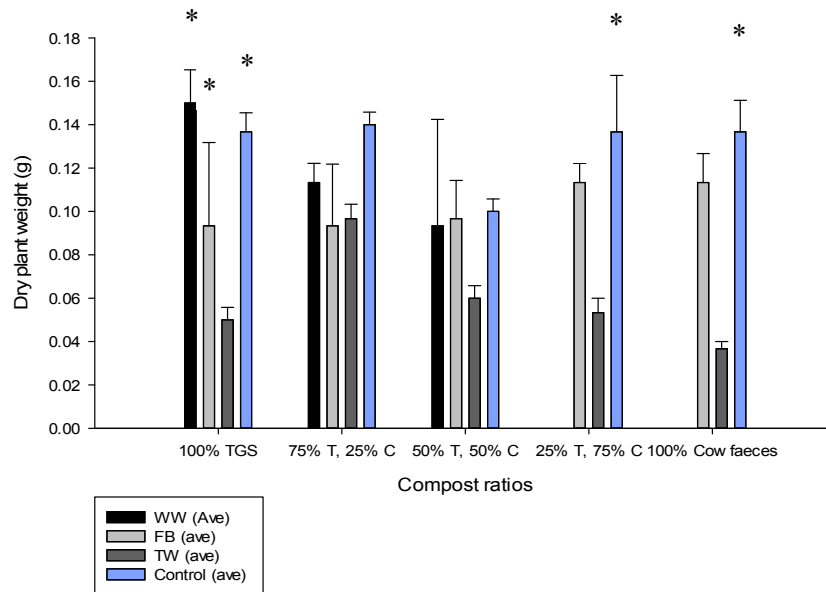
Figure 3.7: Effect of cow faeces treated by FB, Waxworms, Tiger worms and non-treated cow faeces on plant dry weight grown of peas in different compost ratios (Means of triplicate (±)SE log dry plant weight (*Significant difference between groups).
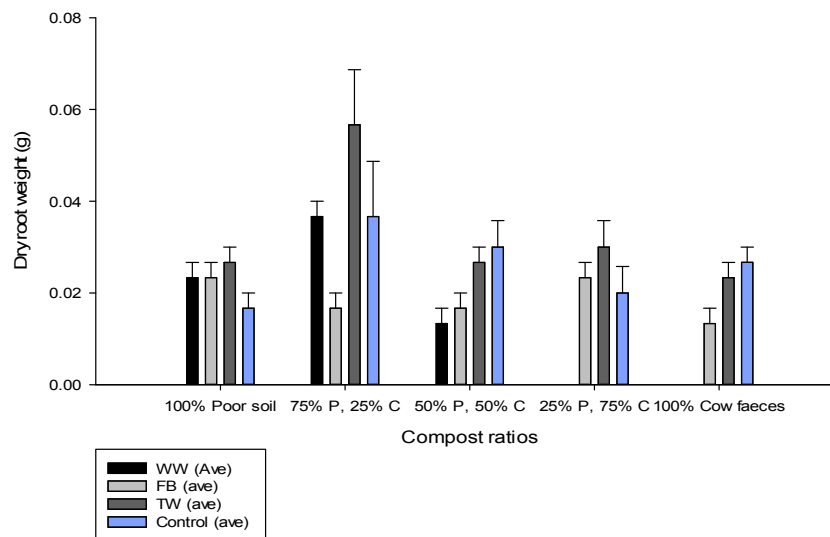
Figure 3.8: Effect of cow faeces treated with FB, Waxworms, Tiger worms and non-treated cow faeces on dry roots weight grown of peas in different compost ratios (Means of triplicate (±)SE log dry roots weight (*Significant difference between groups).

Chapter Five: Studies on the ability of Fruit Beetle Larvae *Pachnoda marginata* to increase the fluidity of cow faeces

5.1. Introduction:

Cow faeces are semi-solid materials which are relatively difficult to handle and move around. These problems become significant when cow faeces are being transferred to fields to act as fertilisers or where, when stored in tanks, the material needs to be moved (Leto *et al*., 2007). During studies on the effect of the various worms used here it was found that cow faeces became more fluid during worm-exposure. This phenomenon was considered to be of potential benefit and so was studied in more detail as described in this Chapter. As far as can be determined, this ability of worms to liquefy cow faeces has not previously been reported upon, as a result there is no reference material available which can be quoted in direct relation to the work described in this Chapter.

5.2. Materials and Methods

5.2.1. Cow faeces and larvae collection and incubation

Fruit beetle larvae were purchased online from Ricks LiveFood. Fresh cow faeces were collected from Butterthwaite cattle farm, Ecclesfield, Sheffield, and distributed into three treatment plastic boxes (30×20×15 cm) with a lid perforated to allow for gas exchange. Ten larvae were added to 100 g cow faeces in each container. All boxes were incubated for 10 days at 25°C.

5.2.2. Liquid material extraction from cow faeces treated by Fruit beetle larvae

After incubation, the three replicates of cow faeces treated with Fruit beetle larvae were placed in each of three sieves (size 2 ml) (bought online from Amazon). The resulting liquid passing through the sieve was collected in plastic containers (Fig.4.3). The cow faeces plus larvae was left in the sieves for an hour in order to collect the largest amount of liquid material possible (Fig.4.2).

5.2.3. Measurement of the amount of liquid and the isolation of bacteria from the faeces liquid

The amount of liquid passing through the sieve was determined using a pipette (10 ml or 25 ml) and the number of *E.coli, Salmonella Sp* and total bacterial count was determined in the liquid sample. Samples (1 ml) were diluted in 9 ml of sterilized water then serially diluted ($10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}$) with 0.1 ml of each diluted sample being spread on to *E.coli* agar, XLT4, LB and Plate Count Agar, and incubated for between 18 and 24 h at 37^0C.

5.2.4. Determination of Nitrate:

Liquid samples (1 ml) were shaken for 15 min with 20ml of water and then the samples were filtered through Whatman No.1 filter paper. Nitrate was determined by using the method of Sims and Jackson (1971). Chromotropic acid (7 ml) was mixed with 3 ml of filtrate and then incubated at 40^0C in a water bath for 45 minutes; the yellow colour formed was measured at 410 nm and the concentration of nitrate was determined by reference to a standard curve of nitrate concentration.

5.2.5. Determination of sulphate:

1 ml of liquid sample was mixed with 20 ml of distilled water for 15 minutes and then samples were filtered through filter paper (Whatman No.1). In order to determine sulphate concentration of the cow faeces samples, the method recommended by Sims and Jackson (1971) was used 5 ml of the resultant filtrate was mixed with 1g of barium chloride and 2 ml of gum acacia. Distilled water was added to make volumes to 25 ml. The extraction was measured at 470 nm using a spectrophotometer. The sulphate concentration was determined by reference to a standard curve (10-100 ug sulphate-ml^{-1}) prepared from a standard solution of $Na_2 SO_4$.

5.2.6. Determination of Ammonium:

Liquid (1 ml): 10 ml KCL was shaken for 30 min and then filtered through a filter paper (Whatman No.1). Filtrate (2 ml) was then added to 1 ml of EDTA (6% w/v), 7 ml of distilled water, 5 ml of phenolate reagent and 3 ml of sodium hypochlorite solution (10%v/v). The reaction mixture was mixed thoroughly and incubated at 25^{0}C for 20 min in the dark. The volume was made up to 50 ml with distilled water and mixed and the concentration of the indophenol-blue ammonium complex was measured at 630 nm using a spectrophotometer (Wainwright and Pugh, 1973). The concentration of ammonium intensity was then determined by reference to a standard curve (10-100 ug NH_4-N ml^{-1}) prepared from a standard solution of ammonium sulphate.

5.2.7. Determination of phosphate

After liquid extraction from cow faeces samples, 2 ml was placed into screw capped glass bottles containing 20 ml of 0.5 N $NaHCO_3$ and all the bottles were shaken for 30 minutes at 70 rpm, using an orbital shaker; the contents were then filtered through a Whatman No.1 filter paper. Phosphorus ions were determined as described by Al-Falih and Wainwright (1995) as follows: Filtrate (3 ml) was mixed with 7ml of working solution and then the mixture incubated at 37^{0}C for 1 hour. The blue colour was measured at 820 nm using a spectrophotometer and the concentration of P was determined by reference to a calibration curve (0-8 μg PO_4-P ml^{-1}).

Figure 4.1: Comparison between the effects of Waxworms on faeces. Normal dry faeces (right), and fruit beetle larvae it's wet (left) on cow faeces.

Figure 4.2: The effect of Fruit beetle larvae on cow faeces fluidity after 10 days treatment.
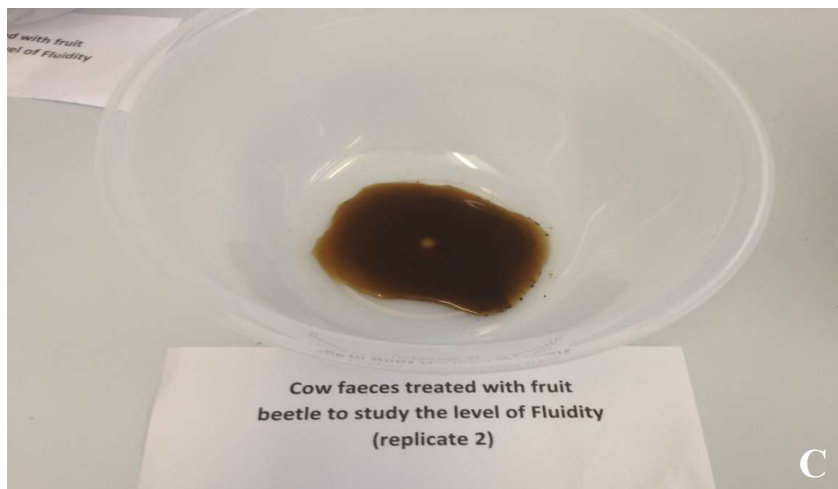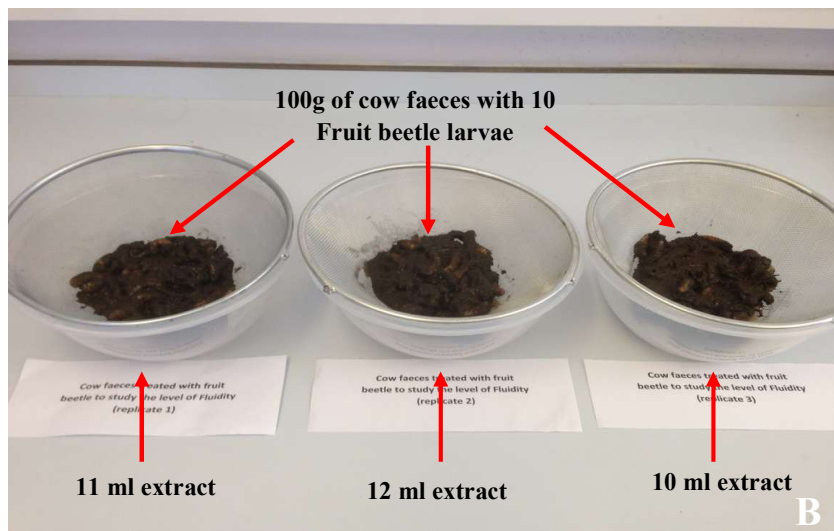
Figure 4.3: A) Sieve for cow faeces, B) Cow faeces with Fruit beetle larvae on sieve as three replicates, C) Liquid extracted from cow faeces.
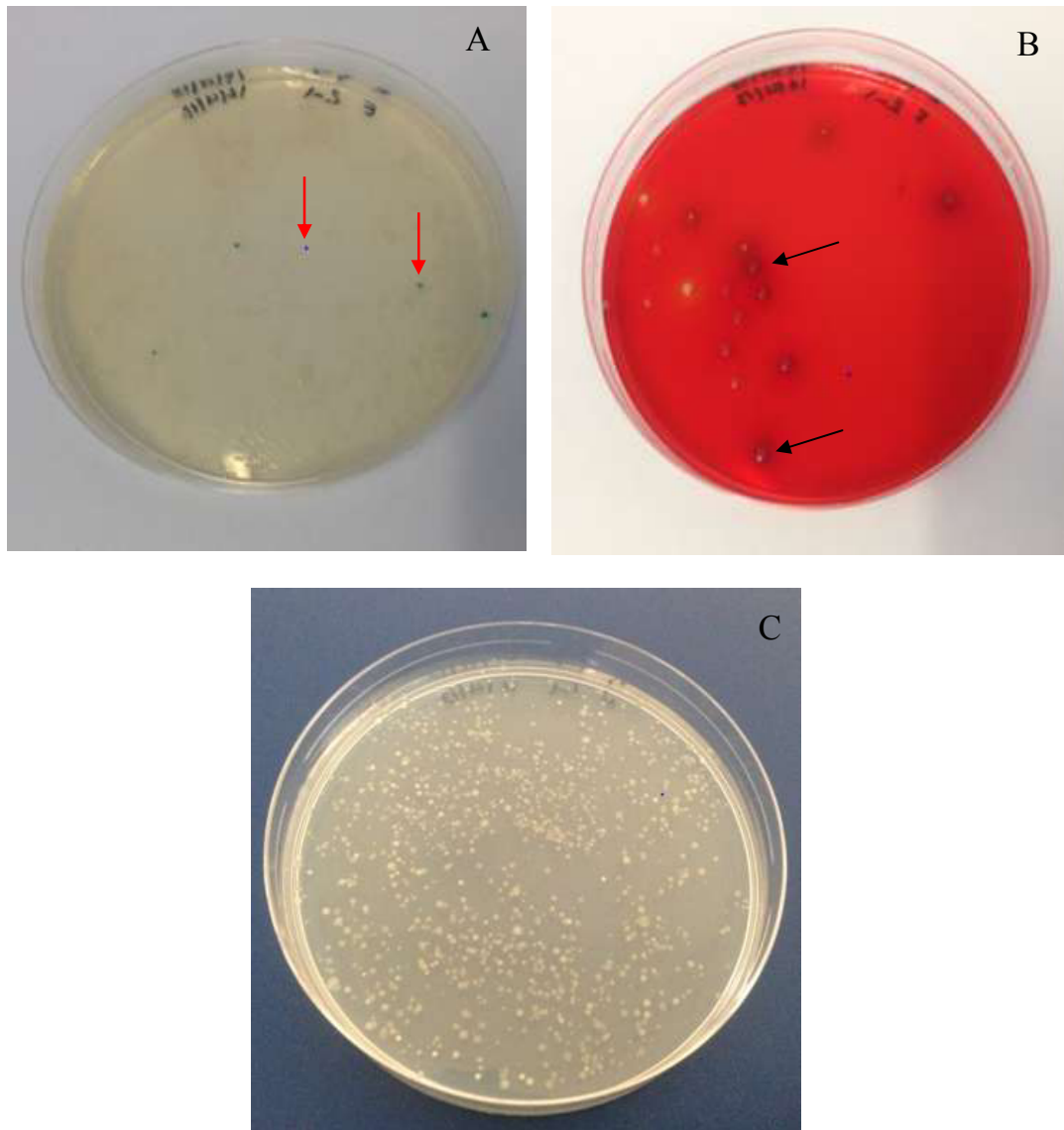
Figure 4.4: Colonies of bacteria grown on A) HiCrome *E. coli* agar showing blue colonies. B) *Salmonella Spp* (black colonies) grown on XLT-4 selective *Salmonella* agar medium. C) Plate count agar medium.

5.3. Results and Discussion

In this study 11 ml of liquid extract was obtained from the first replicate, 12 ml from the second replicate, and 10 ml from the third replicate (Fig 4.3). Numbers of *E.coli*, *Salmonella Sp* and total bacteria in liquid extracts from cow faeces after 10 days of treatment were very low compared with cow faeces in previous studies (Fig.4.4), (Table 4.2). Also the nutrient content in liquid extracts through elemental analysis gave high levels in all nutrients under study (phosphate, sulphate, ammonium and nitrate) (Table 4.1).

Liquid cow and other animal faeces have been effectively used in agriculture (Beauchamp, 1983. Choudhary *et al.*, 1996), particularly in the fertilization of grassland (Leto *et al.*, 2007). This latter approach is often used as means of disposal of feedlot faecal liquids and any fertilizer benefit is seen as a bonus.

Liquid cow manure is available from commercial agricultural suppliers and when homemade is referred to as cow manure tea (made by soaking cow pats in water). Commercially manufactured liquid cow manure that is homogenized (heated) and the smell has been removed is readily available at lawn and garden centres. Liquid cow manure has advantages when used as a fertilizer but it can also be problematic.

5.3.2. Dangers of Overuse

The application of liquid cow manure directly to crops and plants can lead to "burning" and resultant kill-off if the mixture is too strong or is applied too frequently; both commercially manufactured and homemade liquid cow manure fertilizers must therefore be diluted before use. The over frequent application of liquid cow manure can lead to the production of more green foliage than fruit or vegetables, essentially because liquid cow manure is rich in pure nitrogen and lacks the phosphorus and potassium required for a balanced fertilizer. Application of such liquid manures should

be limited to once every week to two weeks for above-ground produce and only once during the growing season for root vegetables (Carpenter, 2007).

Harmful bacteria, such as *E.coli,* occur in cow manure, so it is critical that precautions are taken to avoid or eliminate the possibility of infecting produce with these bacteria. Commercially processed liquid cow manure does not present this problem as it is homogenized, a process which kills all bacteria. Home-made manure tea should only be produced from old or aged cow manure since old manure has lost most of its harmful bacteria content.

Kind of element	The concentration nutrients ($\mu g \ L^{-1}$)
Phosphate	64
Sulphate	717
Nitrate	155
Ammonium	49

Table 4.1: Concentration of the elements in liquid extract from cow faeces treated with Fruit beetle larvae.

Kind of bacteria	CFU/ml
E. coli	300
Salmonella Sp	1600
Total amount of bacteria	25000

Table 4.2: Numbers of bacteria in liquid extract from cow faeces treated with Fruit beetle larvae.

Chapter Six: Studies on the use of Fruit Beetle Larvae and Waxworms to improve the rate of breakdown of paper and waxed cartons (domestic waste) in order to improve the biodegradation of these materials

6.1. Introduction

6.1.1 Recycling-paper breakdown

In Europe, more than 40% of the total paper and board waste is recycled annually and used for re-manufacturing corrugated boards and newspaper (Goddard, 1997; Leppanen -Turkula, 1999).

Lignocellulose used for manufacturing paper contains cellulose, hemicelluloses and lignin. The lignin component of the cell wall strengthens the plants against microbial degradation (Argyropoulos and Menachem, 1997), but is highly undesirable in relation to paper manufacture. The amount of lignin in paper may be up to 20% (Tuomela *et al*., 2000). The white rot fungi (Basidiomycetes) are well known for their ability to biodegrade lignin molecules (Hatakka, 2001; Tuomela *et al*., 2000). White rot fungi degrade lignin using a range of enzymes (Hatakka, 1994; Kirk and Farrell, 1987). Brown rot fungi can degrade cellulose and hemicelluloses but their lignin degrading ability is minimal (Buswell and Odier, 1987; Kirk and Farrell, 1987). Soft-rot fungi, (Ascomycotina and Deuteromycotina) are also capable of degrading lignin (Kuhad *et al.,* 1997) and can also degrade wood in conditions which are unfavourable for white and brown rot fungi (Blanchette, 1995).

Several studies have reported that many bacterial species including Actinomycetes, can solubilise and transform lignin, but are not fully able to mineralize lignin (Buswell and Odier, 1987; Ball *et al.,* 1989; Eriksson *et al*., 1990; Godden *et al.,* 1992).

There are some biodegradable and compostable plastic films obtained from starch which are used in packaging materials (Bastioli, 1997). Vikman *et al.* (1995) examined the compostability of Mater-Bi biodegradable films in two composting experiments. In both experiments, the cellulose material was fully degraded but the total weight loss of

the Mater-Bi films was in the range 40-45 % after 70 days whereas, the manufacturer claimed that Mater-Bi product films degrade after 20-45 days in a composting environment (Bastioli, 1997).

In another experiment, Davie *et al.* (1994) collected paper packaging waste from a fast food restaurant and monitored the decomposition of uncoated and heavily waxed cupstock, waxed burger wrap, and double-sided polyethylene coated paper in poultry manure for a period of 56 days. Maximum dry weight loss was recorded in uncoated cupstock (81%) followed by waxed cupstock (79 %), waxed burger wrap (74 %), and double-sided polyethylene paper (31 %).

Haritos *et al.* (1993) reported that *C. acinaciformes* and *M. darwiniensis,* (termites) *metabolized* and eliminated lipophilic xenobiotics in treated waste paper, particularly M. *darwiniensis*. Their ability to metabolize lipophilic xenobiotics in waste paper suggests their possible use in the biodegradation of waste papers.

Several techniques have been tested to remove wax from old corrugated cartons. Some of them are related to the removal of larger wax particles through screening, reversed hydrocyclones, or with flotation. Other approaches use dispersants along with washing procedures for the removal of wax (McEwen, 1992).

Severtson (2006) studied the potential for using termites for decomposing different kinds of waste papers. This author reported that termites decomposed a total of 5.9 kg of paper in a 20 week span. The most preferred type was newspaper followed by glossy-coated papers and bleached office papers, respectively. In another trial, mixed paper types and added moisture were included. This time, the overall rate of decomposition was increased (i.e., 6.9 kg of paper in 20 weeks).

6.1.2. Paper recycling

Vast amounts of paper are recycled around the world each year. The process is very efficient but can be influenced by paper prices, so that sometimes gluts make the process uneconomic (Leppanen -Turkula, 1999). One of the main environmental problems associated with paper recycling and papermaking in general is the requirement for a cocktail of environmentally unpleasant chemicals, notably chlorine-based bleaches. An alternative use for waste paper would be to use it as a feedstock for Black Soldier Fly and other larvae, the idea being that the waste paper is converted into biomass in the form of worms which can then be fed to livestock, particularly broiler or egg laying poultry (Diener *et al.,* 2011).

The aim of the work described in this Chapter was to determine if the larvae used in this study could effectively and economically break down paper and waxed card to produce biomass

6.1.3. The current process of paper recycling

Although this thesis is not directly concerned with paper recycling (i.e. the conversion of waste paper to new paper) the following brief comment on the process is included to show that environmentally damaging chemicals are used, thereby making desirable alternatives uses for waste paper, such as animal feed production (Leppanen - Turkula, 1999).

Waste paper is collected from domestic and industrial sources and stored in warehouses where the various paper grades are kept separate (e.g. newspaper and card) When the paper mill is ready to begin the process, forklifts move the paper from the warehouse to a system of large conveyors to a large vat called a pulper, which contains water and chemicals (Virtanen and Nilsson, 1993). The paper is chopped into small pieces and heated so that it breaks down into extremely small strands of cellulose (i.e.

organic fibres). Eventually, the old paper is turned into a mushy pulp (Virtanen and Nilsson, 1993). This is forced through screens with various sized holes. The screens remove small contaminants such as plastic and pieces of glue (Virtanen and Nilsson, 1993). This process is called screening. The clean pulp is next treated by spinning it around in large cone-shaped cylinders, where heavy contaminants like staples are thrown to the outside of the cone and fall through the bottom of the cylinder. Lighter contaminants collect in the cone's centre and are removed; this process is called cleaning. During refining the pulp is beaten to make the recycled fibres swell, making them perfect for papermaking. If the recovered paper is coloured, colour stripping chemicals remove the dyes from the paper. The pulp may then need to be bleached with hydrogen peroxide, chlorine dioxide, or oxygen to make it whiter and brighter. If brown recycled paper is being made, like that used to make industrial paper towels, then the pulp does not need to be bleached (Virtanen and Nilsson, 1993).

The recycled fibres can be used alone, or blended with new wood fibre (called virgin fibre) to give it extra strength or smoothness. The pulp is then mixed with water and chemicals to make it 99.5% water. This watery pulp mixture enters a giant metal box at the beginning of the paper machine and then is sprayed in a continuous wide jet onto a vast flat, quickly moving wire screen and passes through the paper machine. While on the screen, water drains from the pulp, and the recycled fibres quickly begin to bond together to make a watery sheet. The sheet moves rapidly through a series of felt-covered press rollers which squeeze out more water and form paper (Virtanen and Nilsson, 1993).

6.1.4. The Tetrapak problem

A major problem in relation to paper recycling is what is to be done with waxed paper cartons or so-called Tetrapaks (Tetra Pak trade name) (Chong and Hamersma, 1995). This packaging material is widely used for packing liquids such as fruit juices.

Chong and Hamersma (1995) reported that the amount of packaging material sold by Tetra Pak in 1999 was equivalent to 86 billion packages, of various sizes; this amount will only increase in the future. Most of the packages sold were suitable for aseptic packaging. Tetra Pak has 66 production plants and serves more than 165 countries. Currently, there are approximately 8200 packaging machines in operation. Tetra Pak is one of the largest buyers of paper, processing over 1 million tons of paper worldwide per year. Tetra Pak does not buy paper made from (or with) recycled fibres, because of the possibility of food-contact and the high requirements on stiffness (Chong and Hamersma, 1995). Almost all of the board used in North America, Japan, Korea and Australia comes from bleached sulphate pulp. In South America it is mostly unbleached, duplex board from sulphate pulp. Europe, Africa and Asia also use unbleached sulphate pulp, but recently the utilization of a three-layered board, with CTMP, has increased due to the need to promote stiffness (Chong and Hamersma, 1995).

In conclusion, waxed cardboard is relatively difficult to recycle, simply because the ability of microorganisms to breakdown the complex waxes used is limited to only a few species of bacteria and fungi (Chong and Hamersma, 1995). As a result, some local authorities require residents not to put waxed cartons in their recycling bins. Chemical pre-treatments can be used for this purpose but these tend to be expensive and relatively environmentally damaging. The use of larvae to remove wax, would on the face of it, be attractive because, not only would the wax covering of these

cardboards be removed but a valuable biomass types, in the form of worms, would be produced which could be fed to poultry, an approach which would reduce the overall cost of waxed cardboard recycling.

Tetrapaks present a major problem because the wax-coated cardboard of which they are made is difficult to process and recycle; it also breakdowns slowly in landfill. The aim of this part of the work described in this Chapter was to determine if the worms used in this study could be used to strip the wax from wax coated card in order to increase its availability for pulping and also increase the rate at which it breaks down in landfill. The research was conducted as a prelude to determining if worms can be used a pre-treatment in the large scale recycling of such cartons.

6.1.5. Waxed paper breakdown

Packaging is used for the safe supply of finished products to the consumer. An efficient packaging must be cost effective, protect product in shipping, and provide producer and consumer information, be environmental friendly and recyclable (Waite, 1995). Glass, steel, paper, fiberboard and plastic are some of the most commonly used packaging materials (Davis and Song, 2005).

Annual EU packaging waste production is more than 67 million tons (Klingbeil, 2000), whereas in the United Kingdom, the annual packaging waste is 3.2 million tons (Wasteline, 2002). About 85% of the municipal waste in the UK is landfilled (Linstead and Ekins, 2001). The UK Government- Industry Forum has strongly recommended the use of nonfood crops for the manufacture of biodegradable packaging (Defra, 2002, 2004).

Generally speaking, there are two types of cardboard, so-called corrugated card board which contains a corrugated/wavy inner layer held between two plan layers of thin card. The other so-called plain card board is made up of a single card board layer.

Some papers and card board used for the packaging of juices and dairy products are coated with resin/wax, including paraffin, microcrystalline and polyethylene; these effectively inhibit moisture permeability (Asadchii *et al*., 1986).

Wax-coated paperboard had been extensively used for packaging of food materials. But now, largely because of difficulties in recycling, most waxed containers have been replaced with plastic (Popil and Joyce, 2008).

The literature shows that research has mostly been focused on biodegradation of plastics (Albertsson and Karlsson, 1995; Forssell *et al.*, 1996) whereas, paper products, many of which are more suitable candidates for biodegradation and composting, have yet to be extensively explored in relation to biodegradation.

The Wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) causes severe damage to the honeybee colonies as it feeds on wax (Garedew *et al.,* 2004). Wax moth larvae can be tested for the decomposition of wax cardboard for subsequent utilization in compost production.

6.2. Materials and Methods

6.2.1. Collection and incubation of worms and larvae and waxed paper in relation to waxed paper breakdown

Fruit beetle larvae and Waxworms were purchased online from Ricks Livefood Ltd. Pieces of waxed paper equal in weight and size taken from Tetra-Pak were placed in plastic boxes (Fig.5.1) with small holes in lids to allow for gas exchange, and incubation was for 50 days at 25°C. Ten Fruit Beetle larvae and 100 Waxworms were used (3 boxes Waxworms and 3 boxes Fruit Beetles), the weight of the waxed paper was determined every three days.

6.2.2. The ability of larvae and worms to break down paper in relation to the recycling of waste paper

Larvae and worms were bought online from Ricks LiveFood Ltd. Paper was collected from a paper recycling bin and the shredded using an office shredding machine. The paper was distributed into 6 plastic boxes with a lid perforated to allow for gas exchange (3 boxes Waxworms and 3 boxes Fruit Beetles), Waxworms (100 P) and Fruit Beetle larvae (10 P) were added to 100 g of shredded paper in each box (Fig.5.2). The boxes were then left to incubate for 28 days at 25°C. Larvae and worms waste weights were measured every 7 days (e.g. 0, 7, 14, 21 and 28 days).

6.2.3. Bacterial isolation from cow faeces

Bacteria was isolated every seven days from WW and FB waste from zero time to 28 days in order to determine the populations of *E.coli, Salmonella Sp* and total number of bacteria. Samples (1g) of WW and FB waste were diluted in 9 ml of sterilized water and then a serial dilution ($10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}$) was performed and 0.1 ml of each diluted sample was finally spread onto *E.coli* agar, XLT4, LB and Plate Count Agar and incubated for between 18 and 24 h at 37^0C.

6.2.4. Statistical analysis

All data are given as means ± SE (Standard Error). Results were analysed using t-tests between two groups at P≤0.05, and compared significance of means between FB and WW by Sigma Plot 12.0$^©$ software.

Figure 5.1: Pieces of waxed paper in plastic box treated by A) Waxworms, B) Fruit

beetle larvae (author's images).

Figure 5.2: Waxworms (A) and Fruit beetle larvae (B) added to shredded paper

(authour's image).

6.3. Results and Discussion

6.3.1. Effect of Waxworms and Fruit Beetle larvae in waxed paper breakdown

This experiment shows that the Waxworms feed on wax that covered waxed paper, causing holes in the paper as a result of eating parts of paper (Fig.5.3), which decreased significantly the waxed paper weight during the experiment. There was no change or weight loss of waxed paper with Fruit Beetle larvae and most of the larvae died within 7 days from when the experiment started (Fig.5.4). Through statistical analysis, significant differences occurred in all the readings during the experiment (Fig.5.5).

6.3.2. The effect of Waxworms and Fruit Beetle larvae on the recycling of paper

The waste weight of Waxworms after feeding on recycling paper was increased through the 28 day incubation period, where it was the highest at day 21. The Fruit Beetle larvae waste weight decreased during the experimental period, and the sharp decline was between day 21 and day 28. The results show significant differences between WW, FB waste weight after feeding on recycling paper at days 14, 21 and 28 (Figures 5.6, 5.7). The numbers of *E.coli*, *salmonella Spp* and total bacteria in WW and FB waste after recycling paper feeding were very low and nil in most of days over the entire incubation period (Table 5.1-5.2).

In conclusion, it is clear that, although Waxworms consume wax and the underlying card when exposed to waxed cardboard as the sole nutrient source, they did not do so on a scale and with the efficiency necessary to remove these components as an aid to the breakdown or recycling of this widely used packaging material; the same can be said for shredded paper. The results of this study clearly show that there is limited potential for the use of Waxworms in biotechnology for such purposes.
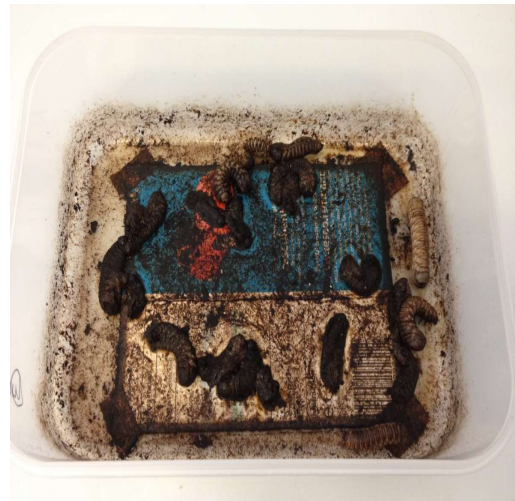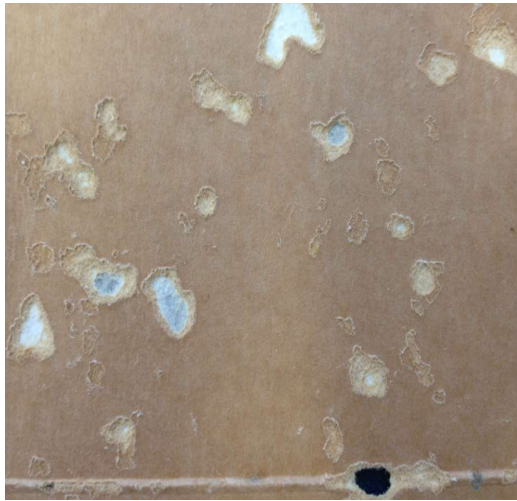
Figure 5.3: Waxed paper undergoing larval breakdown (author's image).

Figure 5.4: Dead Fruit beetle larvae on waxed paper (author's image).
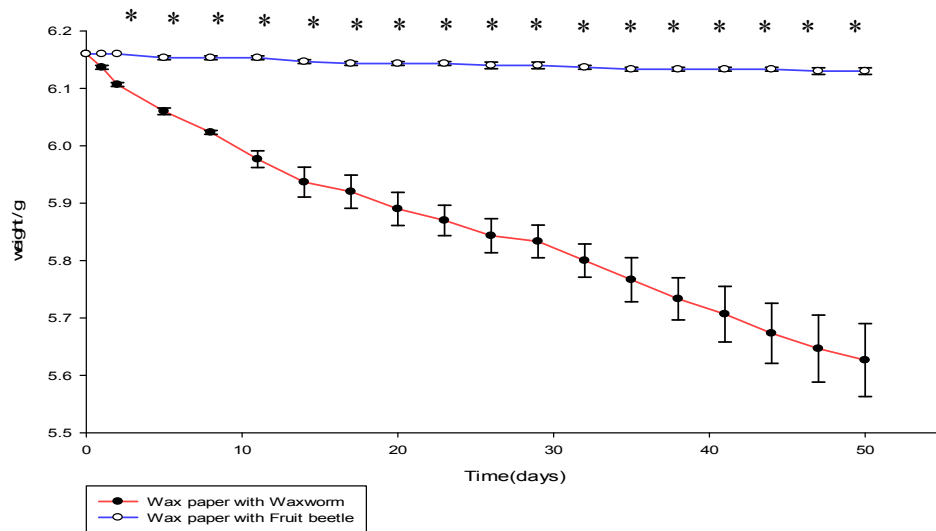
Figure 5.5: Effect of Waxworms and Fruit beetle larvae on waxed paper breakdown (*Significantly different between means of waxed paper weight for FB and WW).
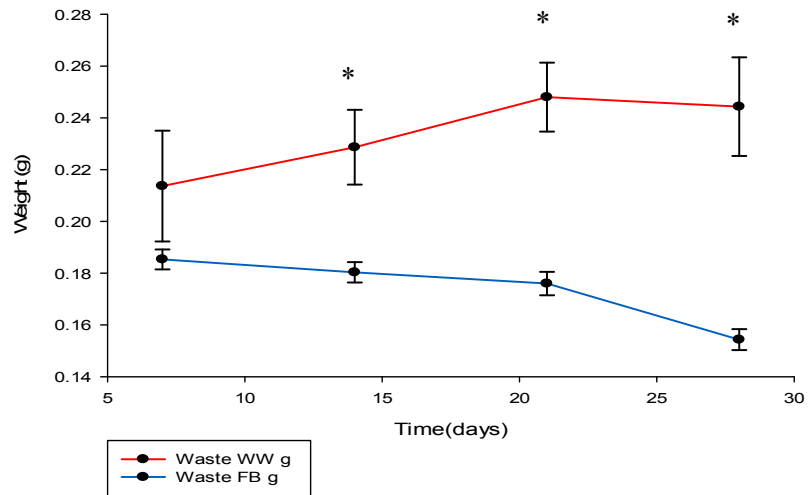
Figure 5.6: Treated paper weight (g) of Waxworms and Fruit Beetle larvae feeding on recycling paper (*Significant differences between means of waxed paper weight for FB and WW).
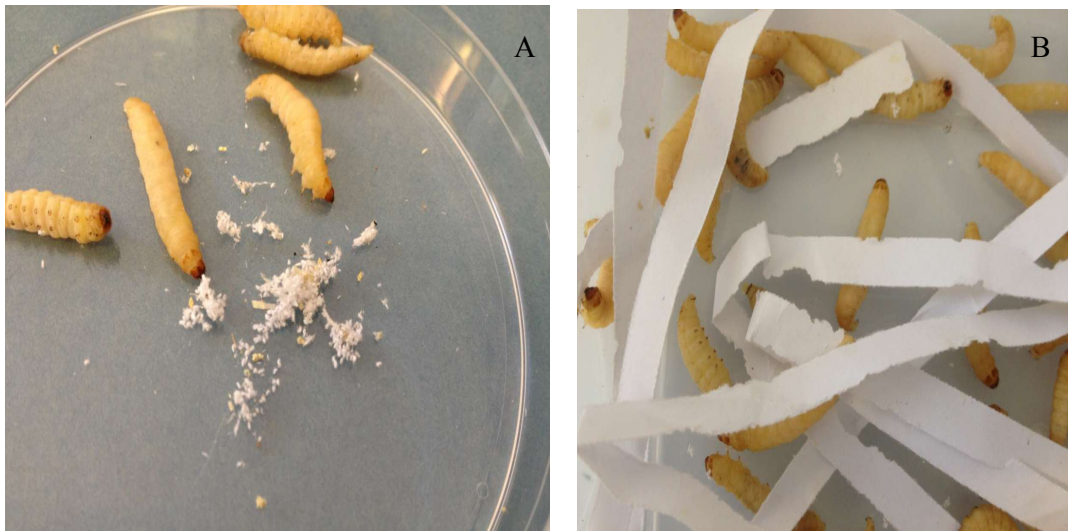
Figures 5.7: A) Waxworms breaking down paper, B) Waxworms eating shredded paper (author's image).

Days / Kind of bacteria	0	7	14	21	28
E. coli	Nil	266	2200	Nil	Nil
Salmonella Sp	Nil	Nil	Nil	Nil	Nil
Total bacteria	3400	1500	200	Nil	160

Table 5.1: The numbers of bacteria (CFU/g) in WW waste after feeding on paper for 28 days.

Days / Kind of bacteria	0	7	14	21	28
E. coli	Nil	Nil	400	Nil	Nil
Salmonella Sp	Nil	Nil	Nil	Nil	Nil
Total bacteria	3200	1000	1000	900	700

Table 5.2: The number of bacteria (CFU/g) in FB waste after feeding on paper for 28 days.

Chapter Seven: Detection of any antibacterial activity of the haemolymph and whole body extracts from Fruit beetle larvae and Waxworms

7.1. Introduction

7.1.1. The Antibacterial Activity of the hemolymph and whole body extract from Fruit beetle larvae and Waxworms against bacteria

Microorganisms have the ability to develop resistance against verities of antibiotics leading to emerging of new fungi and pathogenic bacterial strains. Thus triggered exploration of alternative antimicrobial products for the control of infectious microorganisms (Fredericks, 2001; Sonnevend *et al.,* 2004). Recently, several natural antimicrobial peptides (AMPs) isolated and characterised from amphibians, insects, mammals, plants and prokaryotes have been reported which play an important role in anti-pathogenic microorganisms (Zasloff, 2002).

Insect antimicrobial peptides are the derivatives of innate immune systems and differ in vertebrates having the acquired immune systems which implicate antigen-antibody reactions. Insect innate immune system can be subdivided into humoral and cellular defence responses (Boman and Hultmark, 1987; Bulet *et al.,* 1999; Hoffmann, 1995; Hoffmann *et al.,* 1996; Lavine and Strand, 2002). Humoral defences involves antimicrobial peptides production, reactive intermediates of oxygen or nitrogen, and the complex enzymatic cascades which control coagulation or melanization of hemolymphs (Ekengren and Hultmark, 1999). When insects are infected by microorganisms they trigger the synthesis of antimicrobial peptides in the infected insects fat bodies and secreted into the hemolymph (Bulet *et al.,* 1999; Hoffmann and Reichhart, 2002). General features of AMPs include: a low molecular weight, a positive charge, are stable (Bulet *et al.,* 1999; Oizumi *et al.,* 2005), and are in agreement with the amphiphilic α−helices or hairpin-like β− sheets or mixed structures. Generally, AMPs are non-cytotoxic and have broad-spectrum antimicrobial action against bacteria, yeasts, enveloped viruses, filamentous fungi, and parasites.

These characteristics along with low metabolic cost for the insect, speed of action and low specificity, AMPs become highly versatile components of the insect immune response. Antimicrobial peptides are belonging to a unique and diverse group of molecules that have been classified into numerous subgroups depending on the composition and structure of amino acids (Meister *et al.,* 1997). Based on sequence and antibacterial activities, insect AMPs can be classified into five major classes (Meister *et al.,* 1997): (i) cecropins, deprived of cysteine residues; (ii) cysteine-rich peptides; (iii) glycine-rich peptides; (iv) proline-rich proteins; and (v) lysozymes. Beside this, insect AMPs can also be classified into three major groups depending on the structure (Bulet *et al.,* 2004): (i) peptides with α−helix conformation; (ii) cyclic and open-ended cyclic peptides with pairs of cysteine residues; and (iii) peptides with an extended structure. AMPs are short proteins commonly comprised of 12 to 100 amino acids and are typically small cationic and amphiphilic moieties presenting a wide range of antimicrobial actions (Gough *et al.,* 1996; Ouellette and Paneth, 1996; Lamberty *et al.,* 2001; Lapis, 2008). AMPs contain two or more positively charged residues provided by arginine, lysine, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues (Reddy *et al.*, 2004; Izadpanah and Gallo, 2005; Miyashita *et al.,* 2010).

The expression of AMPs could be constitutive or inducible upon infectious stimuli (Lamberty *et al.,* 2001; Lapis, 2008; Peng *et al.,* 2008). AMPs serve as the basic type of innate immunity plentifully accumulating in epithelial and immune cells. AMPs serve as naturally occurring moieties fighting with pathogenic microorganisms through their microbicidal activities (Braff and Gallo, 2006). AMPs directly influence bacteria (Dimopoulos *et al.,* 1997; Putsep *et al.,* 2002) by injury or disrupting them and

involved in the composition of the innate immune and antimicrobial immune-modulatory responses (Kruszewska *et al.,* 2004; Cotter *et al.,* 2005).

Fat bodies of insects respond to bacterial infection by producing either a range of antimicrobial substances or by synthesizing very few molecules depending on the insect species as dragonfly (*Asechna cynanea*) only produces a single peptide (Bulet *et al.,* 1999). Insects occupy verities of niches; insect species synthesizing rich antimicrobial peptide (e.g. *Drosophila*) are capable of occupying niches with greater microorganism population, whereas, the species synthesizing few of deprived of antimicrobial peptide such as dragon fly prefer hygienic niche, which is due to process of adaptation. Out of over one million described insect species only 170 antimicrobial peptides/polypeptides have been fully characterized from different insect species (Bulet *et al.,* 1999; Saito *et al.,* 2004). Antibacterial peptides have been isolated and purified from different insects orders including dipterans, isopterans, lepidopterans and coleopterans (Peng *et al.,* 2008). There are some well characterized antimicrobial peptides in insects such as mastoparan, poneratoxin, cecropin, moricin and melittin (Bulet *et al.,* 2004).

Insect order Coleoptera (Sheathed Wing) includes "beetles" (Whiting, 2002). It represents, about 40% of all known insects and more than 25% of animal life present on the earth (Hunt, 2007). Coleopterus have wide range of adaptability and except marine and polar they have been reported from all habitats (Hammond, 1992). The fruit beetle (*Pachnoda marginata*), order "Coleoptera", family "Scarabaeidae" and subfamily "Cetoniinae", contain a number of subspecies mainly present in West and Central Africa (Rigout, 1989).

Waxworms are the Wax Moths caterpillar (*Galleria mellonella* and *Achroia grisella*). The Greater Wax Moth (*Galleria mellonella*) belonging to the Order

"Lepidoptera", family "Pyralidae" and Tribe "Galleriini" is only member of the genus "Galleria". *G. mellonella* has been reported from all over the world including Asia and Europe as native whereas, an introduced species in North America and Australia (Grabe Albert, 1942). Waxworms feed on honeycomb. They are good source of protein for animals and are commercially reared/produced.

Studies revealed that fruit beetles (*Pachnoda marginata*), waxworms (*Galleria mellonella*) and their other close relatives belonging to same families synthesize different kinds of antimicrobial compounds. Some antimicrobial compounds have already been isolated, purified and characterized as potential antibacterial agents. They varies in respect to their synthesis and mode of action and are usually produced in response to a microbial stimuli (Bulet *et al.,* 1999; Hoffmann and Reichhart, 2002). Antimicrobial compounds are mainly found in hemolymph however, small traces may also be present in other body parts. Larval stage of insect is comparatively more vulnerable to pathogenic microbial attack due to absences or weak exoskeleton. Mostly the pathogens are bacteria therefore, the antimicrobial/ antibacterial activity are greatest at this stage (Steinhaus, 1949; Reddy, 1978). Waxworm and fruit beetle larvae can be used as a potential source of antibacterial chemical synthesis to cure diseases in humans as well as other animals and plants. However, comprehensive studies are needed on their effect, nature, mode of action and the harm to the victim organism.

Kinoshita and Kozo (1977) reported that cell-free hemolymph of silkworm (*Bombyx mori*) larvae have bactericidal properties and can kill *Escherichia coli* B/SM. This bactericidal property is due to lysozyme-like enzyme and another cofactor (anionic factor) of low molecular weight. They further described that egg-white lysozyme can increase the bactericidal properties of the blend of silkworm enzyme and

cofactor whereas, Mg_2+ and Ca_2+ can prevent the bactericidal property (Kinoshita and Kozo, 1977).

Gross *et al.* (1998) reported that exocrine glands of the larval and adult stages of mustard leaf beetle *Phaedon cochleariae* (Coleoptera: Chrysomelidae) release substances for defence against predators. They examined hemolymph and larval regurgitate against microorganisms and found that larval glandular secretion can inhibit the growth of Gram-negative bacterium *Escherichia coli*, but did not show any *lytic* effect against cell walls of the Gram-positive bacterium *Micrococcus luteus*. But on the other hand the adult glandular secretion of *P. cochleariae* exhibited lytic effect against the cell walls of the Gram-positive bacterium M. *luteus*, but did not presented any inhibitory activity against the Gram-negative bacterium *E. coli* and eukaryotic cells. They also described that the hemolymph and the larval regurgitants of *P. cochleariae* showed the similar properties, presented by tested glandular secretion of adults (Gross *et al.*, 1998).

Bulet and Stocklin (2005) have shown that antimicrobial peptides (AMPs) are part of defence mechanism developed in the insect to combat with pathogenic microorganisms. AMPs are cationic in nature and usually comprise of below 100 amino acid residues. AMPs are varied in structures and can be allocated to a fewer number of families. Common AMPs structures include peptides, which assumes a α-helical conformation in organic solutions or disulfide-stabilized β-sheets with or without α -helical domains. These diverse AMPs structures predict their modes of action (Bulet and Stocklin, 2005).

Dang *et al.* (2006) isolated one antibacterial activity fraction from a resistant Dipteran species, *Bactrocera dorsalis* and purified by pre-purification, ion-exchange chromatography, gel filtration chromatography and reverse-phase high performance

liquid chromatography (HPLC) (Dang *et al.*, 2006). The purified fraction was examined on the HPLC and used for subsequent analysis. The physical and biological analysis indicated that the fraction was stable to heat and showed promising antibacterial activities against Gram-positive bacterial growth (Dang *et al.*, 2006).

Eight peptides from hemolymph of immune-challenged the Greater Wax Moth, *Galleria mellonella* (Gm) larvae were isolated and characterized (Cytrynska *et al.,* 2007). The peptides varied considerably in amino acid sequences, isoelectric point values and antimicrobial activity spectrum. Five peptides, Gm proline-rich peptide 2, Gm defensin-like peptide, Gm anionic peptides 1 and 2 and Gm apolipophoricin, had not been reported previously in *G. mellonella*. While three others, Gm proline-rich peptide 1, Gm cecropin D-like peptide and *Galleria* defensin, were identical with already known G. *mellonella* peptides. Gm proline-rich peptides 1, 2 and Gm anionic peptide 2, presented unique amino acid sequences with no reported homologs. Antimicrobial activity of above peptides was tried out against Gram-negative and Gram-positive bacteria, yeast and filamentous fungi. Results indicated that Gm defensin-like peptide inhibited fungal and sensitive bacterial growth at 2.9 and 1.9 mM concentrations, respectively (Cytrynska *et al.,* 2007).

Ladybird Beetle *Harmonia axyridis* (Coleoptera: Coccinellidae) has been extensively reported for immunity against microbes. Eleven body components from the headspace and hemolymph were examined against yeast (*Saccharomyces cerevisiae*), Gram-negative bacteria (*Escherichia coli*) and Gram-positive bacteria (*Bacillus subtilis, B. thuringiensis ssp. tenebrionis, and Micrococcus luteus*). Results indicated that hemolymphs inhibited the growth of yeast as well as Gram-positive and Gram-negative bacteria (Gross *et al.,* 2010).

Phenol oxidase (PO) is an important factor in insect resistance to diseases. Prophenol oxidase converts into its active form, phenol oxidase when an insect is attacked by pathogenic microorganisms. PO was purified and its molecular mass was determined as 33 kDa. Present study has revealed some insecticidal properties of Phenol oxidase. PO can replace the traditional insecticides (Ajamhassani *et al.*, 2012).

Several insect species protect their offspring from a variety of environmental hazards. But there is paucity of knowledge about the mechanisms and fitness consequences of parental defences against pathogenic microorganisms (Arce *et al.*, 2012). Microbiology and behavioural ecological strategies were used to investigate the role and mechanistic basis of antibacterial secretions of burying beetle *Nicrophorus vespilloide*. Burying beetles rear their larvae on vertebrate carcasses, where larvae bear a strong competition with bacterial decomposers. Studies indicated that anal secretions of burying beetles had vigorous bactericidal properties and specifically effective against gram positive bacteria (Arce *et al.*, 2012).

Antibacterial peptides were isolated from immune haemolymph of Rhinoceros beetle (*Oryctes rhinoceros* L.) larvae and tested against bacterial pathogens, viz., *Klebsiella pneumoniae*, *Micrococcus luteus*, *Bacillus subtilis*, *Paenebacillus macerans* and *Pseudomonas* sp to determine their antibacterial properties. The peptides revealed strong antibacterial properties and stabiloty up to 37°C (Rabeeth *et al.,* 2012).

The objective of this review was to find out if entire larval bodies, or specifically their haemolymph contained antibacterial properties.

7.2. Materials and Methods

7.2.1. Culture of larvae

Fruit Beetle larvae and Waxworms used in this experiment were obtained from a commercial company (Ricks, Livefood, Ltd. UK).

7.2.2. Antibacterial properties of FBL and WW larvae

7.2.2.1 Collection of excretion/secretion from larval wholebody and haemolymph

The larvae were placed in small boxes (10×15x30 cm). Sample of 20g of each larval species (approximately 150 larvae of WW and 15 larvae of FB) were left unfed for 3 days until they were free from gut residues in order to collect the whole body and haemolymph. Sterile deionized water will be used to wash the larvae and to extract samples for whole body extracts and larval secretions. The larvae were homogenized by manual grinding using a mortar and pestle. The anterior end of the larva, near the cephalopharyngeal skeleton, was clipped and the haemolymph was collected by gently squeezing the abdomen. A disposable, sterile vacuum filter unit was used to filter (0.2µml) the extract to remove large particles and bacteria (Fig.6.1). The excretion/secretion (ES) from each group of insect larvae was dissolved in 0.1 µ g/ml protein in 0.1% trifluoroacetic acid (TFA) 1: 10 v/v. The suspension was placed on ice for 6 hours following centrifuging on four occasions (14000 g for 30 min). The supernatant was then harvested and separated into 15 ml centrifuge tubes; samples were used fresh, or kept frozen at -80°C for a few days until required (Sahalan *et al*., 2006; Huberman *et al*., 2007).

7.2.3. Bacterial culture, preparation of extract and antibacterial disc assay

Cow faeces (1g) were diluted in 9 ml of sterilized water and then a serial dilution was performed and 0.1 ml of the final diluted sample was spread onto *E.coli* agar, XLT4, LB and Plate Count Agar and incubated for between 18 and 24 h at 37^{0}C.

Cultures of *E. coli, Salmonella Sp and* total bacteria were grown overnight on LB medium, *E.coli* agar, XLT4, LB and plate count agar. Then 0.1 ml of samples was spread onto Nutrient agar medium. Extract (25 µml) was added to a 1 cm paper disk was applied to Nutrient Agar on which the test bacterium was spread; a disc containing gentamicin (10 µg), was used as a control and all were incubated for between 18 and 24 h at 37^0C (Fig.6.2).
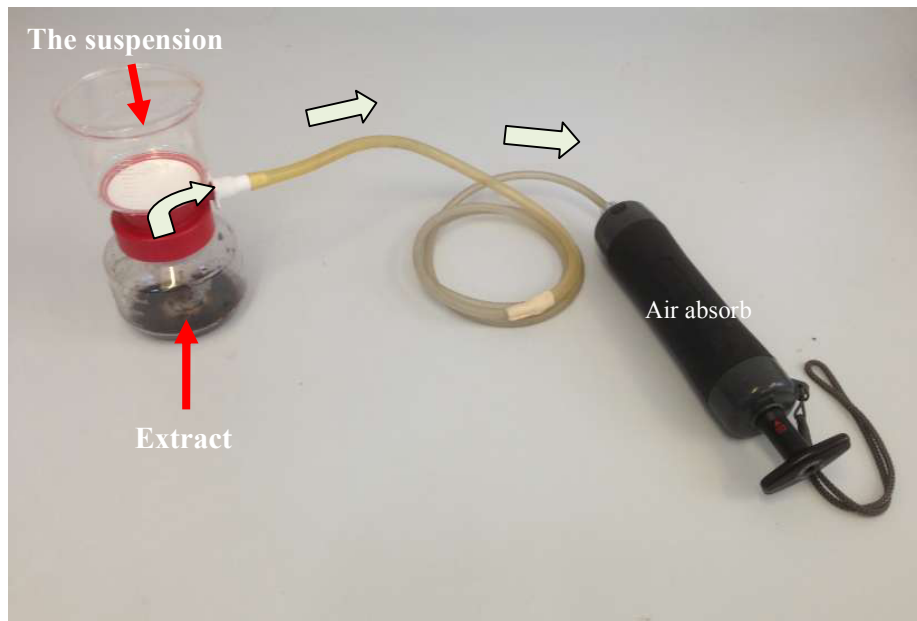
Figure 6.1: Disposable sterile vacuum filter Units. This unit was linked with an air pump to complete the extraction process.
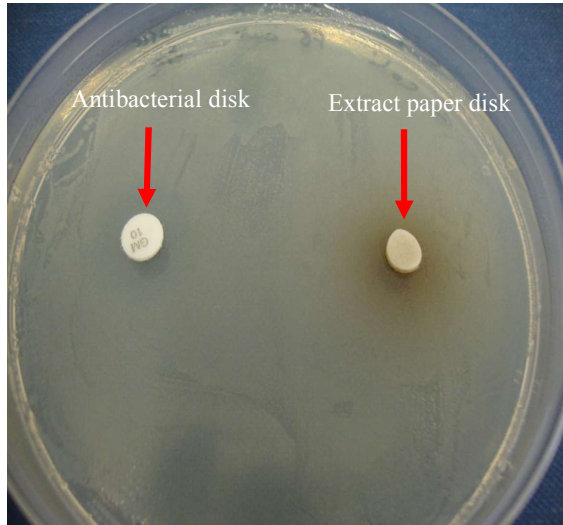
Figure 6.2: Antibiotic extract paper and antibacterial disk on nutrient agar.

Figure 6.3: FB haemolymph disk (left) gave a small zone; compared with the antibacterial disk (right) producing a large inhibition zone against the same *E.coli* culture.

Figure 6.4: FB whole-body disk (left) gave a small zone, antibacterial disk (right); compared with a large zone for the same *E.coli* culture.
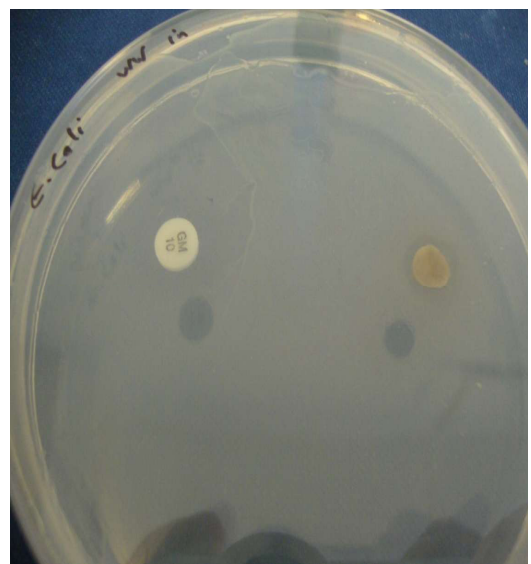
Figure 6.5: WW haemolymph disk (right) gave a small zone; compared with the antibacterial disk (left) against *E.coli*.

.
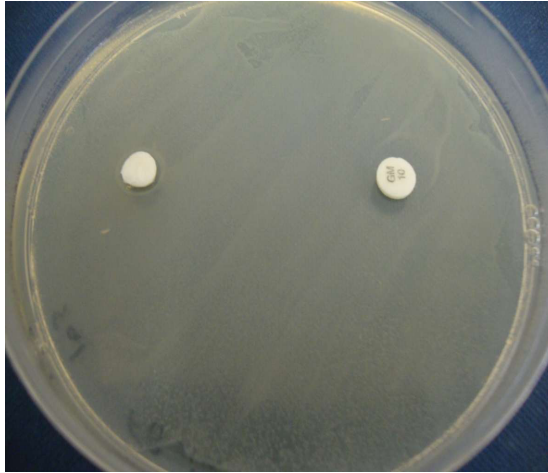
Figure 6.6: FB haemolymph disk (left) gave a very small zone, compared with the antibacterial disk against same *Salmonella Sp* culture.
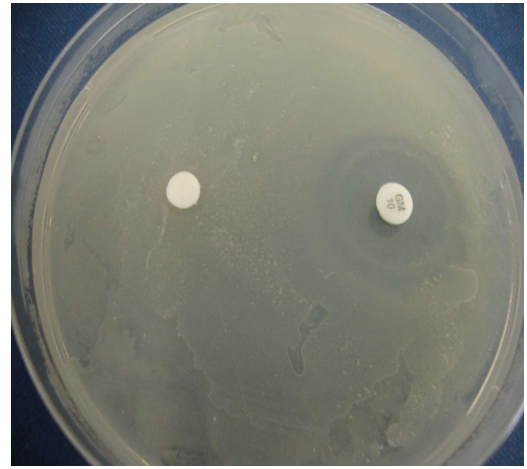
Figure 6.7: FB haemolymph disk (left) produced no zone; compared with a large zone produced by control antibacterial disk (right) on nutrient agar (total bacterial culture).

7.3. Results and Discussion

FB haemolymph and whole-body disks produced only small zones (2-4 mm) when tested against an *E.coli* culture compared with an antibacterial disk which gave an inhibition zone of 7-8 mm (Fig.6.3). With the *Salmonella* culture only, the FB haemolymph gave a zone 2 mm (Fig.6.6). Waxworm haemolymph also produced a small zone (2mm) against *E.coli* (Fig.6.5), and also an antibacterial disk gave a bigger zone in the same culture (Fig.6.4). Waxworm whole-body extract gave no zones against both *E.coli* and *Salmonella* cultures. All of the larvae (FB, WW) haemolymphs and whole-body extracts didn't give any zones with total count of bacteria (Fig.6.7).

In conclusion, the results show that none of the body extracts from the larvae gave substantial antibacterial inhibition zones. Despite this, it must be noted that in order to produce any detectable zone in this inhibition test, there must be antibacterial activity present and that this may manifest itself as effective inhibition to an individual

bacterium or bacteria present in low numbers. In retrospect, it would have been better to conduct this experiment using a very dilute bacterial culture and then determine the MIC for each bacterium against each extract.

One indirect possibility which was postulated when this work was initially considered was the potential use of the worms used here to produce medical antibiotics, or even to use them directly in wound treatment. The results of the study discussed here suggest that neither of these uses is likely to be an effective approach to wound management.

Chapter Eight: Final Discussion

8.1. Final Discussion

This work described in this Thesis is devoted to a study of the biotechnological application of insect larvae, notably in relation to resultant changes in microbial populations and plant nutrient ions, in various substrates, resulting from their activity. The studies reported here rely of course on the ability of these various worms to break down organic matter and, with the aid of internal and external microbes, bring about the mineralization of organic carbon and nitrogen and the subsequent cycling of the mineralization products. The individual Results and Discussion sections of this Thesis have discussed the findings of the individual areas of research. This final Discussion chapter will deal succinctly with the general implications of the findings.

The obvious aim of any research on biotechnology is to apply findings to agriculture or industry (i.e. to move from theoretical work to practical applications). The studies reported here were aimed at conducting preliminary work on the use of insect worms to various potential biotechnological applications. Since this work has, in the main, not been attempted before, or at least not reported in the literature, the work is by its very nature preliminary, and so essentially, a broad look at possible uses of these worms. For example, as far as can be determined from the literature, no one has reported the practical use of larva to digest the wax coatings of TetraPaks as a preliminary step to improving their breakdown and, or recycling. Clearly it a necessary prerequisite for attempts to use these worms for this specific purpose on an industrial (and therefore economic scale), and so that their ability to achieve this breakdown is determined at the outset. If they do not eat and breakdown the wax surface of the waxed cardboard in the laboratory, then there is little, if any likelihood, that they could do so in an industrial-scale process.

The result of these studies have thrown up some interesting potential biotechnological applications, but it remains to be seen whether any of the ideas can be scaled up to become commercially viable, A major advantage of working with these kinds of worms is that there is an economic benefit built into their possible application from the start. This obviously relates to the fact that the worms can act as a source of high quality, protein-rich biomass, which is particularly useful as poultry feed. This is an area of potential biotechnical application of the work reported here which we consider to be particularly viable (i.e. the relatively small scale production of biomass worms for use as feed in the poultry and egg producing industry). Here we envisage wormeries being set up adjacent to poultry farms where waste materials, notably cow manure would act as a cheap substitute for the production of high quality protein in the form of the worms used here. The products would be protein-rich worms and high quality compost which could be used in horticulture or for household use (i.e. sold to the high end of the market where profit margins are likely to be high). An obvious potential extension of this approach is to use chicken waste from the poultry industry as the source of feed. Although chicken faeces were not included in this study, there is no reason whatsoever to expect that these would not be amenable to breakdown and act as a source of nutrients to support the production of high quality protein-rich worms. This approach could also be used on a small garden scale by ecologically minded people who wish to take a more balanced ecological approach to small scale agriculture and poultry or egg production. There already exists an excellent example of the use of larvae in small scale protein feed production in the form of the black soldier fly *Hermetia illucens* (Leclercq, 1997), a fly (Diptera) of the *Stratiomyidae* family. The adult fly is black and wasp-like and about 15-20 mm long (Hardouin *et al.*, 2003) and the larvae can reach 27 mm in length and 6 mm in width and weigh around 220 mg in

their final larval stage. The larvae feed quickly, from 25 to 500 mg of fresh matter per larva per day an wide range of decaying organic materials including rotting fruits and vegetables, coffee bean pulp, distillers' grains, fish offal and especially animal manure and human excreta (van Huis *et al.*, 2013). The larvae become mature after two months, when the larvae empties its digestive tract and stops feeding and turns into the pre-pupal stage which migrates to a dry and protected pupation site where it pupates for about two weeks (Hardouin *et al.*, 2003). *Hermetia illucens* can be used as an efficient way to dispose of organic wastes by converting them into a protein-rich and fat-rich biomass suitable for various purposes, including animal feeding for all livestock species, biodiesel and chitin production (Diener *et al.,* 2011). The black soldier fly is a very resistant species and can deal with all kinds of demanding environments, such as drought, food shortage or a lack of oxygen. The larvae are sold for pets and fish bait, and they can be easily dried for longer storage (Leclercq, 1997). Several methods have been proposed for rearing black soldier flies on pig manure, poultry manure and food wastes (Barry, 2004).

Rearing facilities use the fact that the larvae migrate by climbing up a ramp out of the growth container to eventually end up in a collecting vessel attached to the end of the ramp (Diener *et al.*, 2011). Temperatures need to be maintained between 29 to 31ºC and relative humidity at between 50 and 70%. Black soldier fly larvae can be used live or chopped or dried and ground, and there have also been attempts to create a defatted meal from the larvae (Kroeckel *et al*., 2012). Finally, the black soldier fly can be used commercially to solve a number of environmental problems associated with manure and other organic wastes. Adult flies are also not attracted to human habitats or foods, do not bite or sting and are not considered to be a nuisance. Black soldier fly larvae are fast eaters and process organic waste extremely quickly and in so doing limit

microbial growth and reduce bad odours substantially, especially by the fact that they aerate and dry the manure (van Huis *et al.*, 2013). Black soldier fly larvae are a high-value feed source, rich in protein and fat and contain about 40-44% protein and 15-25% fat; the larvae are also rich in calcium (good for egg production) (van Huis *et al.*, 2013).

Large scale Black soldier fly larvae production then provides an ideal example of what can be achieved with insect worms in relation to high quality protein production and provides a good framework for protein production using the larvae studied here.

The use of larvae to liquify cattle waste has not to our knowledge been previously suggested, or published upon. We regard this as a potentially highly useful finding since it would allow farmers to produce a liquid product which could be readily pumped between large-scale waste storage containers and also be used directly for liquid manure application to fields. Similarly, we consider that there is potential for the use of the larvae used here to improve cow faeces for use as a potting compost (or supplement) for household or horticultural use. On the debit side, we cannot recommend further large scale investigation of the use of larvae to improve the breakdown of wax on wax-coated cartons, simply because the amount of wax broken down over a reasonable time period is so limited that we cannot envisage the approach finding a practical application in the biotechnology of waste recycling.

8.2. Future Studies

The suggestion proposed here for future studies obviously relates to the last paragraph of the above General Discussion in which it is suggested that there are applications of the above findings which could find a use in biotechnology. As a result it is suggested that:

1) The ability of the larvae used here (and others) to liquefy cattle, and other faeces-wastes should be further studied. In particular it is important to know if the larvae can survive and be active in large–scale waste containers, and at depth could they continue to liquify the wastes. If our findings in this regard could be scaled up, then we may have an efficient means of liquifying animal wastes to allow them to be pumped and spread onto fields as liquids. The production of a high quality protein, fat and calcium-rich larvae for use as an animal feed would be an added bonus and we envisage that the larvae could be readily filtered out from the liquid waste. These possibilities should form the basis of further study.

2) Although the large-scale improvement of animal waste by the use of the larvae studied here might be uneconomic, the relatively small scale production of larval-improved manures for household or horticultural use for potting composts or compost additives may be a practical proposition which needs further study.

References

References:

Adegunloye, D.V., Adetuyi, F.C., Akinyosoye , F.A. and Doyeni, M.O. (2007). Microbial Analysis of Compost Using Cowdung as Booster. *Pakistan Journal of Nutrition*. **6:** 506-510.

Ajamhassani M., J. J. Sendi, M. J. Farsi and A. Zibaee (2012). Purification and characterization of phenoloxidase from the hemolymph of Hyphantria cunea (Lepidoptera: Arctiidae). *Invertebrate Survival Journal.* **9:** 64-71.

Albertsson, A.C., Karlsson, S. (1995). Degradable polymers for the future. *Acta Polymer* **46:** 114-123.

Al-Falih, A. M and Wainwright, M. (1995). Nitrification, S-oxidation and P-solubilization by the soil yeast *Williopsis californica* and by *Saccharomyces cerevisiae*. *Mycological Research* **99:** 200-204.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J., (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

American Public Health Association. (1992). *Standard Methods for the Examination of Dairy Products. 16th Edn. APHA Inc. Washington DC.*

Anderson, J. M. and Baird-Parker, A. (1975). A rapid and direct plate method for enumerating *Escherichia coli* biotype I in food. *Journal of Applied Microbiology* **39:** 111-117.

Arce, A. N., P. R. Johnston., P. T. Smiseth and D. E. Rozen (2012). Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology*. **25:** 930–937.

Argyropoulos D.S., Menachem S.B. (1997). Lignin. In: Eriksson K.-E.L. (ed.) *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlag, Germany, pp.127-158.

Aryantha,I.P., Cross,P., Guest, D.I. (2000). Suppression of *Phytopthora cinnamoni* in potting mixes amended with uncomposted animal manures. *Phytopathology* **90:** 775-779.

Asadchii, O.G., Pereverzev, A.N., Zakharova, Z.G. and Gladyshev, V.P. (1986). Development of formulations for wax compositions for paperboard manufacture. Tekhnologiya Topliv I Masei **1:** 32-33.

Atiyeh, R.M.,Subier, S. Edwards, C.A., and Bachman, C. (2004). Effect of vermicompost and composts as potting media for growth of tomatoes. *Journal of Plant Nutrition* **6:** 1107-1123.

Ball A.S., Betts W.B., McCarthy A.J. (1989). Degradation of lignin-related compounds by actinomycetes. *Applied and Environmental Microbiology*. **55:** 1642-1644.

Barry, T., 2004. Evaluation of the economic, social, and biological feasibility of bioconverting food wastes with the black soldier fly (*Hermetia illucens*). PhD Dissertation, University of Texas, August 2004, 176 pp.

Bastioli, C. (1997). Properties and applications of Mater-Bi starch-based materials. Polymer Degradation and Stability **59:** 263-272.

BBC News, Germany: Ten die from E.coli-infected cucumbers. Web site: http://www.bbc.co.uk/news/world-europe-13587182. Accessed October 22, 2011.

Beauchamp, E.G. (1983). Response of corn to nitrogen in pre-plant and side dressing applications of liquid manure. *Canadian Journal of Soil Science* **63:** 377-386.

Beever R.E, Bollard E.G., (1970). The nature of the stimulation of fungal growth by potato extract, *Journal of General Microbiology* **60:** 273.

Bertone, M., W. Watson, M. Stringham, J. Green, S. Washburn, M. Poore and M. Hucks (2004). Dung beetles of Central and Eastern North Carolina cattle pastures. NC State University Extension.
http://www.cals.ncsu.edu/entomology/guidetoncdungbeetles.pdf. Accessed October 17, 2011.

Blanchette, R.A. (1995). Degradation of lignocellulose complex in wood. *Canadian Journal of Botany.* **73:** S999-S1010.

Boman, H. G. and D. Hultmark (1987). Cell-free immunity in insects. *Annual Review of Microbiology*, **41:** 103-126.

Borneman, J. and Hartin, R. J., (2000). PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and Environmental Microbiology* **66:** 4356-4360.

Braff, M. H. and R. L. Gallo (2006). Antimicrobial peptides: an essential component of the skin defensive barrier. Curr*ent Topics in Microbiolgy Immunology*. **306:** 91 – 110.

Pain, B. Creedy Associates and Jessica Buss (2007). Applying Manure. *British Grassland Society.* p. 1-2.

Bulet, P. and R. Stocklin. 2005. Insect Antimicrobial Peptides: Structures, Properties and Gene Regulation**.** *Protein and Peptide Letters*. **12** (1): 3-11.

Bulet, P., C. Hetru, J. L. Dimarcq and D. L. Hoffmann (1999). Antimicrobial peptides in insects; structure and function. *Developmental and Comparative Immunology*. **23:** 329- 344.

Bulet, P., R. Stocklin and L. Menin (2004). Anti-microbial peptides: from invertebrate to vertebrates. *Immunological Review*. **198:** 169- 184

Buswell, J.A. and Odier, E. (1987). Lignin biodegradation. CRC *Critical Reviews in Biotechnology* **6:** 1-60.

Carpenter, K. (2007). Problems of Liquid Cow Manure. Garden Guides**.** http://www.gardenguides.com/103803-problems-liquid-cow-manure.html. Accessed July 12, 2013.

Callaghan, F. J., D. A. J. Wase, K. Thayanithy and C. F. Forster (2002). Continous co-digestion of cattle slurry with fruit and vegetable wastes and chicken manure. *Biomass and Bioenergy* **1:**71-77.

Centers for Disease Control and Prevention (CDC). 1600 Clifton Rd., NE, Atlanta, GA 30333. Web site: www.cdc.gov. Accessed October 20, 2011.

Chen,Y., Inbar,Y., Harder,Y. (1988). Composted agricultural wastes as potting composts for ornamental plants. *Soil Science* **145:** 298-302.

Chong,C. and Hamersma, B. (1995). Growing plants with recycled waxed card. *Biocyle* **36:** 86-87.

Choudhary, M., Bailey, L.D., Grant, C.A. (1996). Review of the use of swine manure in crop production. *Waste Management Research* **14:** 581-595.

Cotter, P. D., C. Hill and R. P. Ross (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*. **3:** 777-18.

Cytrynska M., P. Mak, A. Zdybicka-Barabas, P. Suder and T. Jakubowicz (2007). Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph. *Peptides*. **28:** 533–546.

Davie, I.N., Winter, J.P. and Varoney, R.P. (1994). Decomposition of coated papers from a quick-service restaurant. *Tappi Journal*. **78**(5)**:** 127-130.

Davis, G. and Song, J.H. (2005). Biodegradable Packaging Based on Raw Materials from Crops and Their Impact on Waste Management, *Industrial Crops and Products,* **23**(2)**:** 147–161.

DEFRA, (2002). The First Annual Report of the Government—Industry Forum on Non-Food Use of Crops (PB7470). Department of Food, Environment and Rural Affairs, DEFRA Publications, Admail 6000, London, August (www.defra.gov.uk/farm/acu/).

DEFRA, (2004). A Strategy for Non-Food Crops and Uses—Creating Value From Renewable Materials (PB10188). Department of Food, Environment and Rural Affairs, DEFRA Publications, Admail 6000, London, October (www.defra.gov.uk).

Dickey, Elbert C. and Bodman, Gerald R. Management of Feedlot Runoff Control System (1992). *Biological Systems Engineering: Papers and Publications.* Paper 262.

Diener, S.; Zurbrügg, C.; Roa Gutiérrez, F.; Nguyen Dang Hong; Morel, A.; Koottatep, T. ; Tockner, K. (2011). Black soldier fly larvae for organic waste treatment – prospects and constraints. WasteSafe 2011 – 2nd Int. Conf. on Solid Waste Management in the Developing Countries, 13-15 February 2011, Khulna, Bangladesh, 52-59.

Diener, S., Zurbrugg, C. Gutierrez, F.R. (2011). Black soldier fly larvae for organic waste treatment. *Proceedings of the Waste, 2011, Meeting*, Bangledesh **52:** 1-8.

Dimopoulos, G., A. Richman, H. M. Muller and F. C. Kafatos (1997). Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences* USA. **94:** 11508 - 11513.

Dnag, X. L., J. H. Tian, H. Y. Yi, W. X. Wang, M. Zhengi, Y. Fengli, Y. Cao and S. Y. Wen (2006). Inducing and isolation of antibacterial peptides from oriental fruit fly, Bactrocera dorsalis Hendel. *Insect Science*. **13:** 257-262.

Dusch, H. and Altwegg, M. (1995). Evaluation of five new plating media for isolation of *Salmonella* species. *Journal of Clinical Microbiology* **33:** 802-804.

Edimeades, D.C. (2003). The long term effects of organic fertilisers on soil productivity and quality: a review. *Nutrient Cycling in Agroecosystems* **66:** 165-180.

Ekengren, S. and D. Hultmark (1999). Drosophila cecropin as an antifungal agent. *Insect Biochemistry and Molecular Biology*. **29:** 965- 972.

Erickson, M.C., M. Islam, C. Sheppard, J. Liao and M. P. Doyle (2004). Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Enteritidis in chicken manure by Larvae of the Black Soldier Fly. *Journal of Food Protection*. **67:** 685-690.

Eriksson K.-E.L., Blanchette R.A., Ander P. (1990). *Microbial and Enzymatic Degradation of Wood and Wood Components.* Springer-Verlag, Berlin, Germany.

Evanylo, G., C. Sherony, J. Spargo, D. Starner, M. Brosius and K. Haering. (2008). Soil and water environmental effects of fertilizer, manure, and compost-based fertility practices in an organic vegetable cropping system. *Agriculture, Ecosystems and Environmen*t. **127:** 50-58.

Fabre, J.H. (1911). *The Life and Love of the Insect*. A and C Black, London.

Forssell, P., Mikkila, J., Suortti, T., Seppala, J., Poutanen, K. (1996). Plasticization of barley starch with glycerol and water. J. Macromol. *Pure and Applied Chemistry*. A**33:** 703-715.

Fredericks, L. P. 2001. Isolation and characterization of an antimicrobial peptide from Plethodon cinereus. PhD dissertation, Graduate Faculty of the University of Louisiana at Lafayette, USA. 165.

Gannon, V.P.J. *(*2004*)* Control of zoonotic waterborne pathogens in animal reservoirs. *In Waterborne Zoonoses: Identification, Causes and Control ed.* Cotruvo, J.A*, Dufour, A.,* Rees, G.*,* Bartram, J.*,* Carr, R.*,* Cliver, D.O*,* Craun, G.F*,* Fayer, R. *and* Gannon, V.P.J. *pp.* 380–408. *London, UK: IWA Publishing.*

Garedew, E. Schmolz, I. Lamprecht (2004). Effect of the bee glue (propolis) on the calorimetrically measured metabolic rate and metamorphosis of the greater wax moth *Galleria mellonella*. *Thermochimica Acta* **413:** 63–72.

Goddard, R. (1997). Packaging 2004. Pira International.

Godden B., Ball A.S., Helvenstein P., McCarthy A.J., Penninckx M.J. (1992). Towards elucidation of the lignin degradation pathway in actinomycetes. *Journal of General Microbiology*. **138:** 2441-2448.

Gough, M., R. E. Hancock and N. M. Kelly (1996). Antiendotoxin activity of cationic peptide antimicrobial agents. *Infect Immun*. **64:** 4922 -4927.

Grabe, Albert (1942). Eigenartige Geschmacksrichtungen bei Kleinschmetterlingsraupen ["Strange tastes among micromoth caterpillars"]. *Zeitschrift des Wiener Entomologen-Vereins* **27**: 105-109.

Gregory, P. J. and Nortcliff, S., eds. (2013) *Soil conditions and plant growth. 12th edition*. Wiley-Blackwell, Chichester, pp461. ISBN 9781405197700.

Gross, J., A. Muller, Vilcinskas, Hilker (1998). Antimicrobial activity of exocrine glandular secretions, hemolymph, and larval regurgitate of the mustard leaf beetle phaedon cochleariae . *Journal of Invertebrate Pathology*. **72** (3):296-303 .

Gross, J., A. Eben, I. Müller and A. Wensing A. (2010). A well protected intruder: the effective antimicrobial defense of the invasive ladybird Harmonia axyridis. *Journal of Chemical Ecology.* **36**(11)**:**1180-8.

Hammond, P. M. (1992). "Species inventory", *Global Biodiversity, Status of the Earth's Living Resources*, B. Groombridge. Chapman and Hall, London. 17–39.

Hansen, W. and Yourassowsky, E. (1984). Detection of beta-glucuronidase in lactose-fermenting members of the family Enterobacteriaceae and its presence in bacterial urine cultures. *Journal of Clinical Microbiology* **20:** 1177.

Haque, K., Pfeiffer, R., Beerman, M., Struewing, J., Chanock, S. and Bergen, A., (2003). Performance of high-throughput DNA quantification methods. *BioMed Central Biotechnology* **3:** 1-10.

Hardouin, J.; Mahoux, G. (2003). Zootechnie d'insectes – Elevage et utilisation au bénéfice de l'homme et de certains animaux. Bureau pour l'Echange et la Distribution de l'Information sur le Mini-élevage (BEDIM), 164 p.

Haritos, V.S., French, J.R.J., Ahokas, J.T. (1993) The metabolism and comparative elimination of polychlorinated biphenyl congeners in termites. *Chemosphere*, **26:** 1291-1299.

Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiology Reviews* **13:** 125-135.

Hatakka, A. (2001) Biodegradation of lignin. In: Hofrichter M. and Steinbuchel A. (eds.) Biopolymers: Biology, Chemistry, Biotechnology, Applications, Lignin, humic Substances and Coal (vol.1). Wiley VCH, Weinheim, pp. 129-180.

Hesse, P.R. (1971). *A Texbook of Soil Chemical Ananylsis,* J. Murray, London.

Hoffmann, J. A. (1995). Innate immunity of insects. *Current Opinion in Immunology*. **7:** 4-10.

Hoffmann, J. A. and J. M. Reichhart. 2002. Drosophila innate immunity: an evolutionary perspective. *Nature Immunity*. **3:** 121- 126.

Hoffmann, J. A., J. M. Reichhart and C. Hetru (1996). Innate immunity in higher insects. *Current Opinion in Immunology*. **8:** 8- 13.

Hunt, T. (2007). "A Comprehensive Phylogeny of Beetles Reveals the Evolutionary Origins of a Super radiation". *Science* **318** (5858): 1913–1916.

Huberman, L., Gollop, N., Mumcuoglu, K., Block, C. and Galun, R. (2007). Antibacterial properties of whole body extracts and haemolymph of *Lucillia sericata* maggots. *Journal of Wound Care* **16:** 123-127.

Izadpanah, A. and R. L. Gallo (2005). Antimicrobial peptides *Journal of American Academy of Dermatology.* **52(3):** 381 - 390.

Kuan,Z.U., Ahmad, S.Brazda, A. And Chnady,B. (2009). *Mucor circinelloides* as a cause of invasive maxillofacial zygomycosis: an emerging dimorphic pathogen with reduced susceptibility to posacanozle. *Journal of Clinical Microbiology*. **47:** 1244-1246.

Kinoshita, T., and K. Inoue (1977). Bactericidal Activity of the Normal, Cell-Free Hemolymph of Silkworms (Bombyx mori). *Infection and Immunity*. **16:** 32-36.

Kirk, J. H. (2011). Patogens in Manure.http://www.vetmed.ucdavis.edu/vetext/INF-DA/pathog-manure.pdf. Accessed on October 7, 2011.

Kirk, T.K. and Farrell, R.L. (1987). Enzymatic combustion: the microbial degradation of lignin. *Annual Review Microbiology* **41:** 465-505.

Klingbeil, M. (2000). Working Document of Biodegradable Waste Management. European Commission, Brussels.

Knobeloch L, Salna B, Hogan A, Postle J, Anderson H. (2000). Blue babies and nitrate-contaminated well water. *Environmental health perspectives* **108:** 675– 678.

Kroeckel, S. ; Harjes, A. G. E. ; Roth, I. ; Katz, H. ; Wuertz, S. ; Susenbeth, A. ; Schulz, C. (2012). When a turbot catches a fly: evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute - growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture, **364/365:** 345-352.

Kruszewska, D., H. G. Sahl, G. Bierbaum, U. Pag, S. O. Hynes and A. Ljungh (2004). Mersacidin eradicates methicillin-resistant Staphylococcus aureus (MRSA) in a mouse rhinitis model. *Journal of Antimicrobial Chemotherapy*. **54:** 648 - 653.

Kuhad, R.C., Singh, A. and Eriksson, K.E.L. (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In: Eriksson, K.E.L. (ed.), *Advances in Biochemical Engineering/ Biotechnology*, vol. 57. Springer, Berlin, Germany, p. 46- 125.

Lamberty, M., D. Zachary, R. Lanot, C. Bordereau, A. Robert, J. A. Hoffmann and P. Bulet (2001). Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. *The Journal of Biological Chemistry* **276:** 4085 - 4092.

Lapage, S. P., Shelton J. E. and Mitchell T. G. (1970) *in Methods in Microbiology' Eds. Norris J. R. and Ribbons D. W. Vol.3A. Academic Press. London. p.* 116.

Lapis, K. (2008). Physiologic and pathophysiologic significance of antimicrobial (host defensive) small peptides. *Orvosi Hetilap* **49**(51)**:** 2419 - 2424.

Lavine, M. D. and M. R. Strand (2002). Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*. **32:** 1295- 1309.

Leclercq, M. (1997). A propos de *Hermetia illucens* (Linnaeus, 1758) (Soldier fly) (Diptera Stratiomyidae: Hermetiinae). *Bulletin et Annales de la Société royale belge d'Entomologie,* **133:** 275-282.

Leppanen-Turkula, A. (1999). Association of Packaging Technology and Research. Personal communication.

Leto,J., Percujila, G. and Bosnjak, K. (2007). Effect of liquid manure application on yield, quality and botanical composition of grassland. *Cereal Research* **36:** 637-640.

Li, Q., Zheng, L., Qiu, N., Cai, H., Tomberlin, J. K. and Yu, Z. (2011). Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production. *Waste Management* **31**, 1316-1320.

Linstead, C., Ekins, P. (2001). Mass Balance UK, Mapping UK Resource and Material Flows. *Royal Society for Natural Conservation*, p. 12

Locking, M., L. Browning., A. Smith-Palmer., J. Cowden, and S. Brownlie. (2005). Gastro-intestinal and foodborne infections. HPS Wkly. Rep. **39:** 2-3. [Online.] http://www.show.scot.nhs.uk/scieh/PDF/pdf2005/0501.pdf

Lussenhop, J., R. Kumar, D. T. Wicklow and J. E. Lloyd. (1980). Insect effects on bacteria and fungi in cattle dung. *Oikos* **34:** 54-58.

McEwen, J.G.E. (1992). Progress In Paper Recycling, Wax Contamination In *Paper Recycling: Understanding Wax Chemistry and Recycling Waxed Papers*. **1**(2):11.

Meister, M., B. Lemaitre and J. A. Hoffmann. (1997). Antimicrobial peptide defense in Drosophila. *Bioessays*. **19:** 1019- 1026.

Miller, R. G. and Tate, C. R. (1990). XLT4: A highly selective plating medium for the isolation of *Salmonella*. *Maryland Poultryman*, April p. 2-7.

110

Mishra, K. N., Aaggarwal, A., Abdelhadi, E. and Srivastava, P. C., (2010). An efficient Horizontal and Vertical Method for Online DNA sequence Compression. *International Journal of Computer Applications* **3:** 39-46.

Miyashita, M., A. Sakai, N. Matsushita, Y. Hanai, Y. Nakagawa and H. Miyagawa. (2010). A novel amphipathiclinear peptide with both insect toxicity and antimicrobial activity from the venom of the scorpion isometrus maculatus. *Bioscience, Biotechnology, and Biochemistry*. **74:** 364 - 369.

Muhammad, S. and N. A. Amusa. (2003). In-vitro inhibition of growth of some seedling blight inducing pathogens by compost-inhabiting microbes**.** *African Journal of Biotechnology* **2:** 161–164.

Oizumi, Y., H. Hemmi, M. Minami, A. Asaoka and M. Yamakawa. (2005). Isolation, gene expression and solution structure of a novel moricin analogue, antibacterial peptide from a lepidopteran insect, *Spodoptera litura. Biochimica et Biophysica Acta*. **1752:** 83- 92.

Ouellette, A. J. and M. E. Selsted. (1996). Paneth cell defensins: endogenous peptide components of intestinal host defense. *FASEB Journal*. **10:** 1280 - 1289.

Parkinson, S. M., Wainwright, M. and Killham, K., (1989). Observations on oligotrophic growth of fungi on silica gel. *Mycological Research* **93:** 529-534.

Peng, R., Z. Yang, K. Liu, H. Yao, H. Yang, Y. Cui and H. Hong. (2008). Induction, selection and antibacterial activity of the antibacterial peptides from lepidopteran insect cultured cell lines. *Frontiers of Biology in China*. **3**(2): 203 - 206.

Pennington, J. A., K. Van Devender, J. A. Jennings. Nutrient and Fertilizer Value of
Dairy Manure. Agriculture ans Natural Resourses University Of Arkansas.
FSA 4017. http://www.uaex.edu/Other_Areas/publications/PDF/FSA-
4017.pdf. Accessed October 7, 2011.

Putsep, K., G. Carlsson, H. G. Boman and M. Andersson (2002). Deficiency of
antibacterial peptides in patients with morbus Kostmann: an observation study.
Lancet **360:** 1144 - 1149.

Philips, C. A. (1999). The epidemiology, detection and control of *Escherichia coli*
O157. *Journal of the Science of Food and Agriculture*. **79:**367–1381.

Popil, R.E, and Joyce, M. K. (2008). Strategies for economical alternatives for wax
replacement in packaging. *Tappi Journal*. **4**:11-18.

Rabeeth, M., M. Muthukumar, M. B. Rajkumar and H. K. Kumar. (2012). Isolation,
evaluation and purification of antibacterial peptides from rhinoceros beetle,
Oryctes rhinoceros (L.) *GERF bulletin of biosciences*. **3**(2)**:**16-22.

Rangel, J. M., P. H. Sparling., C. Crowe., P. M. Griffin and D. L. Swerdlow (2005).
Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-
2002. *Emerging Infectious Diseases*. **11:** 603–609.

Reddy, V. M. R. 1978. Studies on the white muscardine disease of silkworm, *Bombyx
mori,* L. M.Sc. (Agri) *Thesis,* University of Agriculture Sciences, Bangalore, p.
93.

Reddy, K. V. R., R. D. Yedery and C. Aranha. (2004). Antimicrobial peptides:
premises and promises. *International Journal of Antimicrobial Agents*. **24:** 536
- 547.

Richardson, P. and Richardson, R. (2000). Dung beetles and their effects on soil. Ecological Restoration **18:**116-117. URL: managingwholes.com/dung-beetles.htm. Accessed on October 7, 2011.

Rigout, J. (1989). The Beetles of the World, (9), *Sciences Nat*, Venette.

Rossen, C. J., and P. M. Bierman (2005). Nutrinet management for fruit and vegetable crop production: Using manure and compost as nutrient sources for vegetable crops. University of Minnesota Extension Service. *www.extension.umn.edu/.../M1192.pdf*. Accessed October 17, 2011.

Sahalan, A. Z., Omar, B., Mohamed, A. Y. and Jeffery, J. (2006). Antibacterial activity of extracted Hemolymph from larvae and pupae of local fly species *Musca domestica* and *Chrysomya megacephala*. *Journal Sains Kesihatan Malaysia* **4:** 1-11.

Saito, A., K. Ueda, M. Imamura, N. Miura, S. Atsumi, H. Tabunoki and R. Sato (2004). Purification and cDNA cloning of a novel antibacterial peptide with a cysteine-stabilized αβ motif from the longicorn beetle, Acalolepta luxuriosa. *Developmental and Comparative Immunology* 28**:** 1- 7.

Shanks, O.C., C.A. Kelty, S. Archibeque, M. Jenkins, R.J. Newton, S.L. McLellan, S.M. Huse, and M.L. Sogin (2011). Community structure of cattle fecal bacteria from different animal feeding operations. *Applied and Environmental Microbiology.* **77:**2992-3001.

Severtson, D. (2006) Bioconversion of waste paper by termites: A landfill of opportunity. Thesis for the degree of Honours in the Bachelor of Science (Environmental Biology), Department of Environmental Biology, Curtin University of Technology, Perth, Western Australia.

Sinton, L. W., R. R. Braithwaite, C. H. Hall, and M. L. Mackenzie. 2007. Survival of indicator and pathogenic bacteria in bovine feces on pasture. *Applied and Environmental Microbiology*. **73**:7917-7925.

Smis, J.R. and Jackson, G.D. (1971) Rapid analysis of soil nitrate with chromic acid. *Soil Science Society of America Proceedings*, **35:** 603–606.

Sonnevend, A., F. C. Knoop, M. Patel T. Pal, A. M. Soto and J. M. Conlon. (2004). Antimicrobial properties of the frog skin peptide, ranatuerin-1 and its [Lys-8]-substituted analog. *Peptides*. **25:** 29- 36.

Steinhaus, E. A. (1949). Principles of Insect Pathology. McGraw Hill Publications, New york. p. 757.

Tauxe, R. V. (1997). Emerging foodborne diseases: an evolving public health challenge. *Emerging Infectious Diseases*. **3:** 425–434

Tuomela, M., M. Hatakka, V. A. and Itavaara, M. (2000). Biodegradation of lignin in a compost environment: a review. Bioresource Technology. **72:** 169-183.

United States Pharmacopeia XXIII, Chapter "Microbial Limit Tests" (1995).

US. Food and Drug Administration, Bacteriological Analytical Manual, 8th ed., AOAC, Arlington, VA. (1995).

van Huis, A. ; Van Itterbeeck, J. ; Klunder, H. ; Mertens, E. ; Halloran, A. ; Muir, G. ; Vantomme, P. (2013). Edible insects - Future prospects for food and feed security. *FAO Forestry Paper* 171.

Virtanen, Y. and Nilsson, S. (1993). *Environmental Impact of Waste Paper Recycling*, International Institute for Applied Systems Analysis. Earthscan Publications: London.

Vikman, M., Itavaara, M. and Poutanen, K. (1995) Measurement of the biodegradation of starch-based materials by enzymatic methods and composting. *Journal of Environmental Polymer Degradation* **3:** 23-29.

Wainwright, M. & Pugh, G. J. F. (1973). The effect of three fungicides on nitrification and ammonification in soil. *Soil Biology and Biochemistry*, **5:** 577-584.

Waite, R. (1995). Household Waste Recycling. Earthscan Publications, London.

Wang, L., K. R. Mankin and G. L. Marchin (2004). Survival of faecal bacteria in Dairy cow manure. *Transactions of the ASAE. .* **47:** 1239-1246.

Wasteline, (January 2002). Information Sheet on Packaging.Wastewatch, UK.

Webb J, Pain B, Bittman S, Morgan J. (2010). The impacts of manure application methods on emissions of ammonia, nitrous oxide and on crop response – A review. *Agriculture Ecosystems and Environment* **137:** 39-46.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J., (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173:** 697-703.

Westover, H.L. (1926) *Farm Manures* p. 193-196.

Whiting, M. F. (2002). Phylogeny of the holometabolous insect orders: molecular evidence. *Zoologica Scripta* **31** (1)**:** 3–15.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, **415:** 389- 395.

Appendices

Appendix one:

1. Nutrients standard curves

1.1 Standard phosphate solution (Hesse, 1971)

0.4393g of potassium dihydrogen (KH_2PO_4) was weights into a litre volumetric flask. This solution was diluted with distilled water to produce 10µg , 20 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg and 100 µg. The control was distilled water without KH_2PO_4.

1 vol. of ascorbic acid was mixed with 6 vol. of ammonium molybdate. The 0.7 ml of working solution was added to 0.3 ml of sample and incubated at 37oC for 1 hour and the blue colour read at 820 nm (Fig 1.1).
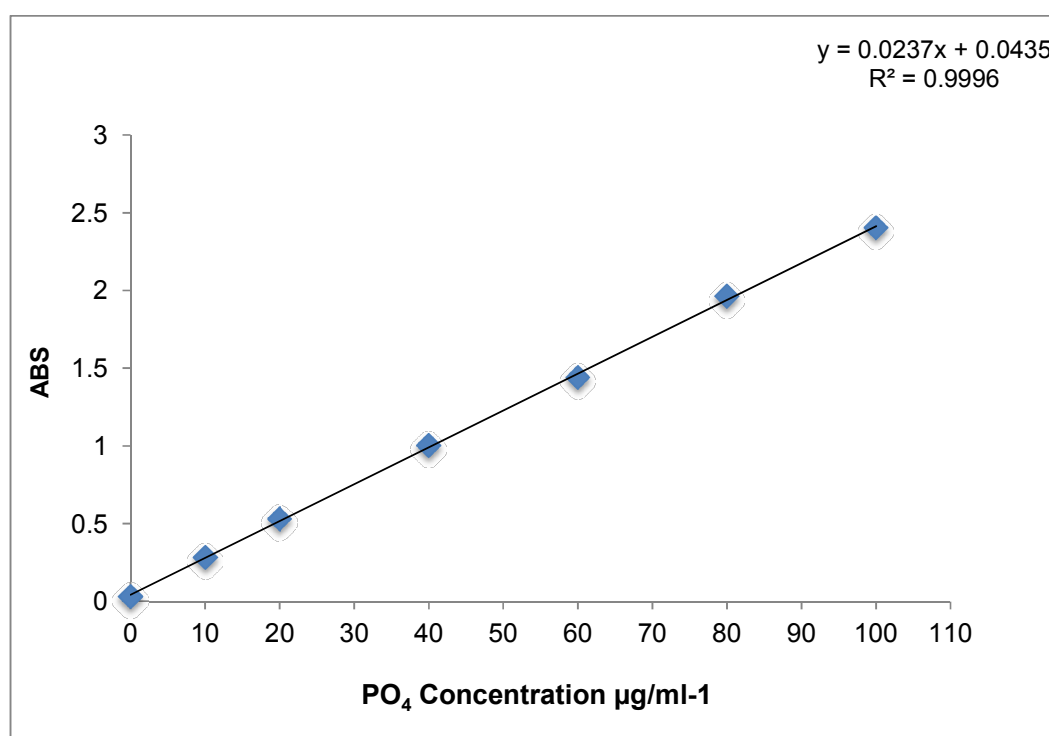
Figure 1.1: Phosphate standard curve.

1.2 Standard sulphate-s solution (Hesse, 1971)

1.47 g of (Na_2SO_4) were dissolved in 1000 ml of distilled water to give a concentration of 1000 µg/ SO_4^{-2}-S ml^{-1} which was diluted 10 times, sodium sulphate solution (10 ml) with of distilled water (90 ml) =100 µ g/ SO_4^{-2}-S ml^{-1}.

5ml of the previous solution was added to 1g barium chloride ($BaCl_2$) and 2ml of gum acacia (0.25%w/v), mixed well and then the volume was made up to 25ml with distilled water. The white suspension resulting from precipitation of sulphate was measured at 470 nm (Fig 1.2).
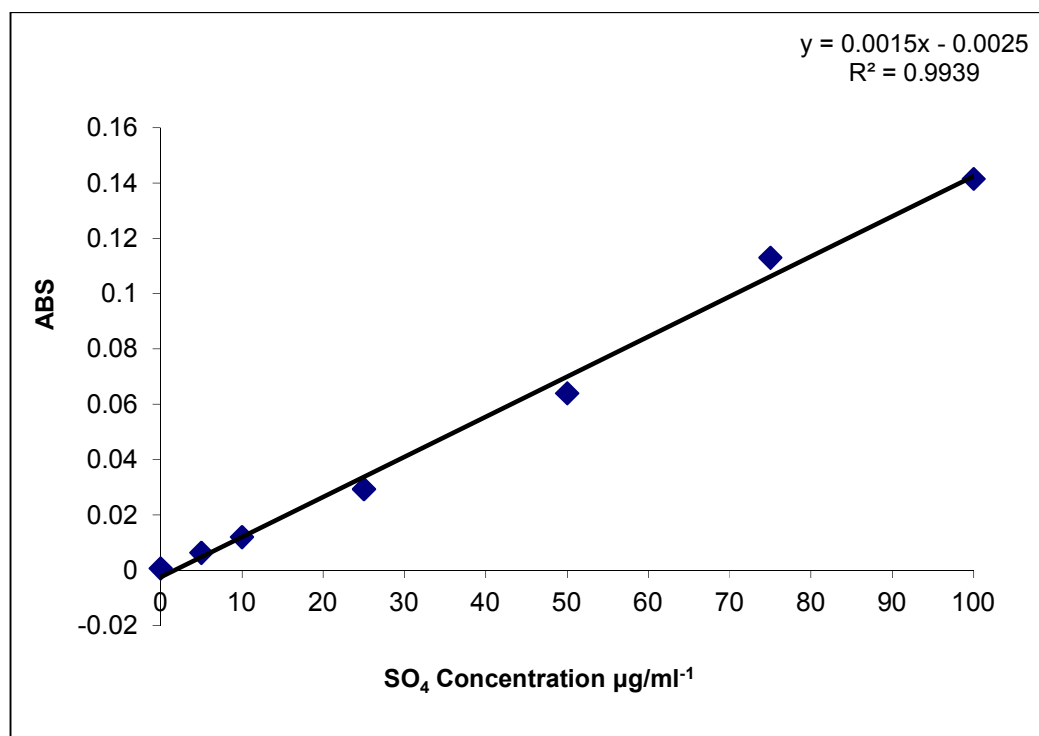
Figure 1.2: Sulphate standard curve.

1.3 Standard ammonium solution (Wainwright and Pugh, 1973)

3.66 g of $(NH_4)_2SO_4$ were dissolved in 1000 ml of distilled water and then diluted 10 times (10 ml of ammonium solution with 90ml distilled water) = $100\mu g/NH_4^+$-N $ml^{-1.}$ 2ml of the previous solution was added to 1ml of EDTA (6%w/v), 7ml of distilled water, 5ml of phenolate reagent and 3ml of sodium hypochlorite solution (10%v/v). The reaction mixture was mixed thoroughly and incubated at 25^oC for 20min in the dark. The volume was made up to 50ml and mixed and the concentration of the indophenol-blue complex was measured at 630 nm (Fig 1.3).
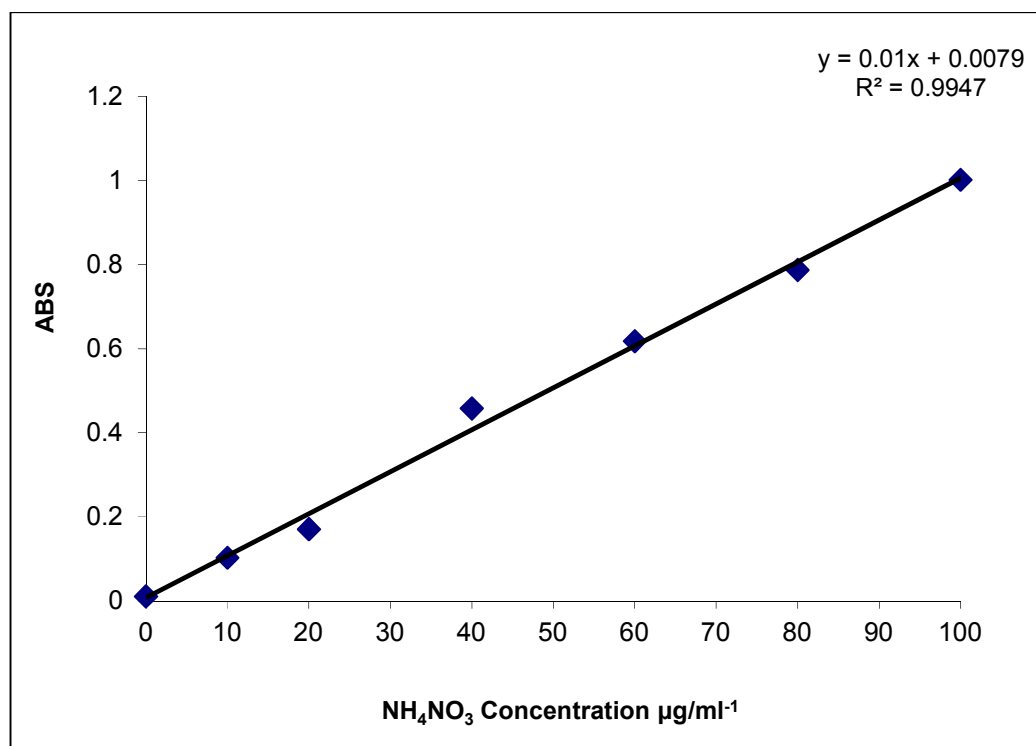
Figure1.3: Ammonium standard curve.

1.4 Standard Nitrate solution (Sims and Jackson, 1971)

0.137g of sodium nitrate (NaN_{O3}) was dissolved in 100 ml of distilled water. The resulting solution is 1mg nitrate ($NaNO_3$) per ml. The solution was pipetted into 6 tubes following volumes of the standard solution: 1µl=1µg nitrate (0µl, 10µl, 20µl, 40µl, 80µl, 100µl). Filtrate (3ml) was mixed into 7ml chromotropic acid (working solution) and then incubated in a water bath for 45 min in $40^{o}C$. The yellow colour formed was measured at 41 nm using a spectrophotometer and the concentration of nitrate was determined by reference to a standard curve of nitrate concentration (Fig 1.4).
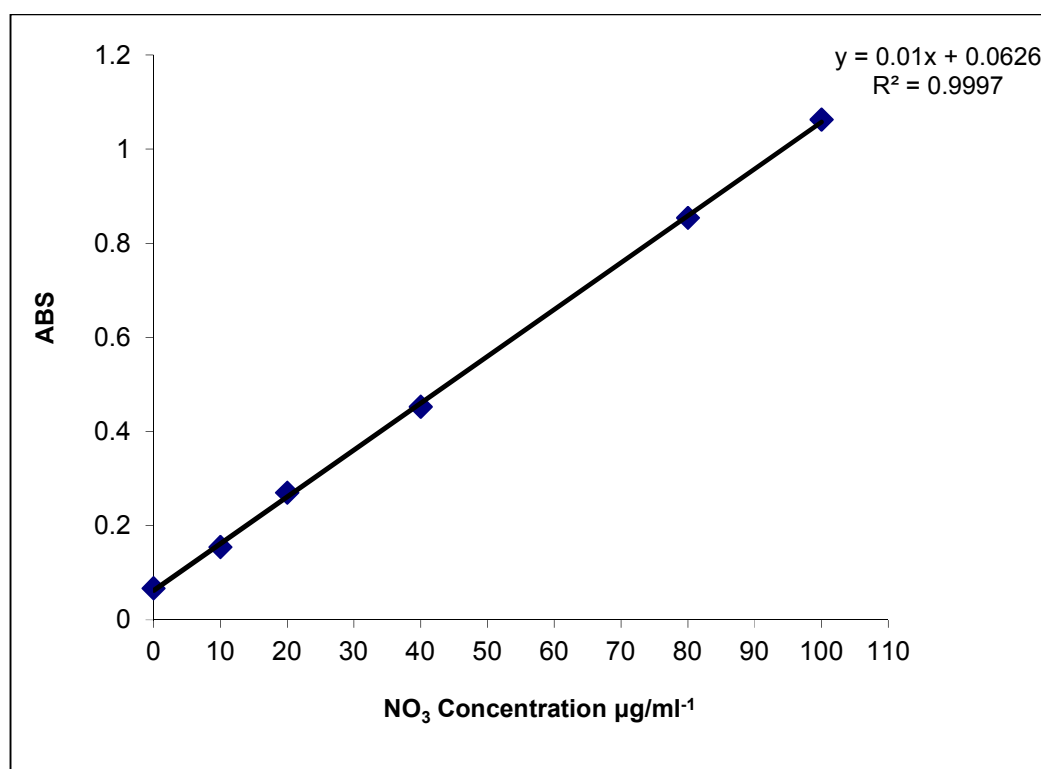
Figure1.4: Nitrate standard curve.

Appendix two:

1. HiCrome (TM) *E. coli* Agar B

HiCrome *E.coli* Agar B is recommended for the detection and enumeration of *Escherichia coli* in foods without further confirmation on membrane filter or by indole reagent.

Table 1: Composition of HiCrome (TM) *E. coli* Agar B

Ingredients	Grams/Litre
Casein enzymic hydrolysate	20.0
Bile salts mixture	1.5
X-Glucuronide	0.075
Agar	15.0

Directions:

36.6g was suspended per litre distilled water. The Sterilize solution was by autoclaving at 121°C for 15 minutes. It was cooled to 50°C and poured into sterile Petri dishes.

Table 2: Culture characterisation after 18-24 hours at 44°C.

Organisms (ATCC)	Growth	Colour of colony
Escherichia coli (25922)	luxuriant	blue
Klebsiella pneumoniae (13883)	luxuriant	colourless, mucoid
Salmonella enteritidis (13076)	luxuriant	colourless
Staphylococcus aureus (25923)	inhibited	

Anderson and Baird-Parker, (1975); Hansen and Yourassawsky, (1984).
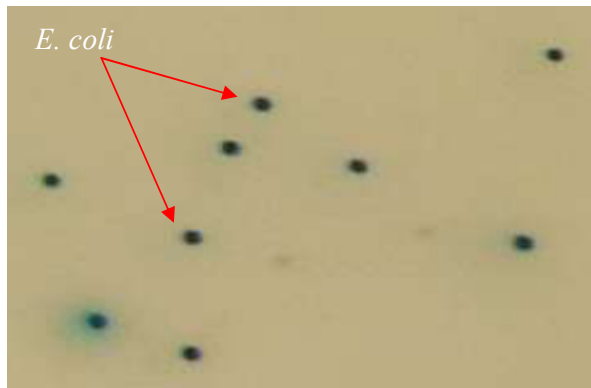
Figure 1: *E. coli* on HiCrome agar / Author's photo

2. Nutrient Agar (Oxoid)

A general purpose medium which may be enriched with up to 10% blood or other biological fluids.

Table 3: Composition of Nutrient Agar.

Typical Formula	g/litre
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.4 ± 0.2 @ 25°C	

Directions

The medium was prepared by suspending 28g in litre of distilled water, boiled dissolve completely. Sterilised by autoclaving at l2l°C for 15 minutes.

Table 4: Quality control.

Positive controls:	Expected results
Staphylococcus aureus ATCC[®] 25923 *	Good growth; straw/white colonies
Escherichia coli ATCC[®] 25922 *	Good growth; straw colonies
Negative control:	
Uninoculated medium	No change

(Lapage *et al.*, 1970)

3. Plate Count Agar (Oxoid)

The medium was prepared by suspending 17.5g in 1000 ml of distilled water, boiling to dissolve completely, pH 7.0 and sterilising by autoclaving at 121^oC for 15 minutes. Silica gel medium (Parkinson *et a1.*, 1989).

Table 5: Composition Plate Count Agar

Typical Formula	g/litre
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar	9.0
pH 7.0 ± 0.2 @ 25°C	

Directions

17.5g was added to 1000 ml of distilled water. It was dissolved by bringing to the boil with frequent stirring, then mixed and distributed into final containers. It was sterilised by autoclaving at 121°C for 15 minutes.

Table 6: Quality control.

Positive control:	Expected results
Escherichia coli ATCC® 25922 *	Good growth; straw coloured colonies
Negative control:	
Uninoculated plate	No change

4. a. XLT-4 AGAR / Oxoid

A highly selective medium for isolation and identification of *Salmonella Spp* from clinical, environmental and food samples.

Table 7: Composition of XLT-4 Agar.

Typical Formula	gm/litre
Proteose Peptone	1.6
Yeast extract	3.0
Lysine	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Ferric ammonium citrate	0.8
Sodium thiosulphate	6.8
Sodium chloride	5.0
Phenol Red	0.08
Agar	18.0
Final pH 7.4 ± 0.2 @ 25°C	

Directions

59g of XLT-4 Agar Base was suspended in 1000 ml of distilled water and 4.6ml of XLT-4 was added as a Selective Supplement, and brought to the boil.

Do not overheat, do not autoclave.

The medium was cooled to approximately 50°C and poured into sterile Petri dishes. It is not advised to hold the medium at 50°C for longer than 1 hour as this may cause the medium to precipitate.

4. b. XLT-4 SELECTIVE SUPPLEMENT.

Table 8: Composition of XLT-4 selective supplement

Supplement available in 100 ml (SR0237C)	per litre
Tergitol™ 4	4.6ml

Appearance

Dehydrated medium: straw coloured, free-flowing powder Prepared medium: clear red gel.

Table 9: Quality control

Positive control:	Expected results
Salmonella enteritidis ATCC®13076 *	Good growth: black or, red with black center
Negative contols:	
Escherichia coli ATCC®25922 *	Reduced growth, yellow
Enterococcus feacalis ATCC®29212 *	No grow

(Miller and Tate, 1990; Dusch and Altwegg, 1995).

Figure 2: *S. enterica* on XLT-4 agar / Author's photo.

5. POTATO DEXTROSE AGAR

Recommended for the isolation, and enumeration of yeasts and molds in dairy products and food. This culture medium complies with the recommendations of the APHA for food (1992), the USP (1995) and the FDA (1995).

Table 10: Composition of PAD medium.

Formula	gm/litre
Potato extract	4.0
Dextrose	20.0
Agar	15.0
pH 5.6 ± 0.2 @ 25°C	

Directions

39g was suspended in 1000 ml of water (purified as requested). It was brought to the boil to dissolve completely. It was sterilised by autoclaving at 121°C for 15 minutes. It was mixed well before pouring.

Appearance

Dehydrated medium: Off-white, free-flowing powder Prepared medium: Light straw coloured gel.

Table 11: Quality Control

Positive control:	Growth	Ascospore formation
Aspergillus niger (16404)	+++	-
Saccharomyces cerevisiae (9763)	+++	+
Penicillium comune (10428)	++	-
Candida albicans (10231)	+++	-

(R.E. Beever *at el* 1990). APHA, Washington, D.C. (1992), USP (1995) and the FDA (1995).
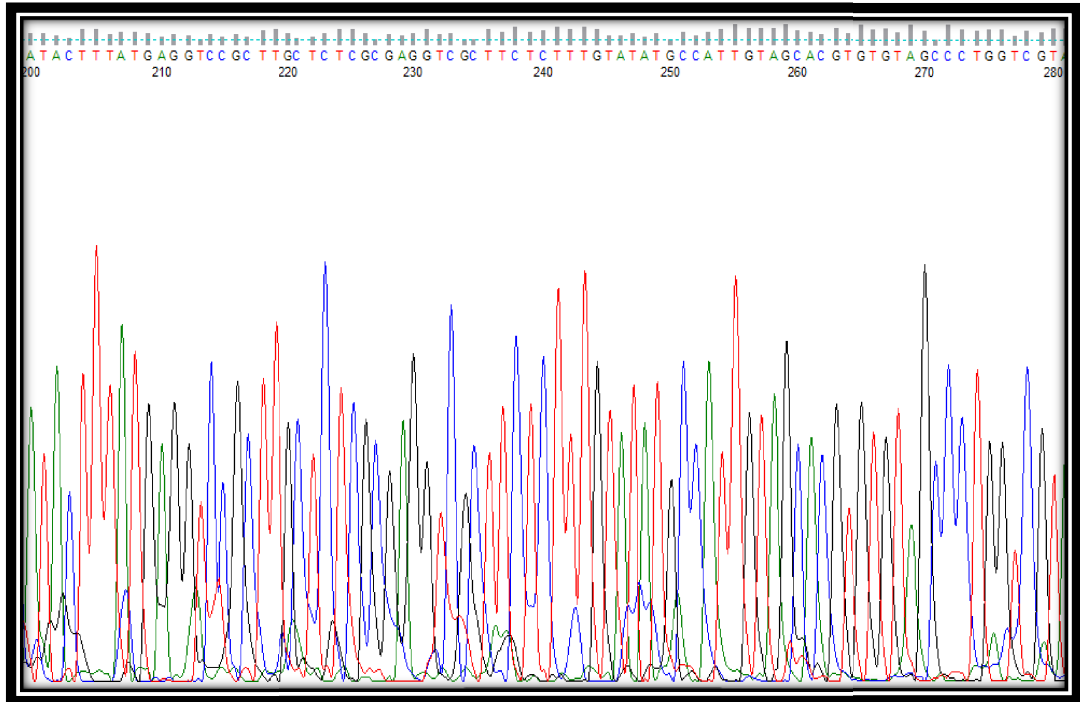
(*S. enterica* sequences shown by FinchTV software before BLASTn NCIMB database).

Escherichia coli O157:H7 str. Sakai strain Sakai 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074891.1| Length: 1542 Number of Matches: 1

Range 1: 1030 to 1193 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
303 bits(164)	5e-83	164/164(100%)	0/164(0%)	Plus/Minus

```
Query  1     CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1193  CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGAC  1134

Query  61    CGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1133  CGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAAC  1074

Query  121   ACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGA  164
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1073  ACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGA  1030
```
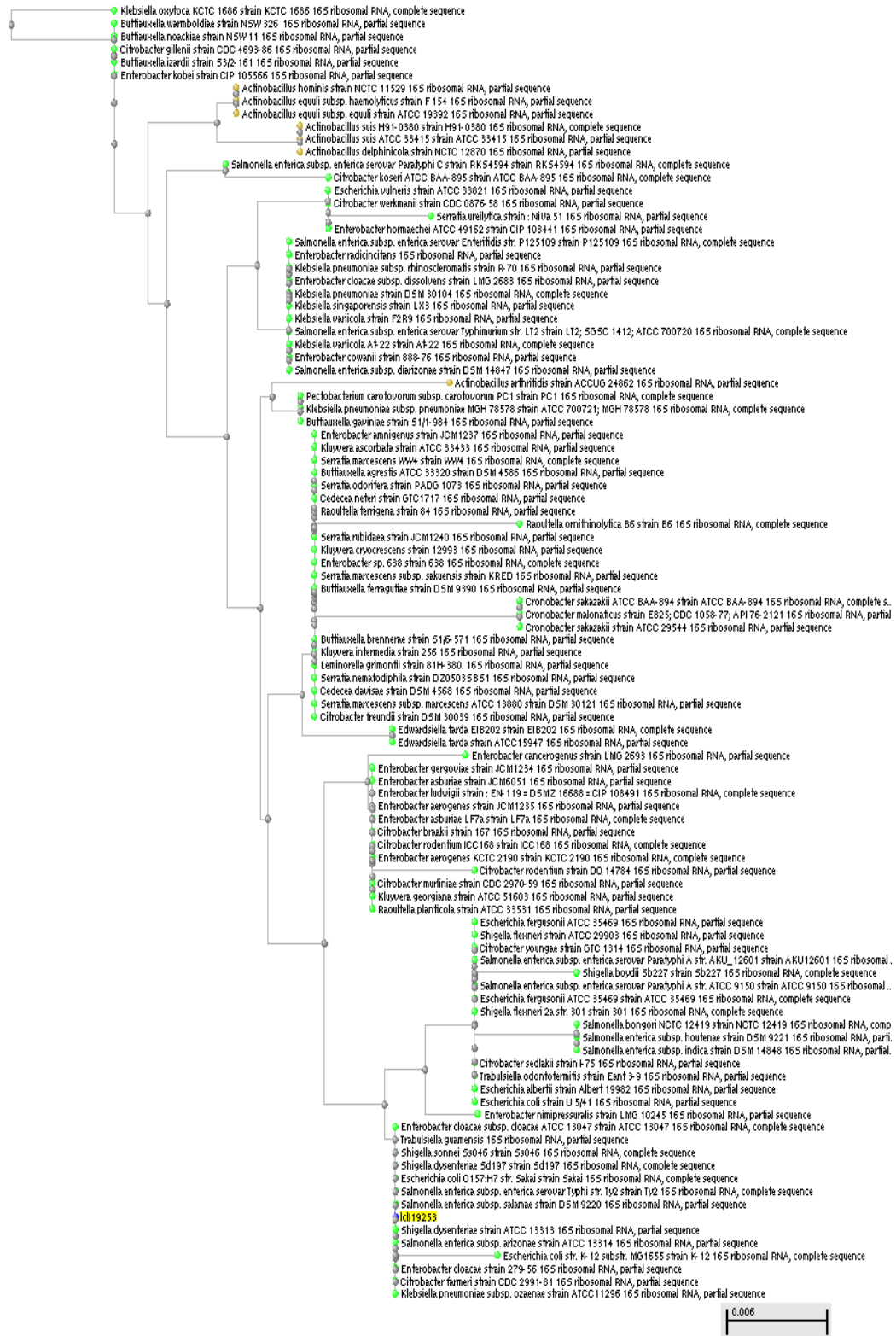
Tree view for *E. coli*.

Salmonella enterica subsp. enterica serovar Paratyphi A str. AKU_12601 strain AKU12601 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074935.1| Length: 1546 Number of Matches: 1

Range 1: 1132 to 1330 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
346 bits(187)	1e-95	195/199(98%)	0/199(0%)	Plus/Minus

```
Query  1     CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGC  60
             |||||||||||||||||||||||||||||||||||||||  |||||||||||||||||||
Sbjct  1330  CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGC  1271

Query  61    TCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTGGTCGTA  120
             |||||||||||||||||||||||||||  |||||||||||||||||||||||||||||||
Sbjct  1270  TCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTA  1211

Query  121   AGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1210  AGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCT  1151

Query  181   TTGAGTTCCCGGCCGGACC  199
             |||||||||||||||||||
Sbjct  1150  TTGAGTTCCCGGCCGGACC  1132
```

129

Tree view for *S. enterica*

Mucor circinelloides strain S2-3 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene.
Sequence ID: gb|JX537952.1| Length: 581 Number of Matches: 1

Range 1: 129 to 398 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
499 bits(270)	4e-138	270/270(100%)	0/270(0%)	Plus/Plus

```
Query   1    TAACCGAGTCATAGTCAAGCTTAGGCTTGGTATCCTATTATTATTTACCAAAAGAATTCA   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   129  TAACCGAGTCATAGTCAAGCTTAGGCTTGGTATCCTATTATTATTTACCAAAAGAATTCA   188

Query   61   GAATTAATATTGTAACATAGACCTAAAAAATCTATAAAACAACTTTTAACAACGGATCTC   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   189  GAATTAATATTGTAACATAGACCTAAAAAATCTATAAAACAACTTTTAACAACGGATCTC   248

Query   121  TTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATT   180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   249  TTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATT   308

Query   181  CAGTGAATCATCGAGTCTTTGAACGCAACTTGCGCTCATTGGTATTCCAATGAGCACGCC   240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   309  CAGTGAATCATCGAGTCTTTGAACGCAACTTGCGCTCATTGGTATTCCAATGAGCACGCC   368

Query   241  TGTTTCAGTATCAAAACAAACCCTCTATCC   270
             ||||||||||||||||||||||||||||||
Sbjct   369  TGTTTCAGTATCAAAACAAACCCTCTATCC   398
```

Penicillium roqueforti strain DF11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|KC167856.1| Length: 581 Number of Matches: 1

Range 1: 132 to 479 GenBank Graphics   ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
643 bits(348)	0.0	348/348(100%)	0/348(0%)	Plus/Minus

```
Query   1    AAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCTC   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   479  AAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCTC   420

Query   61   CAGAATCGGAGGACGAGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGG   120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   419  CAGAATCGGAGGACGAGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGG   360

Query   121  ACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCA   180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   359  ACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCA   300

Query   181  CTGAATTTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAAC   240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   299  CTGAATTTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAAC   240

Query   241  CAAGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTGTTCTCAGACTGCATTCTTC   300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   239  CAAGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTGTTCTCAGACTGCATTCTTC   180

Query   301  AGACAGAGTTCGGGGTGTCTTCGGCGGGCGCGGGCCCGGGGGTGTAAA   348
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   179  AGACAGAGTTCGGGGTGTCTTCGGCGGGCGCGGGCCCGGGGGTGTAAA   132
```

Tree view for *Mucor circinelloides.*
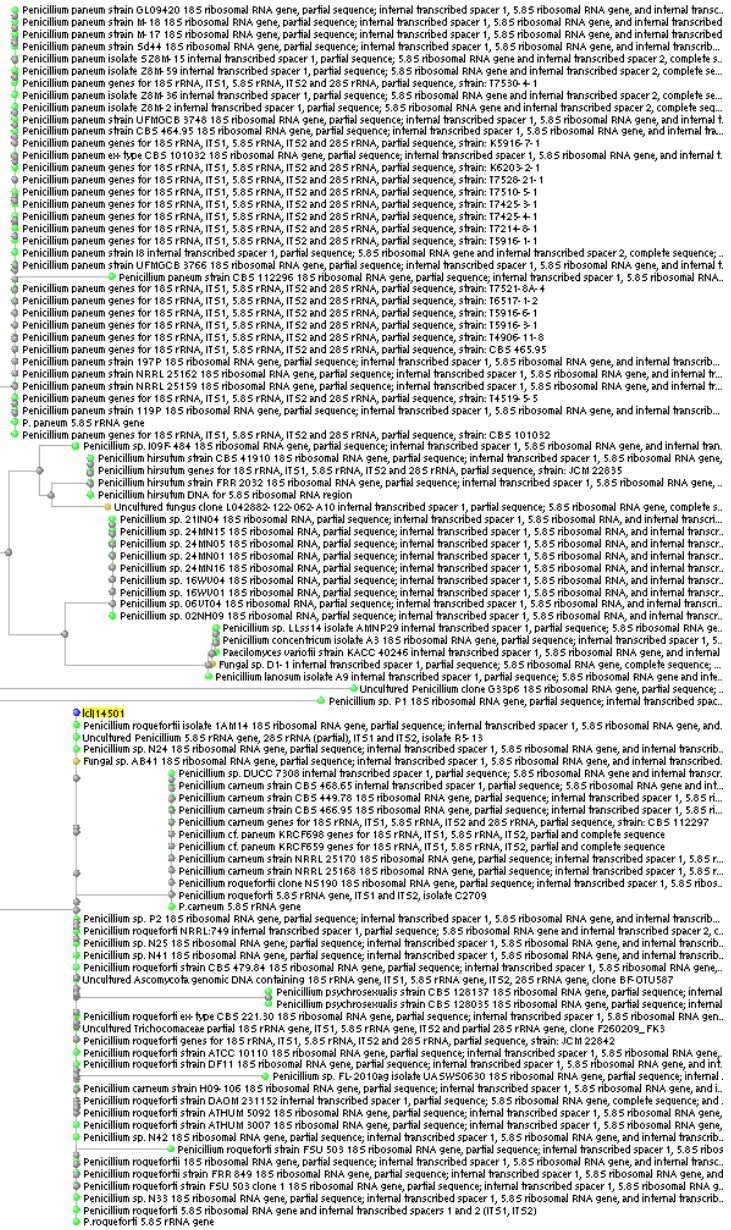
Tree view for *Penicillium roqueforti.*

Appendix Three: t.test between two groups and one way ANOVA analysis:

T-test between two groups   Tuesday, February 26, 2013, 13:50:12

<u>**Data source: Bactria numbers in plate count medium in cow faeces treated by FB (days 14).**</u>

Group Name N   Missing   Mean   Std Dev   SEM
Row 1   3   0   103000.000 2000.000   1154.701
Row 2   3   0   89333.333 6110.101   3527.668

Difference   13666.667

t = 3.682 with 4 degrees of freedom. (P = 0.021)

95 percent confidence interval for difference of means: 3360.939 to 23972.395

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.021).

Power of performed test with alpha = 0.050: 0.759.

T-test between two groups   Tuesday, February 26, 2013, 16:29:10

<u>**Data source: number of *Salmonella Sp* in cow faeces treated by WW (days 7).**</u>

Group Name N   Missing   Mean   Std Dev   SEM
Row 1   3   0   22933.333   1301.281   751.295
Row 2   3   0   35733.333   3971.566   2292.984

Difference   -12800.000

t = -5.305 with 4 degrees of freedom. (P = 0.006)

95 percent confidence interval for difference of means: -19499.362 to -6100.638

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.006).

Power of performed test with alpha = 0.050: 0.970.

T-test between two groups   Thursday, March 07, 2013, 12:10:22

<u>**Data source: number of *E.coli* in cow faeces treated by TW (days 21).**</u>

Group Name N   Missing   Mean   Std Dev   SEM
Row 1   3   0   8333.333   4041.452   2333.333
Row 2   3   0   45000.000   5000.000   2886.751

Difference   -36666.667

t = -9.878 with 4 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: -46972.395 to -26360.939

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0.001).

Power of performed test with alpha = 0.050: 1.000.

T-test   Sunday, May 26, 2013, 08:33:21

Data source: The population of _Salmonella Sp_ between the intestines of fruit beetle and cow faeces (day 7).

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	14400.000	1058.301	611.010
Row 2	3	0	1240000.000	1009554.357	582866.480

Difference   -1225600.000

t = -2.103 with 4 degrees of freedom. (P = 0.103)

95 percent confidence interval for difference of means: -2843897.651 to 392697.651

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.103).

Power of performed test with alpha = 0.050: 0.292

The power of the performed test (0.292) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

T-test between two groups   Wednesday, February 27, 2013, 11:57:42

Data source: The effect FB larvae activity on phosphate in cow faeces (day 21)

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	81.305	6.618	3.821
Row 2	3	0	57.897	10.531	6.080

Difference   23.408

t = 3.260 with 4 degrees of freedom. (P = 0.031)

95 percent confidence interval for difference of means: 3.470 to 43.345

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.031).

Power of performed test with alpha = 0.050: 0.649.

T-test between two groups   Wednesday, February 27, 2013, 12:21:16

Data source: The effect WW activity on ammonium in cow faeces (day 14)

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	24.643	3.016	1.741
Row 2	3	0	54.443	4.286	2.475

Difference   -29.800

t = -9.848 with 4 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: -38.201 to -21.399

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0.001).

Power of performed test with alpha = 0.050: 1.000.

T-test between two groups   Tuesday, March 12, 2013, 17:43:09

Data source: The effect TW activity on sulphate in cow faeces (day 7)

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	374.556	37.341	21.559
Row 2	3	0	508.333	124.736	72.016

Difference   -133.778

t = -1.780 with 4 degrees of freedom. (P = 0.150)

95 percent confidence interval for difference of means: -342.495 to 74.939

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.150).

Power of performed test with alpha = 0.050: 0.203

The power of the performed test (0.203) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Data source: cow faeces treated by FB, Waxworms, Tiger worms and non treated cow faeces on fresh plant weight grown in 75% compost and 25% cow faeces.

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	0.423	0.0252	0.0145
Row 2	3	0	0.520	0.243	0.140
Row 3	3	0	1.193	0.112	0.0644
Row 4	3	0	0.710	0.165	0.0950

Source of Variation	DF	SS	MS	F	P
Between Groups	3	1.056	0.352	14.165	0.001
Residual	8	0.199	0.0248		
Total	11	1.254			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).

Power of performed test with alpha = 0.050: 0.990

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 3 vs. Row 1	0.770	5.983	0.002	Yes
Row 3 vs. Row 2	0.673	5.232	0.004	Yes
Row 3 vs. Row 4	0.483	3.756	0.022	Yes
Row 4 vs. Row 1	0.287	2.228	0.160	No
Row 4 vs. Row 2	0.190	1.476	0.324	No
Row 2 vs. Row 1	0.0967	0.751	0.474	No

One Way Analysis of Variance   Saturday, May 11, 2013, 14:17:55

Data source: cow faeces treated by FB, Waxworms, Tiger worms and non treated cow faeces on fresh roots weight grown in 75% compost and 25% cow faeces.

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	0.260	0.0265	0.0153
Row 2	3	0	0.200	0.0819	0.0473
Row 3	3	0	0.777	0.156	0.0899
Row 4	3	0	0.327	0.117	0.0677

Source of Variation	DF	SS	MS	F	P
Between Groups	3	0.620	0.207	18.209	<0.001
Residual	8	0.0907	0.0113		
Total	11	0.710			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 3 vs. Row 2	0.577	6.632	<0.001	Yes
Row 3 vs. Row 1	0.517	5.942	0.002	Yes
Row 3 vs. Row 4	0.450	5.175	0.003	Yes
Row 4 vs. Row 2	0.127	1.457	0.455	No
Row 4 vs. Row 1	0.0667	0.767	0.714	No
Row 1 vs. Row 2	0.0600	0.690	0.510	No

One Way Analysis of Variance   Saturday, May 11, 2013, 14:01:56

Data source: cow faeces treated by FB, Waxworms, Tiger worms and non treated cow faeces on leaves number grown in 100% compost.

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	3.667	1.528	0.882
Row 2	3	0	3.667	0.577	0.333
Row 3	3	0	5.000	1.000	0.577
Row 4	3	0	3.667	0.577	0.333

Source of Variation	DF	SS	MS	F	P
Between Groups	3	4.000	1.333	1.333	0.330
Residual	8	8.000	1.000		
Total	11	12.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.330).

Power of performed test with alpha = 0.050: 0.090

The power of the performed test (0.090) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

T-test between two groups   Thursday, March 14, 2013, 10:44:58

Data source: Effects of Waxworms and Fruit beetle larvae on waxed paper breakdown (day 20).

Group Name N   Missing   Mean   Std Dev   SEM
Row 1   3   0   5.890   0.0500   0.0289
Row 2   3   0   6.143   0.00577   0.00333

Difference   -0.253

t = -8.718 with 4 degrees of freedom. (P = <0.001)

95 percent confidence interval for difference of means: -0.334 to -0.173

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

T-test between two groups   Thursday, March 14, 2013, 11:09:39

Data source: The waste weight of Waxworms and Fruit Beetle larvae feeding on recycling paper (day 21).

Group Name N   Missing   Mean   Std Dev   SEM
Row 1   3   0   0.248   0.0231   0.0133
Row 2   3   0   0.176   0.00781   0.00451

Difference   0.0720

t = 5.121 with 4 degrees of freedom. (P = 0.007)

95 percent confidence interval for difference of means: 0.0330 to 0.111

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.007).

Power of performed test with alpha = 0.050: 0.960