REMEDIATION OF PULP AND PAPER MILL BIOSOLIDS USING VERMICULTURE

by

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ABSTRACT

Historical wastewater treatment systems at a New Zealand pulp and paper mill, resulted in a biosolid mass in the K-basin at Kinleith mill. Products extracted during the pulping process include resin acids, which are further transformed in the K-basin by microbial activity into recalcitrant end products retene and fichtelite. These products are toxic to fish due to bioaccumulation and subsequent endocrine disruption. Traditional methods for diverting these toxins from waterways were deposition into landfills and incineration, neither of which are considered environmentally sound. The aim of this study was to investigate the viability of vermicomposting as a method for bioremediation of recalcitrant resin acid derivatives from biosolids. Vermicomposting is a cost-effective option for not only reducing toxicity but also reducing biomass. It was hypothesised that earthworms can degrade organic extractives, principally resin acids and derivatives, through microbial, enzymatic, and oxidative mechanisms. A series of vermicomposting experiments were set up, to test the ability of *Eisenia fetida* (the tiger worm) to reduce both the amount of resin acids and overall biomass in a range of substrates. These included the original biosolid collected from K-basin, a "simulated" biosolid containing potting mix with and without additional extractive resins, as well as sterilised and unsterilised controls. Five samples were taken from each experimental composter over 28 days and extracted into dichloromethane after removal of excess water followed by mechanical blending. Samples were concentrated and the amount of each extractive group was determined using gas chromatography mass spectroscopy. The overall biomass in each composter as well as the depurated earthworm mass was measured at the start and cessation of the experiment. A slight reduction in biomass was observed in two out of the three substrates. This reduction was slightly enhanced by the presence of earthworms in the composter, however, it was not significant. The use of methyl bromide to sterilise the substrate was also not a significant

factor in biomass reduction. The overall weight of the earthworms decreased in all cases indicating the unsuitability of any of the substrates as a desirable food source. The addition of supplements such as yeast or manure to the biosolid composter may increase its appeal.

There was a significant reduction in extractive content in all substrates over the 28 day period however no significant difference attributable to the presence or absence of worms was observed. It was hypothesised that the rigorous sampling process encouraged oxidative breakdown of the extractives due to increased exposure to both air and light. This was evident when the extractive content of K-basin measured in 1993 was compared to the samples used in this study collected in 2006.

Whilst vermicomposting does not appear to be an effective treatment for removing resin acids from biosolid mass, the sampling processes used in this study highlighted the effect that rigorous stirring and increased exposure to air and light can have on the natural breakdown of these products. An effective treatment for the removal of resin acids from K-basin may be as simple as regular ploughing.

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LIST OF ABBREVIATION

CHH	Carter Holt Harvey
DCM	Dichloromethane
TMP	Thermomechanical pulping
GC-MS	Gas chromatograph-mass spectrometer
nRasp	Resin acids, neutrals and phytosterols
PMS	Pulp and paper mill sludge
Na_2SO_4	Sodium Sulphate
PM+E	Potting Mix + Extractives

Chapter 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Contamination of the environment by pollutants is of growing concern in New Zealand and worldwide. Pressures from population and productivity growth, as well as increased public consciousness have meant it is no longer acceptable to lockup large areas of land as waste depots. To confront the reality of industrial waste, new and improved treatment technologies are being researched. For organic wastes one such technology is vermicomposting using earthworms. This study focuses on using vermiculture to remediate pulp and paper mill waste into a useable resource.

1.1 The Pulp and Paper Industry

The pulp and paper industry in New Zealand consists of eight mills, predominantly located within the North Island. Carter Holt Harvey (CHH) is a leading supplier of Pulp and Paper products in the Australasian markets and exports throughout Asia and around the Pacific Rim. CHH is listed on both the NZSE and ASX, and employs more than 10,000 people across the New Zealand and Australia.

This study centres on the pulp and paper waste in a retired treatment system at CHH Kinleith. CHH Kinleith is an integrated bleached kraft pulp and paper mill which produces approximately 330,000 tonnes of paper grades annually and 265,000 tonnes of predominantly bleached kraft market pulp. The total product range includes containerboard, paper, pulp and chemicals (http://www.chhwoodproducts.co.nz/).

1.2 The Pulping Process

Papermaking typically begins with trees as the raw material. Softwood trees most used for papermaking include spruce, pine, and fir; common hardwood trees used include oak, maple, and birch. What makes a tree or plant suitable for paper is cellulose fibre. Trees are generally a composite of cellulose fibres bonded together with lignin, plus sugars and other organic compounds. Depending on species, about 40-50% of the tree consists of cellulose suitable for papermaking. Since only the cellulose is needed, the first step in papermaking is to separate the lignin and other materials from the cellulose; a process called pulping. There are two principal pulping processes used in the pulp and paper industry; mechanical and chemical pulping.

The manufacture of pulp for paper and cardboard employs mechanical (including thermomechanical), chemimechanical, and chemical methods (Bailey and Young 1997). Mechanical pulping separates fibers by such methods as disk abrasion and billeting. Thermomechanical pulps, which are used for making products such as newsprint, are manufactured from raw materials by the application of heat, in addition to mechanical operations. Chemimechanical pulping and chemithermomechanical pulping (CTMP) are similar but use less mechanical energy, softening the pulp with sodium sulfite, carbonate, or hydroxide.

Chemical pulping uses chemicals, heat, and pressure to dissolve the lignin in the wood, freeing the cellulose fibres (Bailey and Young 1997). In the kraft (sulfate) process, the wood and chemicals are cooked in a digester to remove the sugars and 90-95% of the lignin. The waste from the digester is known as black liquor and is often burned at the paper mill as an energy source. The pulp may then be bleached to remove all of the residual lignin and to increase the brightness (Cox 1981). In the past chlorine was used as the bleaching agent. Bleaching with chlorine produced large amounts of organochlorine

compounds, including dioxins. Compounds containing a high amount of chlorine may persist for days to weeks in the environment. Furthermore the chlorinated compounds can be biologically degraded or transformed into potentially more persistent and bioaccumulative compounds. As pulp and paper mills are invariably located near large bodies of water, the bioaccumulation of organochlorine has been observed in fish and other aquatic species. In response to the environmental concerns the pulp and paper mill industry has developed 'Elemental Chlorine Free' and 'Totally Chlorine Free' bleaching processes. "In 1991 Kinleith mill undertook process modifications in which the Number 2 bleach plant process was altered from a conventional chlorine bleaching sequence to one employing oxygen delignification and 100% chlorine dioxide substitution." (Sharples and Evans, 1998).

Following the bleaching process the pulp is subjected to alkaline extraction. Chemicals such as alkaline hydrogen peroxide are used to selectively oxidize non-aromatic conjugated groups responsible for absorbing visible light hence improving colour and brightness.

1.3 Mill Description

The combined effluent discharge from New Zealand's eight pulp and paper mills is estimated to be 128,000,000 cubic metres per annum of effluent (Dell *et al.* 1997). Of that total Kinleith accounts for approximately 67,000,000 cubic metres per annum (Sharples and Evans, 1997). At the time biosolids examined in this study were accumulating, the effluent discharged from CHH Kinleith was estimated at 120ML per day (Stuthridge & Tavendale 1993).

The biosolids used in this study were collected from a retired aerated treatment lagoon known as 'K-basin'. K-basin formed part of a two stage biological treatment system. The two systems referred to as 'A' and 'B' operated in different configurations.

Treatment system A was responsible for treating general wastewaters. Before entering treatment system A, the general wastewater underwent primary screening and sedimentation (Stuthridge *et al.* 1991). The treatment system consisted of a series of five lagoons. The first lagoon in the series (K-basin) was approximately 20 metres deep and could hold approximately 540 million litres of effluent. The retention time for effluent in this lagoon was an average of 3.4 days. During this time the effluent received approximately 510 kilowatts of mechanical aerations. The remaining lagoons had a total retention time of approximately one day. "The first of these lagoons has 32 kilowatts of mechanical aeration, whilst the others rely on natural re-aeration" (Suthridge *et al.* 1991). Kinleith's treatment system in its current and previous forms is unique because it utilises the natural topography of the landscape as part of the treatment system. From the mill site to the effluent's discharge point into the Kopakorahi Stream, the effluent travels over 15 kilometres and drops almost 200 metres in elevation. In additional to general wastewaters, treatment system A also received chlorinated stage bleaching effluents and settled stormwater and debarking effluents. Before the chlorinated effluents enter the treatment system it was partially neutralised by direct contact with limerock fines. The stormwater and debarking effluents are screened through a clarifier prior to entering the treatment system (Suthridge *et al.* 1991).

Treatment system B was responsible for treating all alkali extraction stage bleaching effluents and foul condensates. "This system consisted of two lagoons receiving a total of 260 kilowatts mechanical aeration and a naturally aerated storage lagoon giving a total retention time of approximately 45 days"

(Suthridge *et al.* 1991). Effluent from system B mixed with effluent from system A just prior to discharge into the Kopakorahi Stream.

1.4 Resin Acids and Degradation Products

Historically, waste has been deposited in an aeration treatment pond known as the K-basin. Previous studies have found substantial levels of resin acids and their neutral derivatives in K-basin sediments (Stuthridge and Tavendale, 1995). Resin acids occur naturally in coniferous trees and are part of the waste products extracted during pulping (**Figure 1**). They are hydrophobic compounds and bond strongly to suspended particles such as lignin and other organic compounds in the effluent. These particles then settle and become sediment. (Lahdelma and Oikari, 2005). This explains the high concentration of resin acids in sediment from treatment systems such as K-basin. Resin acids are acutely toxic, especially to fish and some of Crustaceans (the Cladocera), whereas bacteria and algae are more tolerant. Dehydroabietic acid, the most plentiful resin acid in effluents from softwood processes, has sublethal effects on salmonids at concentrations of >20 $\mu g\ l^{-1}$ and is acutely toxic (LC50), at around 1 $mg\ l^{-1}$. As an indication of their bioavailability, resin acids have been found at elevated concentrations in caged fish in receiving water areas of the wood processing industry (Karels and Oikari, 2000).

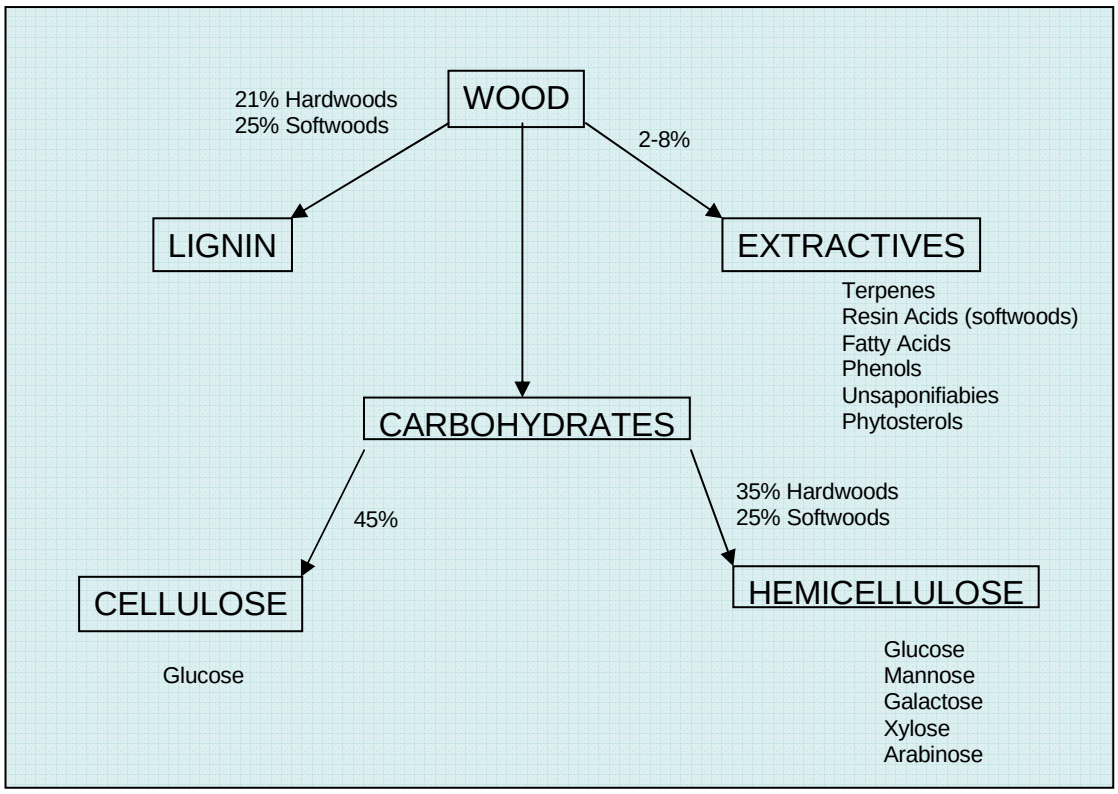

Figure 1 The chemical components of wood. Source: M.J. Robinson

Under anaerobic conditions, the toxicity of resin acids can be increased by microbial transformation (**Figure 2**) (Tavendale, et al. 1997). An example of this process is Retene (7-isopropyl-1-methylphenan-trene), a metabolite created by microbial activity in anaerobic conditions. The saturated structure of retene is very persistent against further transformations under anoxic conditions and therefore represents the final product of microbial transformation. As a consequence, Retene accumulates in old (>20 years) sediments contaminated by pulp and paper mill effluent at a greater rate than untransformed resin acids and other derived compounds (Lahdelma and Oikari, 2005). Retene has been shown to be more bioaccumulative and recalcitrant than the parent compounds. Multiple adverse effects have been detected in fishes exposed to retene, for example the disruption of fish larval development and induction of the cytochrome P-450 1A system (Scott and Hodson, 2008).

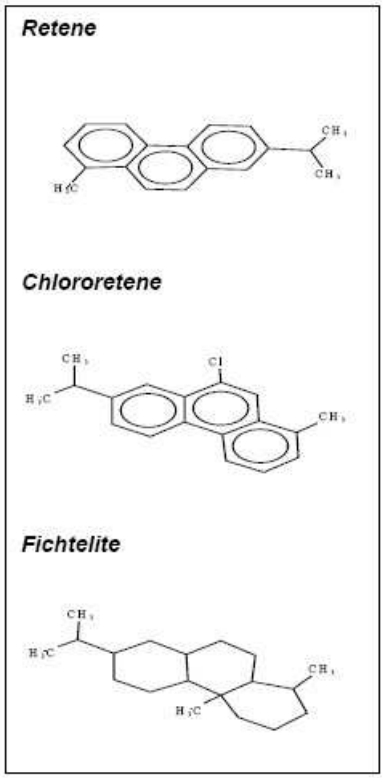

Figure 2 The three main Biotransformation products formed from resin acids. Source: Stuthridge *et al.* 1996.

Previous studies have found that Kinleith's treatment system A removes over 85% of resin acids (as part of total suspended solids) from the liquid effluent waste of debarking and acid bleaching. Treatment system B achieves less than 50% resin acid removal (Stuthridge and Tavendale 1996). The result is accumulation of resin acids in basins, particularly in the second treatment system. Furthermore significant concentrations of biotransformation products were found in K-basin surficial sediments. In the main body of the aerated stabilisation basin, resin acid constituents comprised nearly 20% of the total dry weight of the sediments. It has been estimated that over K-basin's 30 year life, the basin has accumulated 40 000 tonnes dry weight of sediment which contains approximately 1800 tonnes neutral resin acid derived compounds and 3000 tonnes of resin acids. The long term fate and effects of

this accumulated resin acid material is currently unknown (Stuthridge and Tavendale, 1996).

Resin-acid-degrading micro-organisms are widely distributed in the environment. (Martin *et al.* 1999). This may be attributed to the ubiquitous nature of these compounds. Resin acids are released from terrestrial vegetation into the atmosphere or into water bodies from watershed runoffs, and are dispersed widely (Mazurek and Simoneit 1997). Resin acid degrading micro-organisms are phylogenetically diverse. Species of anaerobic and aerobic bacteria and fungi can transform resin acids. Under anoxic conditions, resin acids can be biotransformed, but there is no conclusive evidence that their carbon skeletons are degraded. Furthermore, these anaerobic transformations have been observed only in complex microbial communities such as freshwater sediments and bioreactors (Martin *et al.* 1999). Neutral compounds dehydroabietin and fichtelite are believed to be products of anaerobic transformation. Mass spectral analysis suggests they are derived from abietanic and dehydroabietic acid and transformed by decarboxylation (Tavendale *et al.* 1997). Anaerobic transformation products tetrahydroretene and retene are derived from dehydroabietic acid and are products of an aromatisation process. Some of these neutrals are recalcitrant in the environment (LaFleur, 1983) and are more lipophilic than the parent acids.

1.5 Vermicomposting

A possible treatment for these resin acid contaminated solids is vermicomposting. Vermicomposting is the process by which earthworms are used to convert organic materials (usually wastes) into a humus-like material known as vermicompost (Munroe, 2004). This is much more fragmented, porous and microbially active than parent material due to humification and

increased decomposition (Garg and Kaushik, 2005). As the recalcitrant products are trapped in solid form in the base sediment, vermiculture may offer a viable solution to the remediation of these toxicants. The term vermiculture refers to the culture of earthworms. The use of earthworms in sludge management has been termed as vermistabilisation.

Different organic wastes which have already been converted into vermicompost by different species of earthworms include cattle dung, horse waste, turkey waste, sheep waste, poultry droppings, cow slurry, mango leaves, water hyacinth, and paper waste. Researches at the Environmental Technologies Centre for Industrial Collaboration in Hull, England, have investigated using earthworms to treat sewage sludge and liquid wastes. A pilot wormery achieved a 50-60% reduction in solid waste. The worms were also shown to significantly reduce waste toxicity meaning the water generated by the system as a by-product could be reused (Wiseman, 2005).

1.6 Vermicomposting cost effectiveness

Vermicomposting organic wastes has become increasingly attractive due to the high costs of industrial waste disposal. The sludges resulting from different industrial operations and wastewater treatment plants are typically disposed of through land filling and incineration. Decreasing availability of space for land fill, along with increasingly stringent national waste disposal regulations and public opposition have made land filling expensive and impractical (Garg and Kaushik, 2005).

The action of earthworms during vermicomposting is both physical/mechanical and biochemical. The physical or mechanical processes include substrate aeration, mixing, as well as actual grinding. The biochemical process is induced by microbial decomposition of the substrate in the intestines of the earthworms. In a traditional microbial composting system,

the largest cost is typically associated with the physical/mechanical part of the process. Vermicomposting does not require continuous inputs of energy, reducing costs and aiding sustainability. Therefore vermicomposting is a low cost technology system for the treatment of organic wastes.

Also, as opposed to traditional microbial waste treatment, vermicomposting results in bioconversion of the waste streams into two useful products; the earthworm biomass and the vermicompost (Ndegwa, and Thompson, 2000).

1.7 The Value of Vermicomposting

The value of the vermicompost produced by the earthworm may be the most compelling argument for vermicomposting. Charles Darwin in 1882 noted that earthworms can process huge quantities of plant litter and help convert it into rich topsoil, liberating nutrients for renewed plant growth. Nutrients such as nitrogen, potassium, phosphorus, and calcium present in the waste are released through microbial action into forms that are more soluble and available to plants than those in the parent substrate, while worms themselves provide a protein source for animal feeds.

Other benefits attributed to earthworms include reduced soil compaction, improving permeability and aeration. Earthworms do this through burrowing activities, ingestion of soil along with plant debris, and subsequent excretion of casts. Upon drying, these casts form water-stable soil aggregates. These aggregates are clumps of soil particles bound together by organic compounds, and their presence helps improve soil structure, retain nutrients that might otherwise be leached, and reduce the threat of erosion (Werner and Bugg, 1990). Not only is the cast a highly nutritious and valuable soil additive as an end product, by passing through the gut of the worm, it converts the original

raw material to a safe product whereby harmful pathogens present in animal manures including human excreta (biosolids), are eliminated altogether or significantly reduced.

1.8 Earthworm Taxonomy

Earthworms (**Figure 3**) are scientifically classified as animals belonging to the order Oligochaeta, class Chaetopoda, phylum Annelida. In this phylum there are about 1,800 species of earthworms grouped into five families and distributed all over the world except in arid and arctic regions. The most common worms in North America, Europe, and Western Asia belong to the family Lumbricidae, which has about 220 species. Earthworms range from a few millimetres long to over 330 centimetres, but most common species are 10-15 centimetres in length (Martin et al., 2006).

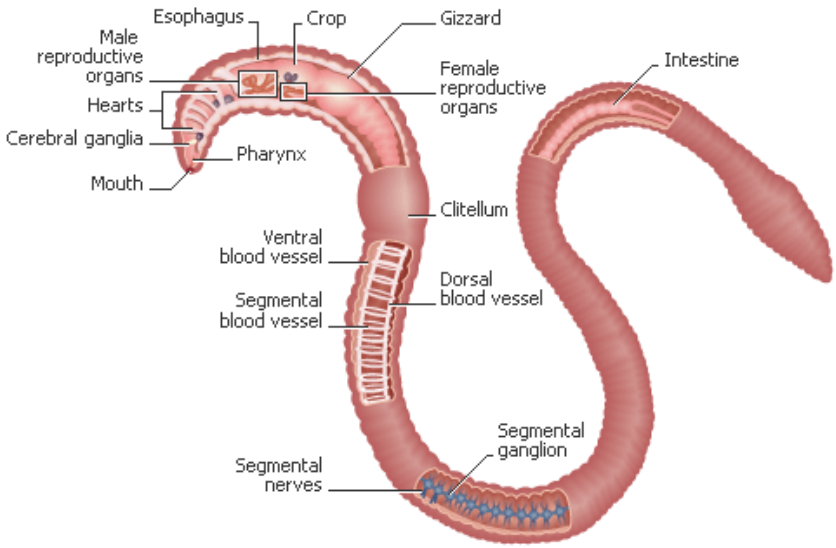

Figure 3 Illustration of generic earthworm http ://www.urbanext.uiuc.edu/worms/index.html

Earthworms are grouped into three types (

Table 1). They come to the surface only on cloudy days and at night unless they are flooded out by heavy rainfalls. In cold and dry weather they retreat into their burrows and remain dormant.

Table 1. General classification of types of earthworm	
Type	**Description**
Anecic (Greek for "out of the earth")	These are burrowing worms that come to the surface at night to drag food down into their permanent burrows deep within the mineral layers of the soil. Example: the Canadian Night crawler.
Endogeic (Greek for "within the earth")	These are also burrowing worms but their burrows are typically more shallow and they feed on the organic matter already in the soil, so they come to the surface only rarely.
Epigeic (Greek for "upon the earth")	These worms live in the surface litter and feed on decaying organic matter. They do not have permanent burrows. These "decomposers" are the type of worm used in vermicomposting.

Table 1 Earthworm types

1.9 Earthworm biology

The segments of the earthworm, visible externally as rings, are separated by internal partitions. The first section of the earthworm, the anterior end or

head, consists of the mouth and the prostomium, a lobe which serves as a covering for the mouth and as a wedge to force open cracks in the soil into which the earthworm may crawl (Hickman *et al.,* 2000). On each segment are four pairs of bristles, or setae, with which the worm anchors itself to the walls of the burrow, drawing itself forward by rhythmic muscular contractions. In addition, various skin glands secrete lubricating mucus which aids movement through the earth and helps to stabilize burrows and casts.

The earthworm's digestive tract is highly adapted to its burrowing and feeding activities. The worm swallows soil (including decomposing organic residues in the soil) or residues and plant litter on the soil surface. Strong muscles mix the swallowed material and pass it through the digestive tract as digestive fluids containing enzymes are secreted and mixed with the materials. The digestive fluids release amino acids, sugars, and other smaller organic molecules from the organic residues (which include living protozoa, nematodes, bacteria, fungi, and other micro-organisms as well as partially decomposed plant and animal materials). The simpler molecules are absorbed through intestinal membranes and are utilized for energy and cell synthesis.

Earthworms have a nerve cord, with ganglia in each segment and an enlarged cerebral ganglion (a primitive brain) at the anterior end. Although they have no prominent sense organs, earthworms are sensitive to light, touch, vibration, and chemicals. The circulatory system is enclosed in vessels; the blood (which contains hemoglobin) is pumped by muscular contractions of five linearly arranged hearts. Earthworms lack specialised breathing devices with respiratory exchange occurring across the body surface.

1.10 Earthworm Reproduction

Earthworms are hermaphroditic, but they cross-fertilize. Two worms exchange sperm cells during copulation; fertilization occurs after the worm's own eggs and the received sperm are encased in a tough sheath secreted by the clitellum, a conspicuous band of tissue near the anterior end (Hickman *et al.* 2000). The sheath slips over the worm's head and is deposited underground, where it serves as a cocoon for the developing young. There is no larval stage; the young hatch as miniature adults. The eggs hatch after about 3 weeks, each cocoon producing from two to twenty baby worms with an average of four.

1.11 Eisenia fetida

This study will focus the earthworm species *Eisenia fetida* (Savigny) commonly known as "tiger worm" (**Figure 4**). This extremely tough and adaptable worm is indigenous to most parts of the world.

Figure 4 *Eisenia fetida* the Tiger Worm *www.amystewart.com/images.html*

Eisenia fetida is the species predominantly used for composting purposes. It can tolerate a wide temperature range (between 0 and 35°C) and can survive for some time almost completely encased in frozen organic material (as long as it can continue to take in nourishment). Its cocoons (eggs) have been shown to remain viable after having been frozen for several weeks (Munroe, 2004). In addition, it can take a lot of handling and rough treatment. Perhaps most importantly, like most if not all litter-dwelling worms, the compost worm has the capacity for very rapid reproduction. This is an evolutionary necessity for a creature whose natural environment is extremely changeable and hazardous and whose natural supplies of food are of the "boom or bust" variety. All of these characteristics make *E. fetida* the natural choice for vermicomposting, year-round, in climates with harsh winter conditions.

1.12 Effects of Stocking Density and Feeding Rate

To create the most efficient and economical vermicomposting system, Ndegwa and Thompson (2000) investigated the optimal stocking density and optimal feeding rate for the vermicomposting of biosolids, with paper mulch provided as bedding. A stocking density of 1.60 kg-worms/m^2 and a feeding rate of 1.25 kg-feed/kg-worm/day resulted in the highest bioconversion of the substrate into earthworm biomass. The best vermicompost was obtained at the same stocking density and a feed rate of 0.75 kg-feed/kg-worm/day. It was noted that whereas individual worms grew more and faster at the lowest population density, the total biomass production was maximum at the highest population density. At higher stocking rates the worms sexually matured faster than in the lower stocking rates.

Worms can consume their own body weight in 24 hours, however, the feeding rate was dependent on the feed as well as the feed preparation or feed pre-treatment. Feed with suitable organic matter tended to encourage higher feeding rates. Therefore the vermicomposting process can be enhanced by supplementation with feed high in organic content such as cow dung.

A study by Butt *et al.* (2005) investigated the habitat preference of two common earthworm species at a field site, when offered a choice between unamended soil and the same soil with boardmill sludge addition. The results showed there to be 1.7 times lower density of earthworms in the soil which had the boardmill sludge addition. This study highlights that while earthworms are capable of consuming a wide variety of organic substrates, their optimality is related to their preference for a substrate. Therefore supplementation of the substrate may be necessary to achieve a higher rate of vermicomposting.

1.13 Earthworm Enzyme Activity

Merino-Tigo et al. (1999) discussed past research that described a direct role for earthworms in the decomposition of plant debris, presuming the existence of their own digestive enzymatic activities. They cite, amongst others, Urbasek and Pizl (1991) who described the presence of active amylase, laminarinase, lichenase, cellulase, glucoamylase and xylanase in the gut of five earthworm species.

The investigation of Merino-Tigo et al. (1999) specifically looked for xylanase in worms fed for 35 days on paper mill sludge amended with pig slurry. Their analyses found that the xylanase in the pulp and paper mill sludge (PMS)-fed worms was 133% of the level in the control (cow manure-fed worms). Other

enzyme levels were also raised with respect to the control. The origin of the xylanase was not clear. It could have been from the gut wall cells, or the digestive glands, or from gut-wall-associated microflora. The main point, however, was that the occurrence of active xylanolytic enzymes in extracts of *E. andrei* indicates that xylan is degraded in their gut and that earthworm activity can contribute directly to the breakdown of hemicellulose (xylan is main component of plant hemicellulose).

1.13 Objectives and Hypothesis

The aim of this study was to investigate the viability of vermicomposting as a method for bioremediation of recalcitrant resin acid derivatives from the pulp and paper industry. Vermicomposting was chosen as an economically attractive process for treating toxic solid waste. Pilot studies with sewerage sludge remediation using vermicomposting in the UK achieved 50-60% reduction in solid waste (Wiseman, 2005). We were interested in seeing if vermicomposting could have a similar impact on reducing solid waste, particularly, biotransformed resin acids such as the toxic bioaccumulative Retene, from K-basin biosolids.

It was hypothesised that earthworms can degrade organic extractives, principally resin acids and their neutral derivatives, through microbial, enzymatic, and oxidative mechanisms.

To prove the hypothesis that earthworms can degrade extractives sterilised controls were established. Potting mix and biosolid substrates were sterilised to kill native microbes that could have degrade resin acids. Therefore any microbes in the system would have been introduced via the earthworms.

Chapter 2

METHODS AND MATERIALS

2.1 Biosolid Collection

Biosolids were collected from K-basin at CHH Kinleith. Approximately 60 kg was taken from the main site (

Figure 5). In addition, six biosolid samples were taken from the same sites documented by Stuthridge and Travendale in 1993.

The samples were collected by using a shovel to dig into the basin to a depth of 20 centimetres. Wearing latex gloves approximately 1 kg of biosolids was scooped from each site by hand and placed into a clearly labelled and sealable plastic bag. New gloves were worn for each site and the shovel wiped with a cloth to remove any residual biosolids.

The biosolids were easily indentified from any other material present in K-basin. The biosolids were dark in colour and made up of fine particles. The consistency tended to be moist and clay-like in nature. The biosolids had a distinctive aroma resembling the smell of pine and citrus.

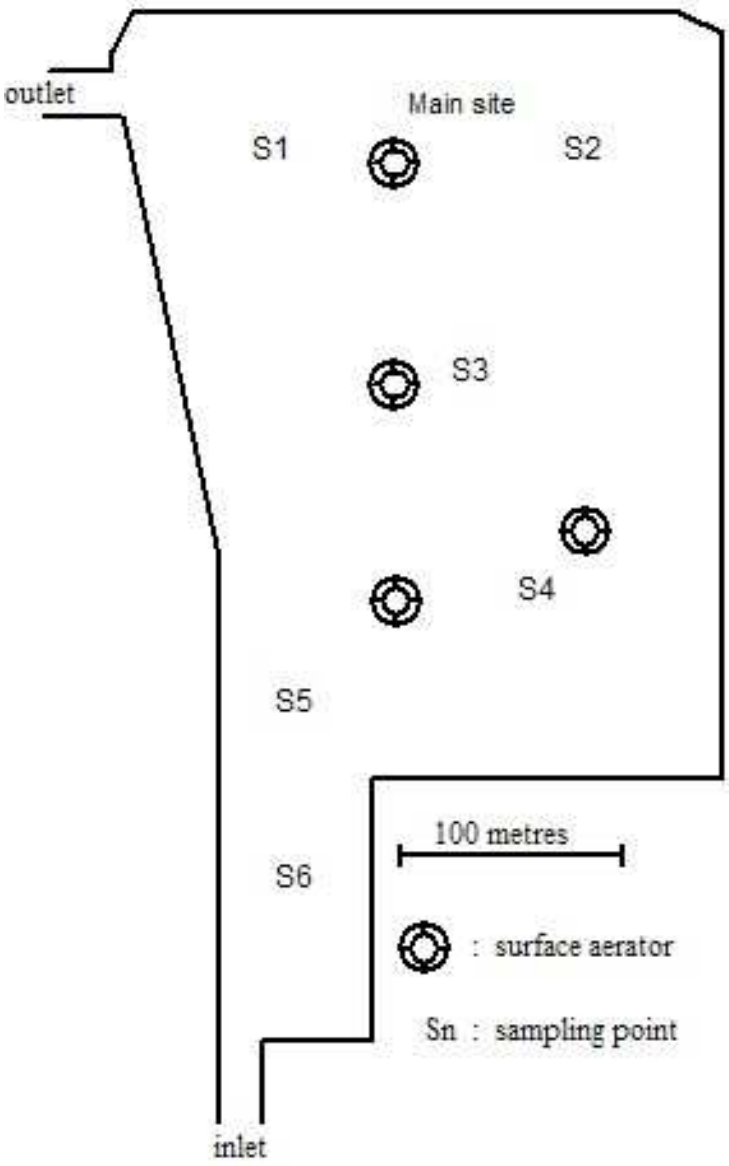

Figure 5 Schematic diagram of K-basin and location of sampling sites

The main sample was transferred into a cleaned concrete mixer and mixed to ensure homogenisation (**Figure 6**).

Figure 6 Concrete mixer used to homogenise biosolid samples

All the samples were stored in a -20° freezer until needed.

Figure 7 is a composite panoramic photograph of k-basin showing sampling locations.

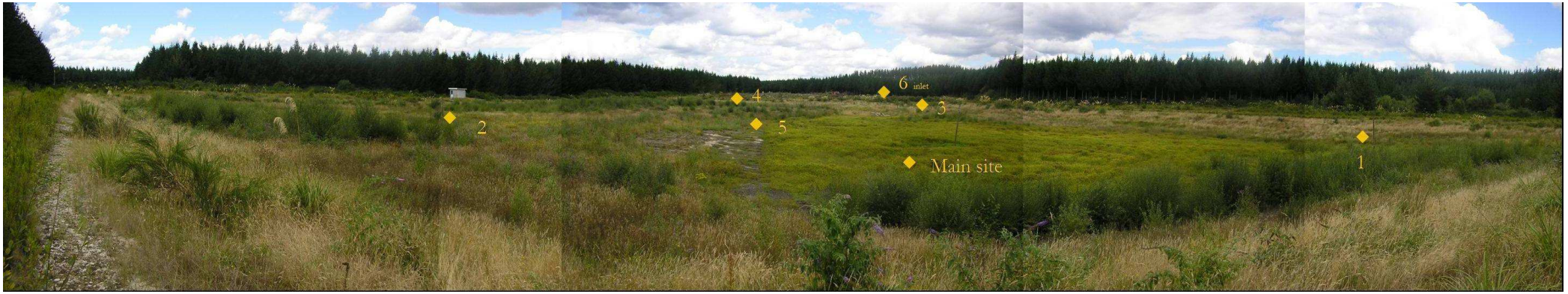

Figure 7 Composite panoramic photograph of K-basin

2.2 Biotransformation and Bioaccumulation

2.21 Potting Mix

Daltons potting mix was chosen as the neutral substrate in this experiment. Two potting mix controls were established to investigate matrix effects; plain potting mix and potting mix with added biosolids extractives. To investigate the effect of microbial biotransformation, sterilised samples for each of the potting mix controls were prepared.

The amount of potting mix used was equivalent to the amount of biosolids; in this case 1.5 kg was chosen. Extract from 1.5 kg of biosolids was added to sterilised and non-sterilised potting mixed.

2.22 Large Scale Soxhlet Method

A soxhlet extraction was used to extract the organics from the biosolids. Glassware was cleaned and sterilised in a muffle furnace at 450°C overnight prior to the extraction.

Figure 8 Large soxhlet extraction apparatus

Approximately 300 g of biosolids was measured out and placed into a thimble. The thimble was placed into the soxhlet extractor (**Figure 8**). One litre of dichloromethane (DCM) was poured over the sample and allowed to drain into the round bottom flask below. A mantle heats the DCM to vapour. The vapour rises up the soxhlet, condenses to a liquid, and runs back through the sample. The cycle was left to run for approximately 3-4 days.

The procedure was repeated until the required weight of biosolids had been extracted.

The extract was transfer to Zymark tubes and place in a Turbovap (**Figure 9**). The water bath in the Turbovap gently warmed the extract while simultaneously blowing nitrogen gas across the samples to reduce the solvent volume by evaporation. DCM is toxic to micro-organisms.

Figure 9 Zymark with biosolid extract tubes in Turbovap

2.23 Methyl Bromide Sterilisation

A quantity of potting mix and biosolids were sterilised using methyl bromide . The sterilisation was performed by Genera, a biosecurity company located in Mt. Maunganui (http://www.genera.co.nz/). The company specialises in the treatment of imported goods for biologically active agents.

Methyl bromide is a general use, broad-spectrum pesticide. It is commonly used as a soil fumigant and kills the vast majority of soil organisms.

The samples were placed in a pressurised cargo container. Methyl bromide was applied at 80 g per cubic metre for a period of 24 hours at a constant temperature of 10°C.

2.24 Preparing Potting Mix and Extractives

The biosolid extract was mixed into a sub-sample from each of the sterilised and non-sterilised potting mix controls. This was done to facilitate the evaporation of DCM to reduce the risk of sample contamination. Approximately 10% or 150 g of the sterilised and non-sterilised potting mix sub-samples were placed into 600ml glass beakers. Extractives from 3.0 kg of biosolids had been reduced to a volume of 160 ml. This volume was carefully portioned into two 80 ml aliquots and was poured over the sterilised and non-sterilised potting mix samples. The beakers were placed under a fume hood and left overnight to allow residual DCM to evaporate. The extract/potting mix sub-samples were thoroughly mixed in with their parent sterilised and non-sterilised potting mix batches.

To satisfy the demands of rigorous scientific method, 80 ml of DCM was added to straight sterilised and non-sterilised potting mix; that is potting mix with no added extractives. By treating all the samples with the same amount of DCM ensures that experimental results can not be attributed to the solvent. To this end 80 ml of DCM was also applied to the sterilised and non-sterilised biosolid samples.

2.25 Preliminary DCM Earthworm Toxicology Assay

A toxicology assay was performed to determine the effect of DCM on earthworm survival. 200 g of potting mix was weighed out and split into two 600ml glass beakers. 10 ml of DCM was thoroughly mixed into each beaker.

Eight earthworms were placed into one of the beakers immediately after mixing. A further eight earthworms were placed into the second beaker after it had been under a fume hood for 24 hours to allow the DCM to evaporate. For the control, eight earthworms were placed into a beaker with 100 grams of potting mix with no DCM.

The duration for this assay was 24 hours.

Earthworm mortality was assessed by a number of factors including;

- Earthworm movement when subjected to a physical stimulus such as a prod

- The condition of the worm's epidermis. An earthworm's epidermis is covered in a layer of mucus to aid locomotion. Mucus production ceases when death occurs resulting in drying of the epidermis and discolouration

However, for this experiment, earthworm mortality was easily indentified as DCM dissolves earthworm flesh.

The results are shown in .

2.26 Substrate Water Content, MHC, and pH

The water contents, maximum water holding capacity (MHC), and pH were measured for each substrate using protocols established by Landcare Research (K. O'Halloran *pers. Comm.*).

The MHC is defined as the maximum amount of moisture that a soil can hold. The ideal moisture content for an earthworm has been determined to be approximately 70% of the MHC. MilliQ water was added to the substrate to achieve 70% MHC. The MHC was calculated by soaking ~30 g of substrate in water for 12 hours. The substrate was then drained for two hours, at which point the substrate was deemed to be at MHC. The water content was calculated by comparing the mass of the water saturated substrate before and after oven drying.

The water content and the MHC were calculated for the following substrates; methyl bromide treated biosolids, methyl bromide treated potting mix, biosolids, and potting mix. The amount of milliQ™ water added to each

substrate was calculated based on the following objectives; to achieve 70% of MHC, and ensuring each substrate was the same mass. In some cases substrate was removed from the sample to offset the mass of the milliQ™ water.

2.27 Experimental design: Biotransformation Earthworm Bioassay

Each of the three substrates; potting mix + extractives, potting mix, and biosolids included both no-worms and worms treatments. In addition each substrate included a sterilised and non-sterilised treatment. Each treatment had three replicates. **Figure 10** illustrates the various treatments.

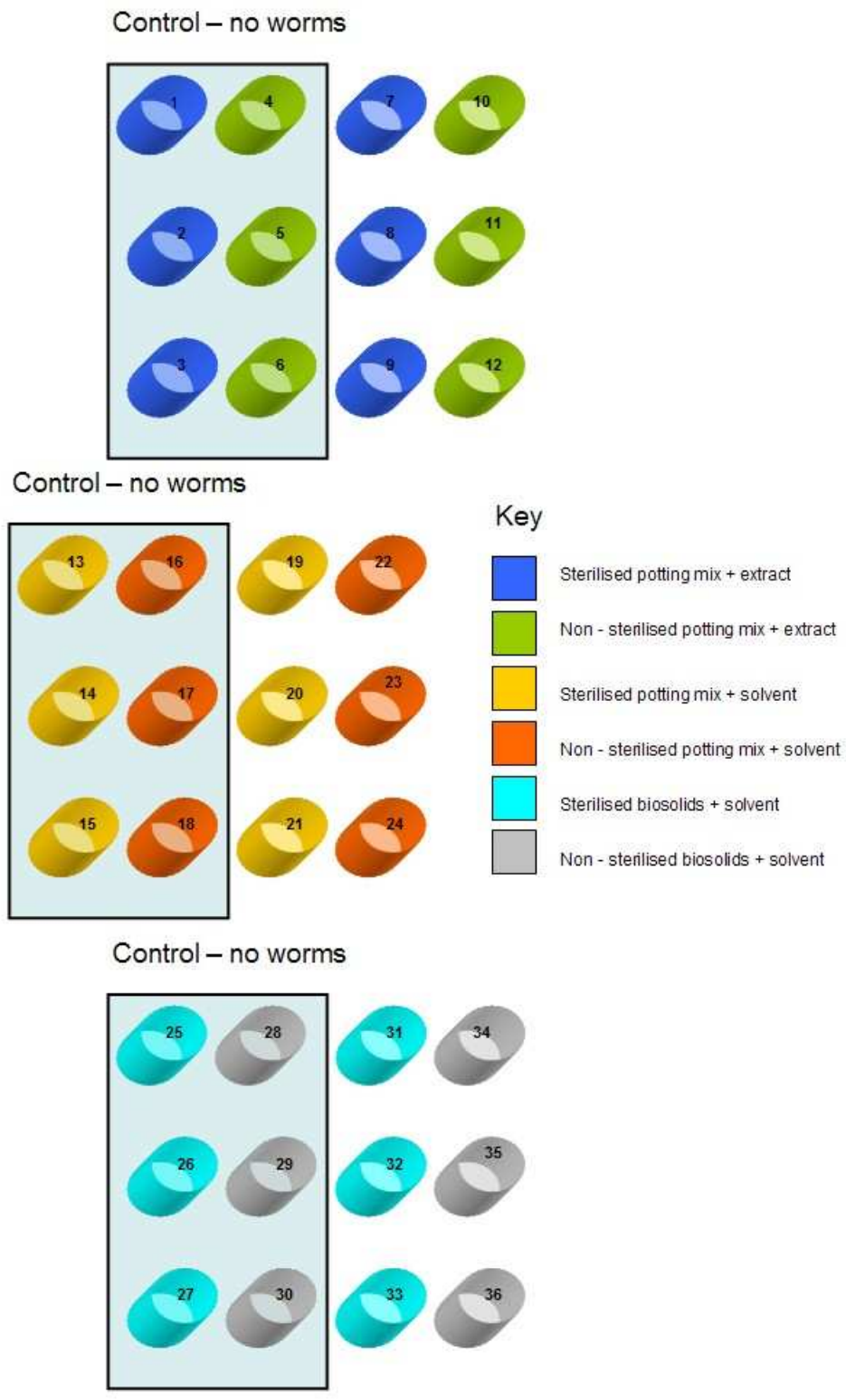

Figure 10 Experimental design

The 36 preserving jars were washed with laboratory grade, biologically inactive detergent (Decon® Registered Office: Conway Street, Hove, E. Sussex, BN3 3LY) and rinsed prior to the addition of substrate and worms. 250 g of sterilised and non-sterilised substrates was placed into each of the jars.

The experiment was performed in a temperature and humidity controlled room set at 20°C and 80%, respectively, under constant 24 h illumination.

2.28 Worm Preparation

The earthworms, *Eisenia fetida,* were gently removed from their interim substrate, carefully rinsed with milliQ™) water to remove soil, and patted dry with a paper towel to remove excess water. The worms were sorted into 18 groups of 20 and weighed to an accuracy of ± 0.1g To clear the gut the earthworms were placed onto moist filter paper and left for a period of 12 h at ambient room temperature. The groups were then re-weighed before being allocated to their jars. The final weight of the jars including content was recorded.

To prevent the earthworms escaping the jars were covered with a film of plastic cling film and secured with a rubber band. Holes were made in the plastic cling film to provide the worms with air.

Figure 11 is a photograph of the jars arranged in the temperature and humidity controlled room.

Figure 11 Jars arranged according to treatment in a temperature and humidity controlled room set at 20°C and 80%

2.29 Sampling schedule

The duration of the experiment was 28 d. Three small substrate samples were collected from each jar on days 0, 3, 8, 15, and 28 and included the following:

1. 2 g for chemical extraction.
2. 2 g for microbial analysis.
3. 2 g for backup.

The samples were place in cryogenic storage tubes and stored in a -20°C freezer for later analysis.

Prior to sampling the weight of each jar including the content was recorded and compared to the last recorded weight. MilliQ™ water was added to jars to compensate for jars that had lost weight through moisture evaporation. After sampling the jars were reweighed.

2.30 Cessation of Biotransformation and Bioaccumulation Experiment

On day 28 the earthworms were removed from the jars, gently rinsed, patted dry with a paper towel, and weighed. The worms were place on damp filter paper for 24 h to empty the gut and then reweighed. The filter paper was stored in the freezer for possible analysis.

The earthworms were placed stored at -80°C freezer for later analysis.

2.3 Extractives Analysis

Two extractive methods; soxhlet and 'shake method' were assessed for consistency and efficiency.

2.31 Mass Balancing

The mass of the material in each jar was tracked throughout the experiment in order to calculate a mass balance. The original mass in each jar was converted to dry weight. The weights for all samples taken for analysis were logged and converted to dry weight. The mass difference was calculated by subtracting the final dry weight mass, including samples, from the original mass. **Table 5** summarises the mass loss for each treatment.

2.32 Soxhlet Method

The substrate samples for analysis were vacuum freeze-dried. Approximately 0.5 g of freeze-dried sample was weighed to 5 decimal places and ground with approximately 10 g of granular sodium sulphate (Na_2SO_4) using a mortar and pestle. The homogenate was transferred to a cellulose extraction thimble, and spiked with a surrogate recovery mixture (Section 2.33). Soxhlet extraction was carried out using 350 ml DCM. The samples were run in a Soxhlet extractor for 8 h; reflux events occurred every 7-10 min.

2.33 Shake Method

Substrate samples from the biotransformation and bioaccumulation experiment were analysed for organic extractives content using the Scion extractable trace organics method.

Glassware and sodium sulphate were muffled at 450°C for 4 h before extraction. Each substrate sample was ground in a blender with sodium sulphate at a 1:10 ratio. Approximately 0.5 g of the mixture was then placed in a centrifuge tube followed by 50 μL of surrogate standard and 20 mL of DCM. The surrogate standard consisted of 2,4,6-tribromophenol, 8(14)-abietenic acid, d_{31}-palmitic acid, D_{10}-anthracene, dihydrocholestrol, and 2,4,6-tribromoanisole in acetone (1.0 $mg.mL^{-1}$ for each compound).

The centrifuge tubes were shaken by hand for ~1 minute to facilitate interaction between the mixture and the DCM solvent. The tubes where then placed into a centrifuge and spun for 5 min at 3500 rpm. The liquid layer was decanted into a Zymark tube via a drying column filled with sodium sulphate. The tubes were rinsed with 20 mL of DCM followed by a further 10mL to bring the total volume to 50 mL.

The anhydrous solution was concentrated to 0.5 mL using a Zymark Turbovap with a water bath temperature of 30°C and using nitrogen as a carrier gas. The solution was then transferred to a GC vial using a micro pipette. 50 μL of an injection standard was added consisting of dibromoanthracene in pyridine at a concentration of 1.0 $mg.mL^{-1}$. The sample was then silylated using the silylation agent N-o-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% TCMS and heated to 50°C in an oven for 0.5 h. The derivatised extract was analysed for organic compound concentrations using a HP 5971A gas-chromatograph-mass spectrometer. Organic extractives concentrations were quantified using an on-line data system.

The extractive results were corrected for recovery using the appropriate surrogate standard. For the resin acids, 8(14)abietinic acid was used, and dihydrocholesterol was used for the phytosterols. The corrected values were then divided by the dry weight of the amount of substrate that was used in the extraction, which resulted in a concentration of extractives (μg) per gram of substrate.

2.34 Surrogate Recoveries

The surrogate standard consisted of compounds that are chemically similar to the analyte group but not expected to occur in an environmental sample. It was used to monitor for unusual matrix effects and analytical errors. The recovery of the surrogate was expressed in percent which is 100 times the amount found in the sample divided by the amount of the surrogated added to the sample. A good percent recovery of the surrogates (usually between 80 and 120%) would indicate that the method was conducted satisfactorily, and target analytes were not present in the sample.

2.35 Comparison between Soxhlet and Shake Method

Two extraction methods were assessed for consistency and time efficiency.

Over 150 soxhlet extractions were performed including the complete data sets for days 0, 8 and 28 (**Figure 16**). The results for potting mix + extractives are graphed Figure 16 in with ±95% confidence interval error bars. A confidence interval is a statistical measurer that gives a range of values around the mean where the "true" mean is located. The soxhlet extraction confidence intervals were calculated by taking the standard deviation of the three replicates for each treatment. This value was multiplied by a t-distribution of 4.30 and divided by the square root of the sample size. As the graph shows the soxhlet method generated variable data as evidence by the wide confidence interval error bars.

Over 450 shake extractions were performed. The graph in Figure 18 shows the results for potting mix + extractives with ±95% confidence interval error bars. In addition to each treatment having three replicates the shake extractions were carried out in duplicate to decrease the degrees of freedom. The deviation for each sample duplicate was calculated by summing the squares of the differences between each observation and the mean. The deviations were then used to calculate a pooled standard deviation for the treatment. This value was multiplied by a t-distribution of 2.57 and divided by the square root of the sample size to calculate the ±95% confidence intervals.

The sample size for both the soxhlet and shake methods was approximately 0.5 g. However to make the potting mix samples more representatives of their respective treatments approximately 2 to 5 g of the samples was combine with ten times the sodium sulphate. The mixture was mechanically ground up into a power. Approximately 0.5 g of the mixture was then transferred into a centrifuge tube with the DCM solvent.

Chapter 3

TEMPERAL DEGREDATION OF ORGANO EXTRACTIVES IN K-BASIN

In 1993 Stuthridge and Tavendale undertook a detailed study of resin acids and their derived neutrals in K-basin. At the time K-basin was the first aerated stabilisation basin in the mill's second treatment system that ceased to be used in 1995. Both wastewater and sediment core samples were collected. The sediment was collected throughout the aerated stabilisation basin from a boat using a semi-automated gravity sediment corer. Cores (1m x 5 cm) were collected in stainless steel tubes and stored at 4°C until analysis. The upper 4 cm of sediment was mixed and used for surface sediment analyses.

Analysis of the sediment was carried out by mixing 1 g portion of the sediment with 9 g of anhydrous sodium sulphate and soxhlet-extracted with hexane/acetone (1:1) solvent. An injection standard (4,4'dibromo-octafluorobiphenyl) was added to the extract and the resin acids were silylated using BSTFA + 1% TMCS prior to analysis by gas chromatography/mass spectrometry.

This chapter compares the concentrations of resin acids and derived neutrals obtained by Stuthridge and Tavendale to the concentrations obtained from samples collect in March 2006. The environment in K-basin in 2006 differs vastly to what it was in 1993. The aerated stabilisation basin has been retired for approximately 11 years when it was abandoned in 1995 in favour of a new treatment system. Since that time the basin has grown a modest covering of vegetation and is largely dry. Parts of K-basin are subject to frequent flooding from rainfall.

The resin acids and derived neutrals are grouped into 11 compound classes (**Table 2**).

aromatic diterpene hydrocarbons dehydroabietin tetrahydroretene retene		*saturated diterpene hydrocarbons* fichtelite
dienic pimarane acids pimaric acid sandaracopimaric acid isopimaric acid	*monoenic pimarane acids* pimarenic acid sandaracopimarenic acid isopimarenic acid	*saturated pimarane acids* pimaranic acid isopimaranic acid
dienic abietane acids abietic acid palustric acid neoabietic acid	*monoenic abietane acids* 13-abietenic acid	*saturated abietane acids* abietanic acid
aromatic abietane acids dehydroabietic acid seco-1-dehydroabietic acid seco-2-dehydroabietic acid	*chlorinated abietane acids* 12-chlorodehydroabietic acid 14-chlorodehydroabietic acid 12,14-dichlorodehydroabietic acid	*oxygenated abietane acids* 7-oxodehydroabietic acid

Table 2 Compound classes for resin acids and resin acid derived neutrals

	1993	2006	
Compound Class	**Sample average (S1-S6) mg/kg.d.w**	**Sample average (S1-S6) mg/kg.d.w**	**Percentage Decrease**
aromatic diterpene hydrocarbons	43545	7063	84
saturated diterpene hydrocarbons	697	373	46
dienic pimarane acids	14057	465	97
monoenic pimarane acids	23270	5167	78
saturated pimarane acids	3229	727	77
dienic abietane acids	3982	861	78
monoenic abietane acids	4736	100	98
saturated abietane acids	35656	11804	67
aromatic abietane acids	8614	542	94
chlorinated abietane acids	501	209	58
oxygenated abietane acids	226	4	98
Total resin acid compounds	138512	27313	80

Table 3 Comparison between extractive concentrations in 1993 and 2006

3.10 Aromatic and Saturated Diterpene Hydrocarbons

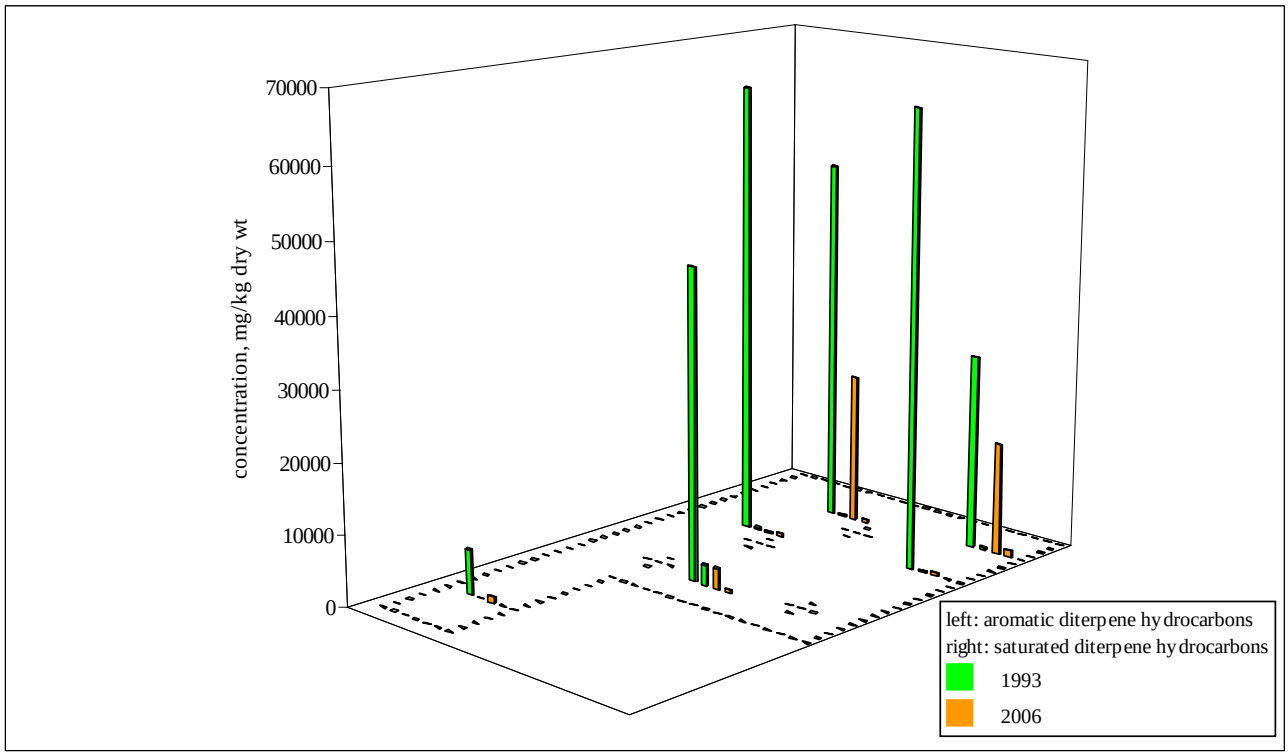

Figure 12 Distribution of aromatic and saturated diterpene hydrocarbons in K-basin

In 1993 resin acids and derived neutrals (nRasp) comprised nearly 14% of the total dry weight of the sediment. By 2006 this figure was reduced to a mere 3%; an 80% decrease. The distribution of aromatic and saturated diterpene hydrocarbons are shown in **Figure 12**. In 1993 aromatic diterpene hydrocarbons, including retene and dehydroabietin, were the most abundant compounds in K-basin. Although there has been an 84% decline; (**Table 3**) the compound class remains abundant second only to saturated abietanic acids. This is largely expected as Retene has been shown to be more bioaccumulative and recalcitrant than the parent compounds (Stuthridge and Tavendale, 1996; Leppanen and Oikari, 1999).

The distribution of retene and dehydroabietin throughout the basin has changed considerably over 13 years. The compounds are in their greatest concentrations at the outlet end of the basin. This reflects the environmental conditions which have ensued over the passing decade. The outlet end is the deepest part of the basin and is frequently flooded. The anoxic environment resulting from low oxygen renewal and high organic content creates conditions amenable to the anaerobic biotransformation of resin acids to neutrals (Brownlee *et al.*, 1977; Zender *et al.,* 1994; Tavendale *et al.* 1997).

Other sampling sites which recorded high concentrations of retene and dehydroabietic acid in 1993 have seen a massive decrease in concentrations. It is not altogether clear why these sites have experienced such a stark change other than oxidation from exposure to air and UV radiation. There may have also been a slow migration of extract compounds to the outlet end. This is confirmed by the fact the outlet end has the highest concentrations for each for the respective compound classes.

3.20 Pimaranes and Abietanes

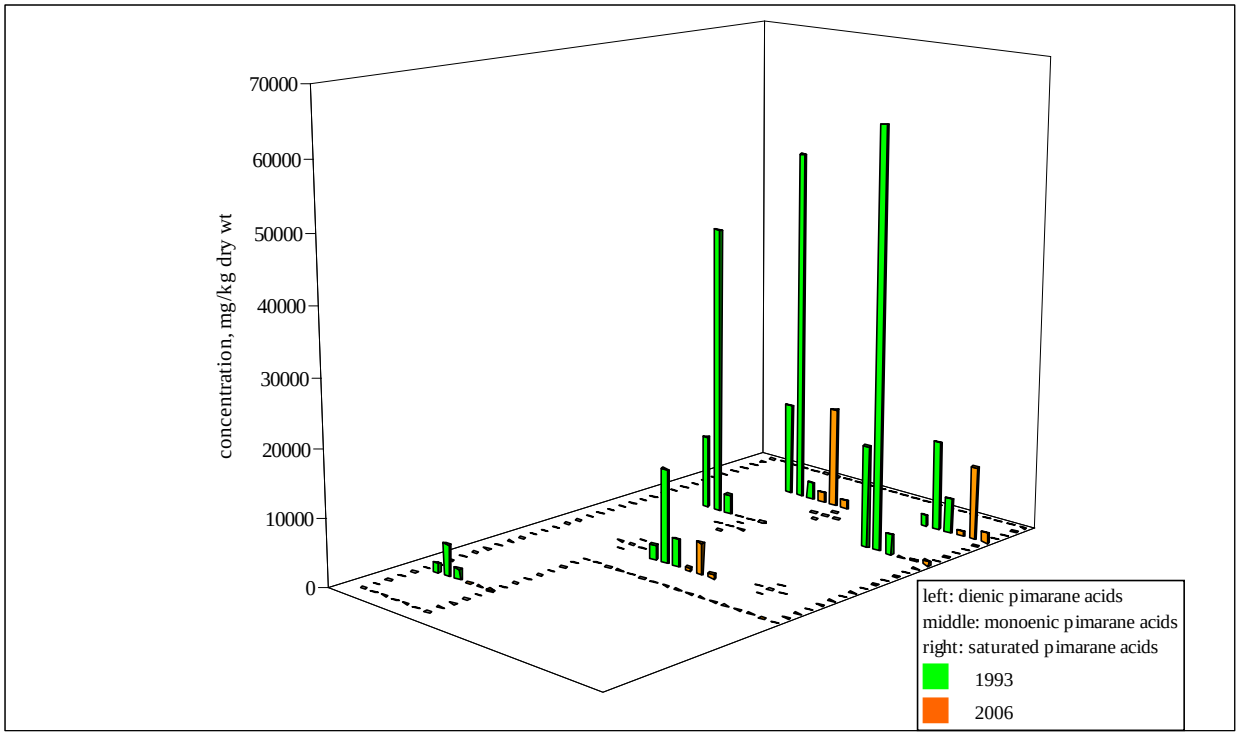

Figure 13 Distribution of pimaranes in K-basin

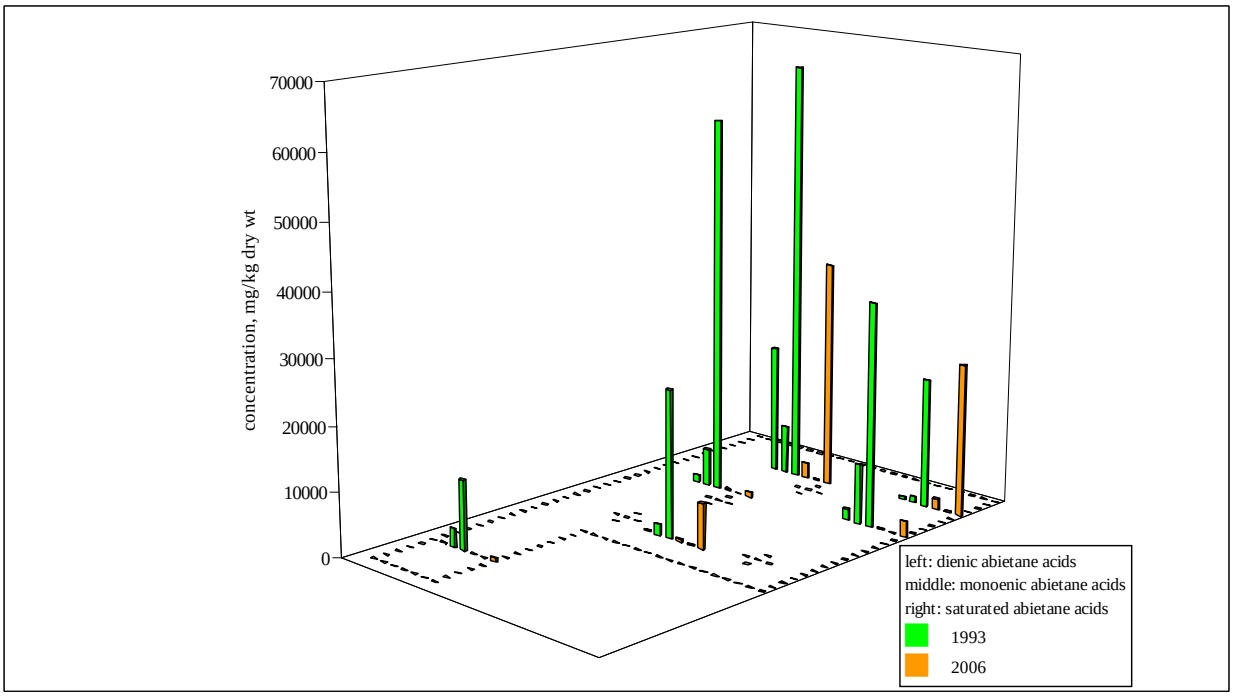

Figure 14 Distribution of abietanes in K-basin

The abietanes and the pimaranes are the predominant forms of resin acids in pulp and paper mill effluent. **Figure 13** and **Figure 14** show the rate of decay for each compound group respectively. Pimaranes are thought to be the most persistent resin acids in pulp mill effluent systems (Zender *et al.* 1994). In 1993 and 2006 abietanes were more abundant than pimaranes but experienced a similar rate in decay. The pimaranes, containing a vinyl group, are more acutely toxic than the abietanes, containing an isopropyl group, based on the inverse relationship between resin acid solubility and toxicity (Peng and Roberts 2000).

3.30 Summary

Over the period from 1993 to 2006 the resin acids and derived neutrals decreased in dry weight by approximately 80%. The decrease can primarily be attributed to oxidation and UV radiation. The highest concentrations of nRasp were found at the outlet end of K-basin. In contrast the other sampling sites showed a greater reduction in nRasp. This can be attributed to anoxic conditions at the outlet end of K-basin due to frequent flooding. Anoxic environments give raise to microbial biotransformation of resin acids. There may also be a migration of nRasp to the outlet end of K-basin due to a slight negative gradient.

Chapter 4

RESULTS

BIOTRANSFORMATION AND BIOACCUMULATION EARTHWORM BIOASSAY

4.10 Preliminary DCM Earthworm Toxicology Assay

	Earthworm survival	**Mortality %**
Potting Mix + DCM	0	100%
Potting Mix + DCM + 24 hours	8	0%
Control	8	0%

Table 4 Results of DCM toxicology assay

Earthworms did not survive in potting mix with unevaporated DCM. All the earthworms survived in the potting mix with evaporated DCM and in the control. This indicates that earthworms were not adversely affected once the DCM has been evaporated.

4.20 Surrogate Recoveries

The graph in **Figure 15** compares the surrogate recoveries for representative day 0 samples. Recoveries for all compounds ranged between 80%-120%.

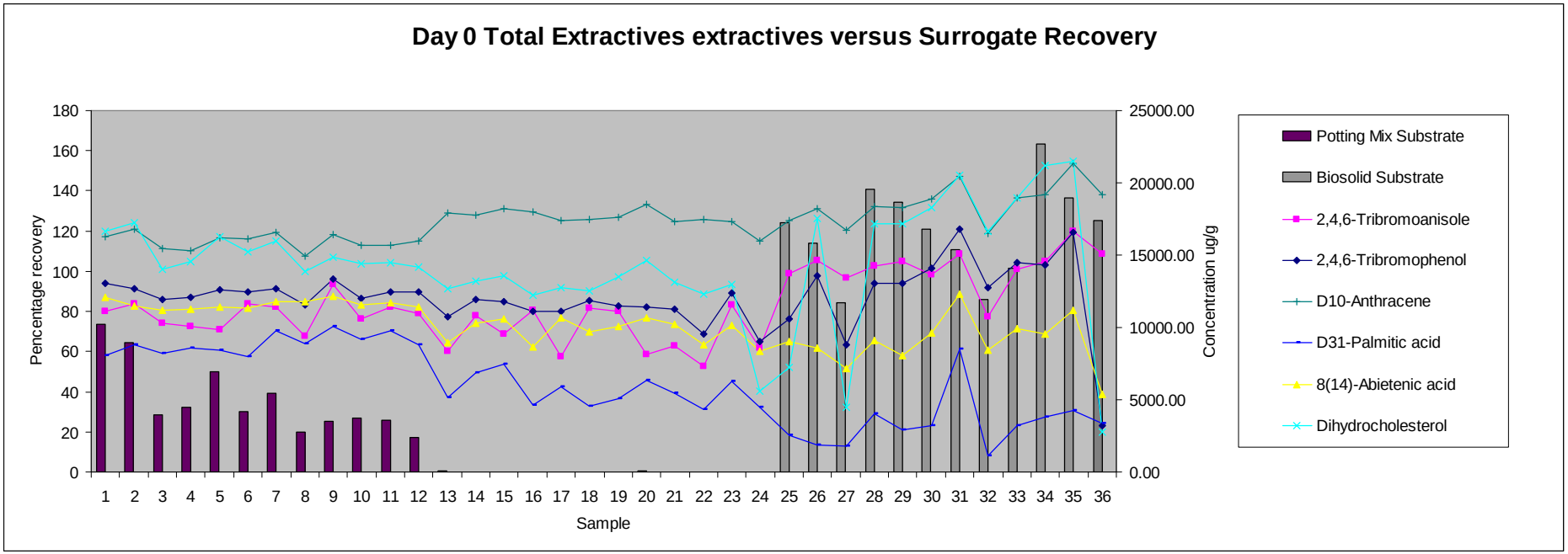

Figure 15 Surrogate recoveries for samples collected on Day 0. Samples 1-12 are potting mix + extractives. Samples 13-24 are potting mix (no extractives). Samples 25-36 are biosolids.

4.21 Comparison between Soxhlet and Shake Method

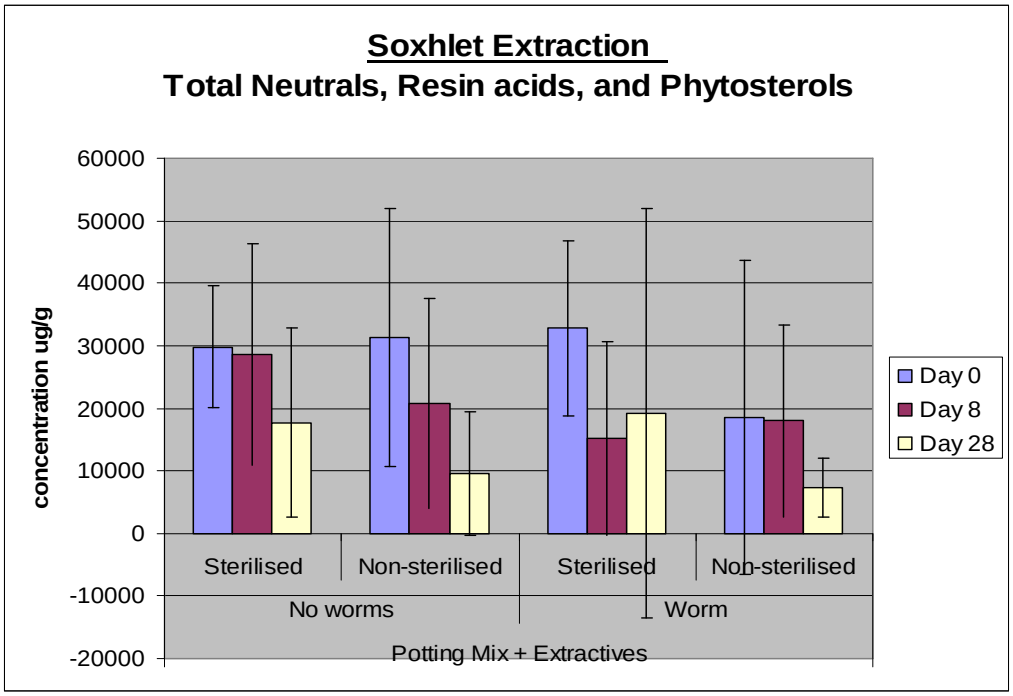

Figure 16 Soxhlet extraction results for potting mix + extractives with ±95% confidence interval

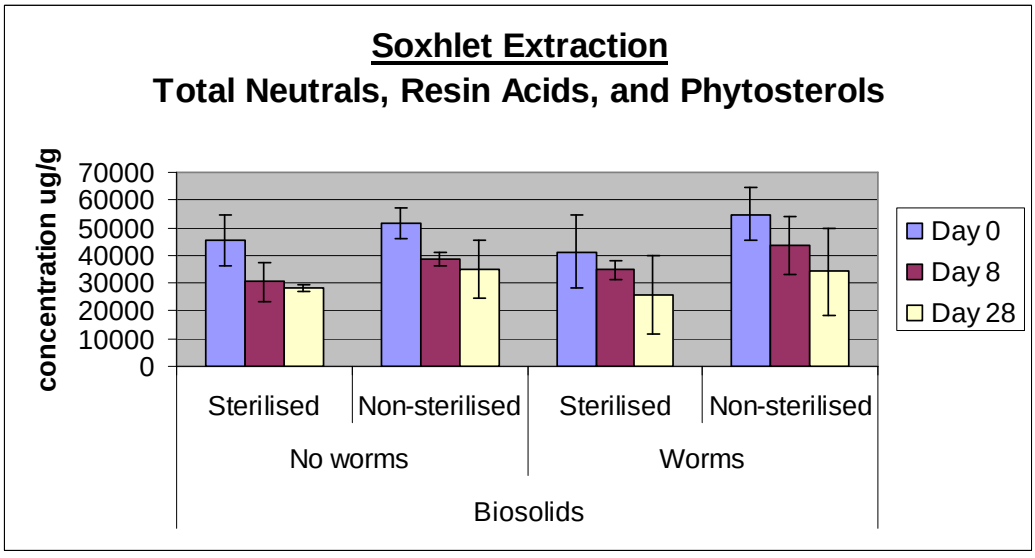

Figure 17 Soxhlet extraction results for biosolids with ±95% confidence interval

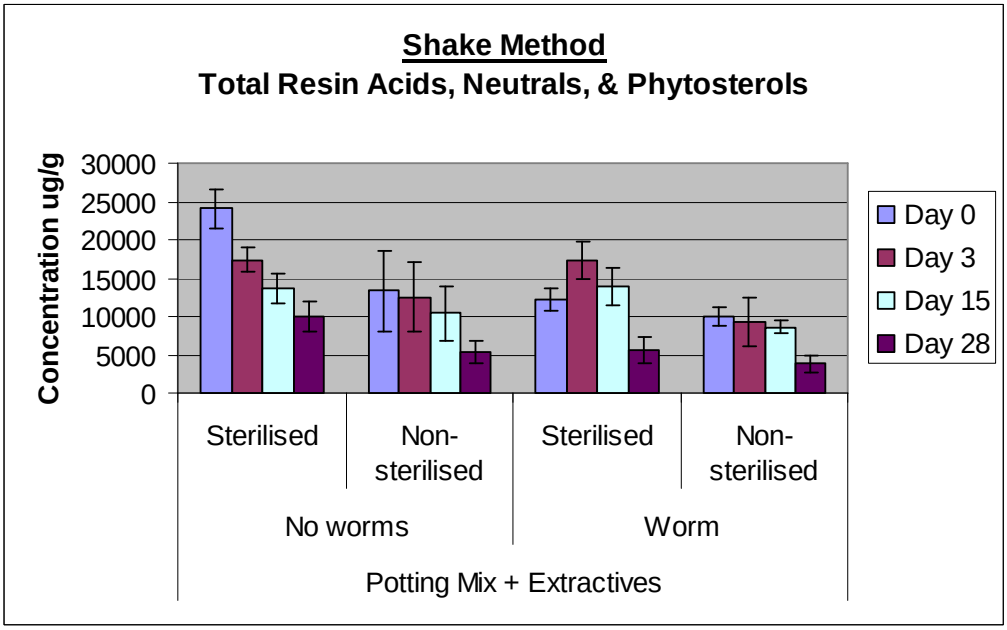

Figure 18 Shake Method results for potting mix + extractives with ±95% confidence interval error bars

The soxhlet method (**Figure 16** and **Figure 17**) produced results with a wide degree of variation as evidence by the huge overlap of the confidence intervals. The shake method (**Figure 18**) produced results with less variability around the mean evidence by the size of the 95% confidence intervals.

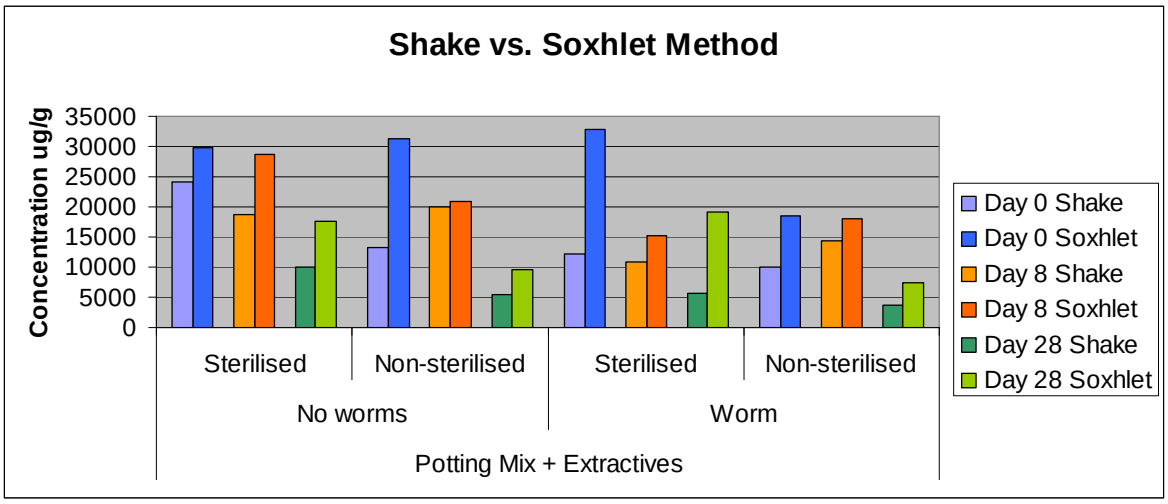

Figure 19 Compares the results for shake and soxhlets by concentration for potting mix + extractives

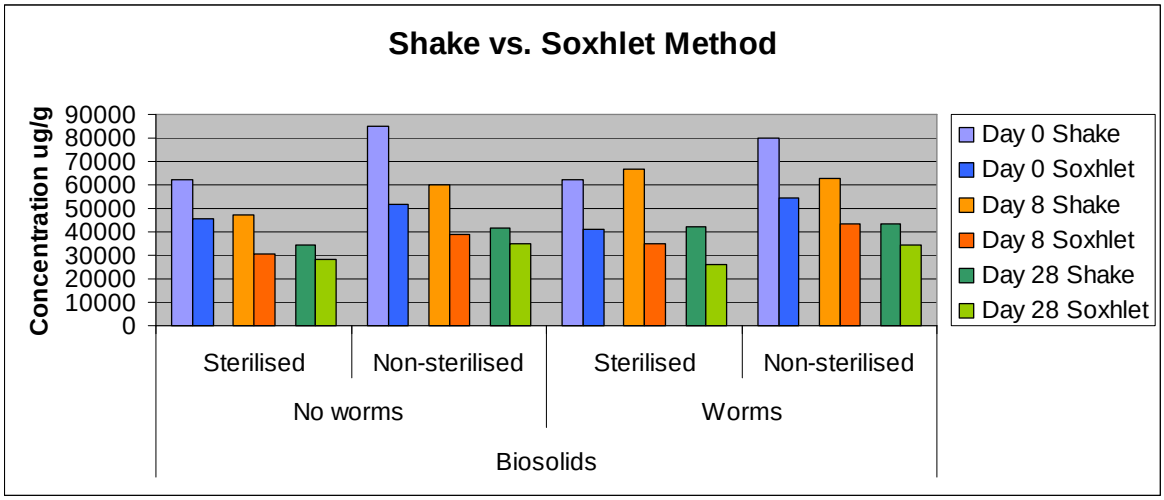

Figure 20 Compares the results for shake and soxhlets by concentration for biosolids

4.22 Mass Balance: Total Resin acids, Neutrals & Phytosterols

Treatments		Sterilised	Original Mass Grams	Final Mass Grams	Difference Grams	% Change
Potting Mix + Extractives	No worms	■	84.8	85.3	-0.6	0.7
	No worms		87.9	84.6	3.3	3.8
	Worms	■	85.1	76.4	8.7	10.2
	Worms		87.8	76.5	11.4	13.0
Potting Mix	No worms	■	79.5	78.1	1.4	1.8
	No worms		86.0	84.9	1.1	1.3
	Worms	■	88.2	86.0	2.2	2.5
	Worms		86.6	77.2	9.4	10.9
Biosolids	No worms	■	114.4	112.5	1.9	1.7
	No worms		116.5	115.2	1.4	1.2
	Worms	■	122.1	118.1	4.0	3.3
	Worms		117.2	112.8	4.3	3.7

Table 5 Average change in mass (substrate mass balance dry weight) per treatment of total substrate.

All treatments, expect for one, showed a reduction in mass. The mass reduction was greatest for the treatments with earthworms. This indicates the worms were metabolising organic material and releasing it as carbon dioxide and water. The original and final mass values were used to calculate the quantities of extract compounds. Therefore the amount of extractives in the earthworm treatments will be less compared to the no-worm treatments.

The decreases in total resin acids, neutrals and phytosterols (nRasp) are graphed on a box and whisker pot in **Figure 21**.

There was an over all decrease in nRasp for all treatments. However the means indicate there is no significant difference between earthworm and no-earthworm treatments. There also appears to be no significant difference between sterilised and non-sterilised treatments.

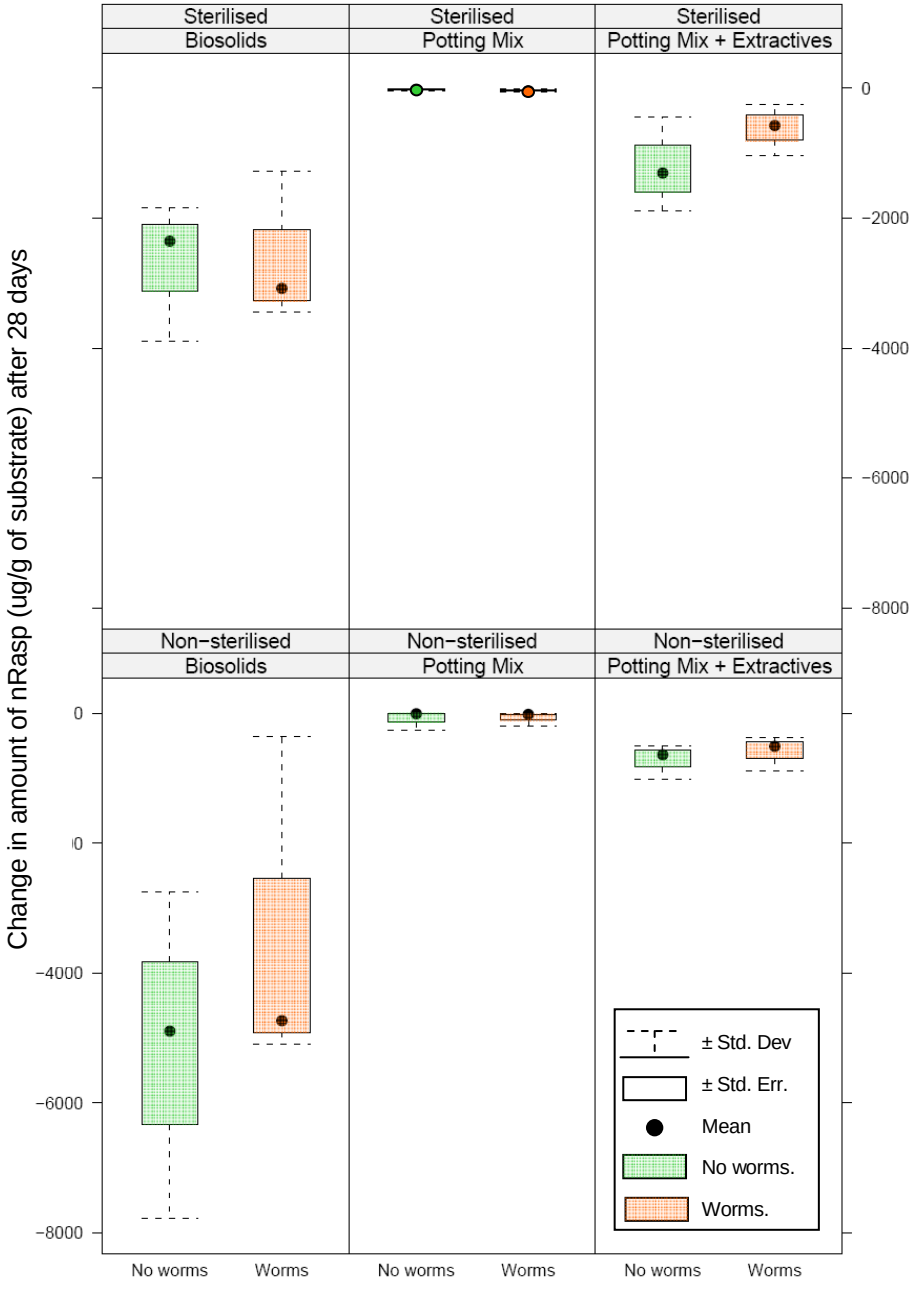

Figure 21 Change in Mass of nRasp

4.30 Earthworm Health

Table 6 compares the weight of earthworms on day 0 versus the weight of earthworms on day 28. On average there was a 38% decrease in earthworm weight. Whilst the earthworms exhibited a weight loss under all six treatment conditions, the only treatment significantly different from the other treatments (*P*-value = 0.0001 in a single factor ANOVA) was treatment 6. Treatment 6 (non-sterilised biosolids) was not exposed to methyl bromide but contained naturally occurring extractives. The factors contributing to increased weight loss in the other five treatments are; (i) methyl bromide sterilisation (the substrate did not appeal to the earthworms and they consequently limited their consumption); (ii) Potting mix (the substrate did not offer sufficient nutrients to maintain weight); (iii) a combination of the two. However, even the non-sterilised biosolids were less than ideal conditions for the health of the earthworms.

	Jar #	No. of worms Day 0	Total worm weight Day 0	No. of worms Day 28	Total worm weight Day 28	Percentage weight loss
1 PM+ Extractives Sterilised	**7**	20	12.844	20	8.95	30.32
	8	20	11.266	20	6.84	39.29
	9	20	10.826	20	6.66	38.48
Average Treatment 1						36.03
2 PM+ Extractives Non-Sterilised	**10**	20	12.949	20	7.28	43.78
	11	20	12.505	19	6.22	50.26
	12	20	10.698	20	6.65	37.84
Average Treatment 2						43.96
3 Potting Mix Sterilise	**19**	20	11.47	20	7.02	38.80
	20	20	12.49	20	7	43.96
	21	20	12.754	20	7.33	42.53
Average Treatment 3						41.76
4 Potting Mix Non-sterilised	**22**	20	12.907	20	7	45.77
	23	20	11.213	20	5.88	47.56
	24	20	11.823	20	7.04	40.46
Average Treatment 4						44.60
5 Biosolids Sterilised	**31**	20	10.819	18	7.03	35.02
	32	20	10.476	19	6.36	39.29
	33	20	11.865	18	5.89	50.36
Average Treatment 5						41.56
6 Biosolids Non-sterilised	**34**	20	10.963	20	9.38	14.44
	35	20	9.588	20	7.94	17.19
	36	20	10.28	20	8.53	17.02
Average Treatment 6						16.22
Overall Average		20	11.54	19.67	7.17	37.90

Table 6 Comparing the weight of earthworm weights at day 0 and day 28

Further evidence for the earthworm's lack of appetite for the substrate was demonstrated by their behaviour. In the dark the earthworms scaled their enclosures in an attempt to escape from biosolids as shown in **Figure 22**. To prevent the worms from escaping the bioassay was performed under continuous light which deterred the light sensitive earthworms from leaving the substrate.

Figure 22 Earthworms attempting to escape

4.31 Bioaccumulation in Earthworm Tissue

After 28 days of exposure, earthworms from each treatment were homogenised with Na_2SO_4 and extracted using the shake method to compare the concentration of extractives in the tissues. The results for total neutrals and total resin acids are shown in **Figure 23** and **Figure 24** respectively. **Figure 25** compares total neutral, resin acids and phytosterols.

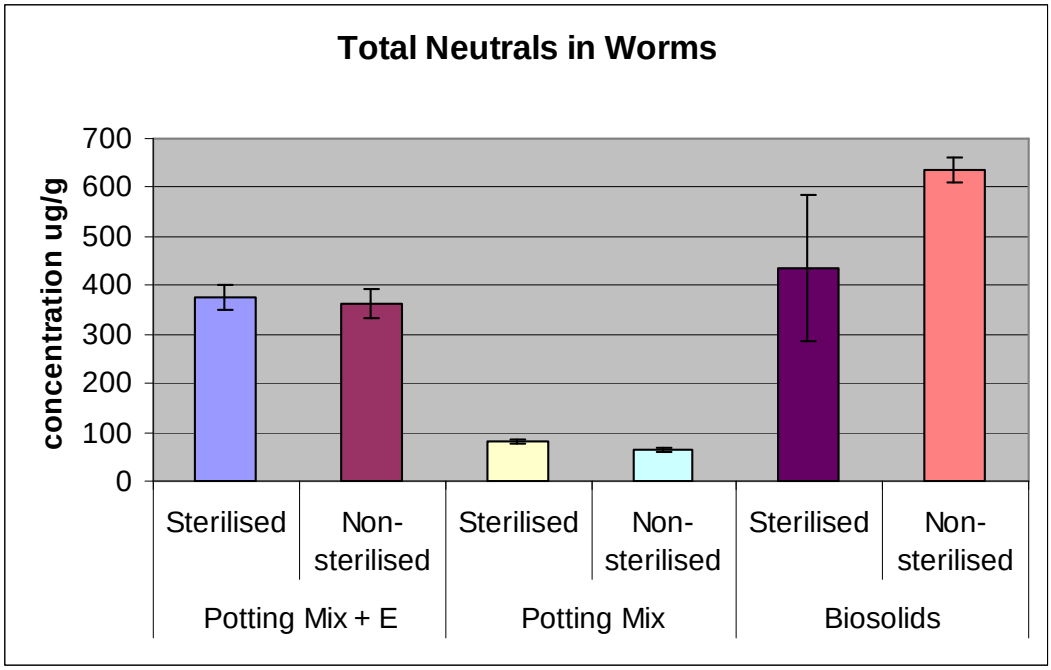

Figure 23 Concentration of neutrals in earthworm tissues cultured on different substrates. Values are means ± SEM. N = 36

As discussed in section 5.12 'Comparative Extractive Efficiencies', at day 0 the concentration of extractives in the PM+E treatment was approximately 80% lower than the biosolids treatment. Therefore it was expected bioaccumulation would be significantly higher in the biosolids exposed worms. Interestingly this does not appear to be the case. While there is a

higher concentration of neutrals in the biosolids, they are an order of magnitude lower than expected. The concentration of resin acids was remarkably higher in the PM+E treatment.

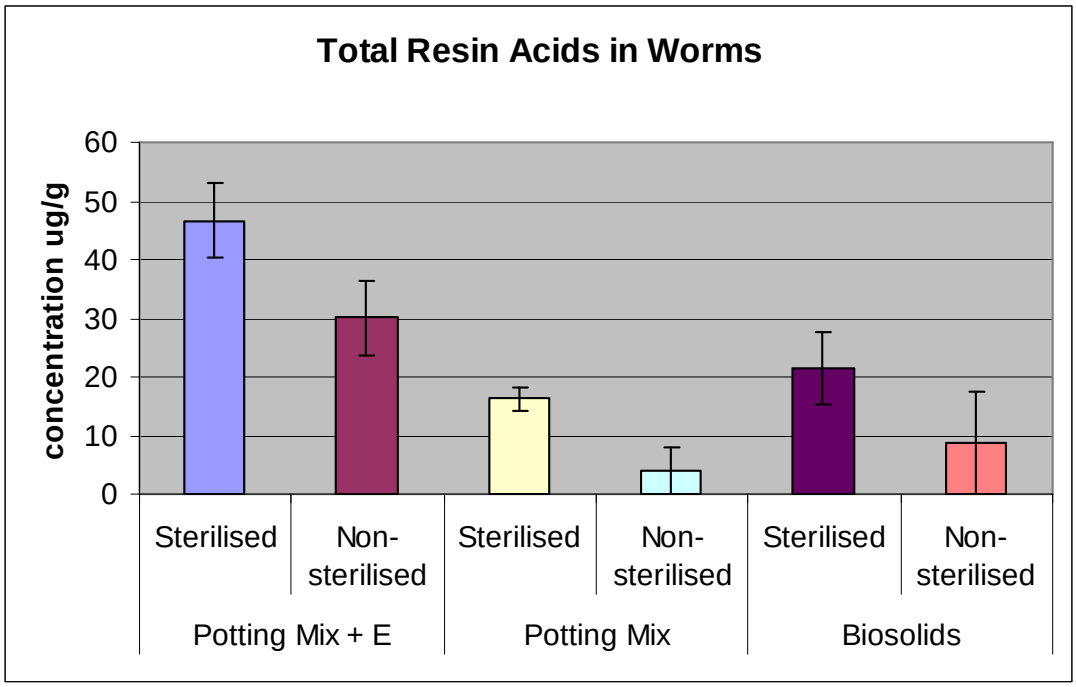

Figure 24 Concentration of resin acids in earthworm tissues with standard error

Figure 24 shows there to be a higher concentration of resin acids in the sterilised samples for each treatment.

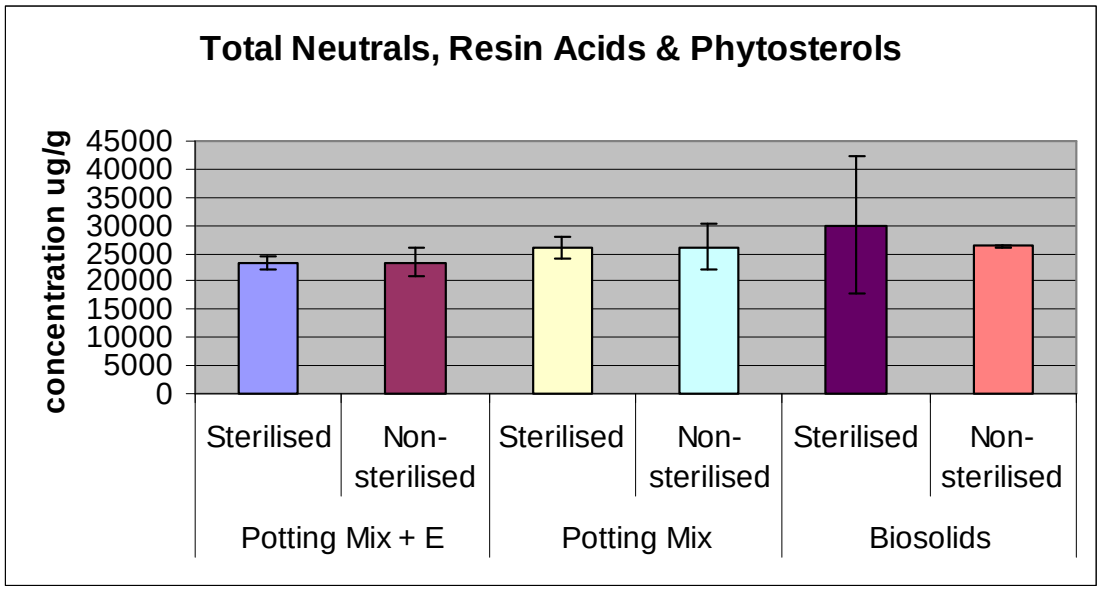

Figure 25 Concentration of nRasp in earthworm tissues with standard error

A relatively flat profile for total neutral, resin acids and phytosterols is seen in **Figure 25**. This is because the cholesterol based phytosterols make up a large proportion of the earthworm tissue extract. The phytosterols 'swamp' the sample hence why all treatments, including straight potting mix, have similar concentrations.

4.40 Concentration Summary: Total Resin acids, Neutrals & Phytosterols

Figure 26 compares the decrease in concentration for total resin acids, neutrals and phytosterols. The graph mirrors the decrease in nRasp mass shown in **Figure 21**. As concentration is expressed in μg/g a reduction in mass invariably affects the concentration. However the graphs illustrate that earthworm related mass reduction is negligible.

If the earthworms had only been able to reduce the mass of the supporting substrate and not the nRASP during the sampling period, then a subsequent increase in relative nRASP concentration would have been seen, whilst the overall mass of nRASP would remain unchanged.

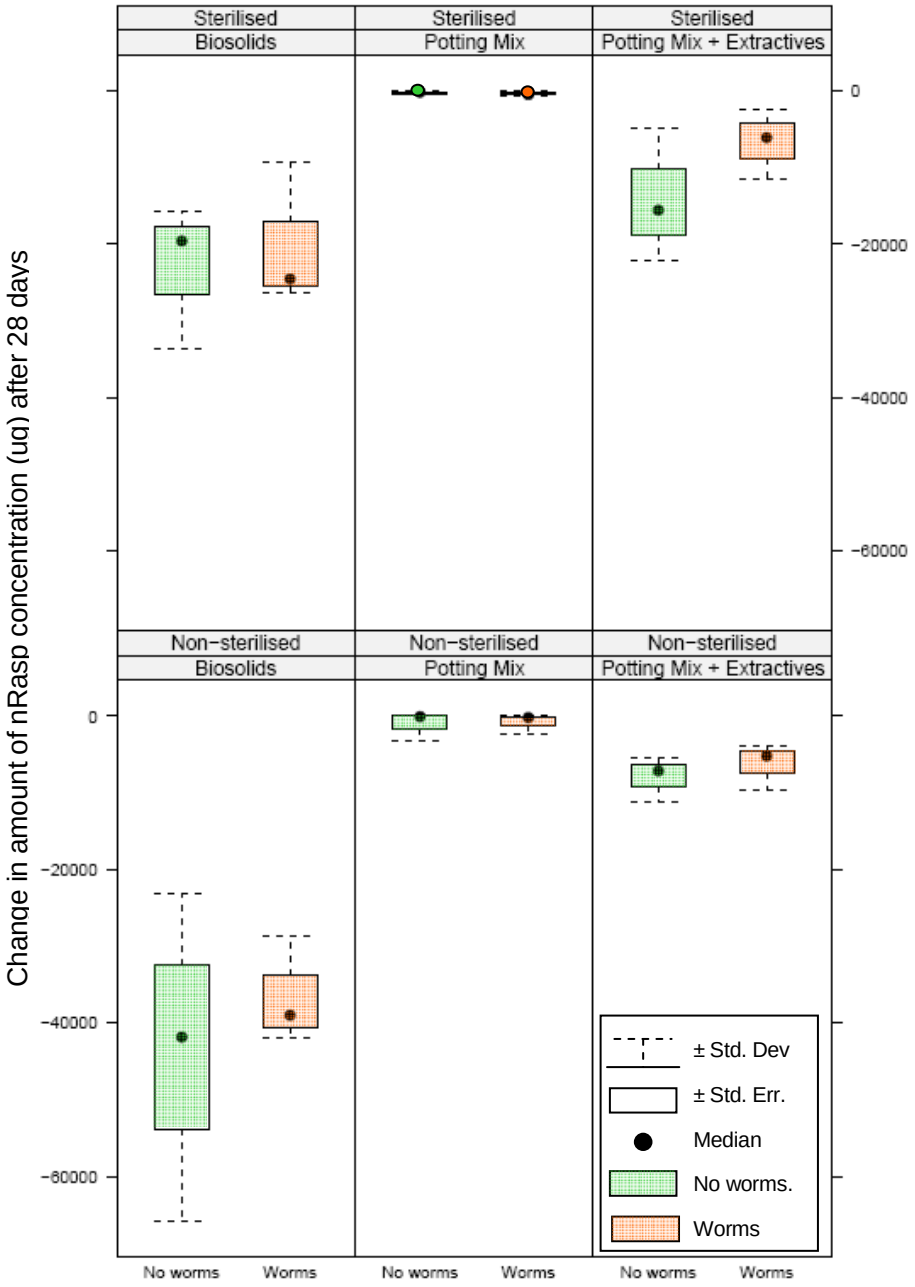

Figure 26 Change in concentration of nRasp

4.41 Resin acids versus Neutrals

Figure 27 shows the relative proportions of resin acid compounds for Day 0 biosolids. The abietic and pimaric type acids each represent approximately 50% of resin acids

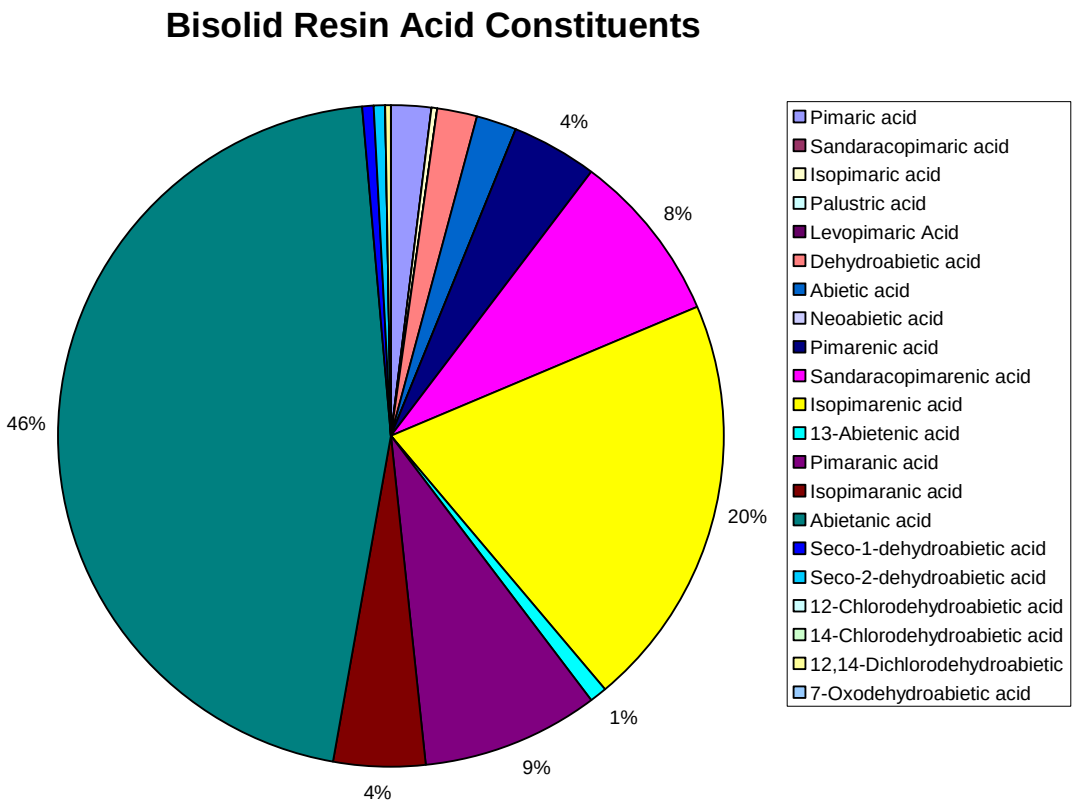

Figure 27 Proportion of resin acid compounds for Day 0 biosolids

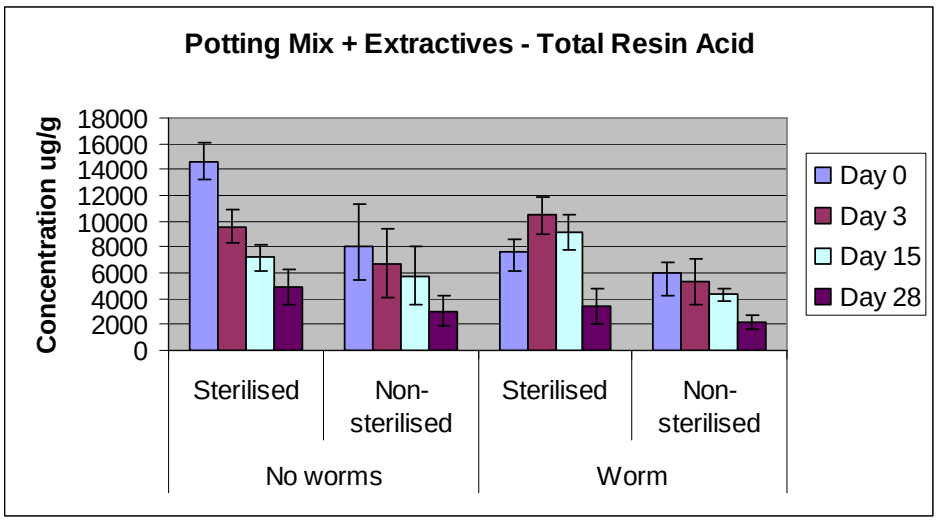

Figure 28 Resin Acid concentrations for potting mix + extractives with ±95% confidence interval

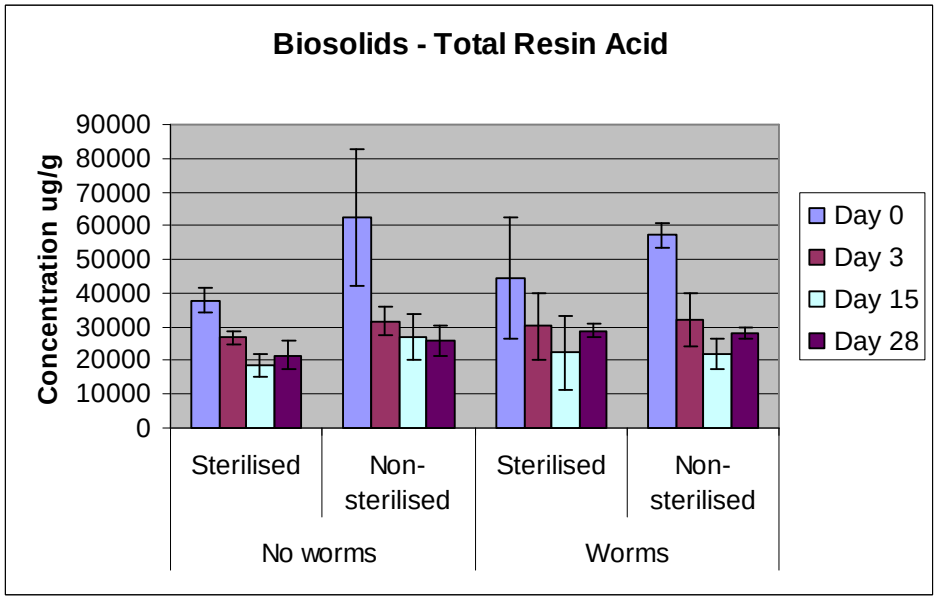

Figure 29 Resin acid concentrations for biosolids with ±95% confidence interval

Figure 28 and **Figure 29** show resin acid concentrations for PM+E and biosolids treatments over 28 day exposures. The error bars are 95% confidence intervals calculated from a pooled standard deviation. The respective graphs for neutrals are shown in **Figure 30** and **Figure 31**. All graphs show a decline in concentrations. The substrates PM+E and biosolids show a similar rate of decline in concentrations of resin acids and neutrals. The substrates are not directly comparable as the biosolids contains significantly higher concentrations of extractives.

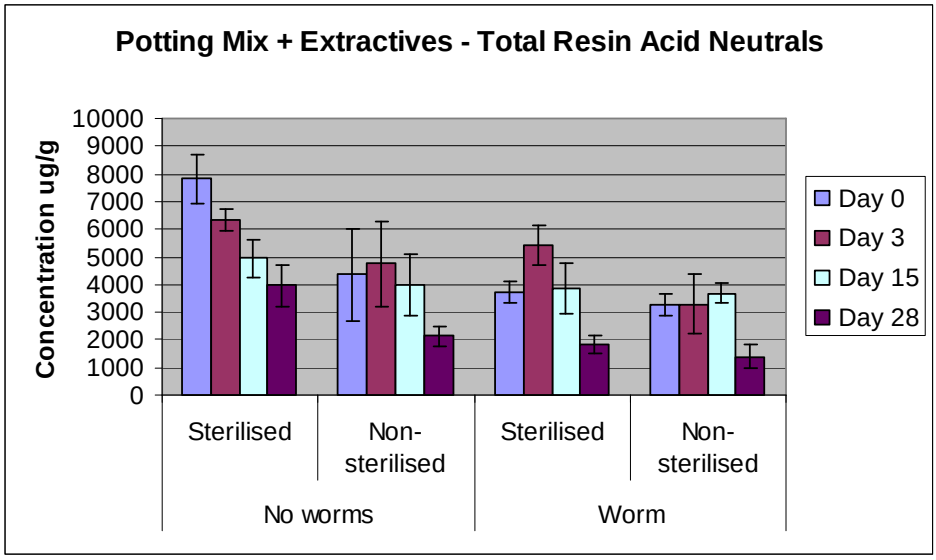

Figure 30 Neutral concentrations for potting mix + extractives with ±95% confidence interval

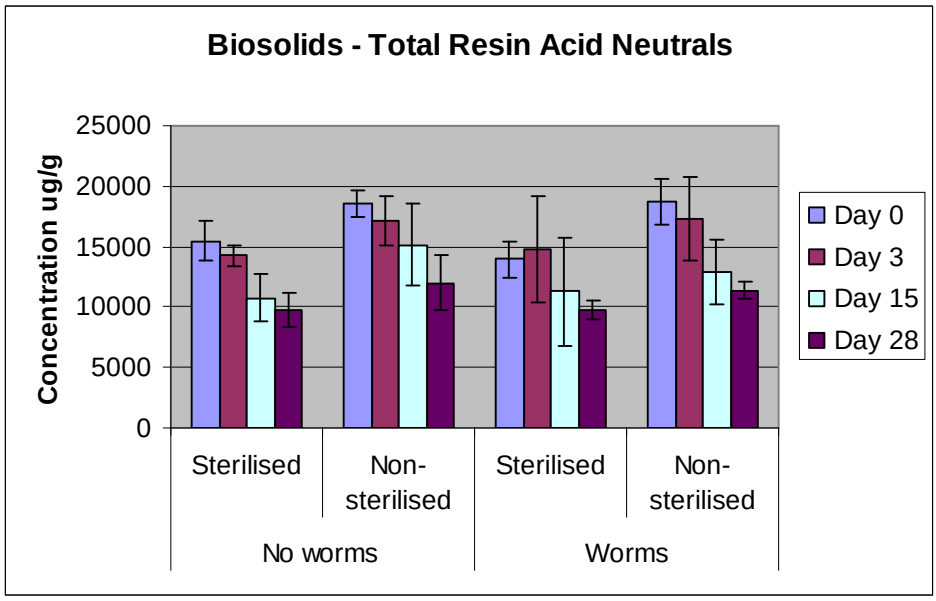

Figure 31 Neutral concentrations for biosolids with ±95% confidence interval

4.42 Retene and Fichtelite

The graphs for retene and fichtelite in **Figure 32**, **Figure 33**, **Figure 34**, and **Figure 35** display a similar trend to the total neutrals graphs.

The average concentration of retene on day 3 exhibits an increase over day 0 for sterilised treatments with earthworms.

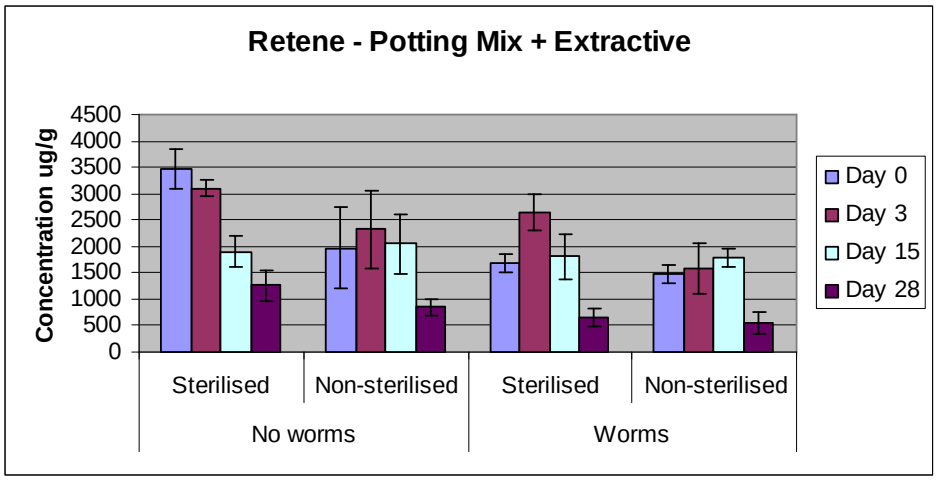

Figure 32 Retene concentrations for potting mix + extractives with ±95% confidence interval

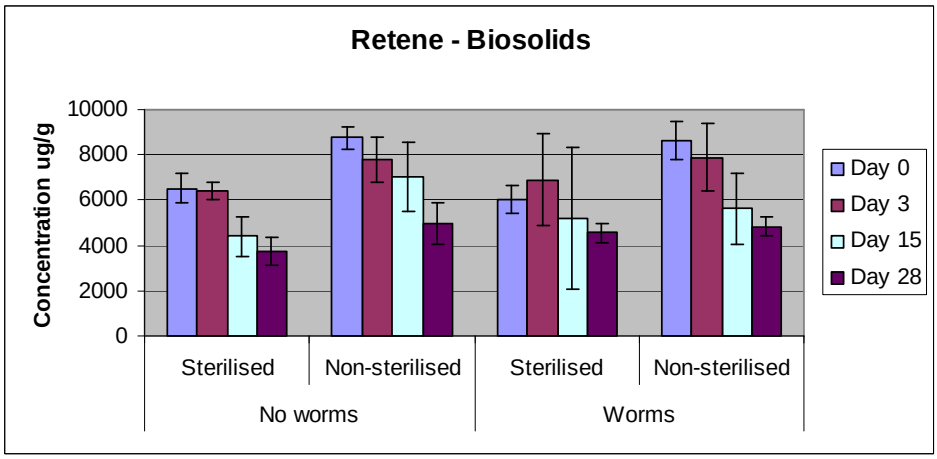

Figure 33 Retene concentrations for biosolids with ±95% confidence interval

While the overall concentrations of retene decreased over the 28 day period, in contrast the fichtelite concentrations showed little or no change. This was most evident in the biosolid treatments.

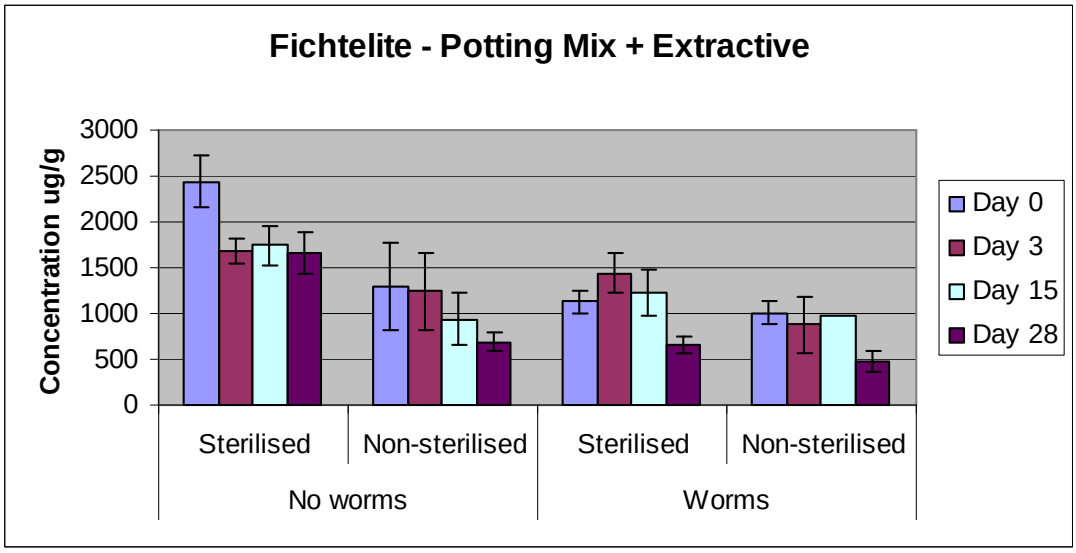

Figure 34 Fichtelite concentrations for potting mix + extractives with ±95% confidence interval

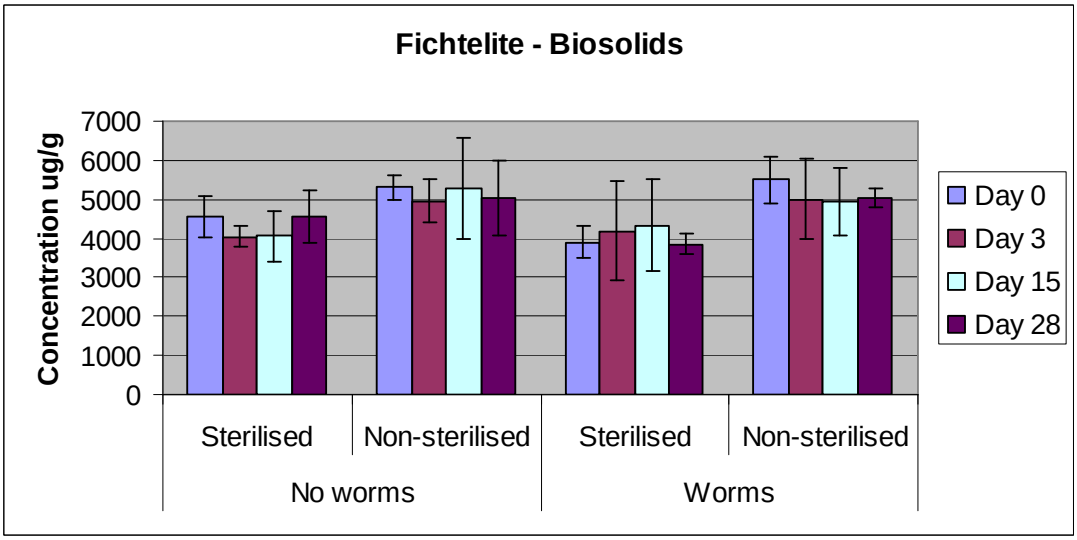

Figure 35 Fichtelite concentrations for biosolids with ±95% confidence interval

4.43 Sitosterol and Sitostanol

Figure 36 and **Figure 37** show the concentrations of sitosterol for PM+E and biosolids. For comparison the equivalent graphs for sitostanol are shown in **Figure 38** and **Figure 39**.

The relative ratio of sitosterol to sitostanol was maintained throughout the 28 day experiment.

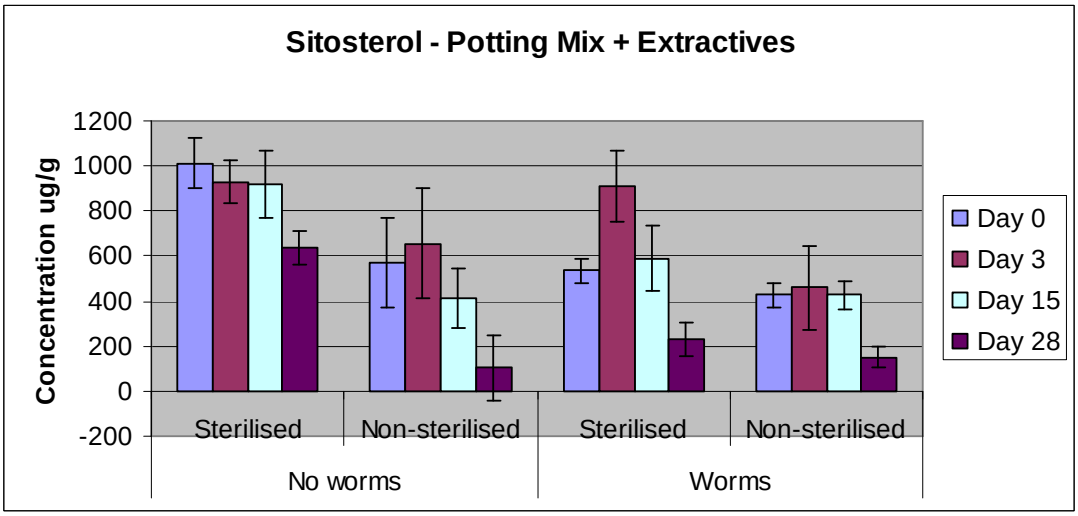

Figure 36 Sitosterol concentrations for potting mix + extractives with ±95% confidence interval

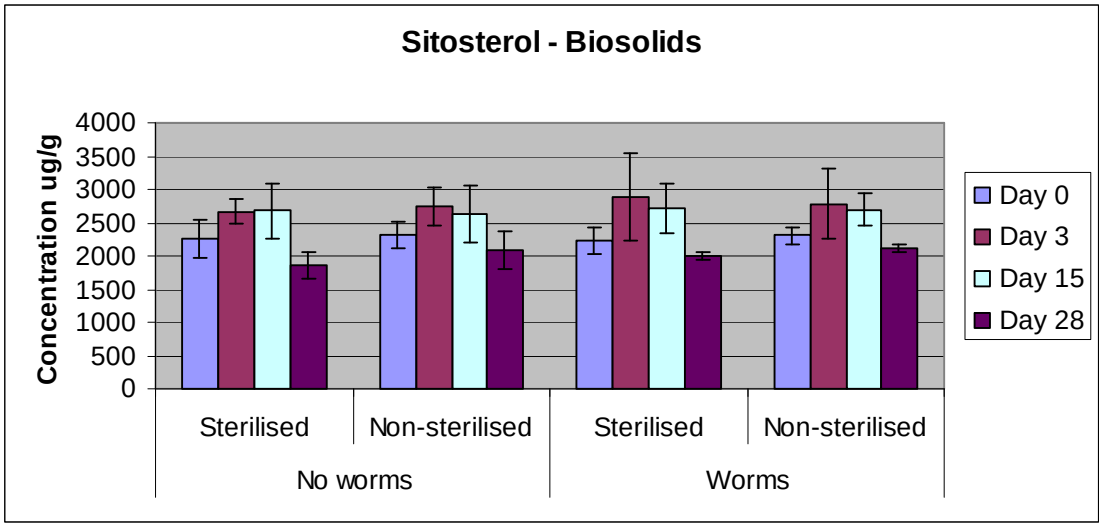

Figure 37 Sitosterol concentrations for biosolids with ±95% confidence interval

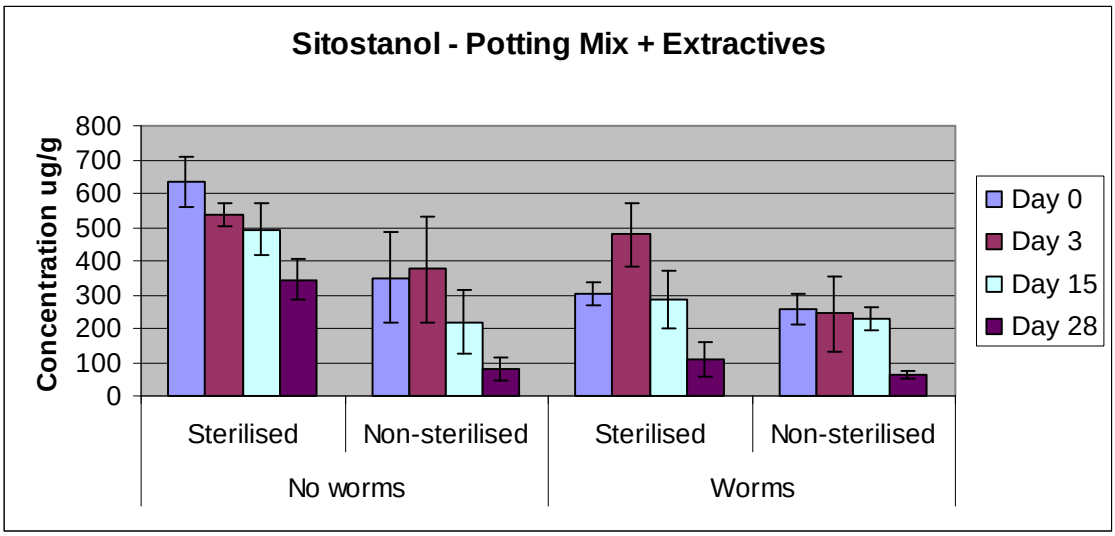

Figure 38 Sitostanol concentrations for potting mix + extractives with ±95% confidence interval

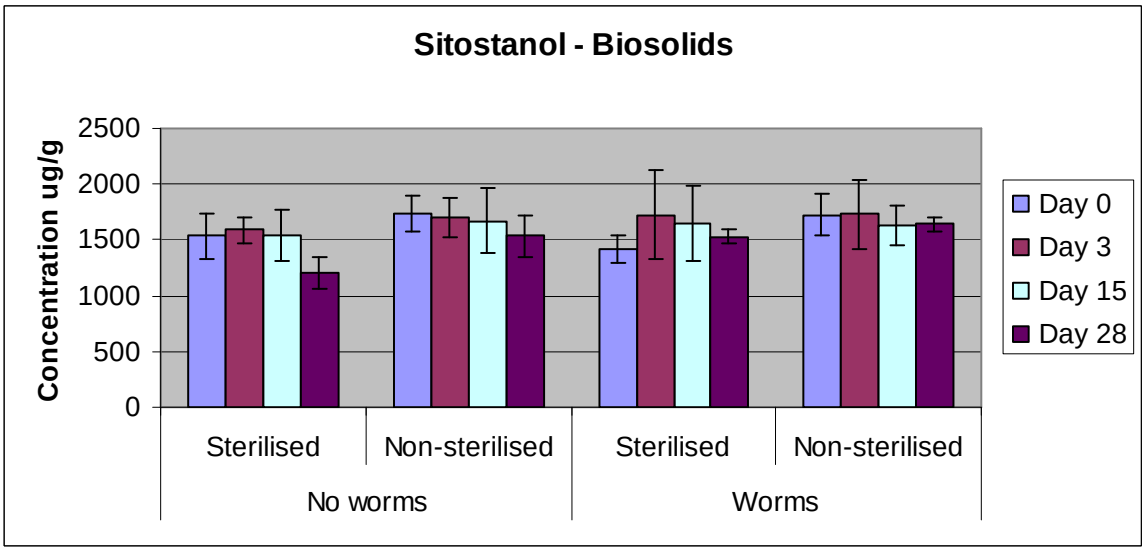

Figure 39 Sitostanol concentrations for biosolids with ±95% confidence interval

Chapter 5

DISCUSSION:

BIOTRANSOFRMATION AND BIOACCUMULATION EARTHWORM BIOASSAY

5.10 Surrogate Recoveries

The variations in surrogate compounds between potting mix and biosolid substrates is indicative of matrix effects. There was greater recovery of surrogate D31-palmitic acid in potting mix compared to biosolids. This may be caused by interference from native palmitic acid found in the biosolids.

For the purpose of this study biosolid extract was mixed in with the potting mix. The concentration of phytosterols in the potting mix was significantly lower compared to the biosolids. This can be attributed to the inefficiencies with the soxhlet procedure used to remove the extract from the biosolids and the strong binding of phytosterols to particulate matter. The fluctuating percent recoveries of extractives seen in **Figure 15** could be explained by the high concentration of phytosterols in the biosolids which may have interfered with the recovery of dihydrocholesterol .

Other factors that may influence surrogate recovery are evaporation of the surrogate standard and handling errors. The surrogate compounds are suspended in DCM and stored in a glass vial. A micro-syringe was used to transfer 50 μL of surrogate standard to each sample. Evaporation of the surrogate standard can occur throughout this process skewing the relative concentrations of the various compounds.

When not in use the surrogate standard was stored in the fridge to prevent evaporation. Under cooling conditions the molecules would partition. To remedy this before use the surrogate was brought to room temperature and shaken to ensure a homogenous solution.

5.11 Comparison between Soxhlet and Shake Method

The potting mix + extractives data (**Figure 16**) showed greater variability compared to the biosolids (**Figure 17**). This could be due to physical differences between the potting mix and biosolid substrates. The biosolids were homogeneous in nature whereas the potting mix was a conglomerate of materials including; bark, soil, fertilisers, and stones. The variable make-up of the potting mix had implications for the soxhlet procedure. The high concentration of extractives limited the samples size to approximately 0.5 g. The smaller the sample size the less likely it is representative of the potting mix treatment.

In addition to the analytical variability the soxhlet technique had disadvantages compared to the shake method.

- The procedure uses large volumes of hazardous and flammable liquid organic solvents (Naude *et al.* 1998).

- The high cost of materials such as cellulose extraction thimbles and high-purity solvents.

- The extraction is non-selective for organic compounds and can overwhelm the GC-MS.

- It is a laborious as the soxhlet glassware needs to be handled with care.

- It is a time-consuming procedure as the extraction cycles for 4-8 hours.

Advantages of the shake method over the soxhlet method include:

- It is less time consuming as the samples are only shook for approximately 1 minute each.

- It is less laborious as no soxhlet extractors required.

- It is less costly with no need for soxhlet thimbles or large quantities of organic solvents.

Overall the shake method is more environmentally acceptable. Soxhlet extractions generate large volumes of contaminated, hazardous solvents and emit toxic fumes (Naude *et al.* 1998). Therefore the shake methods are more inline with the theme of this research.

Figure 19 and **Figure 20** compare the extraction efficiencies for the shake and soxhlet measured by comparing achieved concentrations. The potting mix + extractives graph consistently shows the soxhlet method yields higher concentrations over the shake method whereas the converse is true for the biosolid substrate. However given the soxhlet method has a large error margin it can not be definitively stated that one method is more efficient.

5.12 Comparative Extractive Efficiencies

Figure 40 compares the concentrations of extractives obtained from K-basin biosolids with the standard error. Each bar represents the total resin acids, neutrals and phytosterols for each application prior to the addition of the earthworms.

Each application is as follows:

Biosolids – extracted using the shake method and represents the baseline for K-basin biosolids.

Soxhlet derived extract – biosolids extracted using the large scale soxhlet method for the purpose of mixing with potting mix to simulate K-basin biosolids.

Potting mix + extract – extracted using the shake method.

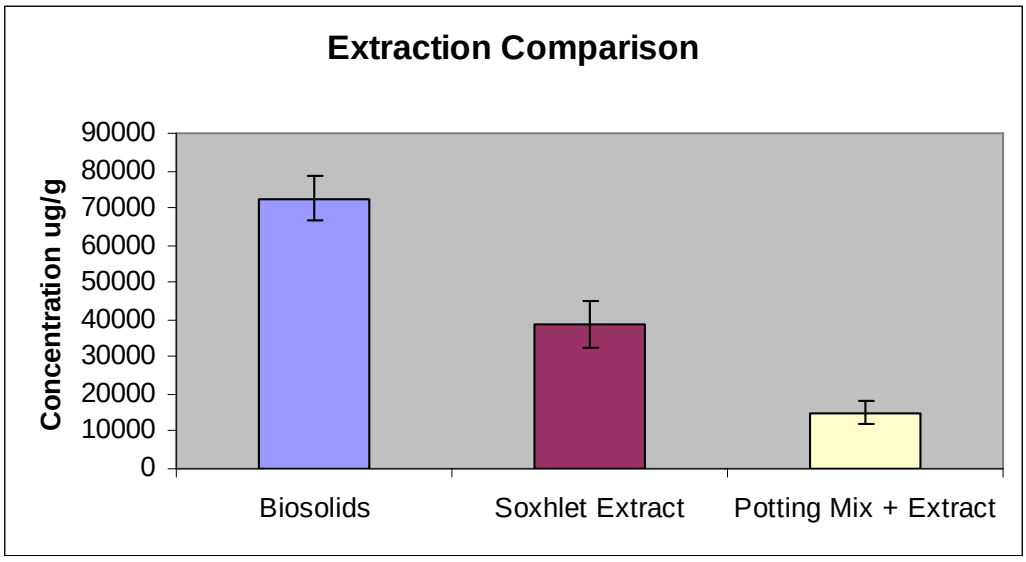

Figure 40 Compares the extract concentrations (ug/g) obtained from three extraction applications

Theoretically each application should be identical given they are from the same batch of biosolids prior to the addition of the earthworms. The differences are likely due to a number of analytical and sample handling factors.

The soxhlet extract achieved an average concentration approximately 47% lower than the biosolids. The difference is due to the sheer scale of the soxhlet extraction. A total of 1,500 g of wet biosolids was extracted using the large soxhlet method. Each soxhlet extraction used approximately 200-300 g of biosolids and cycled for 4 days. The extract volumes were blown down with nitrogen gas. In contrast the shake method used approximately 10-15 grams of freeze-dried biosolids.

It is recommended that samples are freeze-dried prior to extraction as moisture may inhibit contact between dichloromethane and sample (Spigno *et al.* 1997). DCM is a non-polar solvent and is repelled by water. Further confounding the action of DCM was the packing of the biosolids in the soxhlet thimbles. The density of the biosolids may have inhibited the flow of DCM.

The decision not to freeze-dry the biosolids for soxhlet extraction was based on the following two reasons:

- The amount of time required to freeze-dry over 1.5 kg
- The assumption 4 days would provide a sufficient amount of time for extract removal

The potting mix + extract achieved an average concentration approximately 38% lower than the soxhlet extract. This was disappointing considering they are the same extract. The difference can be attributed to a number of factors:

- Analytical error due to the variable nature of the potting mix composition (see section 5.11)

- The lost of extract onto the surface of glassware

The potting mix + extractives achieved an average concentration approximately 80% lower than the biosolids. Therefore the substrate failed to simulate the biosolids on a quantitative level. However there was still valuable information to be gained from comparing trends between the two substrates.

5.20 Mass Balancing

Volume reduction is one of the known benefits of vermicomposting. The vermicomposting process can reduce waste volume by 30 to 50 percent (Elvira, 1996). Earthworms help break apart larger pieces of composting material thus increasing their exposure to microbial degradation. The most easily decomposed substances are oxidized first (such as sugars). Compounds resistant to degradation (such as lignin and non-organic materials) make up the bulk of the finished compost product (Elvira, 1996). Carbon present in the organic materials is used by micro-organisms, transformed into carbon dioxide, and released into the environment. As carbon is lost from the substrate, the compost becomes more condensed and air spaces within the substrate become smaller.

The conclusion drawn from **Figure 21** is that the decrease in nRasp is irrespective of whether the substrate contains earthworms or is sterilised. Therefore, through deduction, the decrease in nRasp concentration is due to factors common to all treatments. As all samples were open to the air the nRasp compounds may have been broken down by oxidation. This process would have been enhanced through the rigors of sampling; the content of

each jar was spread over tin-foil to obtain a representative sample. Ledakowicz *et al.* (2006) investigated the applicability of oxidation processes to eliminate resin acids. The research found the action of ozone on resin acids present in water solutions gives almost complete destruction of the characteristic three-ring structures of these molecules. Of perhaps greater significance was the finding that an increase in UV radiation and temperature enhances the reduction of resin acids. The earthworm bioassay was carried out under a 24 hour constant light photoperiod and a constant temperature of 20°C. The combination of oxidation, UV radiation, exposure to light and constant temperature are the main contributing factors to main factors responsible for the reduction in nRasp concentrations.

A consequence of the oxidation pathway is the formation of partially oxidised intermediates which have lower reactivity with oxygen and could have lower biodegradation ability than the initial components (Laari *et al.* 2000). Therefore the treatments may contain a toxic level of oxidised nRasp compounds not detected by the GC-MS. Further research is required to determine any residual toxicity in the treatments.

5.30 Earthworm Health

Although there was no apparent effect of earthworms in this experiment, it does not preclude the possibility that earthworms could biotransform extractives under different conditions. There is evidence to suggest the earthworms were averse to the biosolid and potting mix substrates, hindering any potential ability to degrade the extractives.

Both the potting mix and biosolid substrates contain high amounts of structural materials cellulose and lignin which are hard for the worms to digest. Research has shown a strong relationship exists between invertebrates and micro-organisms to make use of soil organic matter. It is proposed that mucus excreted by earthworms plays an active role in their mutualistic digestion system; earthworms enhance microbial activities by providing an energy-rich and easily metabolisable intestinal mucus in their gut (Lattaud *et al.* 1999). This allows the ingested soil microflora to recover all its abilities to degrade complex substances such as cellulose and make it digestible (Lavelle & Gilot 1994). The native microbes in the substrates may not possess the capability to degrade cellulose and lignin. This would impact on the earthworm's ability to digest the material. However studies have shown the *Eisenia fetida* species processes a complete enzymatic system for hydrolysing cellulose (Lattaud *et al.* 1999). The nutrient deficient substrates may not provide the necessary energy or microbial activity for enzymatic action.

A possible solution to address the nutrient deficient substrates is through supplementation. Butt (1993), for example, investigated two species of worms fed on a paper mill sludge with yeast extract as a nitrogen source. The sludge was mixed with 3 different yeast extracts at a ratio of 67:1 to bring the C:N ratio down to 25:1. Whilst additions of yeast extract did enhance growth compared with paper only, the responses varied with each yeast.

Other potential supplements investigated by Elvira and Dominguez (1995) include; rabbit manure, sewage sludge, and pig or hen slurries. Each supplement improved earthworm growth dramatically. It was considered that an improved nutrient balance and an increase in micro-organism populations had both contributed. However it may be that the worms gained the bulk of their diet from the supplementary food source relying relatively little on the PMS compost.

5.40 Bioaccumulation in Earthworm Tissue

The result shown in **Figures 23**, **Figure 24,** and **Figure 25** supports the theory that a relationship exists between substrate micro-organisms and the earthworms. Karsten and Drake (1994) compared the aerobic and anaerobic potentials of gastrointestinal microflora of earthworms. Their results showed the guts of the earthworms examined contained similar numbers of culturable anaerobes and aerobes, while soils appeared to have a higher number of microbes capable of aerobic growth. This reinforced the general concept that the earthworm gut is not microbiologically equivalent to soil. It was speculated that certain ingested microbes find better environmental conditions in the intestine relative to that of the soil and hence proliferate during gut passage. Their study suggested that the gut of the earthworm might harbour mobile anaerobic microsites in otherwise well-aerated soil. The microsites may have occurred in response to an oxygen gradient in the earthworm's gut. It is likely that worms ingest oxygen together with food particles and that the oxygen concentration decreases from the anterior gut to the posterior gut due to microbial respiration during passage through the gut. A second oxygen gradient might also occur from the gut wall (blood vessels) to inner gut sites (Karsten and Drake, 1994).

Resin acid neutrals are formed through biotransformation by anaerobic micro-organisms. The biosolids were found to have high concentrations of neutrals which indicates the substrate is host to an anaerobic microbial population. The anaerobic microbes are ingested by the earthworms where they proliferate in the favourable conditions in the earthworms gut. These microbes are native to the biosolids and therefore have the necessary cellular and metabolic mechanisms to biotransform resin acids to their neutral derivatives. The anaerobic microbes in the gut of the earthworm biotransform resin acids to neutrals. As a result the neutrals accumulate in the

tissues of the earthworms. This gut-based biotransformation is supported by the concentrations of neutrals found in the biosolid substrate. Individual neutral compounds are examined in the following sections.

Although the experiment was not performed under sterilised conditions the result suggests; (i) time was required for microbes to recover and re-establish in the sterilised substrates, (ii) that when there is a limited number or an absence of microbes the earthworms ability to degrade resin acids is hindered. Once again the result provides evidence for a relationship between substrate microbes and earthworms. The relationship is also evident when comparing the substrates PM+E and biosolids. The microbes in the biosolids have adapted to the high concentrations of extractives and possess the mechanisms to degrade them. The earthworms enhance the microbes in the biosolids which inturn degrades the extractives at a greater rate than when compared with potting mix.

5.50 Resin Acids versus Neutrals

The resin acids are a class of naturally occurring tricyclic diterpenoid carboxylic acids found in the oleoresin of softwood trees (Hillis, 1985). Native resin acids are divided primarily into abietanes and pimaranes. Abietanes have an isopropyl group, whereas the pimaranes have vinyl and methyl groups.

The accumulation of biosolids in K-basin gave rise to anoxic conditions. Resin acid biotransformation occurs in anoxic sediments. Among the products of these transformations are decarboxylated resin acids (neutrals), including fichtelite and retene (7-isopropyl-1-methyl-phenanthrene) (Stuthridge and Tavendale, 1996). Resin acids and neutrals are mildly

bioaccumulative and are toxic to aquatic organisms. The biological half-lives of resin acids in trout has been identified only as <4 days (Niimi and Lee, 1992). In comparison retene and fichtelite have 14 and 12 day half-lives respectively indicting their recalcitrant nature.

Figure 28 and **Figure 29** compare the total resin acid concentrations and do not communicate the underlying chemical transformation that maybe occurring. For example: the isomerisation of abietane to dehydroabietic acid and derivatives. The graphs for individual resin acid compounds are located in the appendix. The individual compounds followed the same trend as total resin acids and showed no apparent spikes in concentrations to indicate a transformation pathway.

Referring to the neutral graphs in **Figure 30** and **Figure 31**; a commonality exists between PM+E and biosolids in the sterilised treatments containing earthworms. The average concentration of neutrals on day 3 shows a slight increase over day 0. The increase provides further evidence for the hypothesis that anaerobic bacteria are performing biotransformation in the gut of the earthworm.

5.60 Retene and Fictelite

The production of fichtelite and dehydroabietin indicate decarboxylation, whereas tetrahydroretene and retene indicate aromatisation (Tavendale *et al.* 1997). The average concentration of retene on day 3 exhibits an increase over day 0 for sterilised treatments with earthworms (**Figure 32** and **Figure 33**). This indicates the anaerobic bacteria in the earthworm's gut favour an aromatisation pathway. It also suggests that when there is a limited number or an absence of microbes the earthworms rely on gut microfauna. In non-

sterilised treatments the earthworms develop a symbiotic relationship with the native microbial fauna to degrade retene.

The result in **Figure 34** and **Figure 35** conforms to research that fichtelite is more persistent than retene in sediments and in organisms (Fragoso *et al.* 1999). No studies of the toxicity of fichtelite were found, but fichtelite is 18 times more bioaccumulative than dehydroabietic acid, and has a 12 day half-life in mussels (Burggraf *et al.* 1996). The toxicity of retene has been well researched. Billiard *et al.* (2000) found the chronic LC50 for retene to be 177 μg/L. A planar aromatic, retene binds the arylhydrocarbon receptor and induces the cytochrome P450 (CYP) 1A monoxygenase enzymes in juvenile trout (Fragoso *et al.* 1999), and in larval trout at concentrations as low as 8 μg/L.

Retene and fichtelite are the end products of decarboxylation and aromatisation chemical transformation pathways and are more toxic than the parent compounds. Therefore the compounds are important when assessing the hazard associated with biosolid land application practices. The results indicate that retene degrades upon exposure to oxygen and UV radiation. However fichtelite appears to be less resistant to degradation. The toxicity of fichtelite needs to be established to determine the risk for land application.

5.70 Sitosterol and Sitostanol

Phytosterols, sitosterol and sitostanol, are triterpenoids consisting of six isoprene units and are abundant in algae and higher plants. The environmental effects of phytosterols are well documented and include endocrine disruption. The structure of pulp and paper effluent derived

phytosterols is similar to the steroid hormones of vertebrates (Lehtinen *et al.* 1999).

The concentrations of sitosterol (**Figure 36** and **Figure 37**) and sitostanol (**Figure 38** and **Figure 39**) in the biosolid treatments maintained a relatively flat profile over the 28 day experiment. This indicates the phytosterols were not degraded by oxidation, UV radiation, or microbial activity. The relative ratio of sitosterol to sitostanol was maintained throughout the 28 day experiment. This indicates there was little bioconversion between the two phytosterol derivatives.

The concentrations of sitosterol and sitostanol in the PM+E treatments (**Figure 36** and **Figure 38**) showed the phytosterols were degraded over the 28 day experiment. However the concentrations for both sitosterol and sitostanol peaked in the sterilised treatments containing earthworms on day 3 of the experiment. This follows a trend established by the neutral compounds. The day 3 concentration increases maybe attributed to (i) plant material in the potting mix, (ii) enhancement of microbial community by earthworms, (iii) enzymatic action by the earthworms. Bacteria capable of biotransformation of phytosterols have been identified. For example *Mycobacterium* sp can cleave the side-chain for sitosterol (Cabral *et al.* 1997). It is possible that with absence of bacteria in the sterilised potting mix treatments the earthworms enhanced microbes capable of biotransforming phytosterols to sitosterol and sitostanol. The plant material in the potting mix such as bark would have provided an abundant source of phytosterols. Enzymatic action in the gut of the earthworm may also be capable of biotransforming phytosterols. Further research is required to identify bacteria capable of biotransformation.

The apparent lack of phytosterol reduction and the documented negative environmental effects of phytosterol questions the suitability of the treated biosolids for land application.

5.80 Summary and Recommendations

The hypothesis that earthworms could reduce the mass of biosolids was not successfully proven in this study. Although the treatments containing earthworms showed a greater mass reduction compared to treatments containing no worms, the quantities involved were too small to be significant. To further investigate the potential for earthworms to reduce biosolid mass it is recommend a similar study be preformed using much great quantities over a longer time frame. However, the earthworms lost ~50% of their body mass over the 28 day experiment indicating biosolids are either nutrient deficient or the earthworms limited their ingestion. Therefore it is recommended further study is undertaken to investigate options for enhancing the appeal of biosolids. This maybe achieved through supplementation using feed high in organic content such as manure and yeast extract.

The hypothesis that earthworms could reduce the concentration of biosolid extract was not successfully proven in this study. There was also no significant difference between sterilised and non-sterilised treatments. However, the accumulation of neutrals in the tissues of earthworms exposed to biosolids suggests a relationship exists between biosolid micro-organisms and the earthworms. This was further highlighted by the bioaccumulation of resin acids in the tissue of earthworms from sterilised treatments. This suggests that when there is a limited number or an absence of microbes the

ability of the earthworm to degrade resin acids is hindered. Furthermore spikes in the concentration of neutrals on day 3 of the experiment suggest anaerobic bacteria maybe performing biotransformation in the gut of the earthworm. Further research is recommended to establish whether a relationship exists between earthworms and soil microflora.

The most significant finding from this research was the reduction in the concentration of biosolid extracts in all of the treatments. This result suggests that oxidation and UV radiation can degrade extractives. On that basis an effective treatment regime would involve aerating and exposing the biosolids to light. This may be achieved through ploughing the basin with machinery or actively pumping air through the biosolids.

The study investigating the degradation of extractives since 1993 proves even the most recalcitrant compounds can break down over time. However for more immediate land application the following issues require further study; (i) The presence and toxicity of partially oxidised intermediate compounds. (ii) The toxicity of fichtelite. (ii) The biodegradability of phytosterols.

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LIST OF APPENDICES

Please see attached compact disc.