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Die-off of pathogens and assessment of risks following biosolids application in Pine Forests

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Abstract

Timber is one of a few agricultural products that is not linked to the human food chain, and thus can be submitted to a number of practices not usually considered appropriate for other markets such as horticulture. Direct application of biosolids to plantation stands as a substitute for commercial fertilisers is one of these practices. However, there maybe significant health issues in regards to the potential for pathogens to be introduced to the environment through the use of the biosolids. Despite the treatment processes that wastewater sludge undergoes to be classified as biosolids prior to direct land application, some pathogens survive and therefore there is a potential for infection of the local human population.

The research objective of this study is to establish the human health risk of using biosolids in a plantation stand. The die-off of indicator pathogens will be monitored to establish their ability to survive once the biosolids are applied to land. The health risks of airborne pathogens will be determined through studies into the ability of pathogens to be transported via the smoke from a burn off scenario.

A literature review established that the indicator pathogens for this study would be *Escherichia coli*, *Clostridium perfringens*, *Salmonella spp.*, and Coliphage. Preliminary results indicate that there are some colonies of the indicator pathogens present but at very low levels, however further results are needed to establish a firm conclusion. Preliminary results show there is potential for some pathogens to be transported via the smoke initiated from a burn off. Given the low pathogen numbers in the applied biosolids, spiked biosolids samples have also been used to provide a point of comparison. These preliminary results support the current literature, however further analysis is required in order to firmly establish the health risks of this potential occurrence.

Further research to intensively monitor the pathogen die-off in the first year post-application is required and will be combined with the data from the multiple years' post-application samples. Research into the ability of dust generated from the biosolids due to vehicle movement to transfer pathogens is also required to establish the health risk both to members of the general public and also the plantations workers. The effects that the pine trees themselves and specifically the addition of pine needles to the soil organic matter will also be researched to establish if this phenomenon has an effect of the die-off of pathogens.

The results from this study can be transposed to agricultural systems and may aid in future decision making in regards to the use of biosolids particularly areas linked to the human food chain.

Introduction

The use of biosolids as a substitute for fertiliser is an increasing trend worldwide due to the benefits of diverting it from landfill and reducing the use of chemical fertiliser (Epstein, Wu et al. 2001). A study by Meyer *et al.* (2004) found that when biosolids were applied to burnt site undergoing rehabilitation, the plant biomass increased with an increase in the addition of biosolids. Further still, the biosolids also have the ability to improve soil physical properties (Poucher, Francoise et al. 2007).

These findings show that the benefits of biosolids to the agricultural sector could be significant; however there are issues in that the biosolids material does contain pathogens and constituents that are capable of causing adverse human health effects. This is an issue as it is in the public interest and the risk to the end consumer of the agricultural products governs the possible use of biosolids in the production of the produce. Unlike agricultural products, timber producing plantations are not intricately linked to human health and the key issues lie in the risk associated with the application of the biosolids to the land.

Human pathogens that are present in biosolids and are considered a concern include viruses, bacteria, protozoa and helminths (National Water Quality Management Strategy (NWQMS) 2004). With such a significant potential to adversely affect human health, it is unsurprising that there are rigorous guidelines worldwide in regards to the pathogen content of biosolids and their respective potential uses.

The United States Environmental Protection Agency (USEPA) has developed criteria that allows for land application dependant on the biosolids achieving a Class A or Class B standard (United States Environmental Protection Authority 2000). In Australia, a grading system has been developed with 4 different grades that can allow biosolids to be used in residential scenarios (grade P1) down to only being acceptable for landfill (grade P4) with grades P2 and P3 being reserved as acceptable for different forms of agriculture (National Water Quality Management Strategy (NWQMS) 2004).

Whilst there are many studies worldwide that have been concerned with the land-application of biosolids, they have been concentrated on agricultural land with a direct link to the human food chain. Studies on the land-application of biosolids in timber plantations are limited, with few projects worldwide being concerned with the issue. Horswell *et al* (2007) conducted research with biosolids applied to a pine forest and found that microbial die-off was related to the breakdown of the biosolids themselves. Another significant project is being undertaken in the United Kingdom (Scotland and Northern Ireland Forum for Environmental Research), but is unfinished at the present time.

Biosolids Application in Pine Plantations

In Western Australia (WA) wastewater is treated by the Water Corporation and the disposal of biosolids is also their concern. In conjunction with the Forest Products Commission (FPC), the Water Corporation is investigating the possibility of using dewatered biosolids as a substitute for fertilisers in pine plantations. A major concern of the FPC and the Water Corporation are the risks associated with the biosolids that accumulate on or near the roadways within the pine forests and the potential interaction that plantation workers have with these biosolids. Although application of biosolids is not intended on and within the immediate proximity of the roadways, it occurs through ‘spillage’ as the biosolids are transferred from the transporting vehicle and/or the roadside containment vessel to the spreading vehicle that applies the biosolids to the land. This spillage amounts to a significant layer of biosolids building up especially considering that the depth of application in the pine forested areas is only around 10cm.

Dust

The issue of dust originating from the biosolids when they become dry is a concern. This issue is confounded by the fact that the plantation and the application sites are accessible to the public, such as horse riders and motorbike riders. Their safety is of concern as the plantation is public land and therefore restricted access cannot be enforced. The risk to forestry workers and biosolids application personnel is also of concern, however policies can be enforced to ensure that the workers utilise safety equipment whilst working in the vicinity of a biosolids application site.

The activities undertaken by these groups are capable of producing large quantities of airborne dust especially in summer that have the potential to be inhaled, ingested and for contact with the skin to be made, all of which are routes for human exposure (Pillai 2007). Burton and Trout (1999) suggested in a study on biosolids application that the symptoms observed by the workers were probably caused by inhalation of dust from biosolids. Rylander (1999) attributed bioactive organic dust to be the cause of various symptoms and Lewis *et al*. (2002) found that residents living within 1 kilometre of a biosolids land application site complained of a number of symptoms that can be caused by pathogens commonly found in biosolids. Robinson *et al*. (2006) conducted a survey of workers exposed to biosolids on daily basis and whilst coughing, sore throats, headaches and sinus effects were recorded, gastrointestinal, cardiovascular, musculoskeletal, fatigue, fever and flu-like symptoms were not reported.

Smoke

Another issue related to that of dust is, the possibility that the pathogens can be transported via the smoke of fire in the pine plantation. There appears to be no significant literature on the potential of pathogen transmission via the smoke of a fire, but there is significant justification on the serious potential of pathogens to be aerosolised and be transported on the wind. Whilst this method of aerosolisation is widely considered to be unlikely due to the fire destroying the pathogens before they can be transported, there is a possibility for the pathogens to survive. Fires in pine forests can be low-intense burns that can also burn very fast. This combination can result in i) the fire not becoming hot enough to completely destroy the pathogens, and ii) the fire burning too fast to completely destroy the pathogens. The end result is the possibility that the pathogens could become aerosolised and transported by the wind.

Effect of the Pine Trees on the pathogens

Another issue that could affect the ability of the pathogens to survive in the land-applied biosolids is the microclimate found specifically in pine plantations. The dropped pine needles could potentially affect the pathogen survival of the applied biosolids in three ways; biological, chemical and physical. Physical is perhaps the easiest to determine as essentially the pine needles have the ability to form a layer above the biosolids and therefore could not only prevent light and moisture reaching deep into the surface layers of the biosolids and the soil, but also could create an optimum growth environment for the pathogens by creating a warm, humid environment. The biological effects are primarily the soil microorganisms and their interaction with the pathogens. The effect of the soils themselves on the microorganisms is significant with Rogers and Smith

(2007) concluding that the interactions with protozoa and other soil bacteria and bacteriophages do affect the survival of the pathogens. It was also found that the protozoa can limit the colonisation of the soil by the pathogens thus limiting their ability to survive and/or regrow.

However the effects of chemicals from the pine needles are potentially the most suited to affecting the pathogens. Not only can the chemical effects of the pine needles potentially affect the soil microorganisms and therefore their ability to interact with the pathogens, but the chemical interactions may also directly affect the pathogens themselves. Whilst these suggestions are only hypotheses, there does not appear to be significant literature to support the specific interactions between pine needles and their respective biological, physical and chemical effects on the pathogens found in biosolids.

Research Objectives

This research is a preliminary study to assess the human health risk of pathogen survival and re-growth in land-applied biosolids. Biosolids that met the Class B USEPA classification and at least the P3 classification in Australia were applied to sites within the pine plantation. The study will conduct research on the die-off of pathogens within the land-applied biosolids under the conditions of the Myalup Pine Plantation. Research will also be conducted to establish the ability of pathogens to be transported via the smoke of a burn. Both areas of research will estimate the health risks posed to the general public and also to the plantation workers.

Methodology and Results

Pathogen survival multiple years after biosolids application

This study selected *Escherichia coli* (*E.*coli), *Salmonella spp.*, *Clostridium perfringens* and Coliphage for monitoring the pathogen content as they are indicator organisms for contamination by human/animal waste (United States Environmental Protection Authority 2000; National Research Council 2002; National Water Quality Management Strategy (NWQMS) 2004; Eamens, Waldron et al. 2006).

Soil core samples from sites representing 10, 4, 4 and 3 years (site 1 to 4 respectively) post-application of biosolids from the Myalup plantation were taken using an auger to a depth of 10centimetres into the soil profile. The samples were placed into sterile bags and transported to the Murdoch University for analysis using most probable number (MPN) methods for *Salmonella* spp., *Escherichia coli*, *Clostridium perfringens* (Standards Australia 2004; Standards Australia 2004; Standards Australia 2006) whilst coliphage was tested for using methods developed by Adams (1959) and Harrigan *et al*. (1966).

Table 1 shows that the highest level of *E.coli* was recorded at site 4 with 2.9×10^{1} MPN/gram and the lowest recorded was at site 3 with 6.1×10^{-1} MPN/gram. In general it can be seen that there is a decreasing trend in the levels of *E.coli* with an increase in years post-application of biosolids. *Salmonella spp.* did not record a result higher than the minimum MPN/gram value of $\leq 3 \times 10^{-1}$ at any site. *Clostridium perfringens* only recorded values higher than the minimum MPN value represented as $\leq 3\times10^{-1}$ in both its 4 years post-application sites with values of 1.1 MPN/gram and 7×10^{-1} MPN/gram at site 2 and site 3 respectively.

Table1: Post-application of biosolids MPN results for *E.coli, Salmonella spp. and Clostridium perfringens* from samples taken in 2007

Site No. (Years post-application)	*E.coli* (MPN per gram)		*Salmonella spp.* (MPN per gram)		*Clostridium perfringens* (MPN per gram)	
	MPN per gram	95% confidence levels per gram	MPN per gram	95% confidence levels per gram	MPN per gram	95% confidence levels per gram
Site 1 (10)	7.4×10^{-1}	0.13 - 2	$\leq 3 \times 10^{-1}$	<0.05 - <0.9	$\leq 3 \times 10^{-1}$	<0.05 - <0.9
Site 2 (4)	1.1	0.3 – 3.6	$\leq 3 \times 10^{-1}$	<0.05 - <0.9	1.1	0.3 – 3.6
Site 3 (4)	6.1×10^{-1}	0.12 – 0.17	$\leq 3 \times 10^{-1}$	<0.05 - <0.9	7×10^{-1}	0.1 – 2.3
Site 4 (3)	2.9×10^{1}	9 - 99	$\leq 3 \times 10^{-1}$	<0.05 - <0.9	$\leq 3 \times 10^{-1}$	<0.05 - <0.9

NB: 95% Confidence levels taken from supplied MPN methods (Standards Australia 2004; Standards Australia 2004; Standards Australia 2006)

Risk of presence of pathogens in the air samples after a plantation burn

A preliminary test using a wind tunnel was conducted to check the pathogen content of the smoke produced when the biosolids are subjected to a plantation style burn. The study tested the following conditions;
1) Dry biosolids with pine needles;
2) Dry biosolids with pine needles and inoculated with a mixed inoculum (*E.coli*, *Salmonella spp*., and *Clostridium*);
3) Wet Biosolids with pine needles;
4) Wet biosolids with pine needles and inoculated with a mixed inoculum (*E.coli*, *Salmonella spp*., and *Clostridium perfringens*).

The inoculated samples had the mixed inoculum added two days prior to the experiment and were then left undisturbed. This allowed the pathogens time to establish growth and colonies to form within the biosolids. To establish the difference between the levels of pathogens in the biosolids prior to inoculation and after inoculation, a sample of 1 gram was taken; 1) before inoculation and 2) after inoculation. This experiment was done in triplicate. A 'wind tunnel' (1 metre length X 0.5 metre diameter) was constructed that allowed a regulated flow of air produced by a fan to pass from one end of the tunnel to the other. The samples were placed on a tray within the tunnel in the air stream. The air/smoke samples were collected using air samplers known as 'BioSampler' which were placed at the exit of the tunnel so as to ensure that they were in the main air stream and therefore in the path of the pathogen-contaminated air. A fan was placed at the opposite end of the tunnel (not turned on). The pine needles were lit and once the fire had been established, the fan and the Biosamplers were turned on to collect the samples. The samples were analysed for the presence of *E.coli*, *Salmonella spp,. Clostridium perfringens* and Coliphage (Adams 1959; Harrigan and McCance 1966; Standards Australia 1995; Standards Australia 2000; Standards Australia 2007).

The Biosamplers ran for 2 minutes equating to 25 litres of air. Thus all the results represent the number of colonies found within 25 litres of air. The only exception to this standard were the before inoculation and after inoculation samples that were done by weight; 1 gram per sample.

Table 2 shows that neither *Clostridium perfringens* nor Coliphage were detected in any of the samples taken. An average of one colony of *Salmonella spp.* and a reading of 'too many to count' (TMTC) for *E.coli* was detected in the burn of wet biosolids and pine needles. This result indicates that it is possible for *E.coli* to be transported via the smoke of a burn, however the lack of detection of any other pathogens to high levels seems to support the studies by Brooks *et al* (2005) in finding that the down-wind aerosol microbial content is insignificant.

The process of inoculation in both the wet and dry biosolids was successful for both *E.coli* and *Salmonella spp.* as it is clearly shown that there were significant numbers of pathogens present prior to the burn taking place (Table 3). However for *Clostridium perfringens* the inoculation was far more successful in the dry biosolids with the wet biosolids maintaining the same numbers even after inoculation.

Table 2: Average number of pathogens in air contaminated with the smoke from a burn of biosolids and pine needles (TMTC – Too many to count)

	E.coli (per 25lt of air)		*Salmonella* spp. (per 25lt of air)		*Clostridium perfringens* (per 25lt of air)		Coliphage (per 25lt of air)	
		Standard Deviation		Standard Deviation		Standard Deviation		Standard Deviation
Wet Biosolids	TMTC	* (see below)	1	1.73	0	0	0	0
Dry Biosolids	0	0	0	0	0	0	0	0
Inoculated Wet Biosolids	0	0	0	0	0	0	0	0
Inoculated Dry Biosolids	0.33	0.57	0	0	0	0	0	0
* As no specific value was noted, so Standard Deviation can be calculated								

Table3: Levels of pathogens in the biosolids before and after inoculation (TMTC - Too many to count)

	E.coli CFU/gram	*Salmonella* spp. CFU/gram	*Clostridium perfringens* CFU/gram	Coliphage CFU/gram
WET BIOSOLIDS				
Before Inoculation	TMTC	260	80	0
After Inoculation	TMTC	TMTC	80	0
DRY BIOSOLIDS				
Before Inoculation	290	TMTC	15	0

After Inoculation	1010	TMTC	TMTC	0

Discussion

This preliminary study showed a trend that indicates a decrease in the numbers of *E.coli* with a corresponding increase in time after the application of biosolids. According to the guidelines for land-applied biosolids in Australia, the microbial levels of *E.coli* and *Salmonella spp.* would place the samples into the P2 category and possibly even into the P1 category (National Water Quality Management Strategy (NWQMS) 2004). In regards to the USEPA classification, Class A requirements have also been met (United States Environmental Protection Authority 2000).

This means that the biosolids applied to the land in the pine plantation have effectively become safe in regards to their ability to pose a pathogenic health risk to humans. However, Eamens *et al* (2006) found that concentrations of *E.coli*, *Salmonella spp.* and *Clostridium perfringens* were often above soil background levels up to six months post-application and in some cases up to twelve months post-application of biosolids.

Whilst the time frame of Eamens *et al* (2006) is much shorter than those received for this experiment thus far, it is still an important finding that indicates the potential for *E.coli, Salmonella spp* and *Clostridium perfringens* to survive long periods of time when applied to agricultural land. Pourcher *et al* (2007) found that faecal indicators can survive up to two months.

These findings indicate that constant monitoring is required and are confounded by the findings of Zaleski *et al* (2005) who found that rainfall events can allow for reestablishment of both *Salmonella spp.* and faecal coliforms. Importantly, Rogers *et al* (2007) found that the survival of pathogenic microorganisms can be influenced by their interactions with protozoa and other soil bacteria.

The study also found that when biosolids are applied to the land, the protozoa numbers are likely to increase and therefore are able, in some cases, to limit the ability of the pathogens to colonise and grow in the soil. Both Eamens *et al* (2006) and Pourcher *et al* (2007) performed their studies on land used for grazing cattle and so the organic matter from the faeces of the cattle may have had an influence on the results. Zaleski *et al* (2005) suggested that the reestablishment of *Salmonella spp.* in the biosolids was actually due to bird faeces and not from the bacteria indigenous to the biosolids.

The survival of the pathogens in a pine plantation is a scenario that does not seem to have been covered comprehensively, and as it is a use of biosolids that is widely accepted due to timber not being a part of the human food chain, it is likely to be a very useful study.

The assessment of the risk of pathogens being transported through the smoke of a plantation burn has found that there is a potential for pathogens, specifically *E.coli*, to be transferred via the smoke of a burn in a pine plantation. However the results indicate that this action is unlikely to occur, but despite this finding it is essential that all precautions are taken to ensure that minimal human contact is made with the smoke of a pine plantation burn.

It was found that *E.coli* was the only pathogen in this study that was observed being transported in high levels, and it is suggested that *E.coli* be used as an indicator pathogen for future studies that analyse the transfer of pathogens from fires. It must be noted that this result was only received in one of the three replicates and therefore the result cannot be considered insignificant.

To conclude whether this occurrence of *E.coli* being transported in smoke is significant, further replicates are required to test the repeatability of the transfer There have been many papers that show that aerosolised pathogens from biosolids have the ability to cause detrimental health effects (Epstein, Wu et al. 2001; Brooks, Tanner et al. 2005; Brooks, Tanner et al. 2006; Robinson, Robinson et al. 2006) and therefore there is still a need to be cautious and ensure monitoring occurs when a high risk area is undergoing a burn.

Studies undertaken in regards to dust generated from biosolids and its affect on humans and have shown that those most at risk are the personnel working directly with the biosolids (Epstein, Wu et al. 2001; Brooks, Tanner et al. 2005; Robinson, Robinson et al. 2006). The general public are unlikely to be at risk from aerosolized biosolids and more recent studies would suggest that most workers directly involved with biosolids are also at a low risk, although higher than that of the general public not directly involved with the biosolids (Brooks, Tanner et al. 2005; Brooks, Tanner et al. 2005; Tanner, Brooks et al. 2005; Paez-Rubio, Ramarui et al. 2007). Much of the literature discusses the health effects that the biosolids dust can have on humans in confined spaces (e.g. composting factory, sewage treatment plant), but the land-application of biosolids occurs outdoors and as the source of aerosolisation is always moving, little time is spent in one area and thus the exposure would be limited (Brooks, Tanner et al. 2005). The literature certainly indicates that there is a potential risk of exposure from biosolids dust and therefore it is highly recommended that the dust is monitored and tested to establish the risk to the users of the pine plantation.

The literature on the effect that pine needles and their decomposition could have on pathogens and specifically *E.coli, Salmonella spp* and *Clostridium perfringens* is limited and sparse. Whilst the physical and biological effect's that the pine needles can have on the soil and the soil microorganisms are well documented, the effect if any of the chemical composition of the pine needles directly on the pathogens is a question that this experiment is aiming to answer. The literature has not indicated that there is any specific vegetation type that is capable of chemically affecting the pathogens; however the fact that there is such a high input of pine needles into the soil profile and land-applied biosolids it is possible that as the needles break down they may cause an effect.

Conclusion

Whilst current results indicate a low risk, further research is required to establish the human exposure risk of aerosolized pathogens. A major pathogen aerosolisation risk is that of dust and it has been established that an experiment to test the ability of the pathogens to be spread via the wind in the pine plantation, is required. The survival/die-off/regrowth rates of *E.coli*, *Salmonella spp*. and *Clostridium perfringens* after 3 years post-application of biosolids shows that the levels are insignificant, however it is concluded that intense sampling during the first year post-application is required to assess the health risk the site may pose to the general public and plantation workers.

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