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Fate and Effects of Pulp Mill Effluent Solids in the Soil Environment

A thesis
submitted in partial fulfillment
of
the requirements for the degree
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Abstract

The pulp and paper industry in New Zealand annually produces over one hundred thousand dry tonnes of solid waste due to the treatment of pulp and paper mill effluents, the majority of which is currently landfilled. The New Zealand Waste Management Strategy (2002) has set a target for "the diversion of commercial organic wastes from landfill to beneficial use to exceed 95% by 2010".

Effluents produced by softwood pulp mills, such as in New Zealand, contain high concentrations of naturally derived resin extractive compounds that are toxic to fish and other aquatic organisms. Improvements in waste water treatment technology has enabled the industry to meet rigorous discharge standards, however, this has resulted in an increase in the production of pulp mill effluent solids which require disposal. This has become an issue for the pulp and paper industry, especially as current landfill space is limited. Land application has been used for many years as a means of disposal of pulp mill wastes. While most studies investigating land application of pulp mill effluent solids have concluded that the risk posed to the environment is low, few have investigated the potential toxicity of these wastes to soil organisms, and these studies did not directly address the effects of resin extractive compounds.

Resin extractives have been shown to be recalcitrant and to accumulate in anaerobic sediments. It is not known to what extent resin extractives are bioavailable or degradable in land applied Pulp mill effluent solids (PMES), or their potential to bioaccumulate in soil organisms.

This PhD thesis research sought to extend the knowledge on the environmental fate and effects of pulp mill effluent wastes. It focused on terrestrial systems, which have not been well studied in this respect. Four chemically distinct softwood pulp mill effluent solids, a primary treatment solid and three secondary treatment biosolids, were used to investigate their effects on soil organisms and soil functions. An interdisciplinary approach was adopted, which incorporated three main areas of study, as follows:

1. A comprehensive resin extractives analysis of the pulp mill effluent solids undertaken so that effects on soil organisms and soil functions could be related to the resin extractives chemistry of the individual pulp mill effluent solids tested.
2. A battery of bioassays used to investigate the toxicity of the selected pulp mill effluent solids.
3. A field trial set-up to investigate how pulp mill effluent solids affected soil functions and also to investigate the decomposition of pulp mill effluent solids and of resin extractives in these solids.

Three hypotheses tested were:
I. Pulp mill effluent solids are toxic to soil organisms.
II. Resin extractives in pulp mill effluent solids are recalcitrant in the terrestrial environment.
III. Pulp mill effluent solids will cause significant measurable negative impacts on soil functional capacity.

A wide range of resin extractives compounds were identified in pulp mill effluent solids, and concentrations of individual compounds varied widely between the different pulp mill effluent solids tested. During the two years after field application of the pulp mill effluent solids, resin extractives declined, however, decay rates of individual compounds were variable. The decay rate of compounds was influenced by the type of pulp mill effluent solids containing these compounds and not by the initial concentration of compounds. All compounds, including resin acids, degraded rapidly in the applied primary solid and in one of the applied biosolids, with average half-lives calculated ranging from three to twelve months. In the other biosolids, resin acids were recalcitrant with average half-lives calculated to be nearly ten years.

Laboratory bioassays conducted on oats, earthworms and enchytraeid worms indicated that pulp mill effluent solids had low toxicity to these organisms. An aquatic bioassay organism, however, was acutely affected by aqueous extracts from pulp mill effluent solids. Earthworms were shown to bioaccumulate some resin extractive compounds to a limited extent, indicating that resin extractives were bioavailable in pulp mill effluent solids. Enchytraeid reproduction was

reduced by exposure to some pulp mill effluent solids but this was not correlated to resin extractives concentration of the solids tested.

Field applied pulp mill effluent solids significantly enhanced the fluxes of CO_2 from the soil surface. When mass losses of pulp mill effluent solids carbon were taken into account, it was shown that these solids had little effect on soil respiration. Needle litterbags were placed above and below pulp mill effluent solids and in the litter horizon of a control treatment for 12 months. Needle litter decomposition was not significantly different between treatments, however, changes in enzyme activities were detected in litter beneath pulp mill effluent solids compared to control needle litter that had not been exposed to pulp mill effluent solids. The decomposition rate of field applied pulp mill effluent solids was slow, with half-lives extrapolated to be between five and twenty five years. The decomposition rate appeared to be influenced by the pools of carbon available for decomposition, with primary solids decaying significantly faster than biosolids due to a higher cellulose component.

The conclusions of the PhD thesis research are, therefore, that generally, pulp mill effluent solids used in the study were demonstrated to be relatively benign and appear to pose a low risk to the terrestrial ecosystem when applied to soil. However, a cautious approach is still recommended to land application, based on the extensive evidence of disruption to aquatic ecosystems, and because pulp mill effluent solids will take many years to decompose and resin acids are recalcitrant in some pulp mill effluent solids. Further research is recommended to elucidate mechanisms of action by resin extractives in soil organisms and the ultimate fate of these compounds in the soil compartment.

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Abbreviations

ABTS	2,2 – Azino-bis-3 Ethylbenzthiozolin-6 sulfonic acid
ADF	acid detergent fibre
ANOVA	analysis of variance
AOX	absorbable organic halides
ASB	aerated stabilisation basin
BAF	bioaccumulation factor
BOD	biological oxygen demand
CC	carbonate carbon
CHH	Carter Holt Harvey
C_{mic}	microbial biomass
COD	chemical oxygen demand
CTMP	chemithermomechanicl pulp
EC_{50}	effects concentration
ECF	elemental chlorine free
FA	fatty acid
GC/MS	gas chromatography-mass spectrometry
KAB	Kinleith aged biosolids
LC_{50}	lethal concentration (50% of test organisms)
LiP	lignin peroxidase
MFO	mixed function oxygenase
MnP	Manganese dependant peroxidase
NOEC	no observable effects concentration
OM	organic matter
PAH	polyaromatic hydrocarbon
PMES	pulp mill effluent solids
RA	resin acid
RAN	resin acid neutral
RE	resin extractives
SOM	soil organic matter
TAB	Tasman aged biosolids

TB	Tasman biosolids
TCF	totally chlorine free
TLS	Tarawera loamy sand
TMP	thermomechanical pulp
TN	total nitrogen
TOC	total organic carbon
TPS	Tasman primary solids
TSL	Templeton silt loam
USEPA	USA Environmental Protection Agency
VOC	volatile organic compounds
WMC_{max}	maximum water holding capacity
WRE	wood resin extractives

1 Introduction and Literature Review

1.1 Introduction

The manufacture of paper dates to the ancient Egyptians before 3000 B.C., while the 'modern' method of pulping plant material for paper production was developed by the Chinese in the first century A.D. The utilisation of plant fibre for paper production is one of the oldest manufacturing industries and is built upon age-old technologies. It was not until this became mechanised and the scale of production escalated in the early part of last century that many of today's environmental problems associated with the pulp and paper industry emerged. For example, in the industrial manufacture of paper from wood fibre, it was known that natural compounds released during processing caused harm to aquatic populations (Ebeling 1931).

Pulp mill-derived organic compounds, and especially resin extractives, have been studied as a consequence of findings on their aquatic toxicology, which emerged in the middle of last century. From this, wastewater treatment technology developed to deal with many different residuals from the pulp and paper industry, including the aforementioned resin extractives. In the last thirty years, a considerable body of literature has been published on the fate and effects of pulp mill effluent wastes in aquatic environments. A large proportion of this literature, particularly in recent years, has focused on the role that resin extractives (RE) play in the aquatic toxicity of pulp mill effluents. These RE compounds, many of which are hydrophobic, may accumulate and be recalcitrant in pulp mill effluent solids (PMES). Little is known about the behaviour of these compounds in terrestrial systems. The fate and effects of pulp mill derived RE has not been studied in biosolids amended soils.

Sediments in aquatic ecosystems can be 'sinks' and 'sources' of toxic organic compounds derived from pulp mill effluent. How these compounds behave in the soil environment is largely unknown, but is of significance for understanding their terrestrial fate and effects. Terrestrial organisms have not been widely reported to be affected by pulp mill derived resin extractives and an exhaustive literature

search showed that few studies have addressed this issue (Bostan *et al.* 2005). There is some evidence that similar responses may occur in both aquatic and terrestrial organisms exposed to these compounds (Rana *et al.* 2004; Savluchinske-Feio *et al.* 2006).

Increasing awareness globally of the environmental impact of industry has led to a growing movement towards sustainable development of industrial practices. Increasing pressure to reduce and reuse waste has prompted industries such as the pulp and paper industry in New Zealand to look at alternatives to landfill disposal of organic wastes.

Land application of effluent waste solids, which includes primary solids (from untreated effluents) and biosolids (from biologically treated effluents), has been widely used in many countries as a means of dealing with human and industrial organic wastes that threaten aquatic ecosystems. As soil amendments, biosolids offer many advantages and allow industry to beneficially dispose of organic waste. Pulp mill organic wastes have been widely used as soil amendments, with benefits to soil (Henry and Cole 1998). Amending forest soil with wastes from adjacent pulp and paper mills may be an attractive alternative to current landfill disposal practices utilised in New Zealand. However, there are few studies of the potential for these wastes to do damage to soil properties and soil organisms (Bostan *et al.* 2005; Feldkirchner *et al.* 2003; Piearce and Boone 1998). This PhD thesis research focused on how chemically distinct PMES from two softwood pulp mills in New Zealand might effect terrestrial organisms and ecosystems. Mass losses and changes in chemical composition of PMES were also measured to investigate rates and patterns of decomposition in a terrestrial environment.

1.2 PhD Thesis Hypotheses

Three hypotheses were tested in this PhD thesis research in order to extend the knowledge on the fate and effects of pulp and paper mill wastes in the terrestrial environment.

Hypothesis I. Pulp mill effluent solids are toxic to soil organisms

Hypothesis II. Resin extractives in pulp mill effluent solids are recalcitrant in the terrestrial environment.

Hypothesis III. Pulp mill effluent solids will cause significant measurable negative impacts on soil functional capacity.

1.3 Aims

The aims of this PhD thesis research were to test these hypotheses using interdisciplinary methodology and were divided into four objectives. These methods related the chemical characteristics of PMES to their toxicity to terrestrial organisms and their impacts in the terrestrial environment.

1.4 Objectives

The four objectives of this PhD thesis research were as follows:

1. Establish baseline data on the toxicity of pulp mill effluent solids to selected terrestrial organisms.
2. Relate toxicology data to the resin extractives analysis of different pulp mill effluent solids.
3. Relate functional observations at the soil ecosystem level to chemical characteristics of pulp mill effluent solids.
4. Monitor the loss (decay) of pulp mill effluent solids, and specifically the loss of their chemical constituents over time after application to a forest soil.

1.5 Review of Pulp and Paper Mill Effluent Wastes: Generation, Treatment and Discharge to the Environment

1.5.1 Overview

This review will outline the pulp and paper industry and the effluent wastes it generates, with particular attention to the toxicology of these wastes and their handling. The chemical nature of wastewater solids produced by New Zealand mills that utilise softwood feedstock will be presented; generally, this is applicable globally to mills that use similar feedstock and process technology.

Assessment of environmental risk associated with the disposal of wastewater solids using a three component approach will also be outlined. Laboratory testing of single organisms, chemical analysis of wastewater solids and ecosystem monitoring form a quality assessment triad. Details will be given as to how this may be developed as a weight of evidence approach to assessing the suitability of applying these wastes to soil. This will then form the basis for the experimental design that was adopted for the PhD thesis research presented in Chapters Three, Four and Five.

1.5.2 Introduction

Over 300 million tonnes of paper products are produced in the world each year (Blanco *et al.* 2004), including newsprint, writing and printing paper, cardboard/packaging, and tissue (collectively called paper-products). In the manufacture of one tonne of paper, more than 60 cubic metres of water may be required (Ali and Sreekrishnan 2001; Thompson *et al.* 2001). This in turn may lead to large volumes of wastewater being generated if process technology and recycling are not well developed. These effluents require treatment if significant environmental disruption is to be avoided when they are discharged to surface water bodies (Ali and Sreekrishnan 2001). Historically, discharges of pulp and paper mill effluents have caused significant damage to aquatic environments (Hynynen *et al.* 2004; Johnston *et al.* 1996; Thompson *et al.* 2001). Environmental effects have been attributed to chemicals introduced during the

manufacturing process (Hewitt *et al.* 2006), to natural compounds released from plant material used as mill furnish (Fragoso *et al.* 1998; Leach and Thakore 1973), to interactions of these compounds with each other (Couillard and Nellis 1999; Solomon 1996) and interactions with biota in mill effluent during waste water treatment (Hall and Liver 1996; Taverndale *et al.* 1997a). In many modern mills, reduced inputs of toxic chemicals and improved wastewater treatment have resulted in significant reduction of effluent toxicity (van den Heuvel and Ellis 2002), and of environmental impacts (Sandstrom and Neuman 2003).

Solid waste is generated in the process of pulping and paper manufacture, and in effluent treatment. Depending on the type of treatment system utilised, different quantities of solids with widely differing compositions are produced (Ali and Sreekrishnan 2001). These waste solids may be utilised for energy generation (Busbin 1995; Kraft and Orender 1995), used as a raw material in other processes, (Wang *et al.* 2006) or disposed of beneficially to land (Henry and Cole 1998; Pridham and Cline 1988), but because of generally poor energy yield and costs of handling these waste materials are most often land-filled (Feldkirchner *et al.* 2003; Magesan and Wang 2003).

In developed countries, constraints on landfill space and legislation designed to minimise or prevent organic wastes entering land-fills has prompted research into sustainable alternatives to land-fill disposal. Pulp mill effluent solids (PMES) are predominantly organic in nature while containing mineral nutrients and therefore make potentially useful soil amendments. However, these materials may contain high concentrations of compounds that are potentially detrimental in terrestrial ecosystems. This is an issue, which needs to be addressed before routine land disposal of PMES can be considered.

1.5.3 Part I. Production of Pulp and Paper and Associated Effluent Wastes

1.5.3.1 New Zealand Forestry Industry

Forestry is a major industry in New Zealand with a single tree species, *Pinus radiata*, accounting for 90% of harvested production (Lewis and Ferguson 1993). New Zealand is effectively self-sufficient in wood products and exports over 50% of annual harvest (Ministry of Agriculture and Forestry (MAF) 2006). Forestry exports comprise all timber products including saw logs, sawn timber, wood chips and pulp and paper products (Lewis and Ferguson 1993). Forestry and its associated processing industries account for over 10% of total export earnings ($3.1 billion in 2003) with the forestry sector contributing 3.1% to Gross Domestic Product (MAF (2006)). Pulp and paper products account for about a quarter of forestry exports and, in 2005, 1.6 million dry tonnes of pulp and 0.9 million tonnes of paper were produced in New Zealand (MAF (2006)). These figures are expected to increase as more trees come online for harvesting and because increasing areas have been planted in recent years (Walker *et al.* 2000).

1.5.3.2 Pulp and Paper Industry

Plantations grown in New Zealand are primarily for timber production, with the pulp and paper industry utilising inferior saw logs and other residues unsuitable for timber (Uprichard 2002). The major components of wood are cellulose, hemicellulose, lignin and wood extractives. In the manufacture of pulp and paper products, it is the wood fibres that are of primary interest; in softwoods these are tracheids and in hardwoods libriform fibres and vessels. Pulping is the process of reducing wood to a fibrous mass, the type of process determines the extent to which lignin and wood extractives are removed and the choice of pulping process is dependant on the types of products being manufactured (Smook 1982). A wide variety of pulp and paper products are manufactured in New Zealand including newsprint, bleached and unbleached kraft pulp, linerboard, paperboard, tissues, thermomechanical pulp and chemithermomechanical pulp (Uprichard 2002).

Pulping can be achieved by mechanical, chemical or semichemical means (Smook 1982). Mechanical pulps are produced by either stone groundwood or refiner pulping procedures including thermomechanical pulping (TMP). High yields (up to 95% of raw material) are obtained from mechanical pulps and are mainly used for the manufacture of printing grade paper, primarily newsprint (Smook 1982; Uprichard 2002). Chemical pulping principally refers to kraft pulping and uses chemical treatment at high temperature and pressure to dissolve lignin, which binds wood fibres. Chemical pulps have higher fibre strength but lower yield than mechanical pulps, often less than 50% of raw material (Uprichard 2002). Semi-mechanical pulping utilises chemicals to partially digest wood chips before mechanical pulping and yields range from 55% to 90% of raw material (Smook 1982). Of New Zealand's eight pulp and paper mills, two are kraft mills which account for over two-thirds of the total pulp and paper production (Dell *et al.* 1997). Kraft pulp also makes up about two-thirds of pulp production globally (Hewitt *et al.* 2006).

Pulp is bleached to increase its brightness, which is a requirement for many paper products. Pulp colour is due to chromophoric groups, mostly from lignin, that adsorb light, but also extractives and some metal ions. Brightness can be increased by destroying chromophoric groups without removing lignin, but this approach is limited in the brightness values achieved. The effect is not a permanent, because colour returns over time when paper is exposed to light or oxygen, due to chemical reactions in the residual molecule – this is called brightness reversion. To produce high quality stable pulps, bleaching must involve delignification utilising a sequence of bleaching and extraction stages, with washing of pulp in between stages (Smook 1982). Historically, elemental chlorine (Cl_2) was used as a bleaching agent, but since the late 1980's Cl_2 been replaced by elemental chlorine-free (ECF) or totally chlorine-free (TCF) procedures as a consequence of the environmental hazards associated with by-products of chlorine bleaching. Bleaching agents that have replaced Cl_2 include chlorine dioxide, hydrogen peroxide, oxygen and ozone (Ali and Sreekrishnan 2001).

A summary of papermaking is that pulp is diluted to 1% solids or less before being pumped to the headbox of the paper machine. Papermaking is the process

whereby pulp and additional fillers and sizing agents are introduced onto a moving wire cloth where the pulp drains and forms a fibre mat. This is further dewatered and pressed and residual water is removed by passing the paper over a series of steam heated cylinders (Smook 1982; Thompson *et al.* 2001).

1.5.3.3 Pulp and Paper Mill Effluent

Overview

The manufacture of pulp and paper requires large volumes of water from which large volumes of effluent ensue (Ali and Sreekrishnan 2001; Pokhrel and Viraraghavan 2004; Thompson *et al.* 2001). Over the past 25 years, the industry has undergone major process changes. Which are well reflected in changes in the waste streams now generated. Process changes have been driven not only by the need to increase efficiency of resource use, but also to reduce discharges. Most developed countries now have strict discharge standards to which industry must adhere (Ali and Sreekrishnan 2001; Hewitt *et al.* 2006; Pokhrel and Viraraghavan 2004). One of the most obvious changes in modern processes is the reduction in water use per tonne of product with a concomitant reduction in effluent volumes discharged. The closing of process cycles leads to reuse of water while recovering chemical, fibre and dissolved organics (Thompson *et al.* 2001). These changes, while being capital intensive, have resulted in economic and environmental benefits. With the reduction in water use, however, some effluents now carry greater organic loads that bring new challenges in the treatment of effluent.

Generation and Characteristics

Effluents are complex mixtures including organic constituents (Carlberg and Stuthridge 1996; Hewitt *et al.* 2006) and must be treated according to their particular characteristics before discharge to surface water bodies if environmental degradation is to be minimised or avoided. The composition of the effluent produced by pulp and paper mills is largely determined by the type of feedstock and the pulping and bleaching technology employed (Gifford 1996; Pokhrel and Viraraghavan 2004). Pulping generally produces greater quantities of effluent than

papermaking. Paper machine effluent, or white water, tends to have a lower organic load than effluent from the pulping stages. This may have its own problems in that recovery of chemical or organic material is not feasible in such a dilute effluent, unlike some pulp effluent streams that are very concentrated. Mechanical pulping, while releasing less material to process water than chemical pulping, discharges a more concentrated effluent, mostly lignin and wood extractives, despite the chemical process losing up to 50% of raw material. This is due to chemical pulping processes utilising systems whereby chemicals are recovered and dissolved organics are removed such as in tall oil, or retained in recovered liquors, which are then incinerated in recovery boilers (Thompson *et al.* 2001). Effluent from chemical pulping can contain transformed wood extractives. For example, native resin acids are readily isomerised during chemical pulping (Zender *et al.* 1994). The single greatest use of water in modern mills is the bleach stage accounting for 70% of total water use (Johnston *et al.* 1996). Significant effluent streams may also come from the log yard, debarking operations, lime mud and black liquor spills. In Figure 1.1, the various sources of potential wastewater pollution during pulping and papermaking are presented.

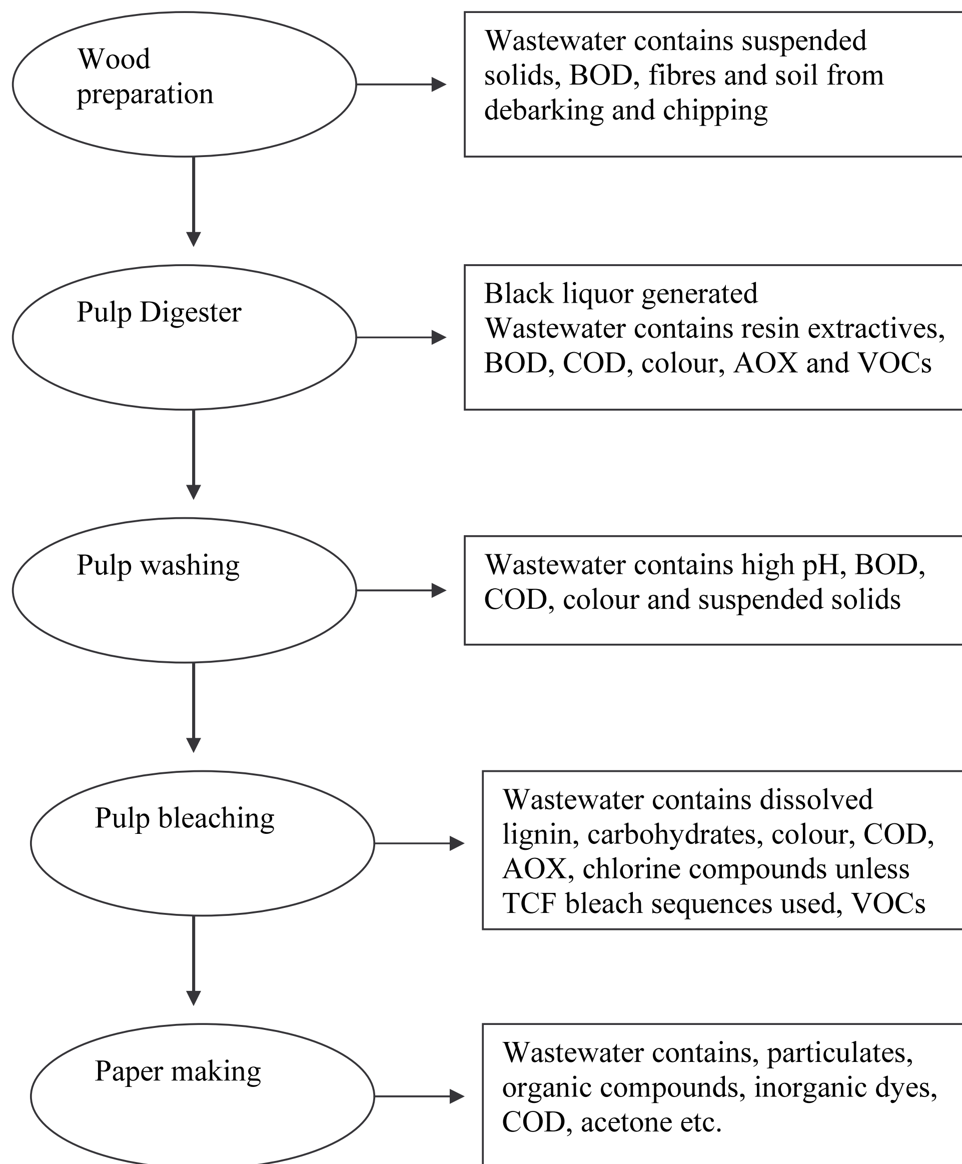


Figure 1.1 Potential pollutants from various sources during the pulping and paper making process (Pokhrel and Viraraghavan 2004)

Discharge to the Environment

Environmental effects and in particular toxicology of pulp and paper mill wastewater discharges will be addressed in detail in section 1.5.4 of this review.

Prior to the 1980's, pulp and paper mill effluents were commonly discharged directly to receiving waters with minimal or no treatment. Gradually, as environmental impacts were demonstrated and as the industry grew, regulations

were introduced to limit discharges (Munkittrick *et al.* 1997; Pokhrel and Viraraghavan 2004). Biological oxygen demand (BOD) and chemical oxygen demand (COD), absorbable organic halides (AOX), suspended solids (SS), wood resin extractives (WRE) and inorganic compounds all contributed to the pollution of water bodies (Ali and Sreekrishnan 2001; Feldkirchner *et al.* 2003; Leach and Thakore 1973; Walden 1976). While not all of these pollutants are toxic they do contribute to environmental degradation (Pokhrel and Viraraghavan 2004), and it is frequently impossible to separate toxic effects from other negative impacts in natural environments (Walden 1976). As the pulp and paper industry developed, the nature of discharges has changed as outlined in section 1.5.3.3 due to process changes and the need to improve discharge quality. Interactions of internal process changes, external process changes, evidence of negative impacts, public opinions and regulatory initiatives have affected discharges in a rapidly changing environment.

The interactions of these factors have made the characterisation of discharges very difficult from an environmental perspective, as continually changing ideas and varying emphasis on environmental issues mean that scientists have a continually moving target of interest to study. The rate of change of effluent characteristics is often much faster than the rate of accumulation of sound scientific evidence for environmental effects. Due to the complexity of these effluents, the problem of how to minimise environmental impacts of pulp and paper industry discharges is unlikely to be completely resolved unless the industry achieves zero discharge. A more realistic solution in the short term is continued improvements in effluent treatment based on incremental increases in knowledge of environmental fate and effects.

Treatment of Effluent

The treatment of pulp and paper mill effluents is based on the technologies that have developed in the treatment of municipal effluents (Liss and Allen 1992). Treatment can broadly be defined as being physical or biological. Primary treatment refers to the removal of solid material by mechanical means wherein effluent may undergo secondary treatment utilising biological means to further

reduce solid and dissolved organic loads. Primary treatment was introduced in many mills to remove suspended solids and reduce oxygen demand. Secondary treatment became widespread as discharge regulations were progressively made more stringent. Today, some mills undertake tertiary treatment of effluent as a final 'polishing' before discharge to receiving waters. Tertiary treatment utilises both physical and biological mechanisms (Thompson *et al.* 2001).

Factors, such as the effluent pollutant loading and the nature of the receiving environment, determine the type of treatment system required to meet discharge standards imposed by environmental authorities (Pokhrel and Viraraghavan 2004). Mills that predominately utilise mechanical and thermomechanical pulping (TMP) release lower pollution loads to process waters than semi-chemical and chemi-thermomechanical (CTMP) processes which produce intermediate pollution loads. Chemical pulping produces concentrated black liquor with high residual lignin (Ali and Sreekrishnan 2001). As previously discussed, concentrated effluents may be effectively treated in the mill itself to recover chemical and organic material with the result that processes that release the greatest load, in terms of mass, to process water do not always lead to the greatest pollution loading in subsequent effluents (Thompson *et al.* 2001).

If receiving waters dilute contaminants sufficiently so that biota is not adversely effected, environmental impacts may be minimal. Most pulp and paper mills have as a minimum, primary treatment, which entails physical removal of particulate matter from the effluent stream through sedimentation, flotation and screening. Primary treatment can reduce suspended solids by more than 80% (Thompson *et al.* 2001), with resulting solids composed mostly of bark, fibres, filler and coating material (Busbin 1995; Pokhrel and Viraraghavan 2004; Uprichard 2002). If discharge is not to a large body of water such as the open ocean then secondary treatment will be required to reduce biological oxygen demand (BOD) and chemical oxygen demand (COD), suspended solids, organic and inorganic pollutants, and colour (Ali and Sreekrishnan 2001; Pokhrel and Viraraghavan 2004; Thompson *et al.* 2001).

The pulp and paper industry utilises numerous methods for secondary treatment of effluent. These are all essentially biological processes that rely on microorganisms using effluent as a growth substrate. Treatment is achieved through the removal of dissolved and suspended material from effluent and the accumulation of biosolids and evolution of CO_2 or methane (Ali and Sreekrishnan 2001; Thompson *et al.* 2001). Organic material may be completely oxidised to CO_2, partially oxidised/biotransformed or fixed as biomass, Alternatively it may absorb to solid material (such as biosolids) or pass through the treatment system unmodified (Mohn *et al.* 1999; Pokhrel and Viraraghavan 2004; Zender *et al.* 1994).
Common secondary treatment systems used in the pulp and paper industry are; aerated stabilisation basins (ASB), activated sludge, and aerobic/anaerobic biological reactors. Generally, activated sludge systems are efficient at removing BOD, COD, adsorbed organic halides (AOX), chlorinated phenolics and other organic contaminants (Thompson *et al.* 2001). These systems operate with lower residence times and therefore have lower system volumes and land area requirements than ASB systems (Thompson *et al.* 2001). Aerated stabilisation basin treatment is the preferred method of treatment in many countries due to lower operating costs particularly if availability of land is not limiting (Stuthridge *et al.* 1991). In high latitude countries, winter freezing can make the operation of ASB systems difficult. ASB systems when working efficiently are able to remove 85 – 90% of BOD, but they are not as effective at removing colour, COD and AOX (Saunamaki *et al.* 1991; Stuthridge *et al.* 1991).

Anaerobic systems require less area and energy to operate and produce less sludge than either ASB or activated sludge systems, and have the added advantage of methane generation. However, they do have the disadvantage that shock loading can reduce treatment efficiency and they also have limited ability to remove poorly degradable toxic compounds (Thompson *et al.* 2001). In order to maintain efficient treatment, anaerobic systems must also be kept active during mill shutdowns.

Resin Extractives

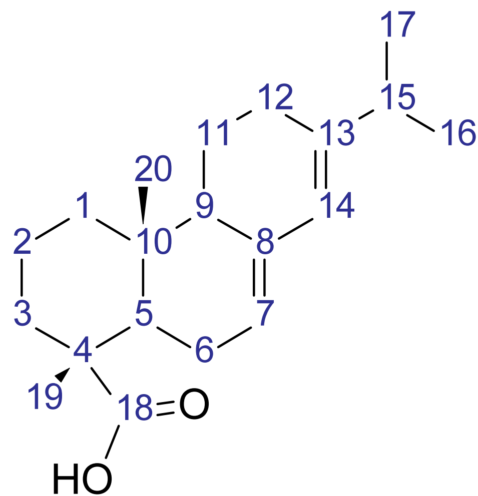
Softwood timber, such as *P. radiata,* contains between 2% and 8 % of dry weight as resin extractives (RE) which include diterpine resin acids, fatty acids,

phytosterols, phenolics and monoterpines (Uprichard 2002). These biologically active compounds (LaFleur 1996) are released during the pulping process, resulting in significant concentrations in effluents if recovery processes are not utilised. In TCF and ECF mills that use softwoods as the predominant mill furnish, most effluent toxicity is associated with RE and their metabolites, namely resin acids (Bogdanova and Nikinmaa 1998), resin acid neutrals (Billiard *et al.* 1999), phytosterols (Lehtinen *et al.* 1999) and fatty acids (Leach and Thakore 1973). Secondary treatment effectively removes the majority of RE from the effluent before it is discharged to surface water bodies (Kostamo and Kukkonen 2003; Stuthridge *et al.* 1991; Taverndale *et al.*1997a). Resin extractives are removed from effluent via several routes, the main ones being biodegradation, biotransformation and sorption to organic matter (Makris and Banerjee 2002; Taverndale *et al.* 1997a). Under aerobic conditions the majority of RE are degraded by aerobic metabolism in a few hours to a few days (Ali and Sreekrishnan 2001). Maintaining the degree of aeration required for this level of degradation, however, may not be possible in all ASB systems. Under low oxygen conditions, sorption and biotransformation mechanisms are responsible for reducing much of the effluent organic load (Hall and Liver 1996).

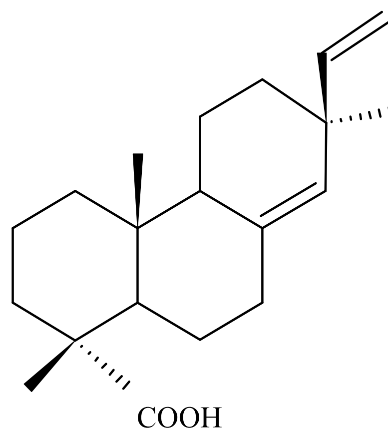
Resin acids are the most abundant fraction of RE and account for between 40 and 70% of total extractives found in *P. radiata* (Uprichard 2002). For this reason, resin acids are major components of pulping effluents. Resin acids are diterpines synthesised by plants and consist of 4 isoprene subunits forming a tricyclic structure (McMurray 1992). Resin acids are classified as abietanes or pimeranes based on the side chains at the C-13 carbon position (Figure 1.2). Abietanes contain an isopropyl group while pimeranes contain a vinyl and a methyl group. Structural stability conferred by these side chains is important in the degradation of these compounds (Martin *et al.* 1999). Dehydroabietic acid is the only resin acid found in wood that contains an aromatic ring (Figure 1.2), and this increases the stability of this resin acid compared to the other native resin acids. Important modifications to resin acids result from the incorporation of chlorine during chlorine bleaching. Resin acids can be chlorinated at the C12 and C14 position and these modifications confer greater toxicity and recalcitrance to the resin acid

(Liss *et al.* 1997). Resin acids can also undergo oxidation, hydroxylation and decarboxylation reactions (Martin *et al.* 1999; Taverndale *et al.* 1997a).

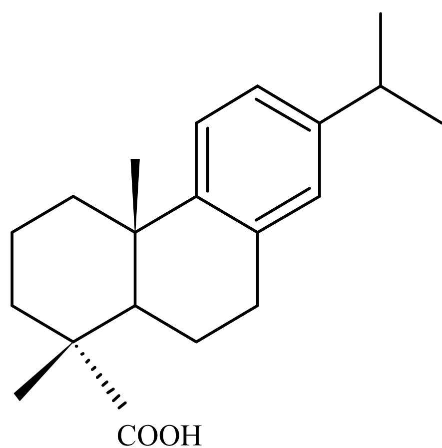
Due to their lipophilic nature resin acids readily partition onto organic sediments (Taverndale *et al.* 1997b). Anaerobic activity occurs in ASB systems, especially in sediments with high BOD and poor oxygen diffusion. Under anoxic conditions resin acids can be biotransformed but it is not clear if they are fully degraded (Liss *et al.* 1997; Martin *et al.* 1999). Transformation reactions form decarboxylated and aromatised products (some structures shown in Figure 1.2) such as fichtelite, dehydroabietin and retene (Martin *et al.* 1999). Resin acids and their anaerobic transformation products are recalcitrant in anaerobic environments as evidenced by their accumulation in wastewater treatment systems and bottom sediments in receiving environments (Leppanen *et al.* 2000).



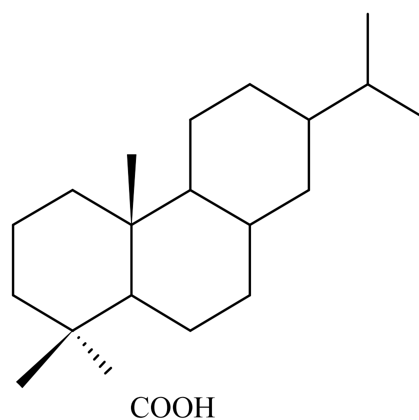
Abietic acid



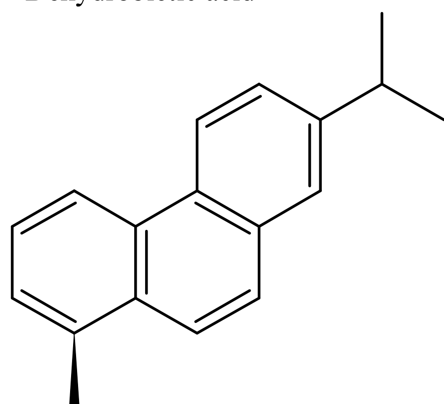
Pimaric acid



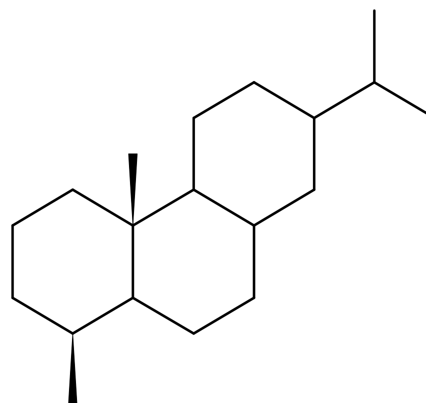
Dehydrobietic acid



Abietanic acid



Retene



Fichtelite

Figure 1.2 Resin acid and resin acid neutrals associated with pulp mill effluents .

Pulp Mill Effluent Solids

Pulp mill effluents and effluents solids are complex mixtures made up of a large number of compounds. The bulk of pulp mill effluent solids (PMES) comprise organic compounds of both high and low molecular weights derived from mill furnish. High molecular weight compounds are those that are structural components of wood such as cellulose and lignin polymers which, particularly the latter, do not undergo rapid degradation due to their un-reactive nature. Low molecular weight compounds such as resin acids and fatty acids undergo much more rapid degradation and may be completely respired to carbon dioxide in a relatively short time if conditions such as high oxygen supply are maintained (Liss *et al.* 1997; Pokhrel and Viraraghavan 2004).

The treatment of pulp and paper effluents results in the removal of much of the pollution load before effluents are discharged. While combined primary and secondary treatment systems may efficiently remove organic compounds from pulp mill effluent, much of this removal can be due to the transfer of organic compounds to solids (Liu *et al.* 1996; Taverndale *et al.* 1997a). Solids are either removed mechanically or retained in the treatment system but ultimately must be disposed in some manner. As treatment technology has improved, increasing volumes of wastewater solids have been produced. In Canada, the amounts have been estimated at 1.7 million dry tonnes per annum (Bostan *et al.* 2005). United Kingdom mills were expected to produce over 1 million dry tonnes per year by 2000 (Piearce and Boone 1998), but this did not reflect the full output per tonne of paper produced, as the UK imports most of the pulp used in its paper industry, and a considerable volume of waste is generated in the countries of origin (Thompson *et al.* 2001). In New Zealand, the amount of wastewater solids was estimated at 112,000 dry tonnes per year (Magesan and Wang 2003).

Wastewater solids are classed as primary or secondary solids, based on the point where they are removed from the treatment system. Primary solids, removed prior to secondary treatment are largely composed of fibres that have been lost to process water. Secondary solids or biosolids, having undergone biological treatment, have a high biomass content. Primary solids have carbon to nitrogen

ratios (C:N) ranging from 100 to 300 whereas secondary solids tend to have much lower C:N, particularly if nitrogen is added to the treatment system to improve efficiency (Vance 2000). The distribution of resin acids found in primary solids reflects the distribution of native resin acids found in softwood furnish, whereas the distribution of resin acids found in secondary solids reflects the microbial transformation processes that occur in the treatment system (Zender *et al.* 1994). Solids often contain resin extractive concentrations significantly higher than are present in the effluent from which they were derived due to the rapid partitioning from the liquid to the solid phase (Hall and Liver 1996).

Solids are dewatered to a dry weight content of between 5-10% before disposal. Wastewater solids from the pulp and paper industry (PMES) are mostly disposed of in landfills (Feldkirchner *et al.* 2003; Magesan and Wang 2003). Pulp mill effluent solids can be used for energy generation through incineration, but energy yields may be low due to the significant energy input required to reduce moisture content, and also if solids have a high ash content (Busbin 1995). The disposal of PMES is a growing problem for a number of reasons including; increased volumes being produced, limited space available in existing landfills, the costs involved in developing new landfills, and increasing pressure by regulatory authorities to reduce material entering landfills.

1.5.4 Part II. Environmental Fate and Effects

1.5.4.1 Environmental Toxicology

Environmental toxicology, as a discipline developed from classical or human toxicology, which has a much longer history (Wright and Welbourn 2002). The study of the effects of toxic substances in the environment has only developed significantly in the later part of the twentieth century, as has public concern surrounding great many environmental issues. The expansion of manufacturing industries, with ensuing pollution, is the basis for much of the public concern and has resulted in the rapid development of environmental toxicology since the mid 1960's (Wright and Welbourn 2002). Assessment of environmental risks posed by toxic substances can not be satisfactorily accomplished using chemical analysis

alone (Rombke and Moltmann 1996), as pollutants may exist in many forms, some of which may not be easily detectable and identified. The fraction that is available to living organisms is of greater relevance to understanding environmental impacts than total amounts of substances. Estimating the bioavailable fraction and its effect on organisms is insufficient by chemical analysis (Debus and Hund 1997) as the bioavailability of chemicals is determined by numerous biotic and abiotic factors. Organisms may be exposed to toxic substances by many different routes, and rates of uptake and excretion can vary widely between organisms. Biological sensors integrate long-term effects of exposure that chemical analysis can not measure (Wright and Welbourn 2002).

In 1969, Truhaut coined the term 'ecotoxicology' to focus on the toxic effects of chemicals and radiation on biological levels of organisation from the individual to the community (Wright and Welbourn 2002). Ecotoxicology is concerned with assessing ecological fate and effects of contaminants in the environment (Moriarty 1988). Truhaut (1977)considered an assessment of effects following a four-part process, as follows:

1. Release into the environment,
2. Transport into biota,
3. Exposure of one or more target organisms,
4. Response of individuals, populations or communities.

Truhaut (1977) differentiated ecotoxicology from classical toxicology as the latter he viewed was primarily concerned with effects from the subcellular level to the level of the individual organism and ultimately on humans. Wright and Welbourn (2002) state "it is fair to say that the modern science of environmental toxicology embraces both these disciplines". Levels of organisation in biological systems range from the molecular to the biosphere and with each higher level of organisation it becomes increasingly difficult to establish clear cut relationships based on cause and effect (Rombke and Moltmann 1996). This has implications for how data collected at one level of organisation is extrapolated to a higher level. The 'dilemma of ecotoxicology' (Rombke and Moltmann 1996), is that of trying to assess the effects of foreign substances at the ecosystem level when the methods available mostly involve describing effects at the level of the individual or lower. Rombke and Moltmann (1996) go further and state that 'these tests,

which are relatively simple and easy to reproduce, have no relevance whatsoever for conditions in the field; the results of laboratory tests are always open to question or in need of further verification' (Rombke and Moltmann 1996). In a toxicity assessment of a complex sewage effluent, Pratt *et al.* (1988) found that laboratory tests, including single species and microcosm tests, did not correspond with observed environmental effects. None of the laboratory tests suggested the severity of in-stream impacts on taxonomic richness and population size. This study highlighted the difficulty in assessing environmental impacts of complex mixtures of chemicals whose characteristics are subjected to many variables including changes in sources of chemicals, process changes before and during treatment, and changes in environmental parameters such as water flows, dilution rates and organism interactions.

Organisms have evolved mechanisms to regulate and protect themselves from damaging chemicals. For some elements that are required physiologically, there is a very narrow range of concentration between deficiency and toxicity and so regulatory mechanisms have evolved to maintain levels below a toxic threshold. Regulatory mechanisms are also responsible for controlling metabolism and detoxification of organic compounds both endogenous and exogenous (Wright and Welbourn 2002).

To establish if a substance is detrimental it is necessary to be able to reproduce a quantitative relationship between exposure to biota and some measure of damage to the biota. Dose-response relationships are used to quantify the amount of a substance that is required to elicit a particular response (Forbes and Forbes 1994). Doses may be administered in a number of ways that allow for different routes or modes of uptake, such as injection, inhalation and ingestion. The concentration of a chemical in an exposure medium such as soil, and the length of exposure time may also be used as a surrogate for dose. This is particularly relevant to environmental situations where the concentration and period of exposure is used to estimate risk to organisms (Wright and Welbourn 2002).

Toxicity bioassays are based on dosing with or exposure to a particular chemical or substance under rigorously controlled laboratory conditions. A variety of

endpoints such as mortality, growth and reproduction may be assessed. For an endpoint such as mortality, results may be presented as a lethal concentration, e.g., LC_{50}, (the concentration required to kill 50% of test organisms in a set time). Other endpoints may be assessed by the concentration at which 50% of a test population are affected, EC_{50}, or the concentration where there is no observable effect (NOEC). Initially, a range finder experiment may be required to establish a concentration threshold for toxicity, which bioassays then bracket (Wright and Welbourn 2002).

Bioassay organisms are chosen to fit a variety of requirements. They must be representative of a class of organisms that you wish to study in the environment. The organisms used should also be well understood in terms of their life strategies and function in particular ecosystems. Of great importance is the ability of an organism to adapt to laboratory conditions so that effects can be related to dose rather than some aspect of the experimental conditions imposed upon the organism. Responses are measured via various predetermined endpoints that may be quantal (such as mortality) or continuous (such as growth). Depending on the duration of the bioassay, effects may be classed as acute or chronic although there is not a clear distinction between the two. Subacute or chronic effect assays are used to investigate toxicity at concentrations below an acute LC_{50} concentration. Chronic effects may be observed at concentrations many times lower than those at which acute effects are seen. When the difference between chronic and acute effect concentrations is wide this suggests that there are different modes of toxic action responsible for the different observed effects.

1.5.4.2 Environmental Fate and Effects of Pulp and Paper Mill Effluents

As early as the 1930's, Ebeling noted negative environmental impacts associated with pulp mill discharges (Johnston *et al.* 1996). Studies by van Horn in 1949 documented kraft mills as sources of compounds toxic to fish (Zanella 1982). The most apparent effects on receiving waters were reduced oxygen levels, eutrophication and deposition of sludges and accompanying microbial growth. Studies conducted in the early 1980's isolated chlorinated organic compounds in pulp mill effluents and receiving environments that could be traced to the use of

elemental chlorine in pulp bleaching processes (Solomon 1996). These compounds were shown to bioaccumulate and were implicated in the toxicity and mutagenicity observed in biota in environments receiving pulp mill effluents (Ali and Sreekrishnan 2001; Hewitt *et al.* 2006).

Many chlorinated organic compounds have been isolated from pulp and paper mill effluents (McCubbin and Folke 1995), in receiving waters (Herve *et al.* 1996) and in biota exposed to pulp and paper mill discharges (Yunker and Cretney 1996). These chlorinated compounds included phenolics, fatty acids and resin acids as well as dioxins and furans, which include some of the most toxic compounds known (Ali and Sreekrishnan 2001; Yunker and Cretney 1996). In the late 1980's and early 1990's, there was increasing pressure to significantly reduce organochlorine discharges in pulp and paper mill effluents, as they were believed to be major contributors to toxicity observed in these effluents (Sandstrom *et al.* 1997; Solomon 1996). Since the 1980's process improvements replaced elemental chlorine bleaching of wood pulps in developed countries, however, elemental chlorine is still widely used in developing countries (Ali and Sreekrishnan 2001). The removal of elemental chlorine from bleaching processes resulted in a suggested reduction in effluent toxicity and also symptomatic changes, but not absence of chronic impacts (Munkittrick and Sandstrom 1997; O'Conner *et al.* 1993). New research also demonstrated that chronic impacts previously associated with the use of elemental chlorine were observed in receiving environments downstream of mills that had never used elemental chlorine and showed that mills that changed to ECF or TCF processing still exhibited acute lethality in their discharges (Munkittrick and Sandstrom 1997; Sepulveda *et al.* 2003; Servos 1996). This was further complicated by the fact that there were also many process changes being implemented continuously so effluents changed significantly over time.

Secondary treatment of effluent became mandatory in many countries and this had a huge impact on the quality of discharges. It became apparent in the 1990's was that these effluents were by nature complex and variable, both temporally and spatially, and that isolating toxic compounds from effluent was not going to be a simple matter (Munkittrick and Sandstrom 1997). As the components of effluent

discharges changed so did their environmental effects (Servos 1996). Extrapolating data gathered from one site to what might be expected at another was problematic, partly due to the great variability of discharges and receiving environments, and partly because the compounds responsible for environmental effects were not known (Hewitt *et al.* 2006; McMaster *et al.* 2006). It was observed that when acute effects disappeared, due to process changes and improvements, chronic effects emerged that had previously been masked (Munkittrick and Sandstrom 1997).

1.5.4.3 Toxicology of Pulp and Paper Mill Effluent Wastes

Over the past 30 years there has been a considerable research effort investigating the environmental fate and effects of pulp and paper mill effluents (Ali and Sreekrishnan 2001; Hewitt *et al.* 2006; Liss *et al.* 1997; Munkittrick *et al.* 1997; Walden 1976). There is a large body of literature on the toxic effects of pulp and paper mill effluents or constituents of these effluents on aquatic organisms (Kovacs *et al.* 1995; Leach and Thakore 1973; Parrott *et al.* 2006; Walden 1976), aquatic populations (McMaster *et al.* 2006; Sandstrom and Neuman 2003) and on aquatic ecosystems (Felder *et al.* 1998; Hynynen *et al.* 2004). As paper production moved away from reliance on elemental chlorine for bleaching, the environmental effects of effluent discharges changed but were not removed. The search for the compounds responsible for effects observed in aquatic ecosystems focused on natural compounds released from wood, rather than those introduced during industrial processes.

Early work indicated that resin acids and fatty acids accounted for 98% of the toxicity observed in unbleached kraft pulping whitewater (Leach and Thakore 1973). Even in 1931, Ebeling had shown that resin acids caused fish death at 5 mg l^{-1}, and van Horn identified fatty acids to be toxic in 1947 (Walden 1976). Early research focused on unbleached kraft effluents, as these were the predominant process wastes being produced. By the 1970's, evidence was beginning to emerge for toxic effects of bleach plant effluents and chlorine was implicated as a contributor to effluent toxicity (Walden 1976). Toxicity was thought to be due to residual chlorine rather than chlorinated organics formed during bleaching and in the effluents (Walden 1976). Leach and Thakore (1975) found toxic chlorinated

guaiacols and resin acids in bleach plant effluents from a Canadian mill. However, Walden concluded that 'chlorine is not normally a toxic constituent of bleachery wastes' (Walden 1976). Evidence accumulated through the 1980's that organochlorines were accumulating in the environment and that this was partly due to discharges from pulp mills (Johnston *et al.* 1996). Chlorinated organic compounds were found to be more toxic and more recalcitrant than non-chlorinated equivalents (Liss *et al.* 1997; Solomon 1996). Major changes were brought about in the late 1980's and 1990's in response to negative publicity and stricter environmental regulations (Pokhrel and Viraraghavan 2004). A combination of in-mill process changes such as ECF and TCF bleaching, recycling and recovery of spent liquors and the introduction of secondary treatment of effluents saw rapid reduction in the toxicity of discharges (Munkittrick *et al.* 1997).

Developments in Toxicology of Pulp Mill Effluents

Bioassays using fish and aquatic invertebrates exposed to pulp and paper mill effluents have been widely used since the 1970's and initially concentrated on short duration (4-7 days) acute toxicity assays (Parrott *et al.* 2006). Process changes and improvements in effluent treatment resulted in chronic rather than acute toxicity, and an emphasis on longer-term chronic assays developed. In the 1990's it became apparent that acute toxicity tests on effluents were not correlated to effects observed in recipient environments. Effluents that were shown to have no acute effects in bioassays had sub-lethal long-term effects on wild populations. Wild fish exposed to pulp and paper mill effluents showed a variety of responses including elevated mixed function oxygenase (MFO) activity, increased liver size, reduced gonad size and reduced sex steroid concentration (Parrott *et al.* 2006). The MFO system is involved in the formation of polar oxygenated metabolites from nonpolar compounds to enable excretion. However, intermediates (metabolites) which are more bioactive, can be more toxic than the parent compounds (Parrott *et al.* 2006). Evidence that MFO induction was caused by readily metabolised compounds began to accumulate when fish that had been exposed to effluents showed a rapid reduction in MFO activity within a few days of exposure to clean water (Fragoso *et al.* 1998). Several wood derived

compounds found in effluents have been identified that cause elevated MFO activity including retene (Billiard *et al.* 1999), stilbenes, juvabione and dehydrojuvabiones (Burnison *et al*. 1999; Martel *et al*. 1997). This list is, however, not exhaustive and other compounds yet to be identified are also implicated (Hewitt *et al.* 2006). While compounds have been isolated that increase MFO activity, considerable debate remains on the relevance to fish health in the environment (Parrott *et al.* 2006). MFO may be stimulated by many compounds and the point at which MFO activity goes from indicating a protective response to indicating a negative impact is not clear (Parrott *et al.* 2006).

More recently the focus of research has moved to reproductive effects linked to depressed sex steroid concentrations. Long-term life cycle studies have provided some of the best evidence linking reproductive changes observed in wild populations to exposure to pulp and paper mill effluents (Parrott *et al.* 2006). Further evidence has been provided by studies using fish caged in pulp mill discharges (Munkittrick *et al.* 1997; van den Heuvel *et al.* 2006).

Toxicity identification evaluation studies have been developed as a means to identify active substances from a complex mixture such as effluents. A three-phase approach has been used to isolate active compounds (Hewitt *et al.* 2006). This involved techniques such as bioassay directed fractionation of effluents (Munkittrick *et al.* 1997). In the first phase, manipulation of the effluent is used to obtain a broad idea of the nature of the active compounds. Phase II involves more specific methods aimed at isolating and identifying active compounds. Phase III then confirmes that a particular chemical was responsible for inducing the condition that had initially been observed (Hewitt *et al.* 2006).

In recent decades, substantial progress has been made in reducing pulp mill effluent toxicity, with many compounds identified that contribute to toxicity. Steps have been successfully implemented to reduce exposure of biota to these compounds, for example, with process changes and improved effluent treatment. However, there are also many conflicting studies, which suggest that environmental impacts are not easily understood from analysis of effluent chemistry and laboratory studies. Many factors obviously affect the way aquatic

organisms respond when exposed to pulp mill-derived organic compounds and the knowledge of these is, as yet, incomplete. Predicting environmental effects due to exposure to complex mixtures of these compounds remains difficult (Hewitt *et al.* 2006).

1.5.4.4 Land Application of Pulp Mill Effluent Solids

In many countries, including New Zealand, municipal and industrial wastewater solids have been used as soil amendments for many years (Cameron *et al.* 1997; Henry and Cole 1997). More recently, concerns have arisen over contamination of soil, plants and animals due to wastewater solids application (Drolet and Baril 1997; Jackson *et al.* 2000; McBride 2003). Ultimately, these concerns also raise issues concerning impact on human health. Industrial and municipal wastes contain many different potential pollutants and a cautious approach has been adopted in many countries to land disposal of wastes, particularly if this land is used for food production or is in a drinking water catchment (Magesan and Wang 2003). While wastewater solids may provide benefits to soil in the form of nutrients and organic matter, long-term environmental impacts should also be considered, for example, the fate and effects of recalcitrant organic compounds (Wang *et al.* 1999).

With increasing use of secondary treatment of pulp and paper mill effluents and improvements in wastewater treatment technology, increasing volumes of wastewater solids are being produced (Bostan *et al.* 2005). Coinciding with this increase is a decrease in the volume of landfill space to dispose of these solids. In areas where development has occurred around existing mills, there is little opportunity for developing new landfills or the costs of establishment makes their development prohibitive. Regulatory controls now also make the establishment of new landfills particularly difficult in many areas (Cameron *et al.* 1997). Regulations, including those being developed in New Zealand under the Waste Management Strategy (Ministry for the Environment 2002), are designed to keep organic materials such as wastewater solids out of landfills unless they pose an environmental hazard (Wang *et al.* 2006).

While PMES are prime candidates for land disposal there are environmental issues that should be addressed (Bostan *et al.* 2005). Environmental impacts documented from PMES in aquatic ecosystems raise concerns for terrestrial organisms. While terrestrial organisms are likely to exhibit different responses to PMES, these responses are largely unknown. Pulp mill effluent solids have been applied to land for many years but little research has been conducted to investigate detrimental effects (Bostan *et al.* 2005). Most studies have focused on beneficial effects related to the nutrient and organic matter contents of these solids (McDonald *et al.* 1994; Piearce and Boone 1998; Zibilske *et al.* 2000).

In some PMES, high concentrations of RE are of concern. While terrestrial organisms may not be as sensitive to these compounds as aquatic organisms, they could nevertheless be expected to be impacted (Hättenschwiler and Vitousek 2000; Savluchinske-Feio *et al.* 2006; Soderberg *et al.* 1996). Many of these RE are synthesised by trees as part of a defence mechanism and have been shown to be toxic to herbivores and inhibitive to decay organisms (Phillips and Croteau 1999). In PMES, the plant matrix in which RE were intimately associated has been removed or largely modified, leaving the extractives in a potentially more concentrated and bioavailable form. In the natural environment these RE existed in the wood, leaves and roots of plants acting as a deterrent against invading organisms (Phillips and Croteau 1999). While they may have been released to the environment upon plant death, this would have been as a slow process of decay. In PMES that are applied to soil, a high concentration of RE may be placed directly on or in the soil where exposed soil organisms may not be adapted to such high concentrations. This has the potential to dramatically change species compositions if these organisms are negatively impacted. Alternatively the addition of PMES to soil may significantly benefit soil biota and soil processes, due to enhanced nutrient and physical conditions. Changes in species composition may have adverse and long-term effects on basic soil functions such as organic matter decomposition and nutrient cycling (Hättenschwiler and Vitousek 2000).

1.5.4.5 Soil as a Receiving Environment for Organic Wastes

The application of organic wastes to soil is an attractive alternative to other forms of disposal such as landfill or aquatic disposal, but, a number of issues arise with this option. Nutrient and contaminant runoff are potential hazards to aquatic environments adjacent to land disposal sites. Groundwater contamination is also possible due to contaminants leaching from land applied wastes. PMES produced in New Zealand mills tend to have high C:N ratios, and application of these types of waste can lead to immobilisation of nitrogen in soil and reduced availability to plants (Feldkirchner *et al.* 2003). Accumulation of toxic compounds may adversely affect natural soil functions (Maliszewska-Kordybach and Smreczak 2003). Reduced microbial biomass C: total organic C ratios and reduced soil enzyme activities have been shown in soils amended with heavy metals contaminated sewage sludge (Renella *et al.* 2005). Organic contaminants, such as PAHs, have been shown to accumulate in soils amended with biosolids and this raises concerns for human health (Oleszczuk 2006).

Organic Contaminants

Pulp mill effluent solids generally have low concentrations of metals and, therefore, organic compounds are of greater concern for land application of these types of waste. With elemental chlorine bleaching, organochlorine compounds were of concern in land applied biosolids (Paasivirta *et al.* 1993). However, in the last two decades with replacement of elemental chlorine bleaching by more benign methods, organochlorines in PMES have been considerably reduced. Resin extractive compounds are, however, found in PMES at high concentrations. Aromatic extractives such as dehydroabietic acid and retene – a poly aromatic hydrocarbon (PAH) - are among the most toxic and recalcitrant compounds found in landfills receiving PMES. While no research has directly investigated the environmental effects of resin extractives in land applied PMES, there have been numerous studies of other organic compounds in soil.

Organic contaminants enter the soil via a number of routes including atmospheric deposition, spills, leaking storage tanks, agricultural applications, and municipal or industrial wastes (Regno *et al.* 1998). Once organic contaminants have entered

the soil compartment the potential to cause environmental damage is controlled by factors such as soil type (particularly organic matter content and clay mineralogy), and the physico-chemical properties of the contaminant compounds (aqueous solubility, polarity, lipophilicity and hydrophobicity) (Semple *et al.* 2003). These factors affect the bioavailability to soil organisms and also determine how long a compound will remain in the soil. Water soluble compounds are generally lost rapidly from soil through leaching and biodegradation. Biodegradation relies on mobility and availability of compounds to individual organisms. Compounds which have a high capacity for uptake and metabolism by organisms also require mass movement through the soil to be bioavailable (Jager *et al.* 2005; Semple *et al.* 2003). The majority of toxic material is often bound to the solid phase of the soil, while it is the concentration in the soil pore water that is usually related to the toxicity experienced by organisms (van Straalen 2002). Readily bioavailable organic compounds may degrade rapidly, however, the initial toxicity to organisms compared to less soluble compounds may be greater (Kapustka 2004).

Hydrophobic and lipophilic compounds may have low bioavailability due to low soil solution concentrations, however, where contacted by organisms they may rapidly cross or associate with cell membranes. Total soil concentrations may have to be quite high to supply the aqueous phase enough material to be toxic to organisms. Some of these compounds, e.g. polyaromatic hydrocarbons (PAHs), have been shown to have low acute toxicity to soil organisms (Sverdrup *et al.* 2002), however, it is not clear if this is related to low bioavailability. Several of these PAHs are on the USA Environmental Protection Agency (USEPA) priority organic pollutants list, being mutagenic and carcinogenic (Samanta *et al.* 2002).

As contact time with soil increases, contaminants usually become less available due to diffusion into soil pores and sorption to organic matter and clay minerals (Semple *et al.* 2003). These ‘ageing’ processes can involve short term sequestering of compounds through weak attractions (hydrogen bonding, van der Waal forces) or involve intimate association with the soil matrix by covalent bonding and occlusion (Semple *et al.* 2003).

There are examples of soil contamination around gasworks, refineries, fuel storage depots, and in locations with long-term application of agricultural pesticides and organic wastes. Difficulties in defining toxic thresholds make setting limits for disposal of families of compounds difficult. Kaptustka (2004) reviewed over 300 papers on PAH's and concluded that there was very little toxicity data useful for ecological-risk assessment as outlined by USEPA criteria. This was partly because data were sparse on individual PAH's; in soil that has been contaminated with organic compounds they are often part of a complex mixture of compounds and separating the effects of individual compounds can be difficult or impractical. Sverdrup *et al.* (2002 a, b) found a wide range of sensitivities to PAHs by different organisms. Different PAHs exhibited different levels of toxicity to the organisms tested, but these differences were not consistent between the different organisms compared. Translating effects on one organism to another or from one PAH to another are problematic. Retene, an alkyl substituted phenanthrene, and phenanthrene were compared in a single organism aquatic test, and showed quite different patterns of toxic action (Hawkins *et al.* 2002). Concentrations as high as 2,000 µg/g have been reported for retene in sediments (dw) contaminated with pulp mill effluent compounds (Fragoso *et al.* 1998). Several researchers have found good correlations between k_{ow} or solubility and toxicity of similar PAH-like compounds (Peng and Roberts 2000; Sverdrup *et al.* 2002).

1.5.4.6 Assessment of Risk – A Weight of Evidence Approach

Due to difficulties in determining adverse effects at the ecosystem level, it has been recommended that a weight of evidence approach be taken to environmental risk assessment (Lowell *et al.* 2000). Chapman (1986) used the concept of a sediment quality triad to develop criteria for the assessment of contaminated sediments. Three types of information were used to assess sediment quality; chemical analysis of sediments, laboratory bioassays with sediments as the substrate for test organisms, and aquatic studies of benthic communities. It was proposed that a similar technique be adopted for soil assessment (van Straalen 2002). By taking a multi-component approach different levels of biological organisation can be compared. A weight of evidence approach can integrate

environmental stressors that range widely spatially and temporally (Lowell *et al.* 2000). When making risk assessments, several lines of evidence have been found to be useful by regulatory authorities. In the assessment of Canadian northern rivers, it was found that stressors can work in conflicting ways. For example, the positive nutrient effects associated with sewage disposal can mask negative toxicological responses of organisms. When individual stressors are used to predict environmental effects, the wrong conclusion may be drawn. These findings gave weight to the need for an integrative approach to environmental risk assessment and regulatory guides (Lowell *et al.* 2000).

While laboratory-based techniques have been criticised for their lack of realism and lack of application to field conditions, they are important as part of an assessment process (Bogomolov *et al.* 1996; Sheppard 1999). Tests done on organisms may give the wrong impression of the effects that would be seen at the ecosystem level. It is important to have a range of repeatable simple assays that provide comparative data that integrates effects on organisms. Laboratory assessments are important for providing causative information for observations made in the field (Kookana *et al.* 2006).

1.5.5 Conclusion

The pulp and paper industry has made many improvements in process technology and effluent treatment in recent years, which have translated into reduced environmental impacts. The improvements are ongoing, and the study of the fate and effects of the discharges from pulp mills continues to develop. There are still documented effects of pulp mill effluents causing adverse effects in the aquatic environment despite these improvements, and much of this is associated with naturally derived organic compounds released from wood during pulping (Ali and Sreekrishnan 2001).

Pulp mill effluent solids have been applied to land, but little is known about the impacts on receiving environments as a result of this practice (Bostan *et al.* 2005). While processes associated with pulp mill discharges in aquatic systems have been well studied this is not true in terrestrial ecosystems. It is still unclear how

terrestrial ecosystems will respond to high loading of RE in PMES, and are methodologies poorly developed to investigate this. A multi-factor approach is needed to generate weight of evidence on the risk posed by PMES to terrestrial ecosystems.

2 Overview of PhD Thesis Research and General Materials and Methods

2.1 Introduction

The New Zealand Waste Strategy (MfE 2002) set a target for industry to divert 95% of organic wastes currently landfilled to beneficial use by 2010.

The Tasman and Kinleith mills, situated in the central North Island of New Zealand, currently use land filling as the primary means of disposal of effluent solid waste. These wastes are candidates for land application to forestry sites under the Waste Strategy, however, investigation of the impact of applying these waste solids to land needs to be addressed before this should be carried out routinely. There is a large body of literature surrounding the aquatic toxicity of pulp mill effluent wastes (Ali and Sreekrishnan 2001; Hewitt *et al.* 2006; Hynynen *et al.* 2004; van den Heuvel *et al.* 2006). Consequently, based on the results in aquatic systems, the effects on terrestrial ecosystems warrant investigation before large-scale land application of these wastes can be recomended.

2.2 General Outline of Methodologies Used for Thesis Research

This research sought to extend the limited knowledge on the terrestrial fate and effects of pulp mill effluent wastes. Pulp mill effluent solids (PMES) were collected from primary and secondary treatment systems and from adjacent landfills at the Kinleith and Tasman mills.

A weight of evidence approach (Section 1.5.4.6), to assessing ecological risk was adopted in this thesis research. To evaluate the risk PMES pose to terrestrial ecosystems, indicators at the organism, community and ecosystem level were assessed. Effects seen at these different levels of biological organisation were related to chemical analysis of the various solids tested. Chapman (1986) described the overlap of multi-disciplinary research that was adopted and was

useful in assessment of the suitability of PMES as soil amendments in this thesis (Figure 2.1).

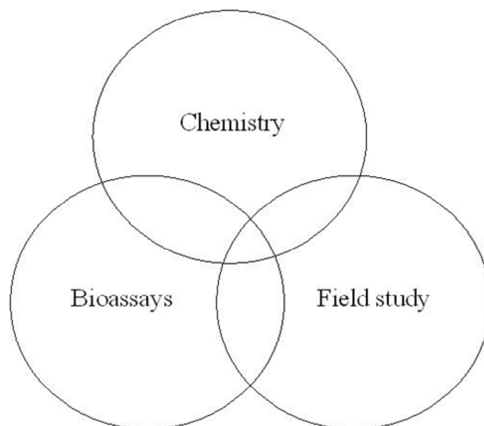


Figure 2.1 PMES quality triad, showing how data from chemistry, bioassays and field studies combine to provide an assessment of the quality of PMES as soil amendments (adapted from Chapman 1986).

There were three areas of study in the PhD thesis research, broadly categorised as chemistry of PMES, toxicology of PMES and ecosystem effects caused by PMES and each area is presented, respectively, in Chapters 3, 4 and 5. Laboratory bioassays were used for their ability to integrate effects of PMES on organisms and capacity to be expressed as simple biological endpoints. Field experiments were undertaken using a number of methods of analysis, and while not having the same level of control as laboratory bioassays, they provide greater 'realism' in terms of environmental effects. Chemical characterisation of PMES and the changes they undergo in a field setting provided information on the fate of various components of PMES in the terrestrial environment. In the following sections, an overview of these methods is presented with reference to the relevant chapters. Four PMES were collected after an initial survey and these same materials were used throughout for all laboratory and field experiments presented in Chapters 3-5, as summarised in sections 2.2.1, 2.2.2 and 2.2.3 respectively.

2.2.1 Organic Chemistry of PMES (Chapter 3)

A diverse group of plant secondary compounds are referred to as resin extractives (RE), or pitch, in the pulp and paper industry. These compounds, found in conifer oleoresin, are by-products of wood pulping. These compounds may be recovered for other industrial processes and, can contaminant paper machinery. They are released in varying amounts in waste water where they contribute to the toxicity of these waste streams.

A supercritical fluid extraction process was used to remove organic extractives from PMES, soil and body tissues. These extractives were resolved by gas chromatography and quantified by mass-selective spectrometry. Chemical characterisation of PMES, of tissues of worms exposed to PMES, and of soil exposed to PMES was achieved using this method. Changes in the chemistry of PMES over two years after application to soil were also analysed by this method.

2.2.2 Toxicology of PMES (Chapter 4)

The toxicology of PMES to soil organisms was investigated under controlled laboratory conditions. Survival, growth and reproduction were measured endpoints. An aquatic organism test utilised change in luminescence, which is linked to respiration, as a measure of toxicity. Basal respiration was used as a means of investigating responses to PMES by the microbial community from a forest soil. This soil was collected from adjacent to the site where PMES were land applied.

2.2.3 Environmental monitoring (Chapter 5)

Ecosystem level effects resulting from the application of PMES to a forest soil were investigated by observing changes in soil function in a field trial. Field measurements were made on soil CO_2 fluxes using infrared gas analysis. Soil and PMES samples were monitored for TOC, TN, mass losses, and acid detergent fractionation. Microbial biomass was assessed by the fumigation extraction method. Soil enzyme activities were measured by extracting the enzyme and measuring the change of absorbance after addition of enzyme substrate.

2.3 Description of Tasman and Kinleith Waste Streams

2.3.1 Norske Skog Tasman Mill

The Tasman mill is situated near the Bay of Plenty town of Kawerau in the North Island, New Zealand (Figure 2.2). The mill draws water from the adjacent Tarawera River and discharges treated effluent back into the river downstream of the mill. The Tasman mill is an integrated bleached kraft and thermo-mechanical pulp and paper mill. There are three pulp mills operating on the Tasman mill site, one of which produces kraft pulp and the other two producing thermo-mechanical pulp. The kraft mill supplies pulp for newsprint as well as for tissue production the balance being sent off site as market pulp board.

Effluent streams are combined and undergo primary treatment through sedimentation and screening whereby primary solids are removed and de-watered by screw press (Figure 2.3). Primary solids are removed from the site and landfilled nearby. Primary treated effluent undergoes secondary treatment through an aerated stabilisation lagoon system (ASB) before discharge. Lagoons are dredged on a regular basis to remove accumulating biosolids. Dredgings are pumped into drainage lagoons where they are allowed to drain over a period of months before being landfilled separately from primary solids (Figure 2.3).

2.3.2 Kinleith Mill

The Kinleith mill, operated by Carter Holt Harvey (CHH), is situated south of Tokoroa in the central North Island of New Zealand (Figure 2.2). This is a kraft mill that utilises oxygen delignification and chlorine dioxide bleaching. The mill produces over 300,000 tonnes of paper and 270,000 tonnes of pulp per annum.

Approximately 85,000 m^3 per day of treated effluent is discharged into the Kopakorahi arm of lake Maraetai; a hydro dam on the Waikato river (Kim and Smith 2006). The Kinleith mill produces about 270,000 m^3 of wastewater sludge per annum, including primary (fibre), secondary (biosolids), lime mud and

stormwater sediments. These sludges are currently disposed of in a dam monofill (Bouma and Mercer 2002).

Historically, Kinleith mill effluent streams were segregated into acid and alkali streams which were treated in separate ASB systems, the final effluent streams were combined before discharge to the receiving hydro lake (Figure 2.4) (Stuthridge *et al.* 1991). In 2002 the alkali effluent was diverted into the acid stream ASB system. The alkali ASB system had received alkali effluent including e-stage bleachery and foul condensate streams. The alkali ASB lagoons were not dredged to remove biosolids and these decommissioned lagoons were drained and contained aged biosolids.

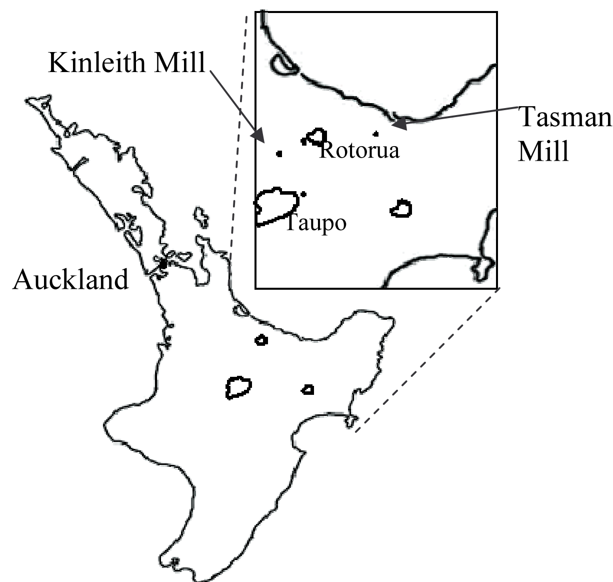


Figure 2.2 Map of North Island of New Zealand showing locations of Tasman and Kinleith pulp and paper mills

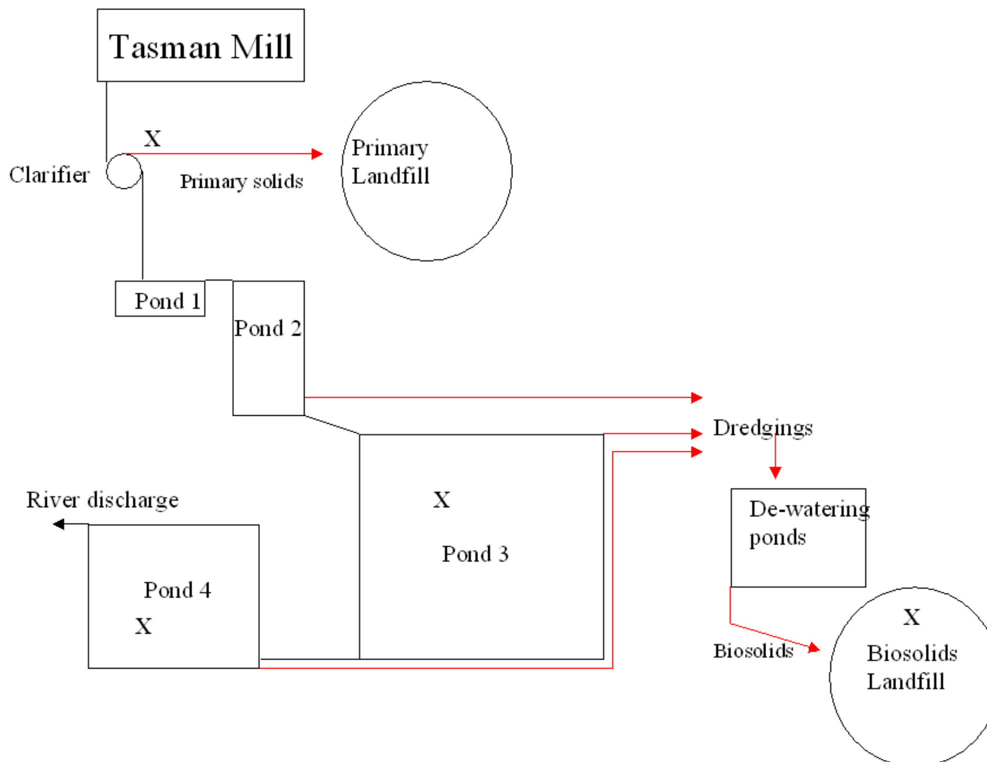


Figure 2.3 General layout of Tasman mill wastewater treatment system and landfills (not to scale). All primary treated effluent enters a series of four ponds; ponds 2-4 were aerated. All ponds were dredged and biosolids dewatered before landfilling separately from primary solids. PMES sampling sites are marked by X.

2.3.3 General Aspects of Pulp Mill Effluent Solids

Solids derived from pulp mill effluents are different from other industrial and municipal solids, due largely to the sources of the effluents. Pulp and paper mill effluents have high organic loading, resulting in high BOD, but unlike many other effluents, contain low levels of nitrogen and phosphorus, unless these nutrients are added during secondary treatment. Heavy metal concentrations are generally low in pulping effluents, while concentrations of calcium and sulphur may be high. The high organic matter (OM) content of pulp mill effluent solids (PMES) confers high water holding capacity.

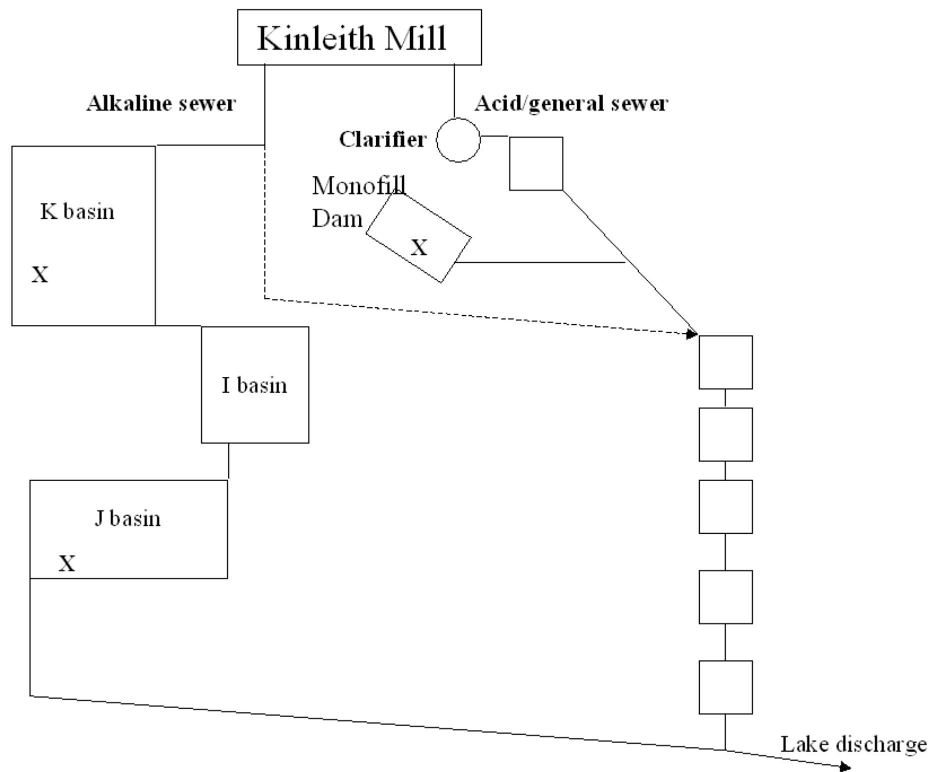


Figure 2.4 General layout of Kinleith wastewater treatment system and landfill (not to scale), showing acid and alkali sewers and ASB basins and Wawa dam monofill. The dotted line is the present (2006) alkali effluent stream. K, I and J basins were decommissioned in 2002. PMES sampling sites are marked by X.

2.4 Pulp Mill Effluent Solids (PMES) used in this Study

Three ASB system biosolids and one primary solid were used in this study. Throughout this thesis, they will be referred to as Tasman primary solids (TPS), Tasman biosolids (TB), Tasman aged biosolids (TAB) and Kinleith aged biosolids (KAB). These PMES were chosen to represent a range of properties relevant to studying fate and effects of pulp mill wastes. *Pinus* species are the most widely used for pulp production globally, while *P. radiata* is the predominant furnish in New Zealand pulp mills. Conifer species have many similarities with respect to the effluents derived from their pulping. PMES derived from New Zealand pulp mills are generally representative of a wide range of PMES produced globally from mills utilising similar effluent treatment technology.

These PMES are characterised by having high concentrations of naturally derived resin extractives (RE) and may also contain transformation products from these extractives. These RE are considered to pose the greatest environmental risk in these types of wastes. The PMES from the Tasman and Kinleith mills were screened for their RE content and the four solids used in this study reflect a variety of different RE profiles representing different mill processes and waste streams, different stages of effluent treatment and different depositional environments. The chemical characterisation of PMES from the Tasman and Kinleith pulp mills will be presented in Chapter Three.

The Tasman and Kinleith mills effluent treatment systems do not have nitrogen or phosphorus added. In many pulp mill effluent treatment systems these nutrients are added to improve treatment efficiencies, but this can lead to problems with excess nutrient levels in discharges to recipient waters and therefore nutrient addition is not utilised in New Zealand mills. The resulting biosolids are, therefore, low in both nutrients compared to some overseas operations (Wang *et al.* 2006).

2.5 General Materials and Methods

2.5.1 Tasman Mill Effluent Solids

Tasman Primary Solids

Approximately 47,000 dry tonnes of effluent solids are produced annually from the Tasman mill of which primary solids comprise about 60% (Garrett and Wang 2006). Tasman Primary Solids (TPS) (Figure 2.3) were the only primary solids used in this study and are distinctly different from biosolids. TPS are composed mostly of fibre that was lost from the manufacturing process, but also contain fly ash and lime mud (Wang *et al.* 2002).

TPS contain high concentrations of resin acids (RA) and fatty acids (FA) released during pulping and paper production. The RE present in TPS are characteristic of those found in the wood furnish as well as those modified (isomerization) by the

pulping process. There is little evidence of biological transformation of RE in TPS. Tasman primary solids have low nutrient levels apart from calcium, which is present due to the high lime content (Garrett and Wang 2006). These solids are high in labile carbon substrates released during pulping. Visually, TPS appear similar to paper pulp and have a considerable water holding capacity and low bulk density. TPS were approximately 77% water when collected from the screw press at the primary clarifier.

Tasman Biosolids

Biosolids accumulate through the sedimentation of biologically treated effluent, and generally, a high proportion of their dry weight, is derived from microbial biomass or microbial metabolites. The Tasman mill uses shallow aerated stabilisation basins for secondary treatment and accumulated biosolids are regularly dredged to maintain treatment system efficiency (Figure 2.3). Tasman Biosolids (TB) were collected in mid August 2004 from the third basin in a series of four ASB basins at the Tasman mill (Figure 2.3). Since these solids were collected this basin has been bypassed and the treatment system only operates three basins, the second and third being aerated. At the time these solids were collected, basin three was the largest of the aerated treatment basins. Biosolids were removed with a digger from the edge of basin three along the causeway that separated basin three and four. This material was placed into two 1000 L perforated containers. Shade cloth was placed inside the containers before they were filled with biosolids so as to allow them to drain before field application. At the time these biosolids were collected they had 95% water content; after two weeks draining, the biosolids were approximately 82% water when land applied. As these biosolids had been situated away from the aerators located in this basin they were anoxic. These solids contained high levels of transformed resin acids and resin acid neutrals (Section 3.5.2).

Tasman Aged Biosolids

Tasman Aged Biosolids (TAB) were derived from the Tasman ASB system and were collected from a landfill site (Figure 2.3), where this material had been

dumped about 12 months previously. These solids were from routine dredging of the Tasman ASB system and they had been allowed to de-water prior to landfilling. These solids were similar in chemical composition to TB except that they only contained only low levels of resin acid neutrals (Section 3.5.2). Tasman aged biosolids had substantially lower water content (68%) than did TB. The TAB also contained a substantial amount of pumice, presumably from the dredging operation.

2.5.2 Kinleith Aged Biosolids

The Kinleith mill I, J, K ASB system was a deeper pond system than the Tasman ponds and dredging was not employed in these ponds; biosolids from this ASB system represent historical accumulation of biosolids since the treatment system was commissioned.

Kinleith Aged Biosolids (KAB) were collected directly from K-basin, which was the first of three stabilisation basins treating alkali effluent from the Kinleith mill (Figure 2.4). These decommissioned basins had been drained for approximately two years when biosolids were collected. Biosolids from K-basin represented accumulations over many years from an aerated treatment basin that was not dredged. This treatment system had received very high resin acid loading compared to the Tasman mill treatment system. Kinleith aged biosolids were extremely high in transformed resin acids, and resin acid neutrals with RE derived compounds accounting for approximately 5% of the dry weight. Kinleith aged biosolids had the highest bulk density and the lowest water content of the PMES used in this study (approximately 50% water content). A summary table of the total concentrations of RE compound groups found in the four PMES outlined above, are presented in Chapter 4 (Table 4.1).

2.5.3 Field trial layout

Chapters 3 and 5 present data collected from a field trial where PMES were applied to soil. A general outline of this field trial is presented below, with further details relevant to individual experiments presented in Chapters 3 and 5.

The four PMES described above were surface applied to soil in a mature pine forest (*P. radiata* D.Don) of trees approximately 25 years of age (Figure 2.5). The soil, Tarawera loamy sand, was a Tephric Recent soil with a loamy sand texture, and overlies Kahoroa and Taupo tephras (Garrett and Wang 2006). The area has a moderate climate and receives approximately 1676mm annual rainfall (Garrett and Wang 2006). The field site was situated on land owned by Norske Skog Tasman near the wastewater treatment system at the Tasman Mill. An area of approximately 0.25 hectares contained 20 three-square meter (m^2) plots. The field trial site was on flat land, which extended into a wetland to the south and was bounded by low hills to the west, north and east forming a sheltered south-facing basin (Figure 2.6).

PMES, which had been collected in the previous two weeks, were applied to the trial site at the end of August 2004, which corresponds to late winter in the central North Island. Four blocks were set out and treatments were assigned in a randomised block design (Figure 2.5). PMES were applied to circular plots two metres in diameter at an application rate of 150 dry tonnes per hectare (7.5-15 cm deep). A control treatment was included, this being an undisturbed soil; therefore, a total of five treatments each replicated four times gave a field trial of 20 plots. PMES stockpiled at the field site were spread on to assigned plots by hand on the 25th and 26th of August 2004.

Field plots were situated between rows of *P. radiata* which were planted at 6x2m spacing. The plot size allowed for five soil respiration cores to be placed approximately 70 cm in from the edge of the plots (Figure 2.5). In the second year (see Section 5.4.7 for description), four needle litterbags were placed in each plot; two were placed in the centre on the surface, while the other two were buried under PMES approximately 50 cm in from the edge (Figure 2.5).

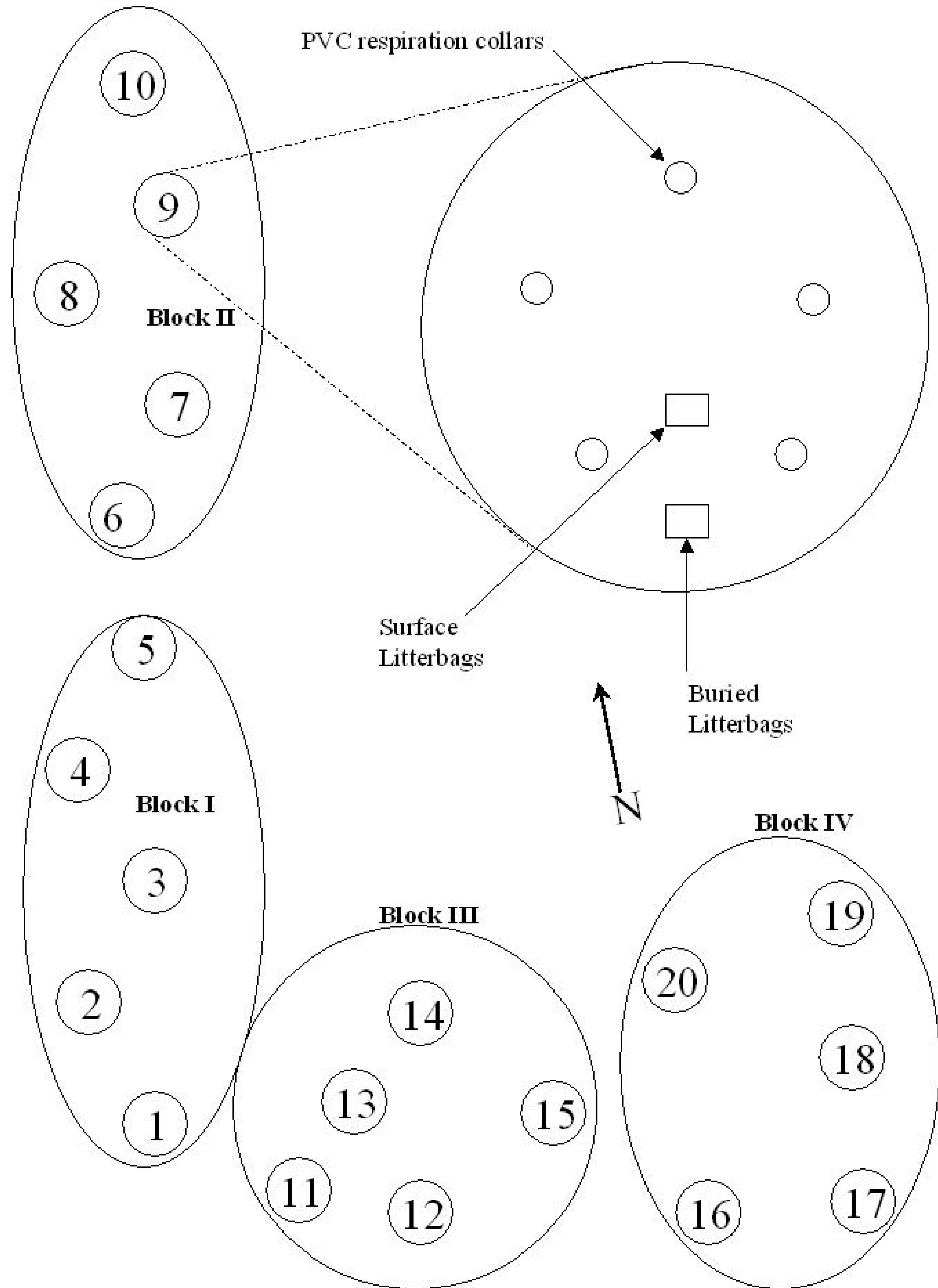


Figure 2.5 Field trial layout showing four blocks containing 20 plots. Each block contained one randomly assigned replicate of each of five treatments. Treatments were: control (plots 4, 6, 15, 19), TPS (plots 2, 9, 11, 17), TB (plots 3, 8, 14, 20), TAB plots (5, 10, 13, 16), KAB plots (1, 7, 12, 18).

Figure 2.6 The field trial was located near to the Tasman mill in a pine forest adjacent to a wetland. Field plots were located in a small valley indicated by circle on left-hand side of this photo.

3 Persistence of Pulp Mill Derived Resin Extractives in the Terrestrial Environment

3.1 Introduction

Conifers produce large quantities of oleoresin, which are distributed about the plant in response to wounding and insect damage. Oleoresin serves as a defence against herbivorous attack and infection by decay organisms. These activities have implications for decomposition processes in forest ecosystems (Kainulainen and Holopainen 2002; Phillips and Croteau 1999). A large number of compounds have been isolated from conifer oleoresins, some of which show toxic or physical action against insects, plants and microorganisms (Kainulainen *et al.* 2003). Other more volatile compounds are involved in the transport of these compounds about the tree (Phillips and Croteau 1999).

During the pulping of wood, lignin and secondary compounds are released making fibres (cellulose) available for further processing. Compounds soluble in water or neutral organic solvents that are removed from wood are generally referred to as resin extractives (Smook 1982). Resin extractives (RE) also include the transformation products of these 'native' RE, which may arise through chemical modification during the pulping process and also through biological activity. In the kraft process, RE released from the wood during pulping are removed in "black liquor" where they may be recovered as turpentine and tall oil. The term "pitch" refers to RE carried over into pulp and paper processes where it can cause contamination of paper machines and product (Smook 1982).

The RE composition of pulp mill effluent is largely determined by the tree species utilised as mill furnish and the pulping process employed by the mill (Ali and Sreekrishnan 2001). Primary and secondary treatment results in the removal of the majority of RE from effluent before it is discharged to surface water. Depending on the efficiency of the treatment system, up to 100% removal of many compounds can be achieved in discharged effluent (Liss *et al.* 1997). While removal may involve metabolism of these compounds to CO_2, a proportion may

be modified, e.g., resin acids may be transformed to resin acid neutrals (Figure1.2). Transformed and native RE readily partition to the solid phase and accumulate in biosolids. The concentration of RE in pulp mill effluent solids (PMES) can be significantly higher than in the effluent due to the hydrophobic nature of RE. Hall and Liver (1996) showed that, within two days, 90% of resin acids in a solution had partitioned on inactivated (with sodium azide) biosolids. The rate of removal by partitioning on biosolids was significantly greater than biodegradation by acclimated biomass, and in anaerobic environments, Hall and Liver (1996) suggested that this would lead to very high concentrations of accumulated resin acids. Phytosterols have also been shown to partition and accumulate in pulp mill biosolids (Mahmood-Khan and Hall 2003).

The ability to degrade resin acids is possessed by a great diversity of microorganisms; bacteria can use resin acids as sole carbon sources, while fungi can transform (hydroxylate), but have not been shown to degrade resin acids (Liss *et al.* 1997). Organisms with the ability to degrade resin acids have been isolated from a wide range of environments including soil (forest and arable), water, biological treatment systems, and sediments (Martin *et al.* 1999). A convergent pathway has been proposed for abietane degradation, in which abietanes are aromatized to dehydroabietic acid. Based on intermediates isolated from culture medium, Martin *et al*. (1999) suggested that this involved hydroxylation followed by oxidation to, e.g. 7-oxodehydroabietic acid – a common transformation product found in PMES. Decarboxylation may or may not occur before ring fission, via an extradiol cleavage reaction. Although pimeranes are rapidly degraded under aerobic conditions, very little is known about the mechanisms of degradation in this class of resin acids (Martin *et al.* 1999). However, similar transformed pimeranes, analogous to abietanes, are common in pulp mill effluent systems.

Proposed hydration (abietenic and abietanic acid) and hydroxylation (Kinleithic acid) products of abietic acid were identified by Zender *et al*. (1994) from an ASB system.

In anaerobic sediments, Taverndale *et al*. (1997b) proposed that abietanes were decarboxylated via dehydroabietic acid to the intermediates dehydroabietin, or

simonelite and then aromatised to tetrahydroretene. Ultimately, these metabolites may be fully aromatised to the PAH retene as evidenced by the predominance of this resin acid neutral in anaerobic sediments derived from pulp mill effluents.

There are no reported studies of the fate of RE from PMES applied to soil. Beauchamp *et al*. (2002) reported rapid degradation of resin and fatty acids when de-inking paper sludge was composted. Wang *et al.* (1999) showed that RE were rapidly removed through a barrel lysimeter when irrigated weekly with TMP effluent over a 16 month period, however, the mechanisms of removal were not investigated and biodegradation of RE was not demonstrated.

RE degradation has been studied in terrestrial environments in plant litter studies. Monoterpenes may be important regulators of carbon metabolism and nutrient turnover in forest systems (Kainulainen *et al.* 2003). Inhibition of soil nitrification by monoterpenes at natural concentrations was demonstrated by Paavolainen *et al.* (1998) in a recently clear-cut forest soil. Kainulainen and Holopainen (2002), studied extractives loss from *Pinus sylvestris* needle litter and concluded that secondary compounds, particularly dehydroabietic acid, were relatively slow to decompose and may be important in the turnover of forest litter.

Bioavailability of RE in PMES, particularly those that are from 'aged' biosolids, is largely unknown. Although resin acids are naturally occurring and are degraded by microbial communities in conifer forests, transformed resin acids and resin acid neutrals are less likely to be present in these environments and may be less degradable by the adapted communities. Transformed resin acids have been reported to be more recalcitrant than native resin acids (Taverndale *et al.* 1997a). The solid matrix that contains RE differs between wood and PMES due to the pulping process where lignin and cellulose components are separated; the resulting PMES being physically and chemically different from wood and other natural inputs to forest ecosystems. Alexander (1995) has noted that the physical matrix as well as the residence time of organic compounds within this matrix can profoundly effect the accessibility of compounds to microorganisms. This accessibility influences the decomposability and also the toxicity of organic compounds.

3.2 Hypothesis, Aims and Objectives concerning Resin Extractives Degradation

Although little is known about the fate of PMES derived RE in terrestrial ecosystems, however, they have been shown to persist in sediments, where they are bioavailable (Merilainin *et al.* 2006; van den Heuvel *et al.* 2006). Transformation of RE to secondary compounds may increase recalcitrance and toxicity (Taverndale *et al.* 1997a).

This PhD thesis research hypothesised that '*RE would be recalcitrant in land applied PMES'*, particularly those RE found in solids derived from anaerobic environments.

Initially a survey of two mills wastewater treatment systems was undertaken to determine the concentration and speciation of RE in typical New Zealand PMES. From the chemical analysis of PMES surveyed, four solids were chosen with distinctly different RE profiles to be tested in field and laboratory experiments (Section 2.4).

The aims of this research were as follows:

1. Determine the decomposition rate of RE in land applied PMES.
2. Determine the extent of RE movement from PMES into underlying soil.

RE concentrations were measured in samples collected every three months from field applied PMES of varying compositions. Soil beneath PMES and a control were sampled once after 9 months to investigate movement of RE.

3.3 Distribution of wood resin extractives in Plant and Pulp Mill Waste Materials

A wide range of RE are found in *Pinus radiata*, the predominant furnish in New Zealand pulp mills. Resin acids comprise the majority of RE found in *P. radiata* (Uprichard 2002) of which over 50% consist of levopimaric, palustric and

neoabietic acid (Table 3.1). Effluent from a New Zealand mill utilising predominantly *P. radiata* has a different resin acid composition to that found in the wood (Zender *et al.* 1994). Levopimaric, palustric and neoabietic are isomerised to abietic acid during mill processes. Transformed resin acids appear as a result of microbial action during effluent treatment. *Pinus. silvestris* needles have a different distribution of resin acids to *P. radiata* timber (Kainulainen and Holopainen 2002).

Table 3.1 Distribution of resin acids in *Pinus radiata* wood and pulp mill effluent - from *(Zender *et al.* 1994) and *P. silvestris* needles - from ‡(Kainulainen and Holopainen 2002).

Resin Acid	Proportion in *Pinus Radiata** (%)	Proportion in source effluent* (%)	Proportion in senescent needles‡ (%)
Levopimaric/Palustric	48.7	22.4	18.0
Neoabietic	20.5	11.0	32.6
Pimaric	8.1	9.0	<1
Abietic	7.5	33.8	16.2
Dehydroabietic	7.2	11.1	28.9
Isopimaric	5.4	6.0	<1
Sandaracopimaric	1.0	1.9	3.6
Unidentified	1.6	-	-
Transformed resin acids	-	4.7	-
Dichlorodehydroabietic	-	0.1	-
Total	100	100	100

3.4 Materials and Methods

3.4.1 Preliminary survey of pulp mill wastes

The first stage of research into PMES chemistry involved a survey of effluent solid wastes generated by the Tasman and Kinleith mills. This was undertaken to scope the types of solids produced and the ranges of concentrations of various extractives present in the wastes from the two mills. In June and July of 2004, PMES samples were collected from the Tasman and Kinleith mills wastewater treatment systems (Figures 2.3 and 2.4).

3.4.1.1Tasman Mill

Primary solids were collected at the screw press adjacent to the clarifer treating the Tasman mill effluent before entry to the secondary treatment system (Figure 2.3). Ponds three and four of the Tasman aerated stabilisation basins (ASB) were sampled (from a barge) near to aerators and from bottom sediments away from aerators. Biosolids dredged from all ponds at the Tasman mill are allowed to de-water before landfilling. Biosolids were collected from a landfill area adjacent to the Tasman mill ASB system where de-watered biosolids had been dumped approximately 12-24 months previously.

3.4.1.2 Kinleith Mill

Biosolids from the Kinleith mill were collected from two ASB basins that had been drained for approximately two years. These basins had historically received alkali effluent. The first in a series of three basins (K-basin) was mechanically aerated while the third basin (J-basin) was a naturally aerated storage lagoon. Solids were also collected from the Wawa dam monofill, which receives clarifier sludge (primary solids), ASB pond sludge and lime dregs from the Kinleith mill (Figure 2.4).

3.4.2 Fate of Wood Resin Extractives in Field Applied Pulp Mill Effluent Solids

Based on the findings of the PMES survey from Tasman and Kinleith mills (results below Section 3.5.1), four PMES were field applied in August 2004 (see Section 2.5. for description of PMES and Section 2.5.2 for the field trial set-up). Samples of PMES from the time of field application and at three monthly intervals over two years were collected from field-plots for analysis of RE. After one year, samples collected from all field applied PMES, at time 0, 3, 6 and 9 months, were analysed for RE concentration. Soil samples were also collected at nine months for RE analysis of soil from all plots including the control (no PMES). After two years, samples (three monthly) of Tasman biosolids (TB and TAB) up to 24 months were re-analysed for RE. Samples were re-analysed

because for these biosolids initial extractions had low recoveries of spiked resin acids.

PMES samples were collected by removing the surface layer of field applied solids and then a sample was taken from the middle of the PMES profile (approximately 5cm deep). For each treatment, samples were collected from each of four replicate plots and these were pooled into one composite sample for analysis. Samples of biosolids (TB, TAB and KAB) and soil were sieved to 2 mm, while TPS samples were hand mixed. Samples were kept at –20°C until analysis.

3.4.3 Extractions on PMES with Super Critical CO_2

Extractions were carried out on PMES and field applied PMES over two years from June 2004 – September 2006 using a HP 7680T Supercritical Fluid Extractor. Extracted samples were analysed by gas chromatography/mass spectrometry. Spiking samples prior to extraction with surrogate standards representing all major compound groups allowed extraction recoveries to be monitored (Appendix 1). For extractions on field applied PMES (0 - 9 month and 0 - 24 month samples) all samples of a particular treatment were extracted in succession, allowing direct comparisons to be made of concentrations at different stages of the field trial within each treatment. This allowed decay curves to be fitted to the extractives data and half-lives to be calculated for individual compounds.

Over the period from June 2004 – September 2006, several issues regarding the extraction process arose and were addressed. Method development over this time meant that measured concentrations of compound classes at different times might not be directly comparable. These different times relate to the following:

Initial survey of pulp mill effluent solids.

Field applied PMES after nine months, field trial soil samples.

Field applied PMES after 24 months.

Sections 3.4.3.1-3.4.3.3 describes changes made concerning the extraction process at these three times.

3.4.3.1 Extraction Process used for Initial survey of pulp mill solids (June 2004)

- Frozen samples were thawed and used at field moisture content. A sub-sample was used to determine moisture content.
- Approximately 5g of material was ground in a mortar and pestle with 35g of anhydrous sodium sulphate.
- Duplicates samples were weighed into extraction thimbles with 50 μl of surrogate recovery standard (D10-anthracene for resin acid neutrals, 8(14)-abietenic acid for resin acids, dihydrocholesterol for sterols, D31-Palmitic acid for fatty acids, 2,4,6-Tribromophenol for phenolics and 2,4,6-Tribromoanisole for monoterpenes).
- After an initial five minute holding period at the extraction pressure, three 25 min extractions were performed with a supercritical fluid pressure of 227 bar, flow of 3 ml/min, CO_2 density of 0.7 g/ml and a thimble extraction temperature of 71ºC.
- Extractives were removed using a C18 trap that was eluted using three rinses of methylene chloride/acetonitrile (1:1)
- In the extracts, the solvent volume was reduced over N_2 gas, samples were dried by flushing through anhydrous sodium sulphate and then derivatised (silylation) for analysis by gas chromatography/mass-selective detection (GC-MSD) using dibromoanthracene as an injection standard. All concentrations were corrected for surrogate standard recoveries and extraction blanks.

3.4.3.2 Extraction Process used for Field Applied PMES after nine months, and for field trial soil samples (July 2005)

- Sub-samples (thawed) from all sampling periods were placed in 50ml falcon tubes, frozen to -20° C, then freeze dried.
- Triplicate PMES samples were acidified (10 drops of conc. HCl), ground with anhydrous sodium sulphate and spiked with surrogate recovery standards as above.
- After an initial five minute holding period at the extraction pressure, three 25 min extractions were performed with a supercritical fluid pressure of 227 bar,

flow of 3 ml/min, CO_2 density of 0.7 g/ml and a thimble extraction temperature of 71ºC.

- Extractives were removed using a C18 trap that was eluted using three rinses of methylene chloride/acetonitrile (1:1)
- Extracts were blown down to dryness with N_2 gas, reconstituted with dichloromethane (DCM) and flushed through anhydrous sodium sulphate.
- Injection standard was added and samples derivatised before GC/MS analysis as above

3.4.3.3 Extraction Process used for Field applied PMES after 24 months (September 2006)

Due to poor resin acid recoveries, extractions of TB and TAB were repeated and extended to 24 months after field application, making a total of nine sampling times (surrogate recoveries are presented for all samples in Appendix I). Modifications made to the July 2005 method were as follows:

- The acidification step using HCl was replaced by adding 100µl methanol to increase polarity of the extraction fluid, and 100µl of formic acid immediately before SFE extraction.
- After an initial five minute holding period at the extraction pressure, one 40 minute extraction was performed with a supercritical fluid pressure of 383 bar, flow of 3 ml/minute, CO_2 density of 0.95 g/ml and a thimble extraction temperature of 71ºC
- Extractives were removed using a C18 trap that was eluted using four rinses of methylene chloride/acetonitrile (1:1).
- Extracts were flushed through anhydrous sodium sulphate, then blown down to dryness before reconstituting to 1.5 ml in DCM.

All compound concentrations are presented as µg/g dw. The following compounds were analysed for but not detected in any extracts: Palustric acid, levopimaric acid, fenchone, eugenol, homovanillic acid, ferulic acid, gallic acid, syringol, acetosyringone, syringylaldehyde, syringic acid, conifieryl alchohol, coniferyl aldehyde and pinosylvin monomethyl ether.

3.5 Results

3.5.1 Pulp Mill Treatment System Survey

The survey of the Tasman and Kinleith mills waste water treatment systems revealed a wide range of primary solids and secondary biosolids distinctly different from each other in their organic compound (RE) analysis (Table 3.2). Primary solids (P) from the Tasman effluent stream were predominantly fibre lost from the pulping process and were chemically distinct from secondary treated solids. Native resin acids and fatty acids comprised nearly 90% of total extractives identified in primary solids whereas secondary solids had less than 40% native resin acids. Biosolids that had been de-watered for more than 12 months (B1, B2 and B3) had low fatty acids, while biosolids collected from the secondary treatment systems of the Tasman mill (B4) or mixed primary/secondary solids from Kinleith mill (P/B) were intermediate in fatty acids.

K and J basins were decommissioned ASB basins from the Kinleith mill which historically received e-stage bleachery and foul condensate streams before 2002. K basin (B1), the first of three treatment basins, had solids with the highest total extractives concentrations of any solids sampled, with resin acids making up 65% of the total. J basin (B2) is the last basin in the series of three and its solids were different from those from K basin in the resin acid signature, being dominated by abietic and open ringed (seco) resin acids; chlorinated resin acids were also more abundant than in other solids.

Biosolids removed from the treatment ponds at the Tasman mill were different from combined primary/secondary solids collected from the monofill at the Kinleith mill. The Tasman mill biosolids were higher in transformed resin acids and resin acid neutrals than were solids collected from the Kinleith monofill, but were quite similar to the K-basin biosolids. The Kinleith mill combined solids had low total extractives, representing a combination of primary and secondary solid characteristics. Aged biosolids (B4) from the Tasman landfill were also low in total extractives, but most of the compounds found in other biosolids were detected in B4 biosolids (Table 3.2).

Table 3.2 2004 Survey of pulp mill effluent solids (PMES) from the Tasman and Kinleith mills. An asterisk indicates where data from only 1 replicate was used.

	P	B1	B2	B3	B4	P/B
Sample Name	**mean** se	**mean** se	**mean** se	**mean** se	**mean** se	**mean** se
	n=3	**n=3**	**n=2**	**n=4**	**n=3**	**n=2**
Fatty Acids						
Decanoic acid (F10:0)	**0.2** ±0.01	**0.2** ±1	**0.2** ±0.1	**0.2** ±0.1	**1.5** 1±0.7	**0.4** ±0.1
Tetradecanoic acid (F14:0)	**1.9** ±0.1	**0.2** ±1	**0.3** ±0.1	**0.3**	**2.8** ±2.1	**1.7** ±0.1
Palmitic acid (F16:0)	**82** ±6	**3** ±1	**3.3** ±0.1	**6.5** ±3	**12.5** ±21.7	**25.7** ±3
Margaric acid (F17:0)	**2.3** ±0.1	**n.d**	**n.d**	**n.d**	**1.2** ±1.0	**2.7** ±0.1
Linoleic acid (F18:2)	**155** ±11	**n.d**	**n.d**	**6.1** ±3	**12.4** ±7.3	**5** ±1
Oleic acid (F18:1)	**172** ±13	**1** ±0.1	**1.0** ±0.1	**10.4** ±6	**13.4** ±6.5	**10** ±1
Stearic acid (F18:0)	**16** ±1	**1** ±0.1	**1.0** ±0.1	**1.2** ±0.1	**1.9** ±1.7	**36** ±4
Docosanoic acid (F22:0)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**9** ±9	**n.d.**
Tetracosanoic acid (F24:0)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**8.5** ±7.6	**n.d.**
Total Fatty Acids	**430** ±28	**5** ±1	**6** ±1	**15.3** ±11	**70** ±60	**85** ±11
Resin Acid Neutrals						
Fichtelite	**0.7** ±0.1	**120** ±8	**564** ±1	**19.8** ±3	**110** ±25	**0.9** ±0.1
Dehydroabietin	**0.7** ±0.1	**120** ±5	**329** ±1	**0.8** ±0.1	**17** ±9	**0.6** ±0.1
Tetrahydroretene	**1.9** ±0.1	**264** ±41	**2012** ±36	**1.8** ±0.1	**168** ±268	**3.2** ±0.1
Retene	**7.1** ±2.3	**2132** ±144	**948** ±12	**26** ±6	**1465** ±1343	**2.0** ±0.1
Methyldehydroabietin	**0.4** ±0.1	**7.1** ±0.1	**9.1** ±0.1	**n.d**	**3** ±3	**0.5** ±0.1
Total Resin Acid Neutrals	**10.6** ±5.8	**2643** ±187	**3454** ±50	**49** ±9	**1763** ±1210	**7** ±1
Native resin acids						
Pimaric acid	**63** ±2	**61.2** ±2	**n.d.**	**11** ±3	**108** ±81	**35** ±9
Sandaracopimaric acid	**6.2** ±0.5	**39.2** ±0.1	**n.d.**	**n.d**	**9** ±4	**3** ±1
Isopimaric acid	**28** ±3	**n.d**	**n.d.**	**3** ±1	**41** ±30	**14** ±3
Dehydroabietic acid	**101** ±8	**68.1** ±1	**n.d.**	**26** ±7	**125** ±100	**40** ±8
Abietic acid	**1171** ±113	**122.9** ±9	**305.9** *	**40** ±7	**620** ±550	**104** ±23
Transformed resin acids						
Pimarenic acid	**4.2** ±0.4	**276** ±13	**n.d.**	**8** ±2	**116** ±120	**6** ±4
Sandaracopimarenic acid	**5.3** ±0.5	**320** ±15	**n.d.**	**15** ±3	**280** ±348	**8** ±6
Isopimarenic acid	**30.2** ±2.4	**1279** ±52	**n.d.**	**6** ±1	**315** ±363	**56** ±43
13-Abietenic acid	**61** ±4	**n.d**	**n.d.**	**22** ±4	**89** ±71	**100** ±18
Pimaranic acid	**5.9** ±0.4	**300** ±14	**n.d.**	**15** ±2	**70** ±16	**12** ±10
Isopimaranic acid	**5.3** ±0.2	**244** ±12	**n.d.**	**6** ±1	**24** ±16	**7** ±5
Abietanic acid	**44.2** ±1.5	**2056** ±71	**n.d.**	**31** ±5	**404** ±444	**82** ±63
Seco-1-dehydroabietic acid	**17.7** ±2.1	**30** ±2	**209.3** *	**4** ±1	**74** ±66	**11** ±3
Seco-2-dehydroabietic acid	**9.3** ±1.4	**41** ±2	**138.3** *	**3** ±1	**58** ±62	**7** ±1
12-Chlorodehydroabietic acid	**n.d**	**3.7** ±0.1	**7.7** *	**n.d.**	**1.9** ±0.1	**1.0** ±0.1
14-Chlorodehydroabietic acid	**1.4** ±0.1	**2.2** ±0.1	**9.5** *	**n.d.**	**17** ±13	**3** ±1
12,14-Dichlorodehydroabietic	**n.d**	**18.4** ±0.1	**83.2** *	**n.d.**	**9.8** ±0.1	**n.d**
Total Resin Acids	**1521** ±80	**4842** ±203	**753.9** *	**158** ±32	**2625** ±2070	**490** ±198
Phytosterols						
Cholesterol	**n.d**	**n.d**	**n.d**	**n.d**	**7** ±1	**n.d**
Campesterol	**2.2** ±0.3	**6.1** ±1	**20.9** ±<1	**n.d**	**8** ±10	**3** ±1
Stigmasterol	**n.d**	**n.d**	**8.5** ±<1	**n.d**	**14** ±10	**2** ±1
Sitosterol	**54.9** ±5.9	**184** ±6	**n.d**	**25** ±5	**257** ±291	**49** ±25
Sitostanol	**8.7** ±1.2	**110** ±4	**n.d**	**17** ±4	**154** ±125	**31** ±13
Total Phytosterols	**65.9** ±6.5	**300** ±8	**29.3** ±0.1	**41** ±9	**428** ±435	**83** ±41
Total Extractives	**2032** ±107	**7533** ±151	**4744.2** ±304	**292** ±41	**4887** ±3775	**665** ±251

3.5.2 Field Applied Pulp Mill Effluent Solids

A primary solid and three secondary solids were field applied in August 2004 (Section 2.5.2) and their analysis is presented in Table 3.3. As with the analysis of P (Tasman primary) given in Table 3.2, approximately 90% of resin acids were native resin acids in the Tasman primary solids (TPS); this was the only PMES with a significant proportion of extractives as fatty acids (approximately 40%) (Table 3.3). The biosolids KAB, TAB and TB were from similar localities to the B1, B3 and B4 biosolids respectively from Table 3.2. The total concentrations of RE are higher in the biosolids presented in Table 3.3 compared to the corresponding biosolids from Table 3.2, most likely due to poor recoveries in the 2004 extractions (Appendix I). However, the relative proportions of individual compounds are quite similar between the respective PMES indicating similar origins (Tables 3.2 and 3.3).

Abietic and dehydroabietic acids made up > 75% of the native resin acids in all PMES (Table 3.3). Levopimaric, palustric and neoabietic acids readily isomerise to abietic acid during pulping, and were not detected in primary solids.

Transformed resin acids, characteristic of biologically treated effluents, particularly the saturated abietane – abietanic acid, were found at high concentrations in the biosolids KAB, TB and TAB. Kinleith aged biosolids and TB had high concentrations of the resin acid neutrals retene and fichtelite, while KAB also had high concentrations of tetrahydroretene, an intermediate resin acid neutral. While KAB had concentrations of native pimeranes similar to those in Tasman solids, it had very much higher concentrations of transformed pimeranes than did the Tasman solids. Zender *et al*. (1994) reported poor removal of pimeranes compared to abietanes in the treatment system that produced KAB was derived from (Zender *et al.* 1994), and pimeranes have been reported to be more recalcitrant than abietanes.

Chlorinated resin acids were detected in all PMES, although only at low levels in primary solids; biosolids concentrations may reflect historical inputs before ClO_2 bleaching replaced elemental chlorine bleaching. In the Tasman solids, 14-

chlorodehydroabietic acid was the predominant chlorinated resin acid, whereas in KAB, 12, 14-Dichlorodehydroabietic acid predominated.

The TB and TAB had very similar resin acid analysis both in total concentration (5777 μg/g and 5806 μg/g respectively) and also proportional distribution (Table 3.3). Both these solids were from the Tasman mill ASB treatment system (Figure 2.3), however TAB had been removed from the ponds for at least 12 months prior to collection suggesting little degradation of resin acids since dredging. A similar trend was observed for fatty acids, although fatty acids were low in biosolids (approximately 5%) compared to the Tasman primary solids. Total resin acid neutrals in TAB (718 μg/g) were approximately 14% of TB total resin acid neutrals (5157 μg/g), indicating significant degradation in the TAB since dredging (Table 3.3).

Phytosterol concentrations were significantly greater in biosolids than in the primary solids, particularly sitostanol in KAB (Table 3.3).

Table 3.3 Concentration of RE in PMES when field applied in August 2004

Concentrations μg/g d.w.	TPS		TB		TAB		KAB	
Monoterpenes	**mean**	se	**mean**	se	**mean**	se	**mean**	se
Beta-pinene	**n.d.**		**n.d.**		**n.d.**		**4**	±0.1
Camphor	**n.d.**		**n.d.**		**n.d.**		**1**	±0.1
Borneol	**4.0**	±0.1	**n.d.**		**n.d.**		**2**	±0.1
Terpinen-4-ol	**n.d.**		**3**	±0.7	**2**	±0.1	**1**	±0.1
Alpha-terpineol	**n.d.**		**6**	±0.1	**3**	±0.1	**3**	±0.1
Total Monoterpenes	**4**	±0.1	**8**	±3	**5**	±0.3	**10**	±1
Phenolics								
Guaiacol	**3**	±0.4	**5**	±0.8	**1**	±0.1	**n.d.**	
Vanillin	**5**	±0.6	**2**	±0.2	**1**	±0.1	**4**	±0.1
Acetovanillone	**1**	±0.2	**1**	±0.1	**n.d**		**1**	±0.1
Vanillic acid	**9**	±1	**1**	±0.1	**1**	±0.1	**2**	±0.1
Total Phenolics	**17**	±2	**8**	±1	**2**	±0.1	**8**	±0.1
Fatty Acids								
Decanoic acid (F10:0)	**n.d.**		**3**	±0.2	**2**	±0.7	**1**	±0.1
Dodecanoic acid (F12:0)	**n.d.**		**2**	±0.1	**1**	±0.2	**n.d.**	
Tetradecanoic acid (F14:0)	**17**	±1	**5**	±0.3	**4**	±0.3	**2**	±0.1
Palmitoleic acid (F16:1)	**n.d.**		**6**	±0.5	**8**	±0.5	**5**	±0.1
Palmitic acid (F16:0)	**1109**	±3	**63**	±1	**57**	±2	**40**	±1
Margaric acid (F17:0)	**12**	±0.1	**2**	±0.2	**2**	±0.1	**n.d.**	
Linoleic acid (F18:2)	**1214**	±10	**19**	±1	**9**	±0.5	**n.d.**	
Oleic acid (F18:1)	**1820**	±7	**37**	±2	**29**	±0.6	**22**	±0.1
Elaidic acid (F18:1)	**109**	±6	**7**	±0.5	**12**	±2	**14**	±0.1
Stearic acid (F18:0)	**89**	±1	**11**	±0.3	**11**	±0.4	**11**	±0.1
Eicosanoic acid (F20:0)	**n.d.**		**7**	±0.3	**13**	±0.5	**n.d.**	
Docosanoic acid (F22:0)	**n.d.**		**33**	±1	**50**	±3	**n.d.**	
Tetracosanoic acid (F24:0)	**n.d.**		**48**	±3	**52**	±4	**n.d.**	
Total Fatty Acids	**4370**	±3	**242**	±9	**251**	±11	**80**	±7
Resin Acid Neutrals								
Fichtelite	**n.d.**		**1567**	±66.94	**385**	±0.05	**2907**	±33
Dehydroabietin	**n.d.**		**26**	±1.09	**2**	±0.04	**493**	±3
Tetrahydroretene	**n.d.**		**33**	±1.24	**6**	±0.35	**1961**	±21
Retene	**n.d.**		**3523**	±88.34	**326**	±31.88	**6978**	±114
Methyldehydroabietin	**3**	±0.1	**8**	±0.32	**0**	±0.05	**45**	±0.1
Total Resin Acid Neutrals	**3**	±0.1	**5157**	±89	**718**	±32	**12384**	±171
Natural resin acids								
Pimaric acid	**322**	±1	**237**	±2.88	**382**	±1.22	**662**	±4
Sandaracopimaric acid	**23**	±1	**25**	±1.21	**39**	±0.46	**6**	±3
Isopimaric acid	**132**	±1	**122**	±3.28	**188**	±2.56	**32**	±18
Dehydroabietic acid	**670**	±5	**482**	±8.41	**863**	±3.75	**205**	±12
Abietic acid	**4559**	±1337	**774**	±75.22	**798**	±30.55	**7145**	±75
Neoabietic acid	**n.d.**		**1**	±0.04	**1**	±0.06	**2**	±0.2

n.d. = not detected (method detection limit 0.01μg/g)

Table 3.3 Continued concentration of RE in PMES when field applied in August 2004

Concentrations μg/g d.w.	TPS		TB		TAB		KAB	
Transformed resin acids	**mean**	se	**mean**	se	**mean**	se	**mean**	se
Pimarenic acid	**n.d.**		**167**	±8	**139**	±1	**1719**	±10
Sandaracopimarenic acid	**n.d.**		**222**	±8	**214**	±2	**2181**	±12
Isopimarenic acid	**n.d.**		**28**	±1	**29**	±1	**10641**	±131
13-Abietenic acid	**660**	±12	**263**	±7	**406**	±0.1	**n.d.**	
Pimaranic acid	**n.d.**		**510**	±15	**424**	±4	**1925**	±15
Isopimaranic acid	**n.d.**		**246**	±8	**189**	±3	**1527**	±10
Abietanic acid	**n.d.**		**2445**	±67	**1869**	±27	**9578**	±294
Seco-1-dehydroabietic acid	**55**	±0.3	**44**	±2	**54**	±0.2	**233**	±0.6
Seco-2-dehydroabietic acid	**25**	±0.2	**19**	±0.8	**22**	±0.3	**169**	±4
12-Chlorodehydroabietic acid	**n.d.**		**24**	±0.9	**23**	±0.3	**19**	±0.1
14-Chlorodehydroabietic acid	**5**	±0.2	**108**	±3	**108**	±2	**30**	±0.2
12,14-Dichlorodehydroabietic	**0**		**51**	±2	**42**	±0.91	**275**	±2
7-Oxodehydroabietic acid	**73**	±7	**10**	±1	**16**	±0.7	**14**	±0.7
Total Resin Acids	**6524**	±1341	**5777**	±206	**5806**	±79	**36364**	±404
Phytosterols								
Cholesterol	**n.d.**		**n.d.**		**12**	±3	**n.d.**	
Campesterol	**2**	±0.5	**n.d.**		**n.d.**		**94**	±2
Stigmasterol	**n.d.**		**n.d.**		**n.d.**		**38**	±0.3
Sitosterol	**n.d.**		**275**	±4	**170**	±0.1	**n.d.**	
Sitostanol	**22**	±0.7	**248**	±4	**191**	±0.4	**1505**	±26
Total Phytosterols	**24**	±1	**523**	±7	**374**	±3	**1637**	±28
Total Extractives	**10941**	±1357	**11714**	±264	**7157**	±60	**50483**	±592

n.d. = not detected (method detection limit 0.01μg/g)

3.5.3 Changes in the Concentration of RE in PMES after Field Application

3.5.3.1 Nine - month extractions

Organic compound analysis of PMES samples collected every three months from field plots, showed that there were considerable losses of RE from all treatments over nine months after field application (Figures 3.1-3.3).

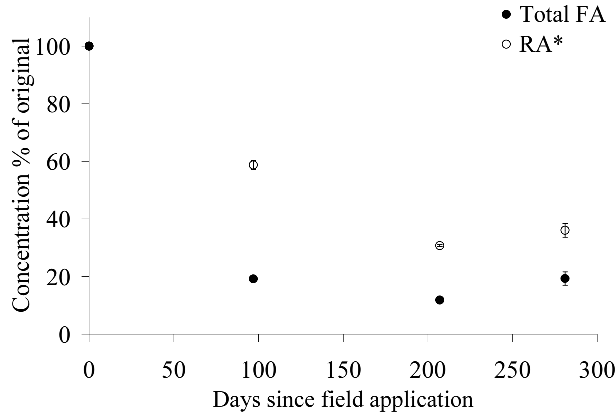


Figure 3.1 Changes in total fatty acids (FA) and resin acids (RA), excluding abietic acid, in TPS during nine months after field application (error bars are standard error). *Abietic acid was not included in total resin acids as abietic acid data had high variability.

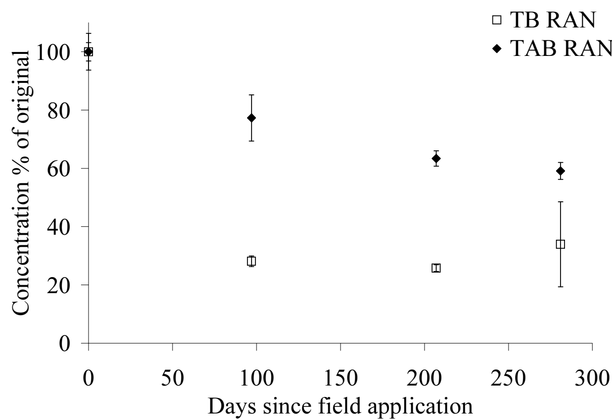


Figure 3.2 Changes in total resin acid neutrals (RAN) in TB and TAB during nine months after field application (error bars are standard error).

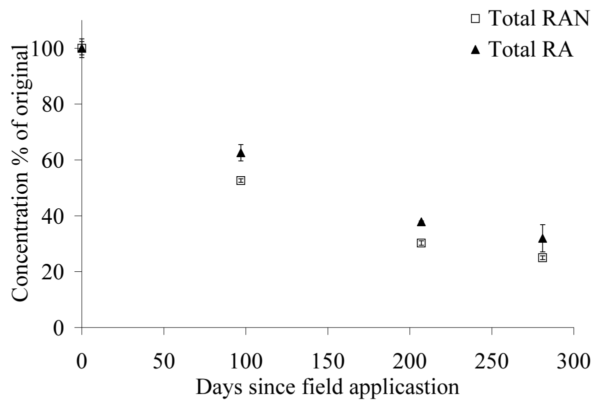


Figure 3.3 Changes in total resin acids (RA) and total resin acid neutrals (RAN) in KAB during nine months after field application (error bars are standard error).

Figures 3. 1 - 3.3 show percentage changes in the predominant compound classes in each PMES over nine months. Total fatty acids and resin acids declined rapidly

in TPS (Figure 3.1), as did resin acid neutrals in TB and TAB (Figure 3.2), over nine months after field application. Resin acids were high in TB and TAB (Table 3.3), but poor resin acid recoveries in nine month extractions from these treatments (Appendix I) did not allow resolution of changes. Good recovery of all surrogate standards in the 24 month extractions of TB and TAB (Appendix I), allowed all compounds to be assessed (Tables 3.4.1 – 3.4.4) and data are presented in Section 3.5.3.2.

The total concentrations of resin acids and resin acid neutrals, making up 97% of total extractives in KAB, declined rapidly as with resin acids in TPS and resin acid neutrals in Tasman biosolids (Figure 3.3).

Exponential decay curves were fitted to the TPS and KAB 9 months data, and TB and TAB 24 months data. Decay was described by the differential equation:

$$dN/dt = \lambda N$$

where N is the compound concentration, λ is the decay constant.

The decay constant was determined from the following equation fitted to the data:

$$N(t) = N_0 e^{-\lambda t}$$

Where $N(t)$ is the concentration at time t and N_0 is the initial concentration. From this a half life was calculated from the equation:

$$t_{1/2} = \ln 2/\lambda$$

The initial concentrations, decay constants and half-lives of selected compounds from each PMES treatment are presented in Tables 3.4.1 – 3.4.4.

All extractives decreased rapidly over nine months in both KAB and TPS; decay rates of individual compounds were not influenced by their starting concentrations (Tables 3.4.1 and 3.4.2). Decay rates did not differ substantially between different compound groups in TPS and KAB, with most compounds having an estimated half-life between 100 and 300 days (Tables 3.4 .1 and 3.4 .2).

Table 3.4.1 TPS Nine month data. Initial concentration (N_0) in μg/g, decay constant (k) and half life ($t_{1/2}$) of RE. The p-value is for the straight line fit to ln = constant – kt, showing the significance of the exponential regression.

	N_0	k	$t_{1/2}$ (days)	p
Fatty acids				
Linoleic acid	1213	0.0058	120	<0.01
Oleic acid	1820	0.0049	141	<0.01
Palmitic acid	1109	0.0054	128	<0.01
Stearic acid	89	0.0025	277	<0.01
Total Fatty Acids	4370	0.0047	147	<0.01
Native resin acids				
Abietic acid	4559	0.0015	462	0.32
Dehydroabietic acid	670	0.0028	248	<0.01
Isopimaric acid	132	0.0037	187	<0.01
Pimaric acid	322	0.0036	193	<0.01
Sandaracopimaric acid	23	0.0042	165	<0.01
Transformed resin acids				
13-Abietenic acid	660	0.0053	131	<0.01
7-Oxodehydroabietic acid	73	0.0053	131	<0.01
Seco-1-dehydroabietic acid	55	0.0022	315	<0.01
Seco-2-dehydroabietic acid	25	0.0022	315	<0.01
Total Resin Acids	6524	0.0041	169	0.03

Table 3.4.2 KAB Nine month data. Initial concentration (N_0) in µg/g, decay constant (k) and half life ($t_{1/2}$) of resin extractives. The p-value is for the straight line fit to ln = constant - kt, showing the significance of the exponential regression.

	N_0	k	$t_{1/2}$ (days)	p
Native resin acids				
Abietic acid	7145	0.0024	289	<0.01
Dehydroabietic acid	205	0.0008	866	0.11
Isopimaric acid	32	0.0038	182	<0.01
Pimaric acid	662	0.0054	128	<0.01
Transformed resin acids				
Pimarenic	1719	0.0056	124	<0.01
12,14-Dichlorodehydroabietic	275	0.0024	289	<0.01
12-Chlorodehydroabietic acid	19	0.0021	330	<0.01
14-Chlorodehydroabietic acid	30	0.0019	365	<0.01
7-Oxodehydroabietic acid	14	0.0010	693	0.12
Abietanic	9577	0.0038	182	<0.01
Isopimaranic	1527	0.0039	178	<0.01
Isopimarenic	10641	0.0067	103	<0.01
Pimaranic	1924	0.0039	178	<0.01
Sandaracopimarenic acid	2180	0.0058	120	<0.01
Seco-1-dehydroabietic acid	233	0.0024	289	<0.01
Seco-2-dehydroabietic acid	169	0.0044	158	<0.01
Total Resin Acids	36363	0.0042	165	<0.01
Resin acid neutrals				
Fichtelite	2907	0.0035	198	<0.01
Dehydroabietin	493	0.0071	98	<0.01
Tetrahydroretene	1960	0.0068	102	<0.01
Retene	6978	0.0052	133	<0.01
Methyldehydroabietin	45	0.0048	144	<0.01
Total Resin Acid Neutrals	12384	0.0050	139	<0.01
Phytosterols				
Campesterol	94	0.0027	257	<0.01
Stigmasterol	38	0.0017	408	<0.01
Sitostanol	1505	0.0021	330	<0.01
Total Phytosterols	1637	0.0021	330	<0.01

Table 3.4.3 TB twenty-four month data. Initial concentration (N_0) in µg/g, decay constant (k) and half life ($t_{1/2}$) of resin extractives. The p-value is for the straight line fit to ln = constant – kt, showing the significance of the exponential regression.

	N_0	k	$t_{1/2}$ (days)	p
Native resin acids				
Pimaric acid	237	0.0005	1386	<.01
Abietic acid	774	0.0006	1155	<.01
Dehydroabietic acid	482	0.0003	2310	<.01
Isopimaric acid	122	0.0005	1386	<.01
Sandaracopimaric acid	25	0.0005	1386	<.01
Transformed resin acids				
12,14-Dichlorodehydroabietic	51	0.0002	3466	.01
12-Chlorodehydroabietic acid	24	0.0004	1733	<.01
13-Abietenic acid	263	0.0003	2310	<.01
14-Chlorodehydroabietic acid	108	0.0005	1386	<.01
7-Oxodehydroabietic acid	10	0.0002	3466	0.28
Abietanic acid	2445	0.0004	1733	<.01
Isopimaranic acid	246	0.0004	1733	<.01
Isopimarenic acid	28	0.0004	1733	<.01
Pimaranic acid	510	0.0005	1386	<.01
Pimarenic acid	167	0.0006	1155	<.01
Sandaracopimarenic acid	222	0.0007	990	<.01
Seco-1-dehydroabietic acid	44	0.0009	770	<.01
Seco-2-dehydroabietic acid	19	0.0011	630	<.01
Total Resin Acids	5777	0.0005	1386	<.01
Resin acid neutrals				
Fichtelite	1567	0.0018	385	<.01
Dehydroabietin	26	0.0046	151	<.01
Tetrahydroretene	33	0.0029	239	<.01
Retene	3522	0.0037	187	<.01
Methyldehydroabietin	8	0.0046	151	<.01
Total Resin Acid Neutrals	5157	0.0027	257	<.01
Phytosterols				
Sitosterol	275	0.0013	533	<.01
Sitostanol	248	0.0007	990	<.01
Total Phytosterols	523	0.0010	693	<.01
Total Fatty Acids	242	0.0008	866	<.01

Table 3.4.4 TAB twenty-four month data. Initial concentration (N_0) in µg/g, decay constant (k) and half life ($t_{1/2}$) of resin extractives. The p-value is for the straight line fit to ln = constant − kt, showing the significance of the exponential regression.

	N_0	k	$t_{1/2}$ (days)	p
Native resin acids				
Abietic acid	800	0.0002	3466	0.87
Dehydroabietic acid	863	0.0002	3466	0.12
Isopimaric acid	189	0.0001	6931	0.48
Pimaric acid	382	0.0001	6931	0.40
Transformed resin acids				
Sandaracopimaric acid	39	0.0002	3466	0.20
12,14-Dichlorodehydroabietic	42			0.86
12-Chlorodehydroabietic acid	23	0.0002	3466	0.40
14-Chlorodehydroabietic acid	108	0.0002	3466	0.27
7-Oxodehydroabietic acid	16	0.0001	6931	0.36
Abietanic acid	1869	0.0002	3466	0.09
Isopimaranic acid	189	0.0004	1733	<0.01
Isopimarenic acid	29	0.0002	3466	0.27
13-Abietenic acid	406	0.0002	3466	0.13
Pimaranic acid	424	0.0004	1733	<0.01
Pimarenic acid	139	0.0004	1733	<0.01
Sandaracopimarenic acid	214	0.0004	1733	<0.01
Seco-1-dehydroabietic acid	54	0.0004	1733	<0.01
Seco-2-dehydroabietic acid	22	0.0005	1386	<0.01
Total Resin Acids	5806	0.0002	3466	0.14
Resin acid neutrals				
Fichtelite	385	0.0017	408	<0.01
Dehydroabietin	2	0.0011	630	<0.01
Tetrahydroretene	6	0.0012	578	<0.01
Retene	325	0.0011	630	<0.01
Total Resin Acid Neutrals	718	0.0014	495	<0.01
Total Fatty Acids	251	0.0008	866	<0.01
Total Phytosterols	374	0.0010	693	<0.01

3.5.3.2 Twenty Four – Month Extractions on TB and TAB

There were significant differences between the decay rates of resin acids and resin acid neutrals in TB and TAB, with resin acid neutrals disappearing 7-8 times more rapidly than resin acids in both biosolids (Tables 3.4.3 and 3.4.4). Tasman aged biosolids had similar resin acid concentrations but less than 15% of the resin acid neutral concentrations of TB. The TB had decay rates approximately twice that of TAB for both resin acids and resin acid neutrals.

Exponential decay curves were fitted to TB and TAB 24-month data and also a subset of these data representing the first 9 months. This was done to compare the decay constants derived from 24 months and 9 months of data, in order to see how well the smaller data set predicted the extended data. When decay constants (*k*) derived from 9 months and 24 months of data were plotted, the linear regression equation (24 months = 0.52x 9 months + constant) showed that the smaller data set underestimated the decay constant by approximately 50% ($R^2 = 0.63$, $p< 0.01$). This suggests that the decay curves fitting the TPS and KAB from 9 months of data may over-estimate the decay rates in these treatments compared to the TB and TAB. However, this is unlikely to account for the magnitude of the differences observed between resin acid losses from Tasman biosolids (TB, TAB) and the TPS and KAB; Tasman biosolids (TB, TAB) resin acids had on average more than 10 fold greater half-lives than TPS and KAB resin acids. Further, the resin acid neutrals in the Tasman biosolids were not substantially more recalcitrant than the KAB resin acid neutrals, showing that only resin acids were recalcitrant in Tasman biosolids (Tables 3. 4.1 – 3.4.4).

3.5.4 Soil Concentration of Resin Extractives

The Tarawera mineral topsoil (Ah horizon) had low levels of RE, with the resin acids dehydroabietic and abietic accounting for greater than 90% of total extractives. In the Tarawera soil litter F/H horizon, RE concentrations were significantly higher than in the Ah horizon, with dehydroabietic and abietic acids accounting for approximately 87% and fatty acids about 10% of total extractives (Table 3.5).

RE concentrations in the Ah horizons of biosolids treatments (TB, TAB, and KAB) were not significantly different from those in the control Ah, however the TPS treatment Ah horizon had significantly ($p<0.01$) higher fatty acid concentrations (Table3.5).

Concentrations of RE were higher in the F/H horizon of PMES treatments than the control F/H. There was a slight increase of fatty acids in the TB, TAB and KAB treatments compared to the control while TPS had a tenfold greater fatty

acid concentrations compared to the control. Resin acids were slightly increased in PMES F/H horizons compared to the control, but there were more resin acids detected, particularly transformed resin acids in the biosolids treatments (TB, TAB and KAB). Phytosterols were also slightly elevated in PMES F/H compared to the control.

Fatty acids were the most mobile in TPS and were the only RE to show significant movement into the soil. The concentration of fatty acids in the F/H horizon beneath TPS was nearly 25% of the concentration in TPS itself nine months after field application. The control organic litter horizons were estimated to contain 25 tonnes per hectare OM, of which 50% was in the F/H horizon (data not shown). Based on the mass of material in the F/H horizon and in the TPS, the F/H horizon beneath TPS was estimated to contain less than 5% of the mass of fatty acids contained in the remaining TPS, after nine months.

Table 3.5 Concentration (µg/g) of fatty acids (FA), resin acid neutrals (RAN), resin acids (RA) and phytosterols (PS) in Tarawera soil sampled nine months after PMES application and in control plots at the same time.

Treatment		**FA**		**RAN**		**RA**		**PS**	
	Horizon	**mean**	se	**mean**	se	**mean**	se	**mean**	se
Control	F/H	**20.7**	±1.76	**0.1**	±0.05	**95.2**	±7.06	**16.8**	±0.95
Control	Ah	**0.6**	± 0.06	**n.d.**		**13.1**	± 5.33	**n.d.**	
TPS	F/H	**198.6**	± 32.7	**n.d.**		**219.1**	±14.85	**39.0**	±7.43
TPS	Ah	**24.0**	±0.67	**n.d.**		**466.4**‡	±100.6	**n.d.**	
TB	F/H	**48.1**	±6.59	**5.4**	± 0.34	**229.2**	±9.98	**32.3**	± 3.80
TB	Ah	**0.7**	±0.01	**n.d.**		**13.0**	±10.87	**n.d.**	
TAB	F/H	**30.7**	±2.35	**3.5**	±0.09	**310.9**	*	**19.0**	±1.61
TAB	Ah	**0.3**	±0.10	**n.d.**		**6.2**	±5.19	**n.d.**	
KAB	F/H	**60.9**	±8.43	**73.8**	±3.49	**139.8**	*	**50.3**	±2.48
KAB	Ah	**1.7**	± 0.88	**2.0**	±0.05	**45.8**	±11.69	**n.d.**	

* data from 1 replicate only

‡ low resin acid surrogate recovery

3.5.5 Discussion/ Conclusions

There are no documented studies of RE decay in land applied PMES, the only comparable datum in the literature are from pulp mill effluent treatment systems. A survey of two New Zealand pulp mill wastewater treatment systems revealed a

range of PMES typical of softwood mills utilising ASB treatment of effluents. Native resin acids and fatty acids dominated the primary solids while transformed resin acids and resin acid neutrals were characteristic of secondary treatment biosolids. In field applied PMES, the concentration of RE decreased over time, presumably due to microbial degradation, but there was also some evidence of movement into the soil profile. Fatty acids in TPS, in particular, were significantly greater in soil and F/H horizons. Fatty acids are more soluble than resin acids and resin acid neutrals, so would be expected to leach more. However, the mass of fatty acids in the TPS F/H horizon did not account for a high proportion of total fatty acids measured in these solids at the time of field application. Slight increases in other RE in F/H horizons under TPS and other PMES could be expected with movement of PMES particulate matter into these layers, as was evident when field samples were collected, without having to invoke leaching as a significant means of movement.

All PMES showed significant losses of resin acid neutrals and/or fatty acids over either nine months (TPS, KAB) or 24 months (TB, TAB). Calculated half-lives from nine months of data over-estimated rates of RE losses when compared to 24 months of data by a factor of two. This may partly be explained by the 9-month data being from samples collected between spring and autumn and therefore not including the winter period when a drop in biological activity was observed (Chapter 5), and presumably decay rates also decline. RE half-lives calculated for TB and TAB integrated 24 months of decay data and suggests that resin acid concentrations in particular will remain high (mg/g) over many years in these biosolids, which themselves have low decay rates (Chapter 5).

Percentage losses were similar for resin acid neutrals from the different biosolids. Fatty acids showed slightly faster rates of loss in the TPS compared to biosolids, and this may have been related to the higher decomposition rate of primary solids (Chapter 5). Resin acids showed variable losses dependent upon the type of PMES. Resin acids from the Tasman biosolids (TB, TAB) had approximately a tenfold longer half-life than resin acids from Tasman primary (TPS) or Kinleith biosolids (KAB). There was little evidence of any major degradation of Tasman biosolid resin acids. Resin acid concentrations were very similar in both Tasman

biosolids, and changed little over two years. The fact that TAB had the same concentration of resin acids to start with as did TB suggests that little degradation of resin acids had occurred between the time TAB were removed from the ASB system and when they were field applied (approximately 1-2 years). In contrast, the resin acid neutrals in TAB were at only 14% of the concentrations in TB at the time of field application suggesting, significant losses had occurred in TAB after dredging from the ASB ponds. Resin acid neutrals which are at high concentrations in ASB sediments apparently degrade rapidly once removed from this anaerobic environment.

Resin extractives were not recalcitrant in TPS or KAB, and exponential decay curves fitted to nine months of data indicate short half-lives. Resin acids were recalcitrant in TB and TAB, having an estimated average half-life of nearly seven years. Resin acids have been reported to be recalcitrant in anaerobic sediments (Liver and Hall 1996), but have been shown to rapidly degrade under aerobic conditions (Kostamo and Kukkonen 2003; Liss *et al.* 1997). Kainulainen *et al.* (2003) studied resin acid decomposition in needle litter and found only 20% remained after 18 months on the forest floor. Most losses occurring in the first six months, how ever, dehydroabietic acid was slower to degrade and concentrations had increased significantly as a proportion of total resin acids after 12 months (Kainulainen *et al.* 2003).

This study is the first to report that resin acids are recalcitrant in biosolids applied to forest soil.

4 Toxicity and Bioavailability of Pulp Mill Effluent Solid Constituents to Soil Organisms

4.1 Introduction

Pulp mill effluent solids (PMES) contain a wide range of compounds associated with aquatic toxicity of pulp mill wastes. The extensive literature on the aquatic toxicity of pulp mill effluent wastes from modern mills indicates that much of the toxicity is associated with naturally derived organic compounds (Billiard *et al.* 1999; Hewitt *et al.* 2006; Peng and Roberts 2000). Due to the hydrophobic nature of many of these compounds, their concentrations can be many times higher in PMES than in the associated effluent (Leppanen and Oikari 1999; Taverndale *et al.* 1995).

Terrestrial toxicity of pulp mill effluent wastes has been investigated in only a limited number of studies (Levy and Taylor 2003; McCarthy *et al.* 2004; Palmer *et al.* 1998; Rana *et al.* 2004) and little (Palmer *et al.* 1998) or no data was presented on the organic chemistry of these wastes. McCarthy *et al.* (2004) found no toxic effects on terrestrial organisms exposed to effluent solids from a thermo-mechanical pulp (TMP) mill, however aquatic organisms exposed to simulated run-off from field plots receiving these wastes were negatively impacted. Levy *et al.* (2003) and Palmer *et al.* (1998) observed reduced biomass production in plants grown in PMES-amended soil, although it was not clear whether this was due to phytotoxicity or nutrient limitation. There are numerous accounts of PMES improving soil characteristics (Chantigny *et al.* 1999; Zibilske *et al.* 2000) and increasing plant productivity (Jackson *et al.* 2000; Phillips *et al.* 1997; Zhang *et al.* 1993), but also examples of nitrogen limitation caused by application of high C:N effluent solids (O'Brien *et al.* 2003; Zibilske 1987). Palmer *et al.* (1998) also showed that microbial respiration, soil dehydrogenase activity and responses of two *lux* based biosensors were significantly reduced in soil amended with pulp mill primary sludge compared to a control soil receiving no sludge. Rana *et al* (2004) supplied laboratory rats with pulp mill effluent as the only source of drinking water, over 15 days and found that there were negative impacts on the

reproductive system of male rats. Significantly lower testis weights, circulating testosterone and sperm count/motility were observed. Haematological and serological analyses suggested that liver and kidney function may be compromised in rats exposed to pulp mill effluent (Rana *et al.* 2004). In vitro cytotoxic effects were observed on human epithelial and fibroblast cells exposed to various resin acids by Soderberg *et al.*(1996). These studies suggested that under certain circumstances PMES could cause negative impacts in terrestrial organisms.

In New Zealand pulp and paper mills, which predominantly utilise the conifer species *Pinus radiata*, the effluents produced are high in resin extractives (RE) including phytosterols, resin acids (RA) and fatty acids (FA) (Judd *et al.* 1996; Judd *et al.* 1998; Wilkens and Panadam 1987). Resin acids can also be biotransformed to resin acid neutrals (RAN), including retene, a polycyclic aromatic hydrocarbon (PAH), during secondary treatment of effluent (Taverndale *et al.* 1997a). Phytosterols (Mattsson *et al.* 2001), RA (Bogdanova and Nikinmaa 1998; Peng and Roberts 2000), FA (Leach and Thakore 1973) and RAN (Billiard *et al.* 1999) have all been implicated in aquatic toxicity derived from pulp and paper mill discharges. The PAH retene, commonly found in secondary treated PMES, is an alkyl-substituted phenanthrene that is toxic to fish at concentrations below 1 mg/L (Billiard *et al.* 1999). PAHs such as phenanthrene are also toxic to terrestrial organisms (Blakely *et al.* 2002; Samanta *et al.* 2002; Sverdrup *et al.* 2002), suggesting that retene may also be toxic to terrestrial organisms.

A wide range of responses to pulp mill effluent wastes and constituents of these wastes have been observed in different aquatic organisms (Kovacs *et al.* 1995; McMaster *et al.* 2006; Sandstrom and Neuman 2003). As terrestrial organisms differ in important ways from aquatic organisms (Kendall *et al.* 2001) and as exposure to organic compounds can differ greatly between aquatic and terrestrial environments, it is difficult to predict effects on terrestrial organisms based on aquatic studies (Kookana *et al.* 2006). However, due to the demonstrated toxicity of pulp mill effluent wastes to aquatic organisms, effects on terrestrial organisms warrant investigation before large-scale land application of PMES proceeds.

4.2 Hypothesis, Aims and Objectives concerning Terrestrial Toxicity Testing of PMES

At the time that this research was initiated a review of the literature indicated a paucity of information on terrestrial toxicity of PMES. The PhD thesis research sought to provide baseline data on terrestrial toxicity of typical PMES from softwood mills. The objective was to test, using a range of chemically distinct PMES, the hypothesis '*resin extractives (RE) are bioavailable and are toxic to selected terrestrial organisms'*. The results from these New Zealand wastes are expected to be applicable to results from pulp mill wastes produced in many countries as pine species are widely used globally, as are similar wastewater treatment systems.

Chemical characterisation of the materials used was undertaken in order to be able to compare these wastes with those produced in other mills both in New Zealand and internationally. Chemical characterisation also allowed results of terrestrial toxicity tests to be compared with aquatic toxicity test results where specific compounds were associated with particular toxic effects.

4.2.1 Overview of Bioassays

The toxicity tests were intended to provide indications as to whether exposure to constituents of PMES were likely to cause harm to soil organisms and to determine what levels of the RE present in the wastes were detrimental to terrestrial organisms. These experiments also sought to determine the bioavailability of the RE to the biota exposed.

A suite of bioassay organisms were chosen that were representative of different functional groups within terrestrial ecosystems. Organisms that had previously been well characterised were chosen and internationally recognised toxicity test guidelines were followed so those findings would be relevant to the international literature. As there were no data available on the toxicity of RE to terrestrial organisms, tests were carried out in a range of PMES that had widely differing concentrations of RE. Organisms were exposed to undiluted PMES to determine if

there was any toxic response. Toxicity endpoints were designed to investigate acute and chronic effects. If toxicity was observed, this would form the basis for further tests using a range of concentrations to establish the threshold concentration where effects where observed.

A monocotyledon plant, oats (*Avena sativa*), which is routinely used in plant toxicity testing was chosen to represent primary producers. An earthworm and an enchytraeid worm species representing two different functional groups, and levels of sensitivity, were used for invertebrate toxicity bioassays (Dam *et al.* 1998; Didden and Rombke 2001). Microtox tests were conducted on liquid phase extracts of the waste solids to evaluate potential toxicity of leachate from the PMES. In addition to these single organism tests, microbial basal respiration, a community level bioassay, was also conducted from soil collected from a field trial site so as to be able to link laboratory responses of soil microbial communities with observations made in the field (Chapter 5).

4.3 Materials and Methods

4.3.1 Experimental design

Preliminary chemical analysis of RE in a range of PMES, indicated a wide range of concentrations of RA, FA, sterols and RAN (Table 3.2). Six different solid wastes were tested from two pulp and paper mills. From this initial survey, outlined in Chapter Two (Section 2.5), 4 PMES were chosen to be used in toxicity bioassays and were also field applied (Table 3.3).

A fifth treatment tested in single organism bioassays was a low RE material, which was generated by treating Tasman primary solids (TPS) with alkali to remove RA and FA - the predominant extractives in TPS (Tables 3.3 and 4.1). The TPS were washed with 0.1 M NaOH to remove readily soluble and acidic compounds. Sequential flushing with water (at room temperature) removed soluble extractives and alkali, and was repeated until neutral pH of the solid was obtained. This treatment provided a reference primary waste material with low resin acid content. All PMES used in bioassays had pH ranging from 6.8 to 7.5.

It was not possible to obtain PMES that did not contain RE to act as matched controls for the waste solids. A silt loam soil (Templton silt loam – TSL) was used as a reference substrate. This soil was removed from a site where there was no known history of chemical contamination and had the following characteristics: 32% sand, 52% silt, 16% clay, 3.4% organic carbon content, and a pH of 6.3. Biological responses of soil invertebrates and plants in this control soil have been previously characterised (O'Halloran *et al.* 2005), hence it was used in these toxicity tests as a reference to evaluate any toxicity that may occur as a result of exposure to the RE in PMES. Microbial community respiration was assessed using Tarawera loamy sand (TLS) soil (75% sand, 20% silt, 5% clay, organic carbon 8%, pH 5.5) which was the same soil used in the related field study (Chapter 5).

Maximum water holding capacity (WHC_{max}) of the silt loam reference soil (TSL) was 0.56g water per dry g of soil (56% water content). This soil was used at approximately 60% WHC_{max} in the bioassays (33% water content). In contrast, the waste solids which had a much greater capacity for holding water (WHC_{max} ranged from 142-550% water content), were used in the tests at 80-90% of WHC_{max}, so as not to limit water availability to the test organisms. The WHC_{max} of the loamy sand soil (TLS) used as a control in the basal respiration was 69% water content and was used at field moisture content (approximately 80% WHC_{max}).

4.3.2 Measurement of Resin Extractives in Tissue and Pulp Mill Effluent Solids

Organic extractives were determined in PMES and freeze-dried whole earthworm tissue (from 20 individuals) and using supercritical CO_2 extraction and gas chromatography/mass-selective detection (GC-MSD). For a detailed description of this method see Chapter 3 section 3. 4.3.2. All extractions were done in triplicate.

4.3.3 Toxicity Bioassays

4.3.3.1 Plant Tests

Plant tests were based on international standards for early seedling emergence and growth tests (ASTM 1994; EPA 1996; OECD 2004) and were conducted under controlled environmental conditions (temperature 20°C, 16 h light at 10,000 lux: 8 h dark, humidity > 80%). Two concentrations of four of the PMES were tested – 50% (diluted with Templton silt loam TSL) and 100% (non-diluted), as well as the reference soil (TSL). Due to a shortage of washed TPS, this treatment was only applied at the 50% concentration. Each treatment had four replicates, with each replicate consisting of five seeds of oats (*A. sativa*), each seed being in a separate pot. Soil moisture was maintained throughout the study by placing the pots on pre-moistened capillary paper and ensuring that the paper remained moist for the duration of exposure. The proportion of seeds germinating per replicate and the time to emergence for each seed was recorded. Plants were harvested 14 days after sowing and individual root and shoot lengths were measured. Root and shoot biomass was assessed, after drying at 70°C for 48 hours.

4.3.3.2 Earthworm Tests

Earthworm tests were conducted according to international standards (ISO 1998; OECD 1984). Sexually mature adult earthworms (*Eisenia fetida)* of similar weight were maintained in 1L glass jars containing approximately 250 ml of PMES (amount of test material was based on volume rather than weight due to the great range of bulk densities of test materials used) over a 28-day exposure period in a controlled environment (temperature 20°C, 16 h light: 8 h dark).

Earthworms were fed uncontaminated fresh cow manure in 5-g portions on day two and weekly thereafter if the manure had been consumed. Soil and PMES moisture was checked and maintained at the initial water content on a weekly basis. There were 10 earthworms per replicate and four replicates per treatment giving a total of 40 earthworms per treatment. Treatments were the five types of PMES, plus the reference soil (TSL), as described in Section 4.3.1. Mortality was assessed at day 14 (to evaluate acute effects) and weight was assessed at day 0, 14

and 28 (to evaluate growth). After 28 days, adults were removed, allowed to depurate for 48 hours and then frozen at –20°C. The tissue was freeze dried before chemical analysis. Bioaccumulation factors were calculated for earthworms, based on the concentrations of RE measured in test material and in whole body tissue from exposed earthworms. Twenty worms from two replicates were pooled from each treatment and three replicate sub-samples were extracted from each pooled sample. The bioaccumulation factor for individual compounds is defined as the ratio of dry weight concentration in earthworm tissue to dry weight concentration in test material. Cocoons produced during the 28-day exposure period were left to hatch over an additional 28 days and the number of cocoons and juveniles per replicate recorded on day 56.

4.3.3.3 Enchytraeid Tests

Enchytraeid tests were conducted according to the international standard (OECD 2004). Adult enchytraeid worms (*Enchytraeus albidus*) from an in-house culture were placed into 120 ml glass jars that contained the PMES treatments (at 100%). There were 10 worms per replicate and four replicates per treatment. The aged biosolids from the Kinleith mill (KAB), which showed the highest levels of RE in preliminary tests (Table 3.2), were considered to be the most likely to cause negative effects. To better define any toxicity that might be observed, a 50% KAB mixed with TSL (three replicates) treatment was included. Templeton silt loam was used as a reference soil and comprised eight replicates. Autoclaved rolled oats (50 mg/jar) were mixed into the test material at the commencement of the test as a source of food. An additional 25 mg/jar was fed at days 14 and 21. Jars were incubated in the dark at 18°C, and aerated on a weekly basis. Soil and PMES moisture were checked and maintained on a weekly basis. After three weeks adults were removed, counted and the treatments were left for a further three weeks for juveniles to hatch and be counted. Juveniles were fed (15 mg rolled oats/jar) on days 28 and 35.

4.3.3.4 Microbial Basal Respiration Tests

Microbial basal respiration differs from the other bioassays used in this study in that it is a community level assay integrating responses of multiple species of microorganisms. Whereas TSL was used as a control or reference soil in all other bioassays, TLS soil was used as a control to investigate basal respiration. Soil respiration was also investigated in the Tarawera soil (TLS) in the field trial (section 5.4.3.). One of the objectives of this experiment was to compare responses of the microbial community in the field and also under laboratory conditions. Microbial responses to different carbon substrates (lignin:cellulose, RE) quantified in the different PMES, were investigated in both laboratory (respiration) and field (respiration, degradation) experiments.

Microbial basal respiration was determined using the method outlined by Sparling (1994). Briefly, PMES were used at 40% WHC_{max} (low moisture content), or 80% WHC_{max} (high moisture content) at 100% concentration. PMES at 80% WHC_{max} were also mixed with field moist Tarawera loamy sand soil (TLS) at 7% or 21% dry wt concentration of solids. Approximately 30 g (wet wt) was placed in a 50 ml beaker in a 1 L sealed glass jar. A second beaker containing 0.5 M KOH was also placed in the jar to act as a CO_2 trap and 10 ml of distilled water was placed in the base of the jar to maintain humidity throughout the incubation. Three replicates of each treatment were incubated at 25ºC for 16 days with KOH being titrated against 0.1 M HCl to determine CO_2 evolved. Titrations were performed on days 2, 4, 6, 8, 12 and 16 with the KOH trap being replaced after each titration. Cumulative CO_2 output was calculated as the sum of CO_2 produced over 16 days.

4.3.3.5 Microtox®

Microtox® is a toxicity test that uses the luminescent bacteria *Vibrio fischeri* to assess toxicity by measuring a decrease in luminescence in response to toxic substances. Toxicity of pulp mill effluent wastes to Microtox® has been widely reported and found to correlate well with toxicity in other aquatic organisms exposed to these wastes (Munkittrick and Power 1991). Microtox® was used to assess the toxicity of leachate from the pulp and paper mill wastes. PMES were adjusted to approximately three times their WHC_{max} with distilled water or 95%

ethanol, and then were shaken on an end-over-end shaker for 24 hours before centrifuging at 12,000xG for 15 minutes. Ethanol extracts were used to determine if toxic components of waste solids could be made more bioavailable to the test organism. The supernatant from the water extraction was used at a starting concentration of 45% and ethanol extracts at 1% so as to be below 2% ethanol, the toxic threshold for the test organism. The supernatant was diluted with osmotic adjustment solution. Distilled water containing 1% ethanol was used as a control to distinguish between effects of ethanol and extractable compounds.

The Microtox® assay was conducted using the Microbics Microtox Model M500 Analyser and Microtox® liquid phase test kit following recommended procedures. Briefly, a dilution series was prepared with test samples in osmotic adjustment solution and included a control, which did not contain test material. Lyophilised *V. fischeri* was reconstituted and introduced to test cuvettes where luminescence was measured before test solution addition. Five minutes and fifteen minutes after addition of test solution, luminescence was measured and the drop in light output calculated. An EC_{50} (at 15 minutes) was generated if light output dropped by 50% (reduction in luminescence) compared to the control. Phenol was used as a reference toxicant to ensure performance of test system was maintained.

4.3.4 Statistics

Toxicity endpoints were examined using Analysis of Variance (ANOVA) for plant, earthworm and microbial respiration bioassays. Where the ANOVA showed evidence of significant treatment-related differences at $p<0.05$, Dunnett's post hoc tests were used to determine which treatments were significantly different to the control. For the enchytraeid data, which was non-parametric, a Mann-Whitney U test was conducted and treatment differences were individually compared to the control using a Bonferroni adjusted p value. All statistical comparisons were done using the Statistica 6 program (StatSoft Inc. Tulsa, USA).

4.4 Results

The chemical analysis of PMES reflected the environments from which the different PMES were derived. The TPS had high concentrations of native resin acids and fatty acids derived from wood as can be seen in Table 3.3. Tasman primary solids show no detectable biotransformation products such as the PAH retene. Washing of TPS with dilute NaOH resulted in 84% removal of resin acids with 30% reduction in fatty acids (Table 4.1). The three biosolids also contained high resin acid concentrations. Kinleith aged biosolids (Kinleith mill) resin acid concentrations were approximately six times those found in TPS (Tasman mill) whilst TB and TAB (Tasman mill) had approximately the same levels found in TPS. The difference between TPS and biosolids is due to the relative distribution of resin acids. Unlike TPS, biosolids contained substantial quantities of resin acid biotransformation products. Resin acid neutrals, presumably derived from microbial metabolism of resin acids in the treatment system, were also present in all biosolids. The Kinleith mill KAB had the greatest concentration of resin acid neutrals, followed by the TB and TAB of Tasman mill, respectively.

Table 4.1 Pulp and paper compound group concentrations detected in waste solids used for this study. For a detailed analysis of all compounds detected see Table 3.3.

Compound Concentrations in μg/g d.w.	TAB	TB	KAB	TPS	Washed TPS
Total Resin Acids Neutrals	718	5,157	12,384	3	4
Total Resin Acids	5,806	5,777	36,364	6,524	1,055
Total Phytosterols	374	524	1,637	24	<1
Total Monoterpenes	5	8	10	4	12
Total Phenolics	2	8	8	17	4
Total Fatty Acids	251	242	80	4370	3218
Total extractives	7,157	11,714	50,483	10,941	4,293

4.4.1 Plant Tests

There were no treatment-related differences in oat seed germination time ($p=0.4$) or on the proportion germinating ($p=0.052$) in the PMES treatments compared to the reference soil (TSL). In all treatments, seeds had mean germination times of four to five days with germination ranging from 16 to 20 seeds out of 20 sown. Root weight, root length and shoot length were significantly enhanced ($p< 0.05$) in oats grown in several of the PMES treatments compared to those grown in the reference soil (TSL), whereas shoot weights were not significantly different (Figure 4.1). Oats grown in TB from the Tasman mill had significantly longer shoots, however, this was not reflected in shoot biomass. Seeds sown in all of the PMES treatments produced oat seedlings with significantly longer roots and there was a significantly increased root biomass in all treatments except the KAB, compared to those grown in the reference soil (Figure 4.1). The PMES treatments in general caused a stimulatory rather than suppressive effect on early seedling development when compared with the reference soil.

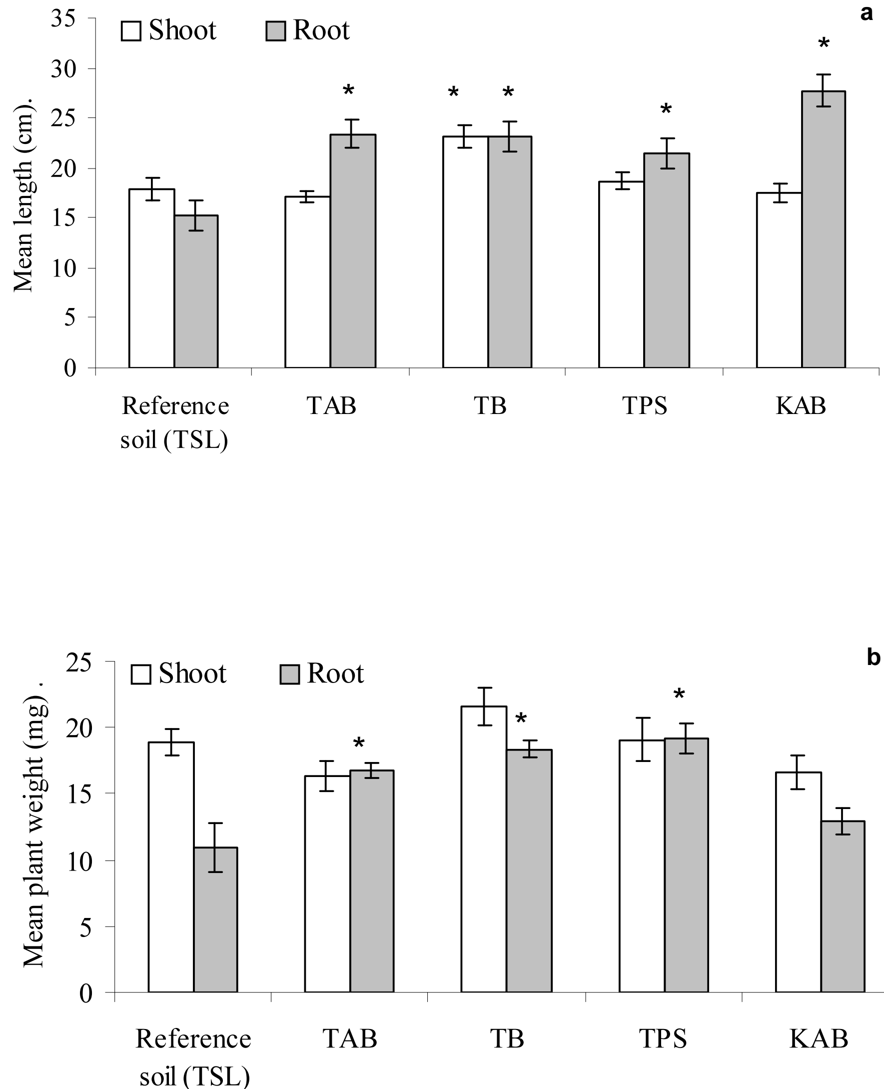


Fig. 4.1 a) Oats shoot length and root length. b) Oats shoot weight and root weight (error bars indicate standard error). Asterisks indicate a significant difference from the TSL reference soil.

4.4.2 Earthworm Tests

There was no significant earthworm mortality after 14 days in any of the treatments. Mortality was highest in the reference soil (TSL), but, it did not exceed 10% which was within the acceptable limits set out in the earthworm test guidelines (ISO 1998; OECD 1984). After 28 days exposure, earthworm weight was not significantly different (p=0.4) in any of the treatments compared to the reference soil (Figure 4.2). The number of cocoons produced in PMES were not

significantly different (Dunnett's post hoc test) from the reference soil. *Eisenia fetida* are known to produce up to 4 juveniles per cocoon (Kula and Larink 1998). The average number of juveniles produced per cocoon was 2.67 in the reference soil, whereas this number increased significantly ($p < 0.05$) to 3.36 in the TB and 3.04 (not significant) in the TAB. This increased fecundity is reflected in significantly ($p < 0.05$) higher juvenile counts in the TB (199 per jar) and TAB (177 per jar) compared to that in the reference soil (124 juveniles/jar) (Figure 4.2).

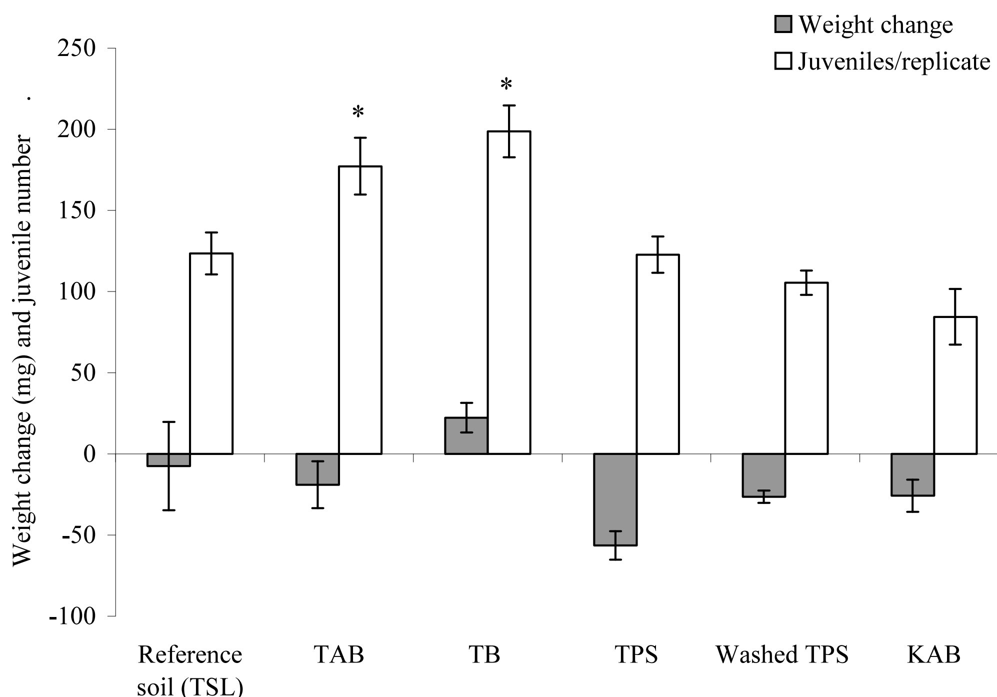


Fig. 4.2 Earthworm body weight changes over 28 days and juveniles produced after 56 days (error bars indicate standard error). Asterisks indicate a significant difference from the TSL control.

Tissue analysis (one pooled sample per treatment) of adults revealed that earthworms had accumulated resin acid neutrals and to a much lesser extent resin acids over the 28 day exposure to PMES (Table 4.2). Resin acids and resin acid neutrals were not detected in worms exposed to the reference soil. The worms exposed to TPS and washed TPS had a small accumulation of resin acid neutrals despite an absence of the compounds in these solids (Table 4.2). The type of waste solid tested influenced the bioaccumulation factor. For example the

concentration of fichtelite was 759 μg/g in TB and 81.5 μg/g in earthworms exposed to these biosolids, representing a bioaccumulation factor (BAF) of 0.107(Table 2). Whereas KAB had a much higher concentration of fichtelite (2093 μg/g), however, the concentration in the earthworms exposed to KAB was only 35.2 μg/g (representing a BAF of 0.017). Retene on the other hand was found to accumulate to a greater extent in earthworms exposed to KAB compared to earthworms exposed to TB with BAF's of 0.032 and 0.005, respectively. For resin acids, earthworms exposed to TPS had a BAF of 0.006 for dehydroabietic acid whereas for abietic acid, which was more than double the concentration of retene in KAB (Table 4.1), the BAF was only 0.001 (Table 4.2). Concentration of some compounds were relatively high in worm tissue due to the high concentration in PMES, however, BAF were not high generally.

Table 4.2 Earthworm bioaccumulation factors and tissue dry weight concentration in brackets (μg/g) in earthworms for resin extractives found in PMES. Worm tissue analysis carried out in triplicate on sub-samples from 20 worms.

Compound	TAB	TB	KAB	TPS	Washed TPS
Fichtelite	0.051 (8.6)	0.107 (81.5)	0.017 (35.2)	n.d. (4.6) ‡	n.d.
Dehydroabietin	n.d.	0.008 (0.1)	0.005 (0.7)	n.d.	n.d.
Tetrahydroretene	n.d.	n.d.	0.018 (8.2)	n.d.	n.d. (0.4)‡
Retene	0.017 (1.5)	0.005 (8.9)	0.032 (218)	n.d. (0.8) ‡	n.d.
Dehydroabietic acid	0.004 (2.6)	0.004 (1.5)	0.002 (1.3)	0.006 (2.1)	n.d.
Abietic acid	0.002 (5.5)	n.d.	0.001 (4.6)	0.001 (11.5)	n.d.
Pimaranic acid	0.003 (2.1)	0.002 (2.0)	0.002 (3.3)	n.d.	n.d.
Abietanic acid	n.d.	n.d.	0.002 (24.1)	n.d.	n.d.

n.d. = not detected (method detection limit 0.01 μg/g)

‡ Detected in tissue but not detected in solids

4.4.3 Enchytraeid Tests

There was no significant mortality of adult enchytraeids after three weeks exposure in any of the treatments (Figure 4.3). Cocoons produced by the adults were left in the treatments to hatch and juveniles were counted after a further three weeks. The number of juveniles produced in individual PMES treatments were

compared to production in the reference soil (TSL). There was significantly ($p < 0.01$) fewer juveniles produced in the TPS (100%), KAB (100%) and the TB (100%) and no juvenile production in the washed TPS. The TAB from the Tasman mill and the KAB from the Kinleith mill that had been diluted (KAB 50%) provided a more supportive medium and enchytraeid reproduction was not significantly different from that which occurred in the reference soil (Figure 4.3).

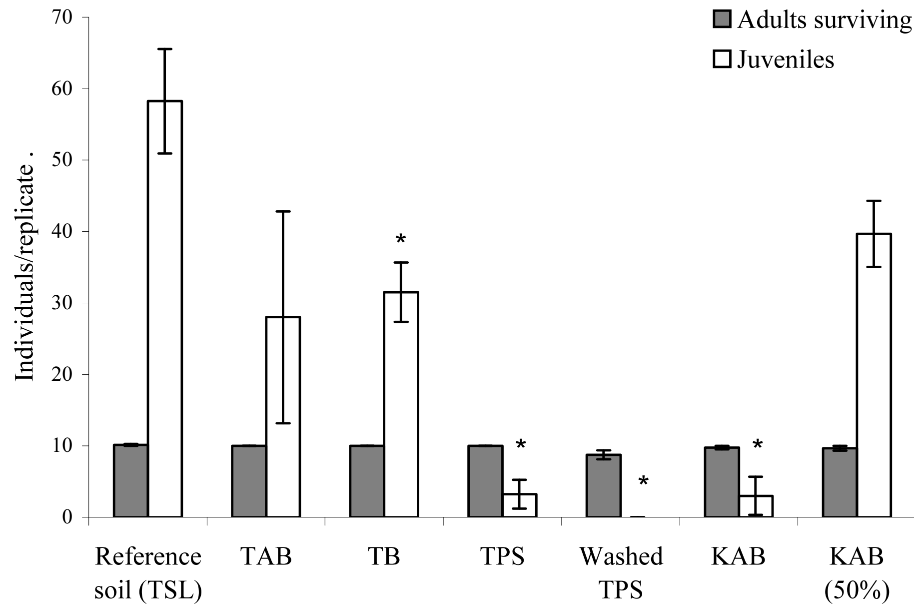


Fig. 4.3 Enchytraeid adults surviving after 21 days and juveniles produced after 42 days (error bars indicate standard error). Asterisks indicate a significant difference from the TSL control

4.4.4 Microbial Basal Respiration

Cumulative CO_2 production over 16 days is presented in Figure 4.4 as respiration rate per day in μg of CO_2 carbon per g dw of test material. All PMES treatments respired significantly more CO_2 ($p < 0.01$) than the control soil (TLS). Increasing the concentration of PMES from 7% to 21% increased respiration, however only in the TPS treatment was respiration significantly greater in the 100% treatment compared to the 21% treatment. There was no relationship between cumulative CO_2 production and RE concentration in PMES treatments ($r^2 = 0.1$, $p=0.2$), or total carbon in PMES treatments ($r^2 = 0.26$, $p=0.06$). However, if 100% PMES

were compared separately from mixed PMES/soil treatments, there was a weak relationship with TOC (r^2=0.56, p<0.01), and there was also a weak relationship between soil/PMES treatments and TOC (r^2 = 0.33, p<0.01). When respiration per unit of TOC was calculated, all biosolids/soil treatments gave significantly (p<0.01) greater values than did 100% biosolids treatments or soil alone. Respiration per unit of TOC for TPS 100% treatment was not significantly different from TPS 7% and 21%.

Basal respiration was significantly higher (p<0.01) in the TPS at high moisture content than at low moisture content, whereas TAB (p<0.01) and KAB (p<0.01) had significantly higher respiration at low moisture content. Basal respiration did not vary between high and low moisture contents in TB (p=0.6).

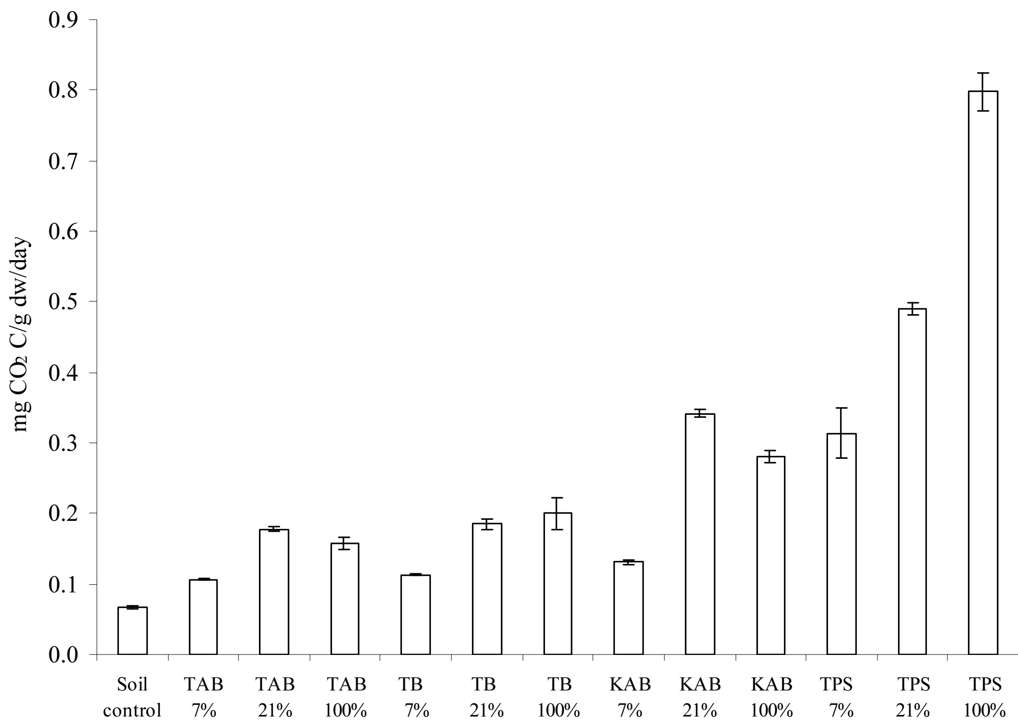


Fig. 4.4 Microbial respiration rate estimated from cumulative CO_2 respired over 16 days (µg CO_2 C produced per g dw) of Tarawera soil control and waste solids at 100%, 21% and 7% concentrations in Tarawera soil (error bars indicate standard error).

4.4.5 Microtox®

Water extracts of the reference soil (TSL) and TAB from the Tasman mill showed no acute toxicity to *V. fischeri* (Table 4.3). Water extracts of Kinleith mill KAB were considerably less toxic (15 minute-EC_{50} = 31.6%) than the other three PMES (15 minute-EC_{50} = 4.4-9.8%, Table 4.3). Ethanol extracts of the reference soil (Table 4.3) and ethanol control (data not shown) were not toxic, however, the ethanol extracts of all of the PMES were more toxic than the corresponding leachate water extracts, with 15 minute-EC_{50}s of 0.1-1.3% (Table 4.3). Of those PMES extracts, KAB was most toxic, followed by TPS, washed TPS, TB and TAB (Table 4.3). Washed TPS had 86% of resin acids removed, but fatty acids were poorly removed (Table 4.1) and the toxicity to *V. fischeri* was only reduced by about 50% compared to TPS.

Table 4.3 Toxicity to Microtox showing concentration of extracts causing a 50% drop in luminescence (EC_{50}) after 15 minute exposure time. Numbers in brackets indicate the 95% confidence range of the EC_{50} concentration.

Treatment	EC_{50} – water extract (95% confidence range)	EC_{50} – ethanol extract (95% confidence range)
TAB	> 100%*	1.3% (0.7-1.9)
TB	7.1% (6-8)	0.9% (0.36-3.3)
KAB	31.6% (28-35)	0.1% (0.07-0.13)
TPS	4.4% (3-6)	0.2% (0.19-0.23)
Washed TPS	9.8% (9-11)	0.6% (0.52-0.68)
Reference soil (TSL)	> 100%	> 2%‡

* The concentration of samples used ranged from 5.6% to 45% (for water extracts) and 0.125% to 1% (for ethanol extracts). EC_{50}'s that were outside the concentration range were determined by extrapolation from dose response curves that were plotted, >100% indicates that the calculated EC_{50} was above the concentration of the undiluted sample.

‡ >2% indicates EC_{50} above the concentration of the undiluted ethanol extract (2% ethanol is the threshold toxicity for Microtox).

4.5 Discussion

The PMES evaluated in this study represented a wide range of pulp mill-related organics, particularly resin acids, and in the case of biosolids, high levels of resin acid neutrals, such as retene, that are formed in the treatment systems. Undiluted pulp and paper waste solids were not acutely lethal to the soil invertebrates and plants tested in these experiments, but, there were reproductive effects on enchytraeid worms. Acute toxicity of aqueous extracts of the solid wastes was observed with an aquatic bacterial bioassay, however, microbial community bioassays showed that PMES increased soil microbial respiration. Differences in basal respiration between high and low moisture contents of PMES suggests that water availability is important for microbial activity, and that different PMES have different optimal moisture contents for microbial activity.

The lack of acute effects in the earthworm and plant tests indicated either tolerance to the contaminants present in the PMES tested, or a limited bioavailability of these compounds in the test material. However, the fact that plant growth, earthworm reproduction and microbial activity were enhanced in the PMES compared to soil controls suggests that some nutritional or habitat benefit may have been derived from constituents of the PMES.

The chemical analysis of residues in earthworms exposed to the PMES for 28 days showed that the resin acid neutrals and to a lesser extent the resin acids were taken up by earthworms and accumulated, though BAF's were consistently lower than 1, indicating much lower levels of those extractives than were found in the exposure material. In fish, compounds such as resin acids are known to be rapidly excreted into the bile (van den Heuvel and Ellis 2002). The ability of soil invertebrates to metabolise resin acids and resin acid neutrals has not been explored, although some studies have shown that they have varying abilities to metabolise other kinds of PAH compounds (Stroomberg *et al.* 2004), and that earthworms such as *E. fetida* apparently have a lower capacity for PAH biotransformation than do other soil invertebrates such as isopods and collembola. Indeed, earthworms have inherently low phase-I metabolic capacity (Booth *et al.* 2002). Earthworms have been shown to accumulate PAH from the soil (Jager *et*

al. 2000; Ma *et al.* 1995), but, after an initial 'peak' in body residue concentration, a subsequent decrease of PAH occurs even when the concentration of PAH in the soil remains the same. This behaviour may be due to biotransformation, excretion or sequestration processes within the earthworm, though it is thought that the main reason is due to a decrease in the pore water concentration of PAH , which is thought to be the main bioavailable phase for earthworms (Jager *et al.* 2000; Ma *et al.* 1995). It should be cautioned that bioavailability to soil invertebrates can vary dramatically with the physical nature of the soil (Lanno *et al.* 2004) the source matrix (Rust *et al.* 2004) and the invertebrate species (Stroomberg *et al.* 2004) and this same phenomenon was likely manifest in the biosolids tested in this study.

Reduced bioavailability of organic compounds (including PAH's) with increasing residence time ('ageing') in soil has been widely reported (Alexander 1995; Hatzinger and Alexander 1995; Northcott and Jones 2001), and this may also apply for organic compounds within waste materials. For example, the bioaccumulation of the resin acid neutral fichtelite was much greater in earthworms exposed to the TB, compared to earthworms that had been exposed to the KAB which contained nearly three times the concentration of fichtelite but which had been left to 'age' for several years. However, retene had a six fold greater BAF in KAB compared with TB even though the difference in retene concentration between the two biosolids was similar to the difference in fichtelite concentration between the two biosolids (Table 4.2). This would suggest that the differences in organic compound bioavailability between PMES do not vary uniformly for all compounds. Differences between fichtelite and retene bioaccumulation in earthworms may also be complicated by different modes of uptake, i.e. fichtelite is more water-soluble and uptake may be preferentially via pore water, whereas retene is more hydrophobic, and uptake may be greater via the ingestion pathway.

The Microtox® EC_{50} data for water extracts indicated that the TB have a greater amount of bioavailable (toxic) contaminants than the two aged biosolids (TAB, KAB), even though the total amount of extractives is far greater in the KAB. By increasing the solubility of contaminants in solid wastes using ethanol, the less bioavailable organics were removed and this can be seen in the low Microtox®

EC_{50} concentrations of all PMES extracts using ethanol. While the fraction that is extracted by ethanol is not likely to be representative of the actual bioavailability experienced by organisms, it does indicate that there are toxic components in PMES that can be made available to test organisms. This further supports the argument that it is the availability rather than the concentration of compounds that is important.

The tests using enchytraeids that showed detrimental effects on reproduction as a result of exposure to the TPS and washed TPS, the TB and the KAB (100%) biosolids must be interpreted carefully. Although the negative impacts on reproduction could be attributed to toxicity of extractives present in the TPS, TB and KAB, the dramatic reduction in fecundity for enchytraeids exposed to the washed TPS solids which was physically similar but without high concentrations of resin acids of the unwashed TPS suggests that suppression of reproduction was via a mechanism other than resin acid-associated toxicity. Washed TPS extracts used in the Microtox® assay also exhibited acute toxicity not attributable to resin acid concentrations (Table 4.3). There is further evidence of this where exposure to 100% KAB almost totally suppressed enchytraeid reproduction, whereas reproduction in the 50% treatment was not significantly less than the TSL control (Figure 4.3), despite the fact that the calculated concentration of resin acids and resin acid neutrals in the 50% treatment would still be well above those in the other treatments.

There were obvious differences in the responses of the organisms tested in this study, particularly where the two species of invertebrates are concerned. This type of pattern has been observed in investigations with other organic contaminants (Semple *et al.* 2003) where differing responses were observed for different organisms. This lends weight to the need for an ecosystem approach to better understand ecological effects of soil contamination (Bogomolov *et al.* 1996). The very nature of bioassays is appropriate to any study designed to probe the effects of variables on an ecosystem; different organisms respond to contaminants differently, otherwise a single organism test would suffice.

Previous studies have shown that aquatic organisms are adversely effected by resin acids and resin acid neutrals at a few µg/L (Leppanen and Oikari 1999). The 96-hour LC_{50} for salmonid fishes has been reported at approximately 1mg/L for common resin acids. Reported sub-lethal effects in trout range from 16-32 µg/L for resin acids and also for retene (Leppanen and Oikari 1999), however, there does not appear to be corresponding detrimental effects on soil organisms. These compounds do have some capacity to bioaccumulate in earthworm tissue although PMES were not acutely lethal to either soil invertebrates or plants, and in many cases appear to have a stimulatory effect on growth of plants, earthworm reproduction and microbial respiration. Some negative effects did occur with enchytraeid reproduction. These effects were ameliorated in the KAB material by dilution with a clean soil. The comparatively low number of juvenile enchytraeids produced in the TPS and washed TPS treatments indicated that the toxicity observed could not be attributed to resin acid neutrals, as these extractives were not present in these treatments. Washed TPS also had most available resin acids removed suggesting that toxicity to enchytraeids and also the Microtox® bacteria in this treatment were not due to resin acids. However, TPS and washed TPS had high concentrations of fatty acids which have been previously reported to contribute to pulp mill effluent toxicity in fish (Leach and Thakore 1973). The negative effects appeared to be caused by different materials in the different PMES that showed these effects.
Studies on microbial function showed that waste solids also exerted a stimulatory rather than an inhibitory effect on soil community level respiration.

This study showed that PMES were relatively benign to the soil organisms tested. The data did not support the original hypothesis that RE were toxic to selected soil organisms, although bioavailability to earthworms was demonstrated. Overall, these data suggest that, contrary to the situation in aquatic environments, the toxicity of resin extractives in PMES is limited in the soil environment, as is the potential hazard posed to soil organisms by these compounds.

5 Effects on Carbon Turnover and Pulp Mill Effluent Solids Decomposition

5.1 Introduction

The turnover of carbon in soil is fundamental to soil development and necessary to maintain ecological functions that soil performs in the terrestrial environment. Soil carbon fluxes are an important component of the global carbon cycle with rates of inputs and outputs determining if a soil is a sink or source for atmospheric carbon (Buchmann 2000). More importantly, at a local scale, soil carbon fluxes influence soil properties such as soil organic matter (SOM) content and nutrient availability (Delaney *et al.* 1996).

Decomposition of plant material and the formation of humus-producing stable SOM, is a key ecosystem function. This process is closely associated with nutrient turnover and nutrient availability, particularly in forested ecosystems (Swift *et al.* 1979). The turnover of carbon through decomposition is primarily controlled by temperature, moisture, substrate quality and the decomposer community (Swift *et al.* 1979). While climatic variables control decomposition at the regional or global scale, substrate qualities are more important at local scales (Delaney *et al.* 1996). Changes in the rate of carbon turnover and the amount of carbon held in soil affect soil qualities that can have profound impacts on primary productivity over the long-term (Jenkinson *et al.* 1992). For example, a decrease in the decomposition rate of plant litter, whether through anthropogenic causes (Johnson and Hale 2004) or natural causes (Albers *et al.* 2004) can result in reduced soil fertility through the immobilisation of nutrients in the litter layer (McEnroe and Helmisaari 2001). Base cations removed from the soil by plants, can be immobilised in plant litter if decomposition processes are inhibited and this can result in decreases in soil pH (Albers *et al.* 2004).

On the other hand, an increase in the decomposition rate can also negatively impact soil through loss of SOM, which can lead to decreased nutrient and water storage capacity (Prescott 1997). The quantity of litter inputs does not necessarily determine the quantity of SOM as the addition of organic substrates to soil can

have a priming effect on the microbial community resulting in the degradation of both the added substrate as well as native SOM (Waldrop and Firestone 2004). This priming effect can substantially increase the decomposition rate of SOM, the long-term effect being a reduction in SOM content and quality.

Decomposition of plant litter is controlled by a number of factors, including the carbon composition of litter inputs (Swift *et al.* 1979). Condensed carbon compounds such as lignin and tannins are important regulators of forest litter decomposition (Vesterdal 1999). Little is known about how related compounds found in PMES may influence forest floor decomposition processes. Lignin is second only to cellulose as the most abundant polymer in nature and its intimate association with cellulose/hemicellulose provides protection from enzymatic hydrolysis of plant material (Kirk and Farrell 1987). Lignin degradation is considered the rate-limiting step in the turnover of carbon in forest ecosystems (Pointing *et al.* 2005). The white rot basidiomycetes, through the secretion of extracellular enzymes, is the only group of organisms that can completely degrade lignocellulosic plant materials (Dedyan *et al.* 2000; Highly and Dashel 1998). There are three enzyme systems that are important in species capable of degrading lignin (Lyons *et al.* 2003); lignin peroxidase (LiP), manganese dependant peroxidase (MnP) and laccase, which mediate lignin degradation in these species (Gallo *et al.* 2004). Due to their broad substrate specificity these enzymes are capable of degrading a wide range of aromatic compounds including PAH's (Baldrian 2006; Lang *et al.* 1998; Majcherczyk *et al.* 1998). Changes in the expression of these enzyme systems or the diversity of organisms possessing them may result in functional changes in litter decomposition and SOM formation (Carreiro *et al.* 2000).

5.2 Potential of Pulp Mill Effluent Solids to Affect Soil Processes

PMES contain mineral nutrients, have high concentrations of carbon (30-40%), and they may also contain significant lime (~10%). There is potential to beneficially use PMES as amendments to improve soil nutrient and SOM status (Chantigny *et al.* 1999; Henry and Cole 1998). However, there could be negative

impacts on soil function that should be investigated before large-scale land application is considered (Bostan *et al.* 2005). While PMES supply nutrient elements and carbon, the high C:N ratio of some solids could lead to nitrogen immobilisation in the receiving soil resulting in plant productivity losses (Feldkirchner *et al.* 2003). Residual aquatic toxicity from high resin extractives (RE) concentrations in PMES may negatively impact terrestrial communities. There is very little information on the toxicity of PMES to terrestrial organisms (Bostan *et al.* 2005). There are, however, studies that indicate plant secondary compounds effect litter decomposition (Kainulainen and Holopainen 2002; Paavolainen *et al.* 1998) and may be important regulators of nutrient cycling in forest ecosystems (Hättenschwiler and Vitousek 2000). Conifer species contain secondary compounds, many of which are thought to act as defences against insect attack and decay organisms (Kainulainen and Holopainen 2002). Pulp mill effluent solids derived from mills that use predominantly conifer feedstock contain high concentrations of these compounds and also related compounds, many of which fall into the category of RE. It is possible that addition to soil of PMES containing these compounds may reduce decomposition processes thereby negatively impacting soil nutrient dynamics.

5.2.1 Community Level Effects Due to Land Applying PMES

The toxicology and chemistry of PMES have been discussed in previous Chapters with reference to their application to soil. The behaviour of single organisms under controlled laboratory conditions when exposed to PMES provides information on the type of effects that might be observed in terrestrial environments receiving PMES. Characterising the chemistry of PMES and particularly, how the chemistry changes in the field, gives some indications of the fate of particular compounds in terrestrial systems. Where studies with laboratory bioassays and chemical characterisation have difficulty, is in integrating the complex interactions of biotic and abiotic variables found at the ecosystem level. It has been well documented that studies conducted at the level of the organism or lower, (i.e. cellular and biochemical, under controlled conditions) have limited ability to predict effects observed at higher levels of organisation such as the community and ecosystem (Wright and Welbourn 2002). Likewise, investigating

chemical characteristics can not on its own inform us of the effects that PMES might have when applied to soil (Debus and Hund 1997). Bioavailability, activity and responses of organisms to chemicals are subject to many variables which are either not practical to measure, not possible to measure, or both. Empirical knowledge gained from ecosystem studies provides additional information that can not be obtained from chemical analysis. A full inventory of the organic compounds present in PMES is difficult as there are thousands of compounds including many that are unidentified (Hewitt *et al.* 2006). It is still unclear which compounds are responsible for the many and varied effects observed in aquatic organisms exposed to pulp mill wastes and how different compounds may interact to affect organisms in natural environments (McMaster *et al.* 2006). While laboratory investigations and chemical characterisation may give useful indications of how PMES will behave in terrestrial ecosystems, these must be seen as preliminary investigations, that indicate where further research might be directed. Ultimately this research should seek to investigate effects at the ecosystem level and be conducted in environments that PMES may potentially impact. Monitoring the turnover of soil carbon may indicate key process changes and be useful to assess the impacts of land application of PMES on soil functions.

5.3 Objectives

5.3.1 Objective I: Hypothesis Testing

In this chapter, a field study investigating the effects of applying PMES to soil is presented. The primary objective of this study was to test the hypotheses that:

- *Applying PMES to soil would negatively impact soil respiration.*
- *Applying PMES to soil would negatively impact soil decomposition processes.*

Significant increases or decreases in soil respiration or needle litter decay rate can both be detrimental, thus a negative impact was defined as a significant deviation from the control treatment. An increase in carbon turnover could result in loss of SOM with time, leading to a decline in qualities such as soil structure, moisture holding capacity and nutrient storage capacity. On the other hand, a decrease in

carbon turnover could indicate negative impacts on soil organisms, such as toxic effects, that may result in lowered nutrient turnover and availability.

To test these hypotheses soil CO_2 fluxes, PMES mass losses, and needle litter mass losses were measured in a field trial described in Chapter 2, Section 2.5.2. These indicators of soil function were chosen to investigate aspects of soil carbon turnover.

Soil CO_2 fluxes give an indication of net belowground respiratory activity and have been shown to decline due to the presence of toxic materials (Baath 1989). Soil respiration was measured in two ways: 1) The respiration from soil below PMES was compared to soil respiration from control plots. 2) The amount of carbon lost due to the decay of PMES was estimated by mass loss measurements and this weight of carbon was subtracted from the total CO_2 carbon respired from the surface of the PMES treatments estimated from monthly CO_2 flux data. The estimated losses of carbon from these two methods were compared in order to investigate if there were net changes in soil respiration following application of PMES.

The decay rate of needle litter declines across contamination gradients in natural ecosystems (Johnson and Hale 2004), and a decline in soil respiration was associated with declines in substrate decomposition (Giller *et al.* 1998). Needle litter decay in PMES treatments was compared to decay of needle litter in control plots that had not received PMES. The lignocellulose ratio of two litter treatments was also measured after twelve months decomposition, as decreases in this ratio have been correlated with decomposition of leaf litter (Osono and Takeda 2005).

Enzyme activities of microorganisms may provide information about different metabolic strategies or pathways to which gross carbon turnover studies may be insensitive (Chantigny *et al.* 2000; Speir 2002). Changes in heterotrophic community structure due to PMES applications may change decomposition pathways, which over long periods may result in changes in soil carbon pools. Changes in enzyme activities may be an early indication of future changes in carbon pools (Sinsabaugh *et al.* 1994). In this study the activity of extracellular

enzymes involved in degradation of lignin was investigated, as lignin degradation is an important regulator of decomposition in forest ecosystems (Berg 2000; Criqueta *et al.* 1999; Leonowicz *et al.* 2001) and PMES also contain significant quantities of lignin-like materials and other recalcitrant compounds that may be substrates for these enzymes.

5.3.2 Objective II: Investigation of PMES Decomposition

A second objective of this field study was to determine the decomposition rate of PMES, to gain knowledge of mechanisms that control this process and understand how PMES might influence soil processes in the long-term. Chemical fractionation of PMES, using the ADF method of Rowland and Roberts (1994) was used to characterise carbon pools in PMES. This method has been used to determine lignin and cellulose proportions (cellulose/ cellulose + lignin) in litter decomposition studies (Ganjegunte *et al.* 2005; Osono and Takeda 2005; Sariyildiz 2003). As plant litter decays the ratio of cellulose to lignin declines as the cellulose fraction is preferentially utilised (Berg 2000). Over two years changes in these carbon pools were tracked to provide information regarding decomposition processes and differences between different PMES.

Microbial respiration, microbial biomass and soil carbon were also measured. Ratios of respiratory CO_2 production per unit biomass, or biomass per unit carbon, have been used to estimate the efficiency with which a community respires carbon. This has been used in studies, such as investigation of heavy metal contamination of soils (Giller *et al.* 1998) and to characterise the utilisation efficiency of plant derived carbon in soil chronosequences (Wardle and Ghani 1995). These methods have the potential to provide an insight on the quality of carbon available for utilisation by the adapted microbial community. The ratio of biomass to total organic carbon (TOC) was compared between different substrates to investigate availability of carbon for respiration.

5.4 Methods

5.4.1 Overview

A field trial was established in August 2004 to assess a range of parameters. The field trial was monitored over a period of two and a half years, including three summer periods. Monitoring included direct field measurements and laboratory analysis on samples of PMES, soil and litter materials. For a full description of the field-trial layout see Section 2.5.2.

5.4.2 Field Sampling

A wide variety of sampling techniques was used to gather field data, many of these at regular time points over a period of 30 months. Table 5.1 shows when various field measurements and samples were collected and for what purpose they were used. A full description of sampling procedures and the purpose of sampling is given below.

Table 5.1. Field trial measurements and sampling regime over 30 months (892 days). Column labels 1 – 12 are; 1 - CO_2 flux, 2 – moisture content, 3 – diurnal CO_2 flux, 4 – mass loss (PMES), 5 – microbial biomass, 6 – carbon and nitrogen, 7 – organic matter, 8 – laccase enzyme activity, 9 – resin extractives, 10 – acid detergent fibre (PMES), 11 - needle litter mass loss, 12 – needle litter acid detergent fibre.

Date	Day	1	2	3	4	5	6	7	8	9	10	11	12
29.8.04	*0*	+	+		+		+	+		+	+	+	+
21.9.04	*24*	+	+										
13.11.04	*77*	+	+										
3.12.04	*97*						+			+			
23.3.05	*207*						+			+	+		
19.4.05	*234*	+	+										
26.4.05	*241*	+	+										
5.6.05	*281*	+	+										
6.6.05	*282*						+			+			
26.8.05	*363*	+	+				+		+	+	+		
9.9.05	*377*	+	+	+					+				
12.10.05	*410*	+	+	+					+				
24.11.05	*453*	+	+	+									
19.12.05	*478*	+	+		+	+	+			+			
6.1.06	*499*	+	+	+									
8.2.06	*530*	+	+				+		+	+	+		
9.3.06	*558*	+	+						+				
4.4.06	*584*	+	+										
2.5.06	*612*	+	+	+	+			+	+				
30.5.06	*640*	+	+				+		+	+			
7.7.06	*678*	+	+										
7.8.06	*709*	+	+						+				
15.8.06	*717*				+		+			+	+	+	+
29.8.06	*731*	+	+						+				
26.9.06	*759*	+	+										
28.10.06	*791*	+	+						+				
21.11.06	*815*	+	+	+	+								
2.6.07	*892*	+	+		+								

5.4.3 Respiration

Short lengths of PVC pipe (10 cm diameter) were inserted into field plots containing PMES and used as permanent soil respiration cores from which CO_2 flux was measured. At the time of field application, a representative sample of approximately 20 kg of each PMES was taken back to the laboratory where moisture content was determined (105°C overnight). Field moist samples equivalent to approximately 130 g dry weight were weighed into zip lock bags -

equivalent to the field application rate of 150 tonnes per hectare (dry weights ranged from 126 – 129 g). On the 29th August 2004 (day 0 of the field trial) the contents of these bags were placed into the PVC pipes that had been inserted in the field plots (Figure 5.1). Each field plot contained five of these respiration cores making a total of 20 cores per treatment. As the bulk density of the PMES differed between treatments, the length of the PVC pipe was determined by the bulk density of material to be placed in it. PVC pipes ranged from 8-15 cm in length. Fibreglass mesh (2 mm) cut into 10 cm discs were placed above and below the PMES to separate them from material outside the cores over the period of incubation. The base of the PVC pipe was approximately 3 cm below the soil surface. Control plots also had PVC pipe inserted to a depth of 8 cm so that CO_2 fluxes could be measured in these plots. PVC pipe heights were adjusted by tapping down with a wooden mallet so that there was 2.5 cm protruding above the surface of the PMES or soil. This was the offset (Figure 5.1) and was entered as an input parameter when making CO_2 flux measurements described in Section 5.4.5.

Figure 5.1. PVC collars inserted in Tasman primary solids (TPS) containing 126 g dw of TPS. Collars were raised (offset) 2.5cm above the surface of the solids.

5.4.4 Destructive Sampling

PVC pipes containing PMES were removed at various times throughout the trial period to determine mass loss. The PVC cores were removed from the field plots with a spade. Soil was removed from the base of the core to expose the fibreglass mesh that separated PMES from the soil. The intact core was then placed in a plastic bag and packed into a plastic box for transporting back to the laboratory. In the laboratory, material was removed from the PVC pipe and the separating mesh and other material was separated from the PMES. Wet weights of PMES and of needle litter that had accumulated above was determined before drying. Mass losses were determined by drying total (except December 2005) PMES to a constant weight at 105°C. In December 2005, a sub-sample was oven dried for mass loss determination, as microbial biomass was also determined from these samples.

5.4.5 Soil CO_2 Fluxes Measurements

Soil CO_2 fluxes were measured on a monthly basis from respiration cores inserted at the beginning of the trial and CO_2 fluxes was analysed using a model LI-8100 portable infrared gas analyser (LI-COR Environmental, Lincoln Nebraska), with an attached survey chamber fitting a 10 cm diameter respiration collar. Generally fluxes were measured each month but the day of measurement was weather dependent; flux measurements were not made during periods of constant rainfall. No measurements were made between November 13th 2004 and April 19th 2005 due to equipment failure. In the 22 months from April 2005 to February 2007 CO_2 fluxes were measured 20 times (Table 5.1).

Measurements were made after needle litter, which accumulated between measurements, was cut from around the PVC pipe to allow the survey chamber to form an airtight seal with the respiration core. Needle litter was allowed to accumulate on top of the respiration cores over the 30 months that measurements were made. The survey chamber was closed for 80 seconds in which time CO_2 concentration was logged every second. A 20-second dead band allowed CO_2 to stabilise inside the chamber immediately after the chamber closed. The data for the 60 seconds that followed was used by the onboard software to calculate the CO_2 flux by fitting an exponential curve to the plotted data with output presented as µmols CO_2/ m^2/sec. Between measurements the survey chamber was blown out with a hand held electric fan to ensure that the chamber CO_2 concentration returned to the ambient concentration. Measurements were made between mid morning and mid-late afternoon although diurnal measurements indicated there was very little diurnal variation (data not shown). Soil temperature at 3 - 4 cm depth was logged every second, as was air temperature inside the chamber. Samples of PMES and soil were collected from each treatment and placed in airtight bags for moisture analysis. Approximately 10 g of material was dried at 105°C overnight to determine gravimetric water content.

Data were downloaded and assessed using the LI-8100 file viewer programme. Flux measurements with a high coefficient of variance were discarded. A

regression plot of the change in CO_2 concentration allowed visual assessment of data before saving to Excel spreadsheets.

After the third destructive sampling for mass loss determination of respiration cores in August 2006, and thereafter, for each destructive sampling, a new PVC pipe was inserted into each plot so that three respiration cores remained for CO_2 flux measurements until the end of the trial period. Preliminary measurements showed that three cores per replicate plot were sufficient to provide a robust data set for statistical analysis. Although five cores were inserted in each replicate plot at the commencement of the trial, CO_2 flux was not measured on all cores every time. This was due to the time constraints involved in measuring all the cores, particularly in winter when daylight hours were short and other sampling procedures were required. PVC pipes that were reinserted were not refilled with a known weight of PMES, rather they were inserted directly into solids already in place. The fluxes from these cores were checked and were not significantly different from cores that had been in place from the beginning of the trial (data not shown).

5.4.6 Carbon Budgets

In order to construct a carbon budget for PMES it was necessary to account for sources of CO_2. Many investigators have endeavoured to apportion CO_2 fluxes into different components such as root respiration, heterotrophic respiration and also by soil horizon (Bouma and Bryla 2000; Certini *et al.* 2003; Taneva *et al.* 2006). To date, there has been limited success in separating the various components that contribute to respiration and there still remains much uncertainty accounting for the sources of CO_2 production in soil (Hanson *et al.* 2000; Kuzyakov 2006).

Surface CO_2 fluxes from PMES cores were measured before cores were removed from the soil, CO_2 fluxes were then measured from cores removed from the soil. CO_2 fluxes were also measured from the soil after the cores had been removed. However, it was not possible to get an accurate measure of the net CO_2 derived from the PMES cores (total CO_2 flux minus soil CO_2 flux) by this method due to

large variability in these measurements. Therefore, to construct a carbon budget, it was assumed that the difference between surface CO_2 flux from PMES treatments and the control treatments were due to CO_2 produced from PMES. The net CO_2 produced from PMES was estimated by subtracting the control soil mean CO_2 flux from the total CO_2 flux from PMES plots. The estimated cumulative CO_2 - C lost from PMES was compared to the mass of carbon lost (Section 5.4.4) over the same time period.

5.4.6.1 Diurnal CO_2 Fluxes

At several times throughout the field trial, continuous measurements were made over a period of 24 hours or more to monitor diurnal variation in CO_2 fluxes. As monthly measurements were made between mid morning and mid-late afternoon any diurnal fluctuations would need to be considered when constructing a carbon budget. Diurnal measurements were made from a respiration core in either the control soil or Tasman primary solids (TPS).

5.4.7 Litterbags

One year after the field trial was established, a litter bag experiment was initiated. Pine needle litter excluding branches and green needles was collected from the top layer of forest floor near to the field site. The top litter layer (representing recent litter-fall) was collected. In the laboratory, needles were mixed by hand and placed in a large plastic bag where they were left for two days so that needle moisture could equilibrate throughout the bag. Moisture content of litter material was determined (70^oC overnight) from several samples throughout the bag. Approximately 3.5 g (dw) of needles (35-40% moisture w/w) were weighed into 10 cm^2 fibreglass mesh (2 mm) bags. A plastic identifying tag was inserted into each bag before it was sealed. There were 16 litterbags for each treatment (four per replicate plot) and these were placed either on top of PMES in the accumulated litter layer or beneath the PMES in the buried F/H layer (Figure 2.6). Litter bags were also placed in the control plots in the surface litter (L horizon), or in the F/H horizon. Litterbags that were buried beneath PMES were put in place with minimal disturbance. A flat metal tray was pushed in from the edge of PMES

plots and then gently tilted up (Figure 5.2), litterbags were placed between the litter layer and PMES before removing the metal tray. The location of buried litter bags was marked with a metal pin. The litter bags placed on the surface of waste solids and in the control horizons were kept in place with a 'U' shaped metal pin. Twelve months later, all litter bags were removed for analysis, each bag was sealed inside a plastic bag and placed on ice before transporting to laboratory where they were stored at 4^0C until analysis. Treatments were separated into the PMES/soil control treatments and then each of these further divided into buried and surface applied treatments. Litter was removed from the litterbags and placed in aluminium drying trays for mass loss determination. Care was taken to avoid contaminating litter samples with soil or PMES adhering to the litter bags. Litter was oven dried at 70°C overnight before weighing.

Figure 5.2. Needle litterbags were placed in the buried litter layer by inserting a metal sheet between waste solids and litter layer which was tilted up to allow access while causing minimal disturbance.

5.4.8 Acid Detergent Fibre (ADF) Analysis

PMES and needle litter were analysed for different carbon fractions, using the acid-detergent fibre method of Rowland and Roberts (1994). This is a sequential extraction developed to fractionate cellulose and lignin components of plant litter in decomposition studies (Rowland and Roberts 1994). Surface applied needle litter from the control and one biosolid treatment (TB) were analysed initially and after 12 months incubation. ADF analysis was also carried out on PMES at five time points over two years. Landcare Research's Environmental Chemistry laboratory at Palmerston North conducted all analyses.

Briefly, finely ground oven dried samples of approximately 1g (W1) were extracted in Cetyl Trimethyl Ammonium Bromide (CTAB)/H_2SO_4, leaving behind acid detergent fibre of which dry weight (W2) was determined. The acid detergent fibre was then extracted with 72% w/v H_2SO_4 and dry weight of insoluble residue was determined (W3). Finally, the sample was combusted at 500°C and the ash weight determined (W4). Four fractions were calculated on a mass balance basis by the following equations:

- Detergent soluble = W1 - W2;
- Acid detergent fibre (cellulose fraction) = W2 - W3;
- Acid soluble (lignin - like material) = W3 - W4;
- Acid insoluble (ash) = W4.

The first three fractions separated represent heterogeneous pools of carbon, which equate to weak acid/detergent soluble carbon, weak acid/detergent insoluble carbon and strong acid soluble carbon. These fractions have been generally defined as a lipid/hemicellulose fraction, alpha-cellulose fraction, and lignin-like fraction, respectively, although these fractions are not mutually exclusive. It is not known how these fractions might be represented in PMES, however, it is assumed that the cellulose and lignin fractions would still represent labile and recalcitrant pools of carbon as it applies to plant litter material (Rowland and Roberts 1994). The ratio of acid detergent fibre: acid soluble fibre + acid detergent fibre, was used to investigate decomposition in needle litter and PMES, as this ratio has been

shown to decrease as decomposition of plant material proceeds (Osono and Takeda 2005). This is commonly referred to as the lignocellulose ratio for plant material; it is assumed that the components of the ratio represent similar pools for the purposes of studying decomposition processes in both litter and PMES.

5.4.9 Enzyme Activity – ABTS Test

The 2,2 – Azino-bis-3 Ethylbenzthiazolin-6 sulfonic acid (ABTS) test has been widely used as an indication of laccase enzyme activity, though it has been realised that peroxidases are also capable of oxidising ABTS (Mayer and Staples 2002). As peroxidase activity was not eliminated as a possible cause of ABTS oxidation, the results of this assay are presented as ABTS oxidation. It is assumed that activity in field samples indicates activity of enzymes associated with lignin or other polyphenol oxidation.

Samples of PMES, forest litter from control plots and from on top of the PMES (L horizon), humus from control plots and beneath PMES (F/H horizon) and soil from 0-5cm in the Ah horizon of all plots were collected for analysis of enzyme activity. All samples were collected wearing latex gloves, with gloves replaced after each sample was collected. Samples were kept in airtight plastic bags and placed on ice immediately after collection until return to laboratory where samples were kept either at $-20^{o}C$ (August 2005 samples), or at $4^{o}C$ (in all later sampling periods) until assayed. Assays were conducted within 48 hours of collection, apart from those samples that were frozen. TPS and surface needle litter (L horizon) were finely cut while all other treatments were sieved to 2mm. Two grams wet weight of each sample and five ml of phosphate buffer (pH 7.0) were incubated on ice in conical flasks for one hour. All assays were conducted in triplicate. Samples were periodically swirled in the flasks during the incubation time. After one hour, one ml sub-samples were placed in 1.5 ml Eppendorf tubes and spun down at 5,000 rpm in a bench-top centrifuge for five minutes. Tubes were then placed on ice until analysed. Enzyme activity of the supernatant was determined by measuring the change in absorbance at 420nm in the presence of the substrate ABTS. 700 μl of citric-phosphate buffer (pH 4.5) and 100 μl of 3 mM ABTS was

added into a one ml cuvette, before adding 200 µl of sample supernatant, then the change of absorbance was determined during two minutes.

Enzyme activity was calculated from the following formulae:

$$c = \frac{\Delta A/\min \times V_t}{\in_{420} \times d \times V_s}$$

Where,

$\Delta A/\min$ = difference of Absorption per minute

V_t = total volume

$\in_{420}$ = extinction coefficient (36 cm^2 / μmol^{-1})

d = thickness of cuvette

V_s = sample volume

The enzyme activity is given in units or milli-units (U or mU), one unit being equivalent to the enzyme quantity that can oxidise one µmol of substrate in one minute. Activity was then corrected to a dry weight basis. A positive control was included each time assays were conducted, using laccase extracted from *Trametes versicolor*. This laccase extract was also spiked into field samples to determine that the field sample could support active enzyme.

To establish if microorganisms, with extracellular ABTS oxidising ability, were present in F/H material, extracts from humus samples were grown on agar plates as follows. A sub-sample of F/H humus (from all treatments) assayed on day 791 (October 2006) was used to make a dilution series that was plated onto YM and BAS agar plates (Appendix II). A dilution series was prepared by adding 1 g of humus to 10ml of saline (0.5% w/v), vortexing briefly, then diluting 0.2 ml of this solution in a further 2 ml of saline (10^{-2}); this being plated onto BAS agar. A further dilution (10^{-3}), was plated onto YM agar. After 7 days incubation at room temperature, colonies were transferred onto ABTS underlay agar (Appendix II) to determine if any isolates were producing enzymes capable of oxidising ABTS in the agar.

5.4.10 Carbon and Nitrogen

PMES total organic carbon (TOC) and total nitrogen (TN) were determined on bulked samples collected at six monthly intervals from each replicate field plot. TOC and TN were determined by dry combustion in a LECO - TruSpec CN analyser (LECO Corporation, St Joseph MI, USA). PMES contain lime so organic matter (OM) and carbonate carbon (CC) were determined by loss on ignition at 500°C and 1000°C respectively on pooled samples from August 2004 and May 2006 (David 1988). All ignition methods used oven-dried samples.

5.4.11 Microbial Biomass

When respiration cores were destructively sampled in December 2005 microbial biomass was determined from samples of the PMES and the F/H and Ah horizons of the control surface soil. Microbial biomass was determined using the chloroform extraction method (Sparling 1994).

Cores were kept at room temperature, approximately 24 hours at 20°C, in the laboratory after returning from field sampling before being analysed for microbial biomass. Pulp mill effluent solids were removed from the PVC pipe, a sub-sample was used to determine moisture content, then biosolids were sieved through a 2-mm mesh sieve while TPS were hand mixed to obtain a more homogenous sample. One of two 10 g dry weight (dw) samples from each of the four replicate respiration cores was placed in a 50 ml beaker and these were placed in glass desiccators with a 20 ml beaker containing 10 ml of purified chloroform and anti-bumping granules. A vacuum pump attached to the desiccator was used to lower the pressure until the chloroform boiled, then the desiccator was left closed for 24 hours to allow fumigation of soil by chloroform. A 0.5 M solution of K_2SO_4 was used to extract organic carbon from both fumigated and non-fumigated samples on an end-over-end shaker before filtering through Whatman number 41 paper filters. Extracts were frozen until TOC was determined on a TOC analyser (LECO Corporation, St Joseph MI, USA). Microbial biomass was calculated as the difference in carbon between non-fumigated samples and fumigated samples and the difference converted to microbial biomass, using a k_{ec} factor of 0.41 (Sparling 1994).

5.4.12 Statistics

Analysis of variance (ANOVA) was used to determine treatment-related differences in CO_2 fluxes and mass losses of PMES and needle litter. Where the ANOVA was significant, Tukeys post hoc test was used to determine which treatments were significantly different. The change in %TOC and %TN were calculated from linear regression of measurements made from field samples collected over two years, if a significant r^2 was observed ($p<0.05$). If no significant trend was observed the mean of the five single time-point samples was used in calculations. The Statistica 7 program (StatSoft Inc. Tulsa, USA) was used to perform all statistical analyses.

5.5 Results

5.5.1 Soil Respiration and PMES Mass Losses

5.5.1.1 Monthly CO_2 fluxes

Monthly CO_2 fluxes measured from field plots receiving PMES were higher than the background soil respiration measured from the control soil plots (Figure 5.3). The CO_2 fluxes increased as soil temperature increased ($r^2 = 0.5$, $p < 0.01$) in the spring of 2004 (days 0 – 77) (Figure 5.3), however, fluxes from the PMES treatments increased by a greater amount than the control soil treatment. CO_2 fluxes were not measured between the 13th of November 2004 and the 19th of April 2005 (days 77 – 241), and for this reason it was not possible to assess the full extent of activity in the first summer after field application (refer to Table 5.1 for flux measurement dates and day numbers). In November 2004 (day 77) TPS, TAB and KAB treatments had high respiratory activity, which declined significantly during the following years for the same period (Figure 5.3, Table 5.2). While the TB treatment also had higher fluxes than the control soil, the mean September - November high for this treatment did not change significantly over the three periods compared (Figure 5.3, Table 5.2).

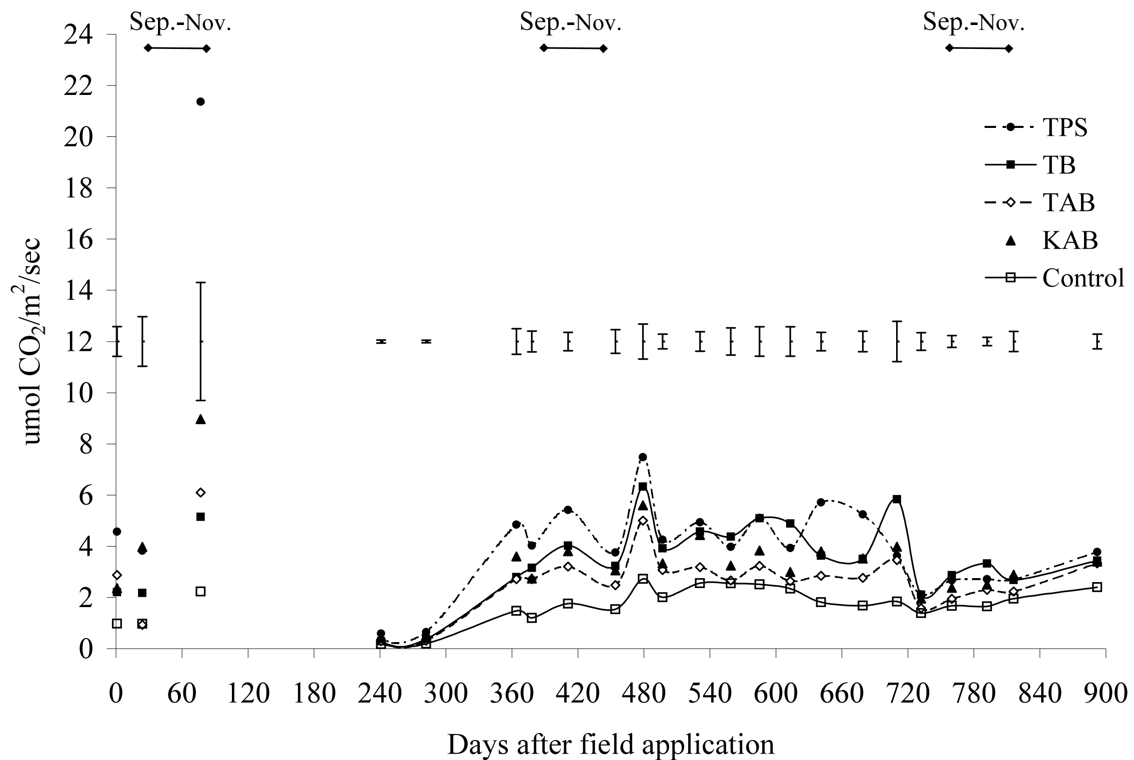


Figure 5.3. Mean fluxes (µmols/m^2/sec) per treatment over 30 month field trial period (August 04 - February 07). Error bars indicate maximum standard error for each sampling period.

As CO_2 fluxes were measured monthly from April 2005 (day 241), a much better indication of respiratory activity was provided for the latter part of the field trial than was available for the early period. Calculations of CO_2 - C losses during this time were improved also because monthly fluctuations were small from spring to autumn when most CO_2 was evolved (Figure 5.3). After a high initial activity in the first November TPS, TAB and KAB plots had a relatively constant level of activity over the following 16 months, apart from lows at days 241 and 281. The summertime mean fluxes from these treatments declined from year two to year three (data not shown), indicating a decline in the decay rate of PMES (Figure 5.3, Table 5.2). Fluxes in the third September – November period were significantly ($p<0.001$) lower than in the first September – November period for TPS, TAB and KAB, with no significant differences ($p=0.14$) in the control soil fluxes over these same periods (Table 5.2). In February 2007 (day 892), after 30 months, CO_2

fluxes from all PMES plots were still significantly ($p<0.001$) greater than from the control soil (Figure 5.3).

Table 5.2. September - November mean fluxes for three years. Different letters indicate significant difference ($p<0.001$) between summertime means within a treatment. See Figure 5.3 for time periods assessed.

Treatment	2004/2005	2005/2006	2006/2007
TPS	12.6 a	4.4 b	2.7 b
TB	3.7a	3.5 a	3.0 a
TAB	3.5 a	2.8 b	2.2 c
KAB	6.5 a	3.2 b	2.6 b
Control	1.6 a	1.5 a	1.8 a

5.5.1.2 Mass Losses from PMES

All PMES showed mass loss during the 30-month trial and there were significant differences ($p < 0.001$) in decomposition rates between treatments (Figure 5.4). The effluent primary solid (TPS) decomposed significantly faster than the biosolids which were all secondary treated pulp mill effluents. TPS lost 26.1% of its original mass over 30 months, while the biosolids TB, TAB and KAB lost 5.2%, 12%, and 6.5% respectively (Figure 5.4). The TPS, TAB and KAB had most of their mass losses in the first half of the field trial, losing between 66% and 76% of the total mass loss between day 0 and 478. The aged biosolids TAB and KAB had no mass loss between May 2006 and February 2007(day 612 – 892).

The TB cores had on average gained two grams (approximately 2%) when sampled in December 2005 (Figure 5.4), and then lost ten grams between December 2005 to February 2007 (day 478 – 892). For the first six months after field application, coincident with a layer of needle litter accumulating, movement of TB material into respiration cores was evident. This appeared to be due to rain splash rather than mass movement.

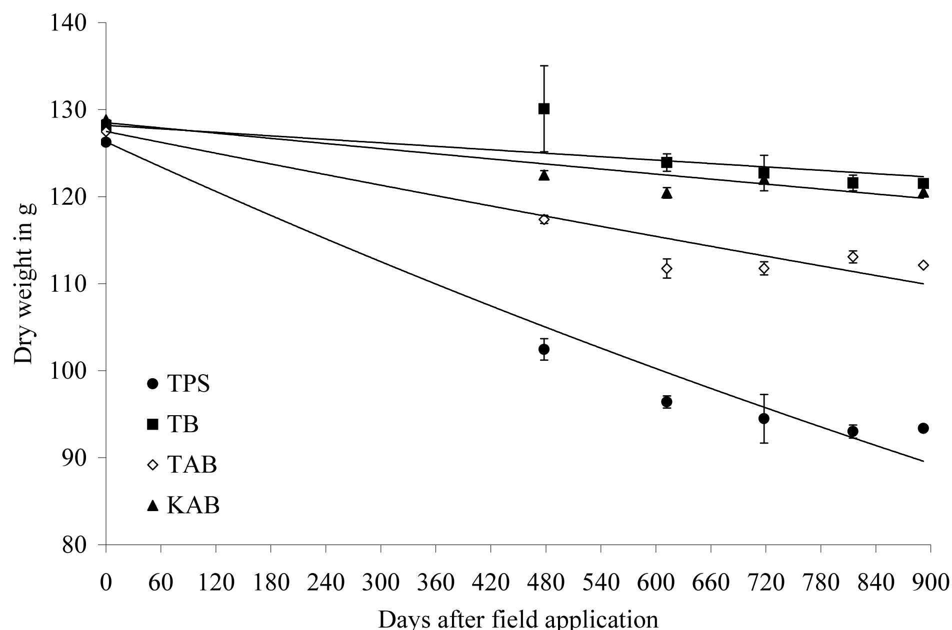


Figure 5.4 Measured mass loss of PMES over 30 months with exponential decay curves fitted. Error bars are standard error.

Exponential decay curves (see Section 3.5.3 for equation) fitted to mass loss data over 30 months showed that PMES, particularly biosolids, are slow to decompose (Figure 5.4). Calculated half-lives of PMES were 5 years for TPS, 10 years for TAB, 26 years for KAB and 27 years for TB.

5.5.1.3 Cumulative CO_2 - Carbon Losses

At four times during the 30-month trial, 24 hour measurements from a respiration core showed very little diurnal variation in CO_2 fluxes (data not shown). Mean daytime fluxes were between 0% and 16% greater than mean night-time fluxes. Generally, it was found that measurements made between mid-morning and late afternoon reflected daily average fluxes. When cumulative monthly CO_2 flux was calculated, no adjustment was made for diurnal variation due to these apparently small fluctuations.

In December 2005, the first destructive sampling of cores indicated mass losses in three of the four PMES (Figure 5.4). For the period December 2005-November 2006 (Days 478-815), CO_2 - C loss and mass loss could be compared as there were regular assessments of the CO_2 fluxes allowing a good estimate of total carbon losses as CO_2 (Figure 5.5). The loss of carbon from each PMES was determined from the net CO_2 fluxes (total CO_2 flux from PMES minus CO_2 flux from control soil), between days 478 and 815 (Figure 5.6). The mass loss of carbon in each treatment (grams/respiration core) was calculated from respiration cores collected at day 478 (December 2005). The mass of carbon in each core was calculated as the product of PMES mass and the %TOC (Section 5.5.4.1). Estimated net CO_2 carbon losses were plotted on the same axes as carbon losses estimated from measured mass losses (Figure 5.7), over the period from December 2005 to November 2006.The net loss of CO_2 carbon (total CO_2 – C from PMES minus control soil CO_2 - C) from PMES treatments was approximately equal to the loss of carbon calculated from PMES decay (mass loss). There were however, minor discrepancies in some PMES treatments (Figure 5.7).

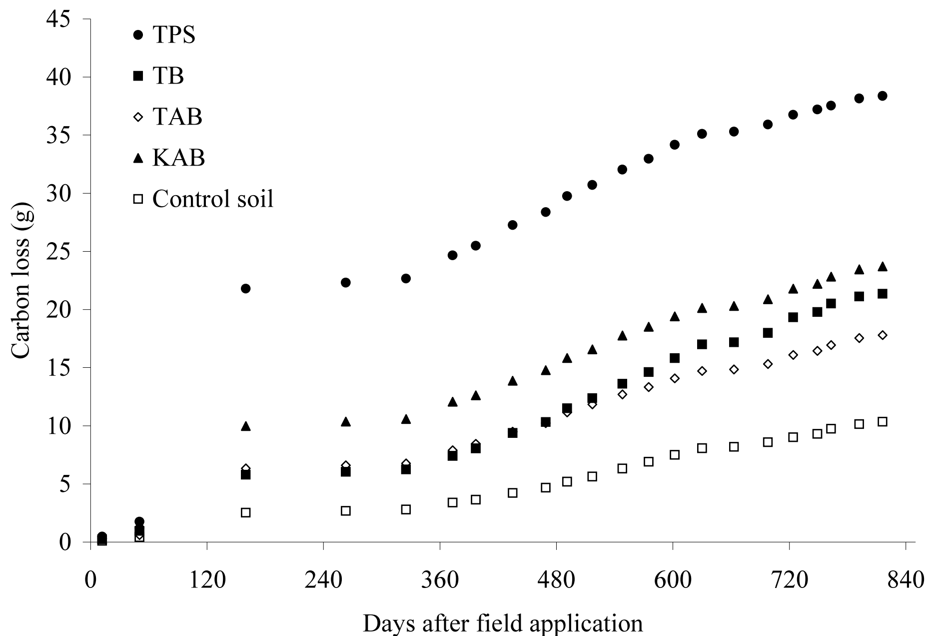


Figure 5.5. Cumulative CO_2 - C loss over 815 days (27 months) from PMES and control treatments

There was no significant difference between mass loss of carbon and cumulative CO_2 losses for KAB and TB (Figure 5.7). Cumulative CO_2 carbon loss from TPS was significantly greater (p=0.02) than measured mass loss of carbon. In contrast mass loss was significantly greater (p=0.04) than cumulative CO_2 loss for TAB (Figure 5.7). The TAB was the only PMES treatment where estimated CO_2 carbon loss over the entire 27 months was less than carbon loss estimated from mass losses (Figures 5.4 and 5.5).

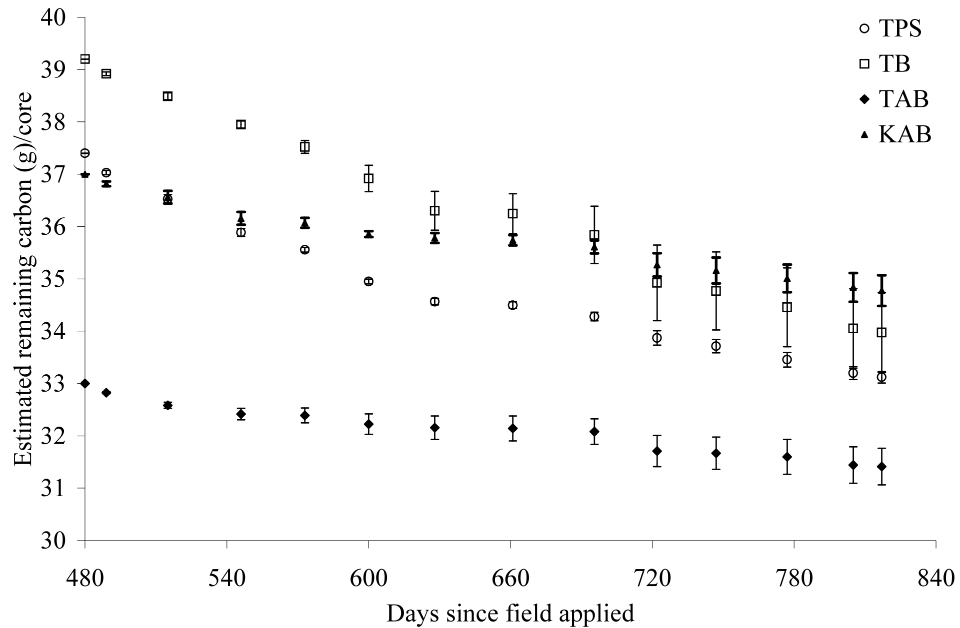


Figure 5.6 Changes in the mass of carbon in PMES, contained in respiration cores, for the period; December 05 - November 06. Net CO_2 - C lost from PMES was calculated as the difference in flux between total monthly PMES treatment and soil control. Error bars are standard error of cumulative carbon loss per month.

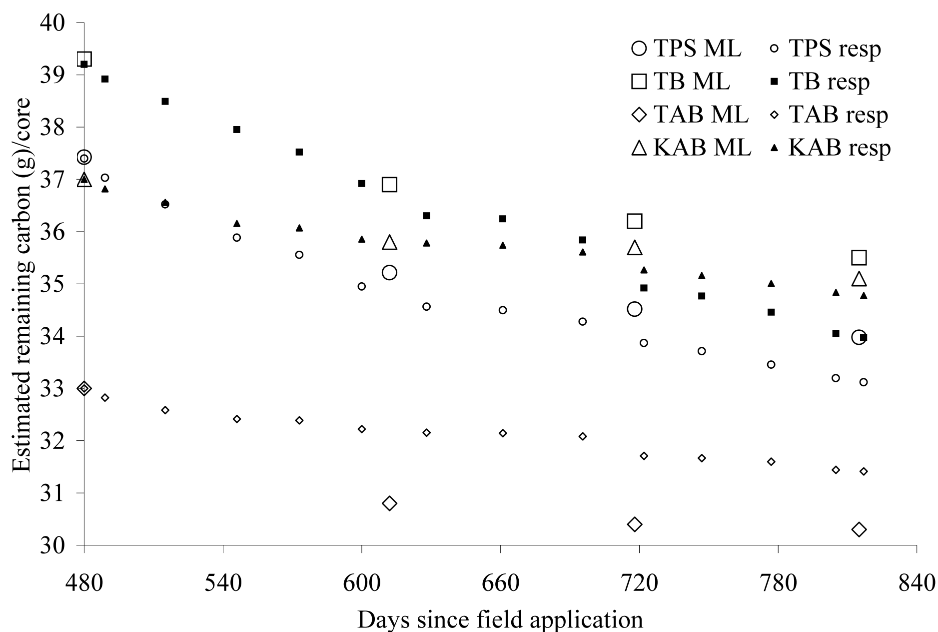


Figure 5.7 Comparison of loss of carbon from PMES calculated from mass loss ML (large symbols) and respired CO_2 (small symbols).

5.5.2 Effects of Waste Solids on Needle Litter Decay

5.5.2.1 Mass Loss of Needle Litter

Needle litter removed from litterbags after 12 months of field incubation had a mean dry weight loss of 31% (Figure 5.8). The mass loss of needle litter ranged from 18 – 47% with a standard deviation of ±6.8%. There were no significant differences in mass losses between buried litter and surface applied litter (p =0.6). There were also no differences in litter decomposition between PMES treatments (p =0.09).

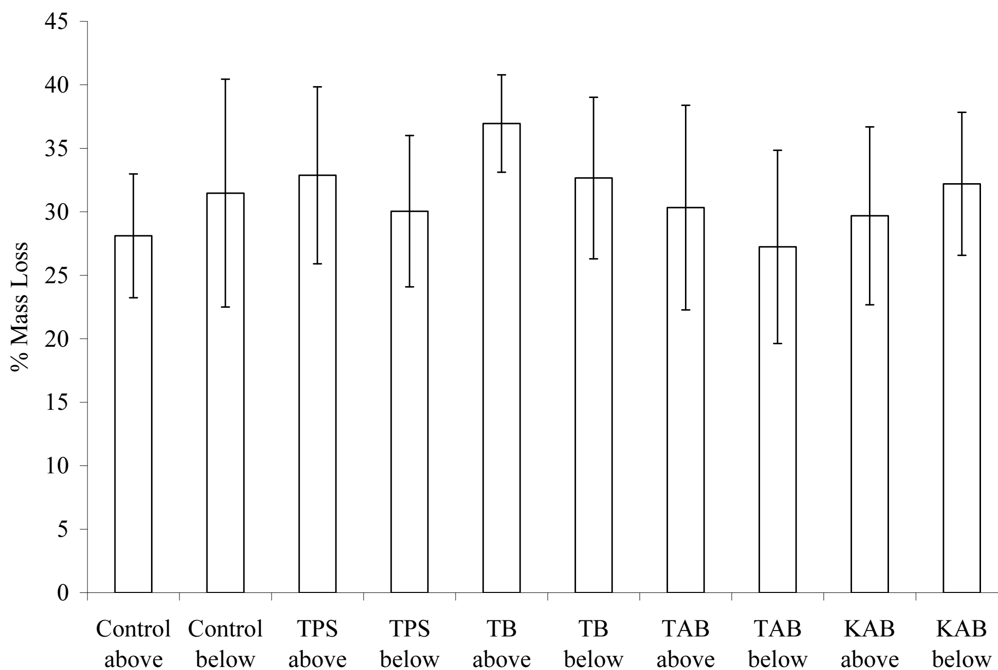


Figure 5.8 Needle litter percentage mass loss after 12 months for all treatments in litterbags placed above and below PMES or in the control humus horizon. Error bars are standard error.

5.5.2.2 Acid Detergent Fibre (ADF) analysis of Needle Litter

ADF analysis was conducted on surface applied needle litter for the control and TB treatments, and also on samples of needle litter prior to field incubation. The change in the lignocellulose ratio (cellulose/cellulose + lignin) showed that needles had lost a significantly greater proportion ($p < 0.01$) of cellulose than lignin during the first 12 months, but there was no difference ($p = 0.9$) in the lignocellulose ratio between the two treatments after 12 months (Figure 5.9). Although there was a significant drop in the lignocellulose ratio after 12 months (Figure 5.9) there was no relationship between the drop in lignocellulose ratio and mass loss ($r^2 = 0.04$, $p=0.5$), as has been implied in published litter decay studies (Berg 2000; Osono and Takeda 2005).

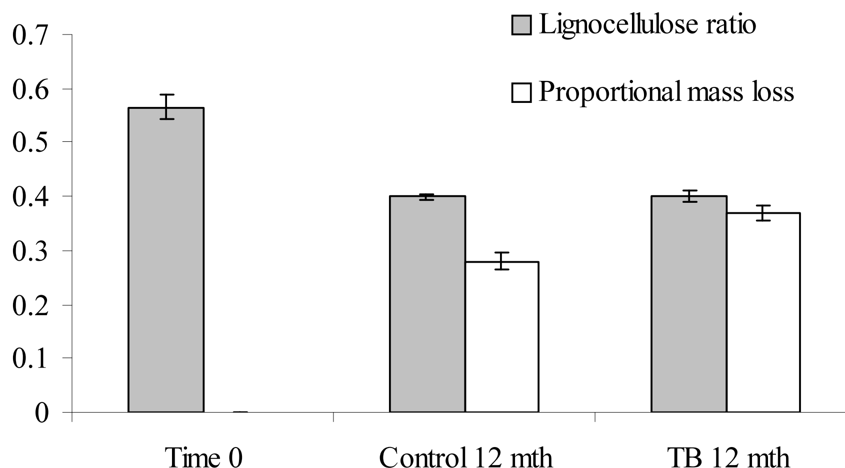


Figure 5.9 Changes in the lignocellulose ratio after 12 months and comparing this with proportional mass loss of needle litter for the same period. Error bars are standard error.

5.5.3 Enzyme Activity – ABTS Test

Preliminary measurements in humus collected from the F/H horizon in spring 2005, showed treatment-related differences in enzyme activity (Figure 5.10). These samples were collected from the same horizon where buried needle litterbags were placed. Activity was observed in all treatments, but the control soil F/H horizon had higher activity than the same horizons beneath PMES. Assays conducted at the same time showed that PMES had no enzyme activity. As the assays were conducted on material that had been frozen for several weeks and later assays indicated that freezing increased activity compared to that of fresh samples (stored at 4°C), on all successive occasions (February – October 2006) fresh material was used.

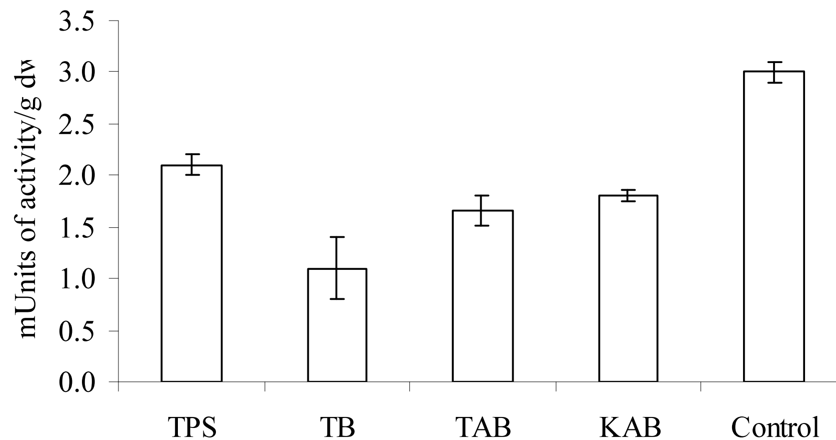


Figure 5.10 Preliminary measurements made in August of 2005 (day 363) from humus collected from the F/H horizon showing ABTS activity (error bars are standard error).

Enzyme activity was measured and detected seven times between February and November 2006 (days 529-791) in the control F/H humus (Table 5.3). Activity was only rarely detected in the F/H humus from under PMES (Table 5.3). On one occasion, May 2006 (day 640), enzyme activity was measured in needle litter (L horizon) collected from above TPS, KAB and the control surface horizon, but no significant differences were detected between treatments (Figure 5.11). Activity, measured in PMES samples at five times in 2006, was only detected in one sample (TPS on day 709) out of a total of 44 PMES samples (data not shown).

Table 5.3 ABTS enzyme activity (Act.) measured in F/H horizon under PMES and in control F/H between February and October 2006 (days529-791).

	Control			TPS			TB			TAB			KAB		
Date/**Day**	**Act.**	s.e.	*n*	**Act.**	s.e.	*n*	**Act.**	s.e.	*n*	**Act.**	s.e.	*n*	**Act.**	s.e.	*n*
8.2.06/**530**	**1.75**	0.25	*2*												
9.3.06/**558**	**4.04**	0.40	*3*	**1.78**	0.21	*3*	**0.00**	0.00	3	**0.00**	0.00	*3*	**0.00**	0.00	*3*
2.5.06/**612**	**3.98**	1.00	*4*	**0.00**	0.00	*3*	**0.00**	0.00	3	**0.00**	0.00	*3*	**0.00**	0.00	*3*
30.5.06/**640**	**7.15**	1.90	*3*												
7.8.06/**709**	**17.6**	2.96	*4*	**1.47**	0.21	*3*							**0.58**	0.39	*3*
29.8.06/**731**	**4.72**	0.82	*2*	**0.00**	0.00	*3*	**0.00**	0.00	3	**0.00**	0.00	*3*	**0.43**	0.43	*3*
28.10.06/**791**	**9.14**	3.41	*3*	**0.00**	0.00	*3*	**1.16**	1.16	3	**0.00**	0.00	*3*	**0.00**	0.00	*3*

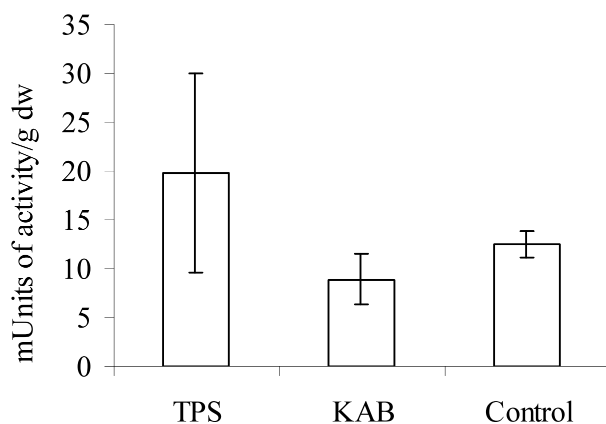


Figure 5.11. ABTS activity in May 2006 (day 640) in needle litter (L horizon) collected from above TPS, KAB and the control surface horizon (error bars are standard error).

Isolates produced from a dilution series from F/H humus collected in October 2006 (day 791) were transferred onto ABTS agar to investigate their ability to oxidise ABTS. Isolates from TPS, TAB and KAB treatments were found to produce a green colour on ABTS agar indicating expression of extracellular enzymes with ABTS oxidising potential. None of the F/H humus in these treatments had shown any ABTS activity when sub-samples of the needle litter were assayed. Interestingly, there were no colonies isolated from the control

humus treatments that were capable of oxidising ABTS, despite this humus having ABTS activity.

5.5.4 PMES Composition and Decomposition

5.5.4.1 Carbon and Nitrogen

Total organic carbon (TOC) ranged from 30 - 37% in PMES before field application and total nitrogen (TN) ranged from 0.19 - 1% (Table 5.4).

Table 5.4 Carbon and nitrogen analysis of PMES before field application, and control soil F/H and Ah horizons sampled in 2006.

Treatment	%TOC	%TN	C: N	%LOI_{500}
TPS	36.6	0.19	193	69.5
TB	31.6	0.64	49	50.9
TAB	29.8	0.58	51	44.6
KAB	31.9	1.00	32	51.4
Soil F/H	33.2	1.59	21	60.3
Soil Ah	6.2	0.56	11	10.7

There was a significant decline in the % TOC in the TB ($r^2 = 0.44$, $p = 0.05$), TAB ($r^2 = 0.55$, $p = 0.02$) and KAB ($r^2 = 0.45$, $p = 0.05$) over two years (Figure 5.12). The TPS %TOC did not change significantly over two years ($r^2 = 0.05$, $p = 0.6$) fluctuating between 35.5% and 37.5% (Figure 5.12). To determine the mass of TOC in the biosolids, TB, TAB, KAB (Figure 5.7), the proportion TOC, derived from the linear equations fitting the data (Figure 5.12), was multiplied by the measured mass of PMES at the relevant sampling time. In TPS the mean proportion TOC over two years, was multiplied by the measured mass of PMES at each sampling time to determine the mass of TOC (Figure 5.7).

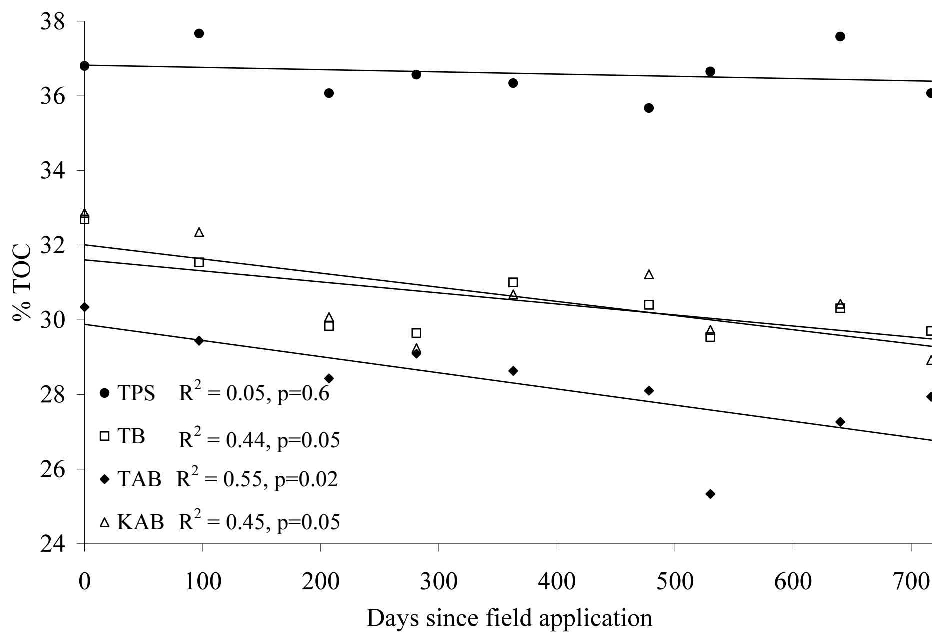


Figure 5.12. Measured %TOC over two years and linear fit equations were used to calculate C change of biosolids TB, TAB and KAB, but not primary solid TPS. Day 530 for TAB was removed for calculation of C loss (new TAB $R^2 = 0.8$, $p < 0.01$).

The concentration of TN increased in TPS ($r^2 = 0.92$, $p < 0.001$), and TAB ($r^2 = 0.5$, $p = 0.03$) over two years (Figure 5.13) while there was no significant change in TB ($r^2 = 0.03$, $p = 0.7$) and KAB ($r^2 = 0.0$, $p = 0.9$). Using the linear equation fitting the data (Figure 5.13), the percent TN for TPS was calculated to be 0.19% initially, increasing to 0.48% after two years. The total mass of nitrogen was calculated to be 0.23 g per respiration core at the beginning of the trial, increasing to 0.45 g after two years (Table 5.5). Extrapolating this increase translates to an increase of approximately 245 kg of nitrogen per hectare which may have been due to translocation of N from the underlying soil (Hart and Firestone 1991) that had a higher TN and much lower ratio of C:N (Table 5.4). Tasman aged biosolids had a weaker trend in TN ($r^2 = 0.5$ $p = 0.03$) and was calculated to have accumulated approximately 90 kg per hectare of nitrogen. There were no significant changes in the mass of nitrogen in the TB or KAB respiration cores over two years.

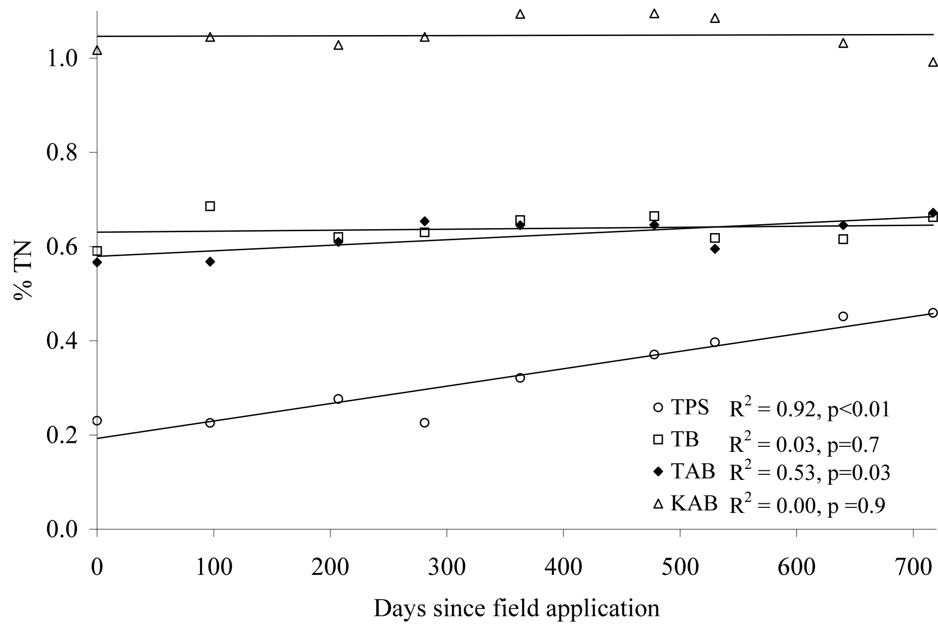


Figure 5.13 Changes in PMES % nitrogen (TN) over 24 months after field application TB and KAB TN did not change significantly.

Table 5.5. Changes in TPS nitrogen (TN) over two years showing percent nitrogen, dry weight of TPS and calculated weight of nitrogen per respiration core.

Day	% N	dw TPS/core	mg N/core
0	0.193	126.3	243
478	0.384	102.5	393
612	0.437	96.4	421
718	0.480	94.5	453

Carbon to nitrogen ratios (C:N) in PMES before field application ranged from 54-160 with primary solids (TPS) having a significantly higher ratio than did biosolids (Table 5.4). Differences in C:N between TPS and biosolids were mostly due to differences in TN rather than TOC (Table 5.4). Tasman primary solids (r^2 = 0.88, p<0.01) and TAB (r^2 = 0.83, p<0.01) showed a significant declines in C:N during the two years (Figure 5.14).

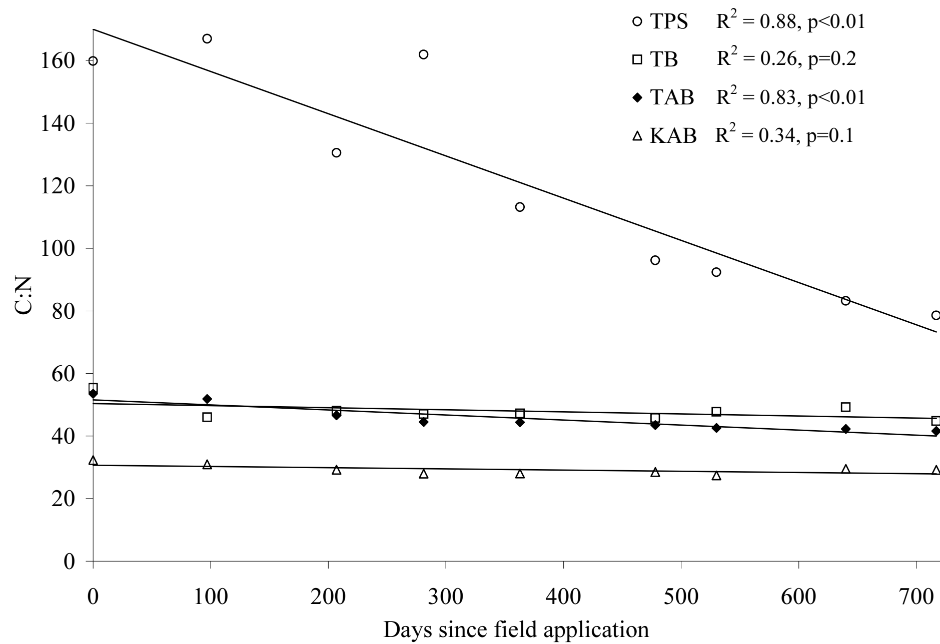


Figure 5.14 Changes in PMES C:N over 24 months after field application

Pulp mill effluent solids had organic matter (LIO_{500}) contents ranging from 45 - 70% when field applied (Table 5.4). The % organic matter (LIO_{500}) had fallen significantly ($p<0.05$) after 20 months (Table 5.6) apart from in TAB, and these declines matched the mass loss changes (Figure 5.4). The organic matter remained the same after 20 months in TAB despite a 12% mass loss (Figure 5.4), suggesting that an equal amount of inorganic matter was lost from this treatment. As outlined above (Section 5.5.1.3) the amount of CO_2 carbon lost from the TAB treatment did not account for all the mass losses (Figure 5.7). Tasman aged biosolids contained about 2% carbonate carbon, but this did not change over two years (Table 5.6) ruling this out as a significant contributor to mass loss.

Table 5.6 TOC, OM, TOC: OM and carbonate C at August 2004 (day 0) and May 2006 (day 612).

Treat.	TOC (%)		OM ($\%LOI_{500}$)		TOC:OM		Carbonate C (%)	
	Aug 2004	May 2006	Aug 2004	May 2006	Aug 2004	May 2006	Aug 2004	May 2006
TPS	36.6	36.6	69.5	62.4	0.53	0.59	2.3	1.8
TB	31.6	29.8	50.9	48.0	0.62	0.62	2.3	2.5
TAB	29.8	27.7	44.6	44.2	0.67	0.63	2.1	2.1
KAB	31.9	29.7	51.4	48.2	0.62	0.62	0.9	1.1

The TPS had significantly ($p<0.01$) higher OM content than biosolids and had a significant ($p<0.01$) decline in OM% over two years, from 69.5% - 62.4% (Table 5.6 % LOI_{500}). In the TPS this drop in percent OM was not accompanied by a drop in percent TOC, which remained stable at around 37% over the entire period, resulting in a change in TOC:OM (Table 5.6).

5.5.4.2 Acid detergent fibre (ADF) analysis of PMES

ADF analysis of PMES collected at approximately six monthly intervals over two years showed that carbon fractions changed with time (Figures 5.15 – 5.18). Different pools of carbon contributed to mass losses for the different PMES. Tasman primary solids had significantly more acid detergent fibre (ADF) than did the biosolids; this fraction represents the cellulose fraction in plant litter decomposition studies (Rowland and Roberts 1994).

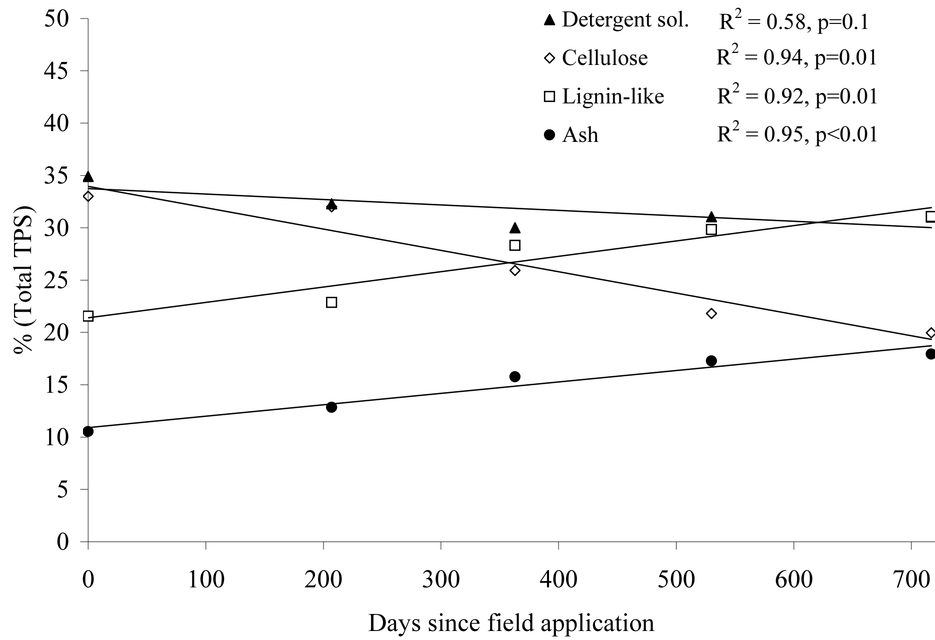


Figure 5.15 Changes in ADF fraction (% of total mass) of TPS during the two years from August 04-06 (717 days).

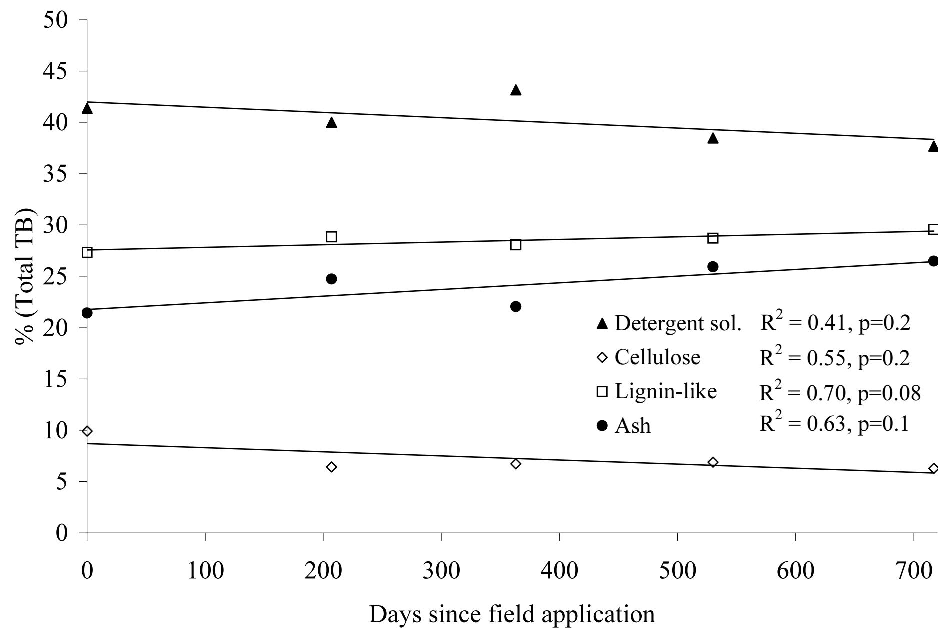


Figure 5.16 Changes in ADF fraction (% of total mass) of TB during the two years from August 04-06 (717 days).

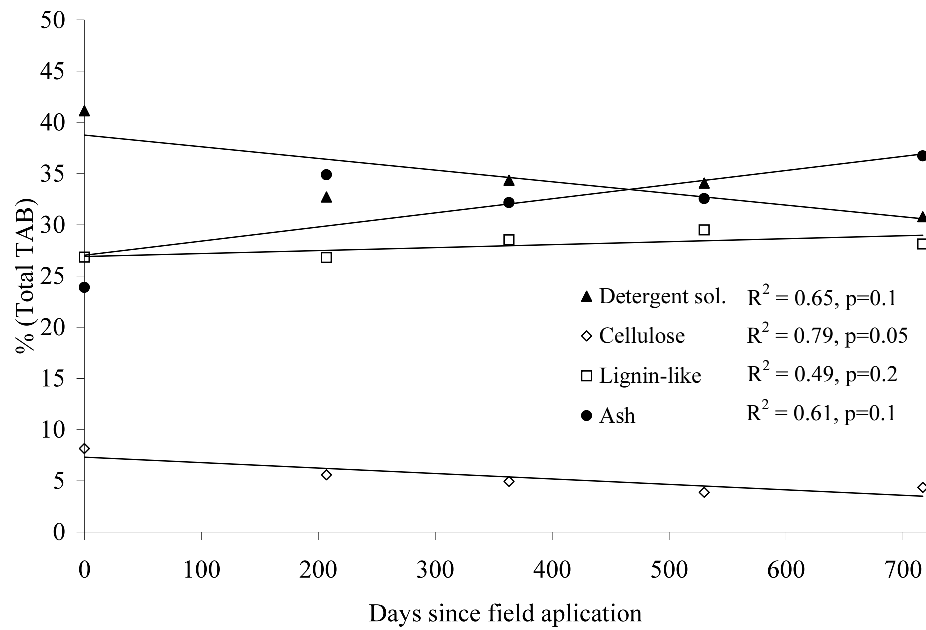


Figure 5.17 Changes in ADF fraction (% of total mass) of TAB during the two years from August 04-06 (717 days).

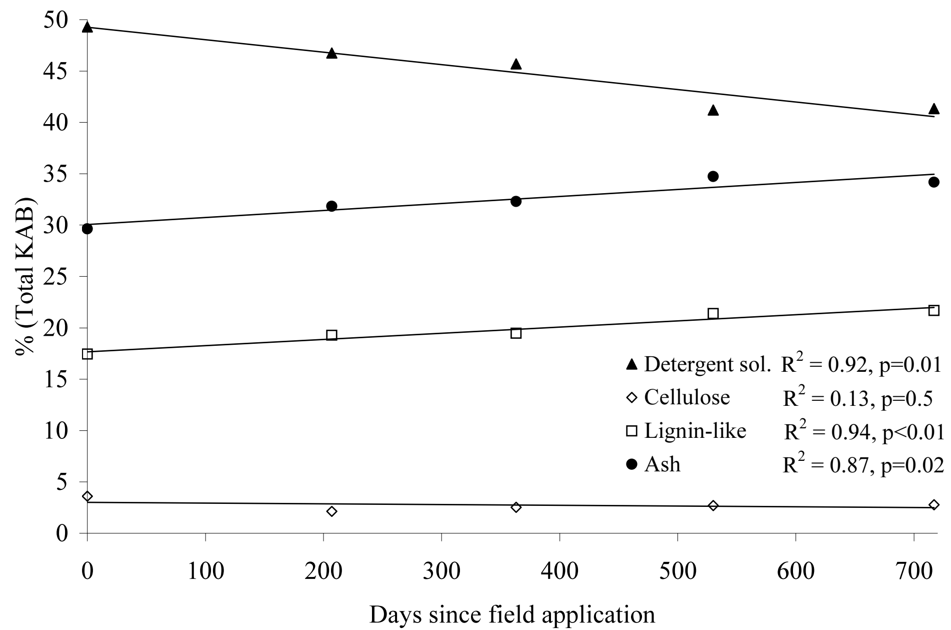


Figure 5.18 Changes in ADF fraction (% of total mass) of KAB during the two years from August 04-06 (717 days).

Due to losses in the cellulose fraction, all treatments had large decreases in the lignocellulose ratio (Table 5.7). As the cellulose fraction is preferentially metabolised compared to the acid soluble fraction, the lignocellulose ratio dropped as PMES decayed. The biosolids had a low lignocellulose ratio initially (0.17 – 0.27), indicating that they were at a late stage of decay, with KAB the most decomposed and TB the least decomposed of the biosolids. The TPS initially had a lignocellulose ratio of 0.6, having significantly more cellulose than lignin-like material and having had little decomposition prior to field application. In all treatments the lignocellulose ratio dropped by about 60% in two years (Figure 5.19).

Table 5.7 ADF fractionation of PMES showing changes (mean/respiration core) in total weight (g) of the different fractions and total dw (g) between August 2004 and 2006 (day 0 and 717).

PMES	Date	Detergent soluble	Cellulose	Lignin-like	Acid insoluble	Total dw
TPS	29.8.04	44.1	41.7	27.2	13.3	126.3
TPS	15.8.06	29.3	18.9	29.4	17.0	94.5
TB	29.8.04	53.0	12.7	35.0	27.5	128.2
TB	15.8.06	45.8	7.6	36.0	32.2	121.6
TAB	29.8.04	52.5	10.4	34.2	30.4	127.5
TAB	15.8.06	34.8	4.9	31.8	41.5	113.0
KAB	29.8.04	63.5	4.6	22.5	38.2	128.8
KAB	15.8.06	50.3	3.4	26.4	41.6	121.6

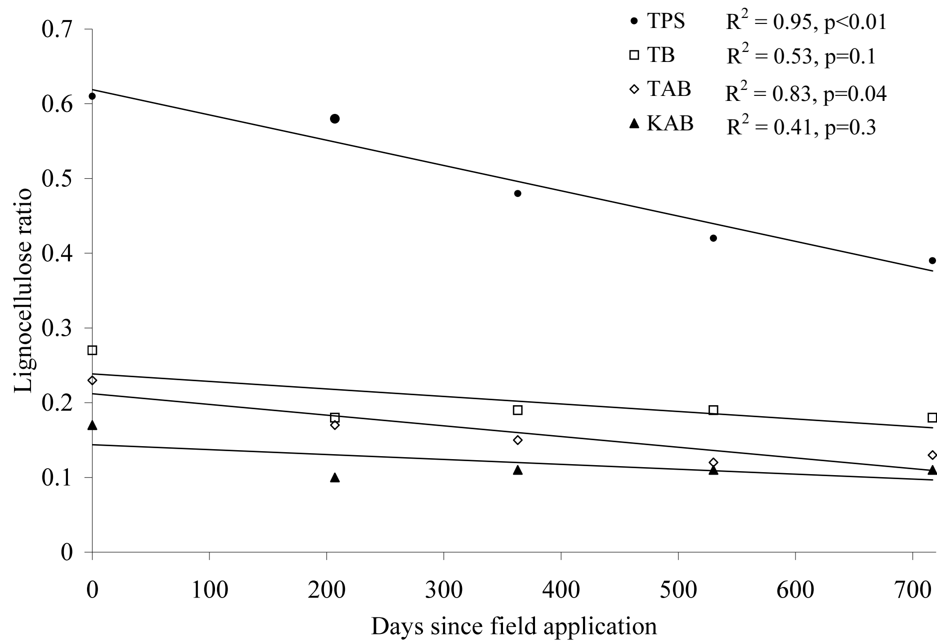


Figure 5 19. Changes in ADF ratios (acid-detergent fibre/acid-detergent fibre + acid soluble material) of PMES over two years.

In TPS, the cellulose fraction represented 34% of the total mass at the beginning of the trial and this dropped to 20% after two years (Figure 5.15). Seventy percent of total mass losses from TPS were from decomposition of cellulose. The remaining mass losses came from the detergent soluble fraction. In KAB the initial cellulose fraction was only 3.6% of total mass and this dropped to 2.8% after two years contributing less than 10% to mass losses, the remaining losses being from the detergent soluble fraction (Figure 5.18). Initially cellulose fractions in TAB and TB accounted for 8.1% and 9.9% of total mass, with these values dropping to 4.4% and 6.3% respectively over two years (Figures 5.16, 5.17). This represented 20% and 45% of the mass losses from TAB and TB, respectively, with the balance of mass losses accounted for by losses in the detergent soluble fraction in both treatments.

5.5.4.3 Microbial Biomass

Microbial biomass in soil or PMES measured in December 2005 was significantly ($p< 0.01$) different between treatments (Table 5.8). The TPS and control F/H

humus had a greater microbial biomass than did the biosolids (TB, TAB and KAB) and control soil Ah horizon. The differences in microbial biomass was not correlated to the total carbon (TOC) of the substrates (Table 5.4). The Ah soil supported the greatest biomass per unit of carbon; TPS and F/H humus were intermediate, while the biosolids supported relatively low microbial biomass per unit of carbon (Table 5.8).

Table 5.8. Biomass C (C_{mic}) letters indicates significant differences in C_{mic} between treatments, TOC and C_{mic}: TOC in December 2005.

PMES/soil horizon	C_{mic} µg/g	C_{mic} s.e.	TOC %	C_{mic}: TOC %
TPS	3015a	±125	37	0.8
TB	838b	±83	30	0.3
TAB	919b	±131	28	0.3
KAB	1011b	±59	30	0.3
F/H	2258a	±19	33	0.7
Ah	896b	±33	6	1.5

5.5.4.4 Resin Extractives

Changes in the concentration of resin extractives (RE) up to two years after field application of PMES were presented in Chapter Three. There was no apparent relationship between the rate of PMES decay and the concentration RE present in the different PMES (Table 5.9). The TPS, which had significantly greater decay than the other solids, had low concentrations of transformed resin acids and no resin acid neutrals (Table 3.3), but these compounds were lost at a similar rate to native resin acids in KAB. The KAB initially had a total RE concentration of 50,000 µg/g representing 15% of the total carbon in this treatment. The RE concentration in KAB reduced by 3.5 fold in nine months and on a mass balance basis (based on estimates from first mass loss determination after 16 months) most of the mass loss in this time appears to be accounted for by decreases in RE. In the TPS, RE (approximately 3% of TOC) had only a small contribution to the mass loss of this treatment (5%) although the mass of RE dropped by about 80%. TB and TAB lost 40% and 33% of RE mass respectively, the RE only accounting for 3% and 2% of TOC in the respective treatments.

Table 5.9 Comparison between percent mass loss and intial resin extractives (RE), resin acids and resin acid neutrals concentration.

Treatment	% Mass loss (30 months)	Total RE µg/g	Resin acid µg/g	Resin acid neutrals µg/g
TPS	26	10,941	6,523	3
TB	5	11,741	5,777	5,157
TAB	12	7,156	5,806	718
KAB	6	50,483	36,363	12,384

5.6 Discussion

PMES applied to a forest soil significantly increased the CO_2 fluxes for more than 30 months. Analysis in the second year showed that these increases were due to decomposition of PMES and there was little net change in soil respiration beneath the PMES. A needle litter study, also in the second year, showed little effect of PMES on needle litter decay processes. Enzyme activities were significantly higher in the control soil litter than litter beneath PMES, however, the implications for litter decomposition were not clear. The different PMES had different rates of decomposition, and in general, were slow to degrade.

5.6.1 Effects on the Turnover of Carbon

The application of PMES to soil greatly enhanced CO_2 fluxes, particularly in the earlier stages of the field trial. However, PMES did not greatly affect background soil respiration, as the extra CO_2 carbon output closely corresponded to the carbon losses estimated from mass losses of PMES. Generally CO_2 fluxes declined during the 30 months of the trial (Figure 5.3, Table 5.2) as did the rate of PMES mass loss (Figure 5.4). CO_2 fluxes from PMES treatments were consistently greater than the fluxes from the control soil plots despite some treatments having no significant change in PMES mass during the latter stages of the field trial. This would suggest that in these treatments (aged biosolids), the greater fluxes were due to some affect other than decay of PMES. It is possible that there was downward movement of PMES, particularly Tasman aged biosolids (TAB), out of the respiration cores early in the trial, that continued to contributed to CO_2 fluxes at later stages. It may also have been that PMES have had a stimulatory effect on respiration in the soil beneath them due to, for example, improved moisture

regime or addition of nutrients. In an adjacent field trial an increase in soil solution pH was observed as a result of PMES application (Garrett and Wang 2006), which may have enhanced soil respiration.

The decomposition of needle litter is fundamental to nutrient cycling in conifer forests (Swift *et al.* 1979), and significant disruption of this function due to PMES application is undesirable (Borken *et al.* 2002). Mass loss of needle litter, assessed after 12 months incubation during the second year of the field trial, provided no evidence that PMES affected decay processes in needle litter. Mass losses of needle litter were not significantly different between control plots, above PMES or below PMES. Comparison of needle litter from the Tasman biosolids (TB) treatment with the control soil treatment litter using ADF analysis showed there was significant decomposition after 12 months incubation, but there were no significant differences between the two treatments. This provided further evidence that PMES did not affect the rate or pathways of decomposition of needle litter.

5.6.2 Decomposition of PMES

A number of factors appear to have influenced the decomposition of PMES. Taken together these different factors show how PMES have changed in the field during the 30 month trial and provide evidence on how decomposition processes are controlled, and the ultimate fate of these types of waste material when land applied. Half-lives calculated for PMES indicate slow assimilation of these materials by receiving soils. The limit value for decomposition, the point at which decomposition stops, will be influenced by the starting OM percentage (Osono and Takeda 2005) which ranged from 45% - 70% in PMES (Table 5.4).

It was hypothesised that high concentrations of RE in PMES would negatively impact carbon turnover in soil and inhibit decomposition of PMES. The basis for this was the acute toxicity observed in aquatic organisms exposed to RE and discussed from the literature cited in Chapter 1, and also the role of many of the parent compounds found in wood that inhibit decay organisms (Hättenschwiler and Vitousek 2000; Phillips and Croteau 1999). While PMES displayed a wide range of decay rates, there was no relationship between initial RE concentrations

and PMES decay rate. Decomposition rates of RE (Chapter 3) and PMES both decreased with time, and if RE had inhibited decay of PMES, decay rate of PMES would have been expected to increase as RE concentration decreased.

Decomposition rates of PMES were not related to the total organic carbon (TOC) or total nitrogen (TN), but the C:N ratios indicate that for all PMES, particularly Tasman primary solids (TPS), decomposition was likely to have been nitrogen limited (Wang *et al.* 2006). In decomposition studies, the C:N ratio tends to decrease as decomposition proceeds due to carbon being lost as CO_2 more rapidly than nitrogen losses through denitrification and leaching (Swift *et al.* 1979). In an adjacent trial established by Scion to investigate nitrogen leaching from PMES, the concentration of nitrate in ground water was not affected by PMES application suggesting that nitrogen leaching was limited (Garrett and Wang 2006). The changes in C:N ratio of Tasman primary solids (TPS) and Tasman aged biosolids (TAB) suggested that significant losses of carbon had occurred. However, in TPS, and to a lesser extent TAB, there was an increase in the mass of nitrogen which also contributed to the decrease in C:N and the greater decomposition observed in these treatments. This extra nitrogen may have been translocated from the underlying soil horizons that had greater TN and lower C:N than the PMES, fungal translocation has been shown in previous decomposition studies (Hart and Firestone 1991). Translocation may lead to nitrogen limitation for plant growth, in soil receiving these PMES. Berg (2002) found that increased nitrogen may limit litter decomposition in the later stages of decay due to formation of recalcitrant aromatic compounds. Kinleith aged biosolids (KAB) had the highest TN of the PMES tested (Table 5.4), and the lignocellulose ratio (Table 5.7, Figure 5.19) showed that this material was in a late stage of decomposition; these factors may explain the recalcitrance of KAB.

The quantity and type of labile carbon substrates largely determined decomposition rates in PMES and the extent to which decomposition precedes. The labile carbon that was respired during the field trial was derived from different pools in different PMES. The Tasman primary solids (TPS) had a large pool of cellulose that supported significantly higher respiratory activity and greater mass losses than the biosolids. An increase in the ratio of TOC: OM

(Table 5.6) in TPS suggests that some pools of carbon had been preferentially lost from TPS, as apparent from ADF analysis (Figure 5.15). Cellulose has approximately equal masses of carbon and oxygen (McMurry 1992), while lignin-like compounds and waxes contain significantly greater mass of carbon than oxygen (Fengal and Wegener 1984). If cellulose is preferentially lost then the ratio of carbon to oxygen in OM will increase with time (Sjoberg *et al.* 2004), which in turn will result in an increase in TOC:OM as a greater proportion of the oxygen is lost, as appears to be the case for TPS. In the biosolids the TOC:OM decreased over two years, which is also consistent with ADF analysis of these materials. ADF analysis showed that for biosolids most of the mass losses came from the detergent soluble fraction, presumably from oxygen poor substrates.

The greater mass losses from TPS compared to the biosolids TB, TAB and KAB was due to the significant size of the cellulose fraction in this treatment compared to the biosolids. Most of the labile carbon respired from biosolids came from the detergent soluble fraction, which appears to support lower respiratory activity than the cellulose rich fraction. The evidence for this was that in all PMES the proportional decline over two years in the cellulose fraction (45-75% lost) was greater than in the detergent soluble fraction (15-35% lost), despite the detergent soluble fraction contributing significantly more to the total carbon pool in the biosolids (Table 5.7, Figures 5.15-5.18).

5.6.3 PMES as a Substrate for Microbial Growth

The quantity and quality of substrate carbon directly relates to the microbial biomass that can be supported by a particular soil or soil amendment (Webster *et al.* 2001). The ratio of microbial biomass carbon (C_{mic}) to total organic carbon (TOC) gives an indication of the quality of the substrate (Sparling 1992; Webster *et al.* 2001). The PMES all had high carbon contents, but the quality of carbon to support microbial biomass was greater in the primary solids (TPS) than in biosolids, in that a greater biomass was supported per unit of carbon in TPS than in biosolids (Table 5.8). The TPS as a substrate to support microbial biomass was similar to F/H humus, while the biosolids that have similar carbon content to F/H humus supported significantly less microbial biomass than TPS or F/H humus

(Table 5.8). The differences between the microbial biomass of different PMES was correlated with higher CO_2 fluxes and mass losses observed in the TPS compared to the biosolids, and was most likely due to the high cellulose content indicated through ADF analysis.

5.6.4 Enzyme Activity

The activity of extracellular enzymes involved in the decomposition of recalcitrant carbon is of great importance in forest ecosystems as much of the carbon input to these systems is in forms that are protected from decay by lignin (Kirk and Farrell 1987). PMES suppressed ABTS activity in the F/H horizon beneath PMES, suggesting that laccase enzyme activity may decrease as a result of PMES application. This, however, did not result in detectable differences in the decay of needle litter placed in the F/H horizon for one year. ADF analysis conducted on needle litter from TB and control soil treatments also showed no differences such as might be expected if lignin degradation was inhibited. Isolation of organisms on solid media (Section 5.5.3) suggested that there were still organisms in the F/H horizon beneath PMES with extracellular ABTS oxidising potential. If laccase enzymes are involved in lignin degradation in the field trial soil, and their activity has been inhibited by PMES, it may be that alternative enzyme systems can still carry out lignin degradation under PMES (Highly and Dashel 1998). It is also possible that differences in enzyme activity indicate differences that may become apparent in TOC and carbon turnover in the longer term (Speir 2002).

5.7 Conclusions

The surface application of PMES to a forest soil had limited impact on the turnover of carbon in the forest system, despite the flux of CO_2 being significantly enhanced. Increases in CO_2 fluxes was accounted for by decomposition of PMES and there were no large net changes in soil CO_2 fluxes. Needle litter turnover is an important source of nutrients in forest systems and while PMES caused no discernible differences in decay of needles, there was a decrease in enzyme activities that have been associated with litter decay. These changes in enzyme

activity suggested that there may have been subtle changes in microbial community dynamics, but these were not sufficient to alter litter dynamics during the year long litterbag study.

PMES did not adversely impact the terrestrial environment to which they were applied, although generally, they were slow to decompose. The biosolids have not lost a substantial amount of their original mass and there was a substantial decline in decomposition rate after two years. Exponential decay curves fitted to mass loss data show biosolids were likely to persist for many years before becoming fully incorporated into the soil. The primary solids (TPS) decomposed significantly more than did the biosolids but the rate of decomposition slowed significantly in this treatment after 27 months (Figure 5.4). Low nitrogen cotent in primary solids may lead to immobilisation of soil nitrogen, an issue that should be examined to determine whether this will cause nutrient deficiency in plants.

When considering the total input of carbon from PMES, the effects on soil respiration were minimal and any loss of soil carbon would be more than offset by the addition of PMES carbon. The carbon in biosolids is of lower degradability to that of natural inputs into the forest, as ADF analysis indicated there was little cellulose material remaining in biosolids. The cellulose component appeared to be important in driving the overall decomposition rate. Differences in rates of decomposition between PMES and needle litter suggested slow assimilation and a long repeat period would be required for land application. In New Zealand, *P. radiata* forests annual needle litter fall has been estimated to be around six and a half tonnes per hectare (Girishaa *et al.* 2003). This represents less than 5% of the inputs from PMES at 150 dry tonnes per hectare so carbon will most likely increase in soil receiving PMES. There may, however, be detrimental effects from this build-up of carbon if the turnover of that carbon is low. This could lead to the immobilisation of nutrients over time and may require addition of fertilisers to balance the nutritional requirements of the soil for forestry use.

On the whole, application of PMES did not alter the ecosystem functions we studied, but PMES only degrade slowly and return times before reapplication of many years are likely at these application rates.

6. Conclusions

6.1 Introduction

The safe disposal of industrial wastes is desirable in order to protect the environment for future generations. For organic wastes such as pulp mill effluent solids (PMES), beneficial reuse is seen as a better alternative than disposal into landfills. If soils are used for disposal, organic wastes may provide benefit to soil properties and forest productivity and at the least, should not negatively impact receiving environments.

This study sought to investigate the potential of PMES as soil amendments. The primary objective was to investigate potential negative impacts on terrestrial organisms and environments, due to organic compounds contained in these solids. A review of the literature revealed a history of environmental impacts associated with effluent discharges from pulp mills. This literature was focused on the aquatic environment because, historically, these wastes have largely been discharged to surface water bodies. There was a paucity of literature addressing application of pulp mill wastes to terrestrial ecosystems, partly because these wastes are not generally disposed of to soil, and partly due to a lack of negative impacts observed where these wastes have been studied.

Early in the development of the pulp and paper industry, it was obvious that effluent discharges negatively impacted aquatic environments (Ebeling 1931; Walden 1976). Gross pollution due to, for example, high BOD, suspended solids and process chemicals were easy to link to the pulp and paper industry (Ali and Sreekrishnan 2001). Many of these issues are not applicable to disposal in terrestrial environments, and so these obvious signs of environmental degradation are not observed. However, as has been shown with aquatic studies, when the industry started to 'clean up' its discharges, there were many previously unrecognised negative impacts on aquatic organisms. As effluent qualities incrementally improved, environmental impacts were reduced, but new adverse effects emerged that had not been observed in heavily polluted ecosystems. These symptoms were subtler and often were only detected over long time periods and

as circumstantial evidence began to mount (Munkittrick *et al.* 1997). To date there are many unanswered questions relating to the aquatic toxicity and mode of action of pulp mill derived organic compounds, much of this due to the complexity of the wastes produced and interactions in the recipient environment (Hewitt *et al.* 2006). The starting point for investigating terrestrial impacts of pulp mill effluent wastes was to design a series of experiments that could be integrated to provide new knowledge on terrestrial fate and effects of PMES. These types of waste contain a complex mixture of organic compounds that behave in unpredictable ways in aquatic receiving environments (Culp *et al.* 2000).

There has been very little investigation of the potential of pulp mill effluent wastes to do harm in the soil environment, with most studies focusing on plant related nutrient effects (Henry and Cole 1998; Jackson *et al.* 2000; Zibilske *et al.* 2000). While results from these studies have often found beneficial effects from land application (Phillips *et al.* 1997; Zhang *et al.* 1993), these studies have rarely looked at laboratory based cause and effect (Levy and Taylor 2003; Piearce and Boone 1998). Where PMES have been assessed for their impact on terrestrial organisms and environments there was no documented chemical analysis to relate effects to resin extractives (RE) concentrations (Bostan *et al.* 2005; Palmer *et al.* 1998). At the time this PhD research was initiated there was no information on the fate and effects of pulp mill derived RE in land applied PMES; the aim of this study was to establish if PMES posed a significant risk to the soil environment, and how these materials behave in soil. Affects were measured using soil organisms and in the soil environment, and were compared to see if there was a relationship to the extractives chemistry of PMES.

A series of hypotheses were formulated concerning the terrestrial toxicity and persistence of RE, and the potential for PMES containing these RE to negatively impact soil processes. Hypothesis testing involved a range of interdisciplinary methods designed to provide information from different levels of biological organisation and evidence from overlapping fields of study (Figure 6.1). This thesis research was inter-disciplinary and conclusions drawn from it are based on a weight of evidence generated through these different observations.

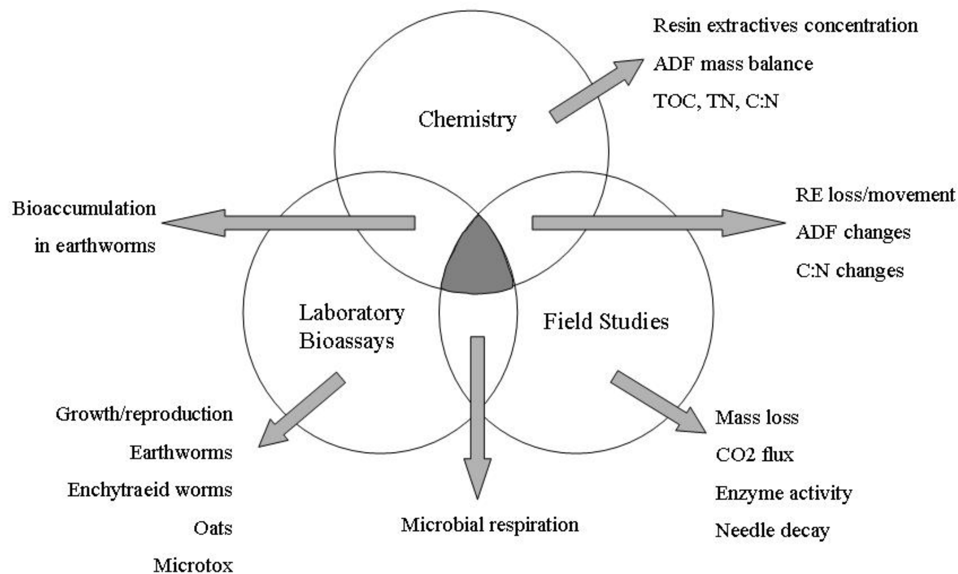


Figure 6.1 Quality triad developed for PhD research to assess the risks of PMES in the terrestrial environment. The arrows indicate where methods fitted into various compartments. There was overlap of methods between two compartments for the main areas of chemistry, bioassays and field studies, however there was no overlap of all three compartments (grey central area), which might be investigated in future work.

6.2 Bioassays Used to Test Toxicity of PMES to Soil Organisms

Much of the knowledge on the toxicity of pulp mill effluent wastes was acquired through the use of aquatic bioassays. While these bioassays have difficulty in predicting environmental effects, they have been used to demonstrate many of the mechanisms involved in the toxicity of pulp mill effluent wastes and environmental impacts observed. Based on the RE chemistry of the PMES tested, it was expected that these materials would pose a significant threat to aquatic organisms, and tests on aqueous extracts from PMES demonstrated that these solids contained bioavailable compounds that were toxic to the Microtox®

organism. Toxicity of pulp mill effluents to Microtox® has been shown to be comparable with toxicity observed in other aquatic organisms (Munkittrick and Power 1991).

A battery of single organism toxicity tests using soil organisms representative of primary producers and heterotrophs from different functional groups showed that undiluted PMES were not acutely toxic to these organisms. Inhibition of enchytraeid reproduction indicated that PMES could impact some terrestrial species, but it was not clear what caused this inhibition. A study of the toxicity of eight PAH's to enchytraeids by Sverdrup *et al.* (2002a) showed that these compounds had low acute toxicity to adults. For example, phenanthrene did not cause significant mortality over a 21 day exposure at concentrations above 2500 μg/g. Reproduction was found to be far more sensitive, with the concentration causing 50% mortality up to 42 fold higher than the concentration causing a 50% drop in reproduction (Sverdrup *et al.* 2002a). RE concentrations in the PMES were not correlated with the number of enchytraeid juveniles produced. The 100% KAB replicates had an average of 3 juveniles compared to 58 in the control, but when KAB were diluted by 50% with clean soil an average of 40 juveniles were produced and this was not significantly different from the control (Figure 4.3). The concentration of retene (an alkyl substituted phenanthrene) was approximately 3500 μg/g in the 50% KAB, well above the concentration of phenanthrene that Sverdrup reported (80 – 160 μg g^{-1}) to reduce reproduction by 50% in a sandy loam soil. There were major differences between these two studies, in OM content (1.6% in sandy loam and 32% in KAB), and in residence time of the compounds, with PAH's spiked into sandy loam while RE had accumulated over many years in KAB. These factors may have influenced the bioavailability of RE compounds in the PMES. It can not be ruled out that different factors may have been responsible for reducing enchytraeid reproduction in the different PMES treatments, as there was a wide range of compound concentrations and speciation between different PMES.

Generally, bioassay results with soil organisms were in contrast to results of aquatic bioassays found in the literature. Bioassays on soil organisms showed that

PMES enhanced some endpoints tested, compared to a reference soil, and had low acute toxicity to the test organisms.

6.3 Soil Respiration

In laboratory incubations, addition of PMES to forest soil significantly enhanced soil respiration. Respiration from PMES was also significantly greater than from forest soil, however, when respiration was normalised per gram of carbon, soil/PMES mixtures had significantly higher respiration than PMES or soil on their own (Section 4.4.4). There are a number of possible factors that might explain this. PMES may have caused a beneficial nutrient response in the soil population adapted to a nutrient limited environment. Conversely, PMES, which were likely nitrogen limited, may have provided labile carbon while soil which had a low C:N (Table 5.4) may have been a significant source of nitrogen. The greater respiratory capability of the soil/PMES community may have been due to greater functional diversity in soil compared to PMES.

TPS treatments respired significantly more CO_2 than biosolid treatments, whether mixed with soil or at 100% concentration (Figure 4.4), and TPS also had significantly higher CO_2 fluxes and mass losses than biosolids in the field trial (Figures 5.3 and 5.4). Associated with this higher respiratory activity was a significantly higher microbial biomass (Figure 4.20) and also C_{mic}:TOC ratio. Acid detergent analysis of PMES may explain these differences, as TPS were the only PMES that had a significant proportion of carbon in the cellulose fraction, this presumably representing the most labile pool of carbon. While TPS initially had significantly less nitrogen than biosolids, nitrogen accumulated during two years in the TPS field plots (Table 5.5 and Figure 5.13), possibly through translocation from the underlying soil. This accumulation could have alleviated a nitrogen limitation in the TPS treatment.

There was general agreement between laboratory and field methods used to investigate microbial respiration and activity. TPS supported greater activity than biosolids while all PMES had significantly higher CO_2 output than the control soil (Figure 4.4 and 5.3).

6.4 Field Study on the Fate and Effects of PMES in a Terrestrial Environment

In the field trial, the significant increase in CO_2 fluxes due to applied PMES appeared to be primarily due to decomposition of PMES, as calculated mass loss of PMES-C and cumulative CO_2 carbon fluxes were similar. Both PMES mass losses (Figure 5.4), and CO_2 fluxes from PMES plots declined during the 30 months trial (Table 5.2). Decay of PMES was approximated by an exponential decay function (Figure 5.4). Compared to needle litter decay (approximately 30% mass loss in 12 months), PMES had a slow decay rate, particularly the biosolids, which lost only between 5 and 12% of their mass during the 30 months trial. PMES have a greater inorganic component than needle litter (30-55% vs <5%), but even if this is taken into account the decay rates of PMES were significantly slower than reported needle litter decay rates (Girishaa *et al.* 2003).

There was no evidence that PMES significantly affected the mass loss of needle litter in the soil F/H horizon, or in needle litter that accumulated above PMES (Figure 5.8). The lignocellulose ratio in needle litter from TB and control treatment litterbags after 12 months incubation was not significantly different suggesting similar decay processes were operating in both treatments (Figure 5.9).

The ability to oxidise ABTS has been used to investigate extracellular laccase enzyme activities in soil (Baldrian *et al.* 2000; Lorenzo *et al.* 2005). Needle litter from the F/H horizon beneath PMES had significantly reduced ABTS activity, suggesting inhibition of, or reduction in, extracellular laccases. The cause of inhibition of ABTS activity was not investigated, however microorganisms with ABTS oxidising capability could be isolated from needle litter beneath PMES that had no ABTS activity (Chapter 5, section 5.5.3).

Analysis of changes in RE revealed that all compounds degraded rapidly in some PMES, but more slowly in other PMES with some compound groups being recalcitrant (Tables 3.4.1 - 3.4.4). The recalcitrance of resin acids in TB and TAB was difficult to explain as resin acids have been shown to degrade rapidly in aerobic environments (Liss *et al.* 1997). Kinleith aged biosolids had substantially higher concentrations of RE than did Tasman biosolids, but a similar range of

compounds. The KAB rapidly lost all compound groups, however KAB had a similar total mass loss to TB, while both these biosolids were significantly slower to decay than TAB (Figure 5.4). The Kinleith mill KAB were generated from a different type of effluent than the Tasman biosolids, and may have had a different community of microorganisms capable of degrading resin acids. The treatment system that KAB was derived from had historically received very high resin acid loading (Stuthridge *et al.* 1991) and may have developed a community better adapted to resin acid degradation. Resident microbial species in the different biosolids at the time of field application may have been responsible for most of the resin acid decomposition during the following two years, as high resin acid concentrations can be toxic to, or inhibit non-acclimated populations (Liss *et al.* 1997; Yu and Mohn 2002).

There was a clearer relationship between RE loss (Table 3.4.1) and mass loss (Figure 5.2) in the TPS, with both declining significantly over two years. A possible mechanism is the substantial amount of acid detergent fibre, indicating high cellulose content, in this treatment which may have provided an easily respired substrate that maintained a high rate of RE degradation.

6.5 Weight of Evidence

A range of techniques was used to assess the risk PMES pose in terrestrial ecosystems. Experiments were conducted under controlled laboratory conditions and in the field under environmentally relevant conditions. Figure 6.1 shows that there were overlaps in three general areas of study. Bioassays and chemical analysis showed that responses by organisms did not reflect PMES RE composition, suggesting either low bioavailability or low acute toxicity of these compounds to the test organisms. Chemical analysis of worm tissue showed that RE were bioavailable and there was potential for some bioaccumulation in earthworms (Table 4.2), although bioaccumulation was generally low compared to what was found in other studies (Hyotylainen and Oikari 2004; Ma *et al.* 1995). Reproductive effects in enchytraeids raise some concerns for field application of PMES, especially as enchytraeids are important species in conifer forests, where earthworms are largely absent (Jansch *et al.* 2005). Similar responses in the field

and laboratory were observed with microbial respiration and indicated that soil carbon turnover was little affected by PMES, although at a lower level of organisation, enzyme activity was reduced under PMES. ADF analysis of PMES helped to explain decomposition rates of PMES and RE.

Table 6.1 provides a summary of the important findings of this research.
Various aspects of field and laboratory studies are given a weighting based on a generally positive effect, no significant effect or a negative effect or perceived risk. Overall, Table 6.1 demonstrates the PhD research findings that PMES pose a low risk in the terrestrial environment with some benefits to soil, but a cautious approach should be adopted as some negative effects were observed that warranted further investigation

Table 6.4 Summary of effects observed through exposure to PMES or behaviour of PMES in the soil environment. Symbols represent (0) no significant affect/low risk, (+) significant enhancement, (-) significant inhibition or some cause for concern.

	TPS	TB	TAB	KAB
Laboratory study				
Oats survival	0	0	0	0
Oats early growth	+	+	+	+
Earthworm survival	0	0	0	0
Earthworm growth	0	0	0	0
Earthworm reproduction	0	+	+	0
Enchytraeid survival	0	0	0	0
Enchytraeid reproduction	-	-	0	-
Microtox	-	-	0	-
Microbial respiration	+	+	+	+
Field study				
Soil respiration	0	0	0	0
Needle decay	0	0	0	0
PMES decomposition*	0	-	-	-
ABTS activity	-	-	-	-
RE decomposition*	0	-	-	0

* based on decomposition rate with no control treatment

6.6 Future Work

The demonstrated toxicity of pulp mill RE compounds to aquatic organisms necessitated a thorough investigation of PMES containing high concentrations of RE, before widespread disposal in terrestrial ecosystems is adopted. Results of the current research suggest PMES pose a low risk to soil organisms and processes, but a cautious approach is still recommended to their disposal. It is also recommended that there be further investigation of the fate and effects of PMES in terrestrial systems to address questions arising from this research.

Future research might focus on three areas including reasons for a decreased enchytraeid reproduction, reduced enzyme activity in forest litter and recalcitrance of resin acids. These were the main indicators that recorded negative impacts (Table 6.1), where some risk to the terrestrial environment was suggested.

Life cycle studies have provided useful information on the toxicity and mechanisms of action of pulp mill effluent wastes, in aquatic systems (Parrott *et al.* 2006). Equivalent terrestrial studies may be useful to determine risk of land applying PMES. Resin extractive compounds in PMES were not toxic to the soil organisms tested and it was not established if this was due to limited bioavailability and uptake or whether these compounds have low toxicity to terrestrial organisms compared to aquatic organisms. This could be addressed by spiking soil with selected extractives in dose response bioassays on soil organisms.

Resin acids are reported to rapidly degrade under aerobic conditions (Kainulainen and Holopainen 2002; Liss *et al.* 1997), but this study showed that resin acids have variable decay rates which were apparently influenced by the source material. Half-lives of ten years were calculated for some resin acids in some biosolids and further research is required to elucidate the factors influencing resin acid decay rates in PMES applied to soil.

It is not known why ABTS enzyme activity was reduced in needle litter beneath PMES compared to control treatment litter. While treatment related differences in carbon turnover were not detected, subtle changes in carbon dynamics would be

unlikely to have been detected. The results of ABTS enzyme assays may indicate changes in community structure and function and further investigation of these lower levels effects is recommended.

The grey region within Figure 6.1, where all three areas of research (chemistry, bioassays and field studies) intersect, would be a useful area to conduct further research. An experiment that provided this sort of information would combine aspects of RE chemistry, exposure of organisms from field relevant populations under controlled laboratory conditions. One such technique that could fulfil these criteria is catabolic response profiles (Degans and Harris 1997). Field soil that had been exposed to RE or PMES could be sampled to determine the metabolic response to a range of simple carbon substrates. Information could be gained from the type and range of substrates that were metabolised by different communities. A reduction in catabolic diversity, compared to a control soil, may result from RE exposure. Resin acids could be included as substrates to gain knowledge on the ability of the microbial community to degrade resin acids.

6.7 Recommendations

Land application of PMES appears to pose a low environmental risk, while there are potential benefits such as increased SOM and mineral nutrients. Factors that need to be considered by land managers are the slow decomposition rate and high C:N ratio of PMES. Combining primary and secondary treated solids may alleviate problems associated with these factors. Other ways to mitigate this could be to combine with other high nutrient wastes such as municipal biosolids or to supplement with mineral nutrients as required. Due to the high RE content of PMES and additionally where these compounds are recalcitrant, care needs to be taken to prevent runoff into adjacent waterways. It is recommended that a buffer zone adjacent to waterways be created and PMES not be applied on land where there is a risk of erosion or mass movement of PMES into aquatic ecosystems.

The pulp and paper industry has reduced and improved the effluent waste stream entering aquatic environments substantially in recent decades. Part of mitigating the environmental impact of pulp mill effluent wastes may involve land disposal, however, a cost benefit analysis should take a holistic approach, including

consideration for all aspects of sustainability. In 2007, accounting for carbon pools contributing to atmospheric CO_2 or as a sink for CO_2 has become topical. Part of the cost benefit analysis should probably include carbon emissions associated with different disposal options. While land disposal offers potential benefits to soil, these benefits should be carefully weighed against potential adverse affects on the greater environment.

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Appendix I Surrogate standard recoveries for SFE

Table 1 Indicating which samples met surrogate standard recovery criteria

Date	Sample	Meet RSD Criteria*	
22.7.04	B3	Yes	
22.7.04	B3	Yes	
22.7.04	B3	Yes	
22.7.04	B3	Yes	
22.7.04	B1	Yes	
22.7.04	B1	Yes	
22.7.04	B1	No	No resin acid recovered
22.7.04	B2	No	No resin acid recovered
22.7.04	B2	Yes	
22.7.04	P/B	Yes	
22.7.04	P/B	Yes	
22.7.04	P	Yes	
22.7.04	P	Yes	
22.7.04	P	Yes	
22.7.04	B4	Yes	
22.7.04	B4	Yes	
22.7.04	B4	Yes	
22.7.04	B4	No	Low resin acid recovery

*Meets recovery standard criteria if recoveries were greater than 25% and less than 140%

Table 2a Surrogate standard recoveries (%) for 9-month SFE on PMES.

Sample Date	2,4,6-Tribromoanisole)	2,4,6-Tribromophenol	D10-Anthracene	D31-Palmitic acid	8(14)-Abietenic acid	Dihydrocholesterol
TPS 26.8.04	70	69	63	72	72	55
TPS 26.8.04	74	76	61	75	76	59
TPS 26.8.04	80	79	55	81	83	67
TPS 3.12.04	60	57	38	55	49	48
TPS 3.12.04	70	67	49	66	64	57
TPS 3.12.04	83	76	57	70	70	60
TPS 23.3.05	62	68	55	62	51	53
TPS 23.3.05	63	70	59	67	60	57
TPS 23.3.05	63	61	51	65	51	58
TPS 6.6.05	68	71	55	68	63	63
TPS 6.6.05	49	51	48	61	47	59
TPS 6.6.05	63	56	49	62	43	55
TB 26.8.04	61	61	59	58	55	39
TB 26.8.04	74	74	72	71	66	47
TB 26.8.04	61	64	61	64	65	50
TB 3.12.04	68	61	61	58	18	61
TB 3.12.04	62	64	62	53	28	62
TB 3.12.04	0	0	0	0	0	0
TB 23.3.05	60	53	55	30	3	25
TB 23.3.05	61	54	55	49	15	35
TB 23.3.05	61	54	55	49	15	35
TB 6.6.05	67	62	60	58	13	37
TB 6.6.05	39	30	25	22	7	25
TB 6.6.05	67	67	61	56	18	47
TAB 26.8.04	61	48	50	23	1	30
TAB 26.8.04	66	54	51	28	1	33
TAB 26.8.04	64	52	50	32	3	35
TAB 3.12.04	47	54	51	49	16	57
TAB 3.12.04	59	65	59	60	32	61
TAB 3.12.04	67	67	62	68	38	64
TAB 23.3.05	53	46	48	47	27	19
TAB 23.3.05	54	52	49	56	45	31
TAB 23.3.05	64	58	54	55	47	35
TAB 6.6.05	48	41	41	40	10	28
TAB 6.6.05	51	48	47	47	24	37
TAB 6.6.05	49	42	42	41	28	31
KAB 26.8.04	89	92	89	107	96	93
KAB 26.8.04	86	88	84	106	94	87
KAB 26.8.04	87	86	81	98	88	77
KAB 3.12.04	84	84	78	100	83	80
KAB 3.12.04	90	88	78	105	88	93
KAB 3.12.04	81	79	74	92	79	81
KAB 23.3.05	88	88	76	103	81	88
KAB 23.3.05	80	79	73	92	71	79
KAB 23.3.05	86	85	78	99	76	86
KAB 6.6.05	85	83	74	95	78	70
KAB 6.6.05	81	81	72	95	81	80
KAB 6.6.05	81	79	71	88	81	77

Table 2b Surrogate standard recoveries (%) for SFE of soil – Ah and F/H horizons on 6.6.05.

Sample	2,4,6-Tribromoanisole	2,4,6-Tribromophenol	D10-Anthracene	D31-Palmitic acid	8(14)-Abietenic acid	Dihydrocholesterol
control Ah	80	88	79	94	17	82
control Ah	1	22	78	90	11	75
control Ah	5	35	75	92	26	82
TPS Ah	77	89	89	62	0	51
TPS Ah	70	76	84	39	0	58
TPS Ah	84	91	92	75	11	63
TB Ah	101	91	92	77	8	52
TB Ah	76	89	93	95	13	78
TB Ah	67	78	87	82	0	67
TAB Ah	73	64	85	6	0	22
TAB Ah	91	96	92	89	3	85
TAB Ah	75	87	83	95	37	79
KAB Ah	90	89	85	91	6	77
KAB Ah	79	67	77	45	0	52
KAB Ah	81	88	86	80	6	78
control F/H	63	45	51	49	25	60
control F/H	88	71	65	88	58	65
control F/H	83	66	63	88	65	76
TPS F/H	76	55	59	80	39	80
TPS F/H	84	73	66	93	62	74
TPS F/H	89	69	64	101	66	71
TB F/H	68	44	52	50	41	83
TB F/H	70	42	48	73	50	79
TB F/H	51	35	40	53	35	79
TAB F/H	73	50	60	36	2	75
TAB F/H	65	47	54	45	7	75
TAB F/H	84	60	67	87	26	81
KAB F/H	46	38	44	33	12	71
KAB F/H	61	40	47	38	7	69
KAB F/H	72	66	54	100	79	79

Table 3 Surrogate standard recoveries (%) for 24-month SFE. Numbers in red were samples with low recoveries where data was not used in calculations.

Sample	Date	2,4,6-Tribromoanisole	2,4,6-Tribromophenol	D10-Anthracene	D31-Palmitic acid	8(14)-Abietenic acid	Dihydrocholesterol)
TB	26.8.04	71	74	69	81	89	78
TB	26.8.04	86	82	81	81	89	80
TB	26.8.04	103	90	88	89	94	85
TB	3.12.04	87	85	82	92	95	90
TB	3.12.04	105	94	87	95	99	90
TB	3.12.04	110	99	90	103	105	99
TB	6.6.05	88	92	81	95	95	86
TB	6.6.05	92	92	82	93	95	89
TB	6.6.05	88	95	88	100	101	92
TB	23.3.05	85	75	86	97	98	91
TB	23.3.05	88	94	85	99	103	91
TB	23.3.05	84	90	85	99	102	91
TB	26.8.05	94	86	88	98	99	88
TB	26.8.05	105	98	92	99	100	90
TB	26.8.05	100	100	91	103	107	93
TB	19.12.05	99	77	91	102	102	96
TB	19.12.05	107	92	90	104	105	97
TB	19.12.05	112	102	93	102	105	97
TB	9.2.06	108	94	89	96	98	89
TB	9.2.06	98	93	84	95	97	86
TB	9.2.06	109	101	93	98	101	90
TB	30.5.06	113	103	93	103	106	93
TB	30.5.06	101	98	93	99	102	89
TB	30.5.06	113	101	93	98	101	89
TB	15.8.06	108	101	98	102	106	92
TB	15.8.06	**17**	**43**	**41**	60	65	**42**
TB	15.8.06	88	94	97	105	107	95
TAB	26.8.04	**6**	**3**	**3**	**14**	**20**	90
TAB	26.8.04	94	94	80	101	103	92
TAB	26.8.04	87	89	74	101	108	89
TAB	3.12.04	110	98	74	99	103	83
TAB	3.12.04	114	103	83	103	104	81
TAB	3.12.04	123	93	94	102	82	93
TAB	23.3.05	118	107	93	103	84	99
TAB	23.3.05	128	113	101	110	85	102
TAB	23.3.05	112	101	76	101	102	90
TAB	6.6.05	**0**	**0**	**0**	**2**	**2**	**1**
TAB	6.6.05	100	99	77	99	101	24
TAB	6.6.05	110	100	78	96	96	93
TAB	26.8.05	119	108	81	107	108	153
TAB	26.8.05	89	99	93	104	107	92
TAB	26.8.05	110	101	98	108	106	95
TAB	19.12.05	119	99	97	105	104	91
TAB	19.12.05	122	104	95	103	105	80
TAB	19.12.05	125	107	100	106	106	62
TAB	9.2.06	116	107	102	107	116	102
TAB	9.2.06	127	113	102	115	116	103
TAB	9.2.06	85	102	90	110	114	100
TAB	30.5.06	100	110	94	116	119	107
TAB	30.5.06	135	110	98	116	116	104

Table 3 Continued surrogate standard recoveries (%) for 24-month SFE. Numbers in red were samples with low recoveries where data was not used in calculations

Sample	Date	2,4,6-Tribromoanisole	2,4,6-Tribromophenol	D10-Anthracene	D31-Palmitic acid	8(14)-Abietenic acid	Dihydrocholesterol)
TAB	30.5.06	136	118	101	118	117	104
TAB	15.8.06	133	88	94	117	116	107
TAB	15.8.06	**18**	**43**	**38**	63	68	53
TAB	15.8.06	88	100	82	109	111	99

Appendix II: Media Recipes

Yeast Media (YM)

Yeast extract 2g
Malt extract 15g
Agar 15g
Water 1 L

Basidiomycete Media (BAS)

Yeast extract 2g
Malt extract 15g
Benlate 60mg
Agar 15g

Water 1L
Autoclave
Streptomycin 10mg
Lactic acid 2ml

ABTS underlay

ABTS 50mg
20x mineral media 5ml
Agar 2g
Water 100ml

Mineral media (20x)
K_2HPO_4 2g
KCl 2g
Mg $SO_4 . 7H_2O$ 2g
Water 1L