The Use of *Pinus radiata* Sawdust or Bark, or Zeolite to Reduce *E. coli* in Stand-Off Pads or Associated Drainage

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Abstract: Cow dung slurry containing a total of 7.4×10^5 *E. coli* was applied to laboratory-scale microcosms of *Pinus radiata* sawdust or bark. Rainfall was simulated by application of water at rates of 25 mm h^{-1} or 50 mm h^{-1} over 30 minutes. For sawdust 5% and 11% (respectively) of the *E. coli* were transferred to drainage compared to 12% and 28% for bark. After collection of drainage less than 10% of the retained *E. coli* were recovered from sawdust compared to about 90% for bark. Stand-off pad drainage (10^2 *E. coli* 100 mL^{-1}) was passed through laboratory-scale microcosms at 0.42 mL min^{-1} for 4 weeks. The *E. coli* concentration was reduced by 1.5 $\log_{10}$ for sawdust microcosms but there was no significant reduction for bark and zeolite. *P. radiata* sawdust seems an unfavourable environment for *E. coli* and its use in stand-off pads could reduce transmission of faecal microbes from dairy farms to waterways.

Keywords: *Pinus radiata*, sawdust, bark, *Escherichia coli*, manure, stand-off pads, stand-off pad drainage, dairy farms.

INTRODUCTION

In a report on environmental issues associated with New Zealand farming [1] the Parliamentary Commissioner noted that many waterways have faecal microbial loadings that are unsuitable for recreation or as source water for drinking. As well, concentrations can increase substantially after heavy or prolonged rainfall due to flow-generated inputs and resuspension of sediments [2,3]. Infectious faecal microorganisms are present in a wide range of warm-blooded animals. When shed by livestock there is potential for these microorganisms to enter waterways by direct deposition or indirectly as contaminants in surface and sub-surface flows following rainfall or irrigation [4]. Concentrations in surface flows are particularly high when animals graze on wet pasture [5-7]. *Escherichia coli* is a consistent constituent of the faeces of all warm blooded animals so that its presence in water "indicates" faecal contamination [8]. Extensive studies of New Zealand recreational freshwaters identified a relationship between *E. coli* concentrations and health risk [9]. New Zealand authorities have produced microbiological guidelines for freshwater that are based on *E. coli* [10].

An increasingly popular winter management strategy on dairy farms is to remove cows from wet pastures to animal houses (herd homes) or stand-off pads. One of the outcomes of such removal is a reduced likelihood of transferring contaminants to natural water bodies [11]. Stand-off pads are typically unroofed structures on which dairy cows are held for various lengths of time. An essential component of a stand-off pad is a drainage collection system that retains liquid seepage and prevents contaminants being transferred to surrounding land. Pads can be constructed from free-draining materials including sawdust, bark, woodchips, lime or soft rock. The type of pad materials may influence capture and retention of contaminants as it has been reported that faecal microbial capture and survival are related to the type of bedding material used in animal-houses. For example, Miller *et al*. [12] reported lower concentrations of coliform bacteria in runoff when straw was used as feedlot bedding material compared to wood chips made from pine and spruce. Zehner *et al*. [13] noted that in contrast to softwood sawdust that had a bactericidal effect, there was rapid growth of three bacterial species associated with mastitis when straw and some hardwood chips were used.

The relationship between bacterial die-off and bedding material may not be straightforward. Davis *et al*. [14] reported that the pathogen *Escherichia coli* O157:H7 grew in cedar chip bedding moistened with urine in contrast to a decline when the bedding was moistened with water. LeJeune and Kauffman [15] measured differences in survival of *E. coli* O157:H7 in sawdust and sand and also prevalence rates in animals using these materials as bedding. These workers reported both higher survival and higher prevalence rates for sawdust than for sand, but the type of wood was not stated. A comparison of the bactericidal properties of wood was carried out by Milling *et al*. [16] who measured the survival of two faecal bacteria, *E. coli* and *Enterococcus faecium*, in the sawdust made from seven wood types. These workers found survival rates were related to microbial species and wood type, and were also affected by environmental factors including ambient temperature and humidity. Under typical ambient conditions (temperature 21°C and relative humidity 55%) and for both test bacteria, the most rapid decline was observed on pine sawdust, followed by oak.

Luo *et al*. [17] investigated the environmental performance of unroofed stand-off pads constructed from either fine crushed bark or sawdust (both prepared from untreated *Pinus radiata*) overlaid with coarse bark. These workers calculated removal efficiency as faecal microbial inputs could only be estimated. They reported that drainage from the bark pad contained consistently higher concentrations of *E. coli* than that from the sawdust pad, with a similar trend observed for the pathogen *Campylobacter jejuni*.

This paper reports a laboratory-based study that was carried out to quantify the retention of *E. coli* by sawdust and

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bark under different levels of rainfall. As well, three natural materials, sawdust, bark and zeolite were tested as possible treatment options to reduce *E. coli* concentrations in stand-off pad drainage to levels that would be acceptable for disposal to the environment.

MATERIALS AND METHODS

The sawdust and bark used in this study were prepared from untreated *Pinus radiata*. These materials were obtained from a commercial supplier (Daltons Ltd, Matamata NZ). The bark had been stored outdoors and the sawdust was freshly milled. To maintain their natural state these materials were not subjected to any disinfection treatment (e.g. heat) prior to use.

Interactions of *E. coli* with Sawdust and Bark

Sawdust and bark (particle size 3-10 mm) were soaked for about 6 h in excess water and drained overnight. Microcosms were prepared by adding the prepared material to plastic sleeves (220 mm diam., 100 mm high). Five microcosms of each material were prepared (sawdust microcosms contained about 1600 g and bark about 2130 g (wet weight)).

To ensure even application across the surface of all microcosms, slurry was prepared by thoroughly mixing 10 g fresh cow dung (faeces) with 1 L of water. Over a 45 minute period 100 mL of slurry was applied evenly (using a Pasteur pipette) over the surface of each microcosm. Three microcosms were treated in the same way for each material. A further microcosm (control) of each material received 100 mL of distilled water applied in the same way.

Each microcosm was placed on a shallow perforated tray containing gravel that was then placed on a drainage collection bucket. After application of slurry, or water, the microcosms were placed in a controlled temperature room at 4°C and left overnight. During this period no drainage was collected, demonstrating that all of the applied slurry remained within the microcosm.

A plastic squeeze bottle fitted with a dropper was filled with water and used to apply simulated rainfall. Two slurry-contaminated microcosms of each material (i.e. sawdust and bark) received simulated rainfall at two rates. To achieve a rate of 25 mm h^{-1} 475 mL of water was applied evenly over the entire surface of a microcosm over 30 minutes. For 50 mm h^{-1} 950 mL was applied over 30 minutes. The "control" microcosms received simulated rainfall at 25 mm h^{-1} in the same way.

Drainage from each microcosm was collected over about an hour after rainfall application (or until no more drainage was collected). The drainage volume was measured and a sub-sample analysed for *E. coli*. The microcosms were returned to the 4°C room and left overnight. The following day a second application of simulated rainfall was applied as described above. Following the drainage collection, the contents of each microcosm were separately and thoroughly mixed and a portion (about 500 g) retained for *E. coli* analysis.

Survival of *E. coli* RR in Sawdust

To determine whether the poor recovery of *E. coli* from sawdust was an artefact of analysis due to "overgrowth" by closely related bacterial species in the microbiological growth medium, a laboratory grown strain, *E. coli* HS (pFamp)R [18]– designated RR, that is resistant to the antibiotics ampicillin and streptomycin, was added to sawdust.

E. coli RR was grown in tryptic soy broth (TSA, Difco, USA) plus antibiotics [18] for 24 h at 35°C and serially diluted in 0.1% yeast extract (YE) to obtain target concentrations of 10^8, 10^6 and 10^4 *E. coli* 100 mL^{-1} (designated high, medium and low respectively). 10 mL portions of each dilution were placed in a Universal bottle as "minus sawdust" controls. A 300 mL portion of each dilution was added to 250 g sawdust and thoroughly mixed. This proportion of liquid to sawdust allowed thorough wetting without separation into a liquid and solid phase.

All treatments were incubated at 15°C for 28 days after which they were analysed for *E. coli*.

Using Natural Materials to Reduce *E. coli* in Drainage from Stand-Off Pads

About 50 L of liquid was collected from the drainage-collection sump at a sawdust stand-off pad located on a dairy farm and stored at 4°C over a 4 week study period.

Small microcosms, (plastic sleeves - capacity 1 L), 4 for each material were prepared using sawdust, bark (particle size 3 - 10 mm) or crushed zeolite (similar particle size to bark). Prior to filling, the materials were soaked in excess water, the sleeves filled and drained under gravity overnight. Stand-off pad drainage liquid (influent) was dripped continuously through each microcosm at a rate of 0.42 mL min^{-1} (i.e. each column received 0.6 L d^{-1}) for about 4 weeks (microcosm contents were not replaced during this time). On 10 occasions, at approximately equal intervals and commencing 24 h after the experiment began, samples of effluent were collected from each microcosm. At the same time a sub-sample was also taken from the influent holding vessel. Two such experiments were carried out.

E. coli Analysis

Liquid samples, and solid samples that contained high concentrations of *E. coli* were analysed by a defined substrate method [19; Method 9223B] using the Colilert/ Quantitray 2000™ technique (IDEXX, Westbrook ME, USA). The limit of detection was 1 *E. coli* 100 ml^{-1} in drainage and 100 *E. coli* 100 g^{-1} in solid material.

For solid samples, in which *E. coli* numbers were too low for detection by the method described above, a traditional Most Probable Number (MPN) method was used [19; Methods 9221B and 9221F-1]. For this MPN method the 3-consecutive dilution series was inoculated by direct addition of 5×: 10 g, 1 g and 0.1 g portions, to the microbiological growth medium (so as to include those *E. coli* that were attached to the solid material in the count). The limit of detection was 2 *E. coli* 100 g^{-1} of solid material.

To confirm *E. coli* identification selected isolates (both positive and negative) were identified using Enterobacteriaceae test kits (Microbact Ltd., Australia), used in accordance with the manufacturer's directions.

Data were $\log_{10}$ transformed and ANOVA (Minitab Inc., USA) performed for statistical analysis.

RESULTS

Retention of *E. coli* by Sawdust or Bark

The total number of *E. coli* in the cow dung slurry applied to each microcosm was 7.4×10^5. A substantial fraction of the water applied as simulated rainfall was collected as drainage, demonstrating that the microcosms were free-draining. At the lower (25 mm h^{-1}) application rate drainage recovery was 92% and 85% of the volume applied for sawdust and bark, respectively, and at the higher (50 mm h^{-1}) rate, the recoveries were 95% and 93%, respectively.

The number of *E. coli* transferred to drainage from the slurry-contaminated microcosms at each rainfall rate, and the number remaining in the material (both results also expressed as a percentage of the number of *E. coli* applied) are shown in Table **1**. No *E. coli* were recovered from uncontaminated sawdust or drainage but "natural" *E. coli* were identified in bark microcosm drainage (2.4×10^3 *E. coli* after the first rainfall application and 1.2×10^3 *E. coli* after the second) therefore results for contaminated bark microcosms were corrected for "background".

The data in Table **1** demonstrate more of the applied *E. coli* were transported to drainage at the higher (50 mm h^{-1}) rate. As well, the percentage of applied *E. coli* leached from sawdust was less than that from bark at both rates of rainfall. As shown in Table **1**, for bark microcosms the majority of the applied *E. coli* could be accounted for in either the drainage or the bark itself (90% at 25 mm h^{-1} and 97% at 50 mm h^{-1}). In contrast, for sawdust microcosms the number of the applied *E. coli* that could be accounted for was only 11% at 25 mm h^{-1} and 16% at 50 mm h^{-1}.

Determination of *E. coli* RR Survival in Sawdust

When *E. coli* RR was incubated at 15°C in yeast extract in the absence of sawdust, irrespective of the initial number of *E. coli* (i.e. high, medium or low) after two days numbers had increased to 10^9 or 10^{10} *E. coli* 100 mL^{-1} and did not decline over the 28 day period. In contrast, when *E. coli* RR mixed with sawdust was incubated under the same conditions numbers declined substantially after 28 days, for all three inoculum levels, as shown in Table **2**, by at least 99.9% (i.e. 3 logs) of the original inoculum number.

Removal of *E. coli* from Stand-Off Pad Drainage

The concentration of *E. coli* in the drainage used as microcosm influent in the first experiment did not vary much (geometric mean 1.9×10^2 *E. coli* 100 mL^1, sd 1.7 $\log_{10}$) over the course of the experiment. As shown in Fig. (**1**), the *E. coli* concentration in the effluent that was collected from the sawdust microcosms decreased by about 1.5 $\log_{10}$ over the course of the 4 week experiment and this reduction was statistically significant ($P<0.05$). There was little difference in *E. coli* concentration in the influent and in the effluent from the zeolite microcosm ($P>0.05$) but concentrations were consistently lower in bark effluent. However, the reduction in *E. coli* due to passage through bark was not statistically significant ($P>0.05$).

In the second experiment the bark was found to have a substantial population of natural *E. coli* that resulted in bark microcosm effluent *E. coli* concentration (overall average 9.8 $\times 10^2$ *E. coli* 100 mL^{-1}, sd 3.3) being five times higher than that of the applied influent. As a consequence, the results for the bark microcosms were not evaluated further. The *E. coli* concentration in the influent remained reasonably constant over the course of this experiment (geometric mean 2×10^2 *E. coli* 100 mL^{-1}, sd 1.3 $\log_{10}$). As shown in Fig. (**2**), there was again a substantial decline in *E. coli* after passage through the sawdust microcosms ($P<0.05$). As for the first experiment, there was little difference between the *E. coli* numbers in the influent and those in the effluent from the zeolite microcosms ($P>0.05$).

Although there was a significant reduction in *E. coli* after passage through sawdust in both experiments, removal rates differed (as determined by simple linear regression) being -0.196 $\log_{10}$ *E. coli* d^{-1} in the first experiment and 0.057 $\log_{10}$ *E. coli* d^{-1} in the second.

DISCUSSION

The application of simulated rainfall transferred some of the *Escherichia coli* from the cow dung slurry layered on top of the sawdust and bark to the microcosm drainage. The percentage transferred to sawdust drainage, under both the low and high rainfall rates, was only about half of that for bark (5% and 11% compared to 12% and 28%, respectively). However, the most notable difference between these two materials was an apparent "loss" of *E. coli* in the sawdust as

Table 1. ***E. coli*** **Recovered in Drainage or Microcosm Material after Application of Simulated Rainfall at Two Rates to Sawdust and Bark Microcosms Contaminated with Cow Dung Slurry**

	E. coli **(100 mL^{-1} or 100 g^{-1})**				***E. coli*** **(100 mL^{-1} or 100 g^{-1})**			
	A	**B**	**Mean**	**% of Applied *E. coli***	**A**	**B**	**Mean**	**% of Applied *E. coli***
Sawdust	**25 mm h^{-1}**				**50 mm h^{-1}**			
Drainage	5.0×10^4	2.4×10^4	3.4×10^4	5	8.7×10^4	7.9×10^4	8.3×10^4	11
Remaining in sawdust	8.0×10^4	2.7×10^4	4.6×10^4	6	3.7×10^4	3.4×10^4	3.5×10^4	5
Bark	**25 mm h^{-1}**				**50 mm h^{-1}**			
Drainage	8.9×10^4	7.3×10^4	8.8×10^4	12	2.5×10^5	1.7×10^5	2.0×10^5	28
Remaining in bark	5.8×10^5	5.8×10^5	5.8×10^5	78	1.0×10^6	2.5×10^5	5.0×10^5	69

Data are the results obtained for each of the duplicate microcosms (i.e. A and B) for each treatment and the geometric mean. The recovery of *E. coli* in drainage or remaining after rain was calculated as a percentage of the number of *E. coli* applied.

more than 80% of the applied *E. coli* were not recovered from either the drainage or the sawdust itself. In contrast more than 90% of the applied *E. coli* were recovered from the bark plus its drainage. The observed high recovery from bark microcosms and the relatively short experimental timeframe suggests that the sawdust result was not due to "natural die-off" during the experiment.

Table 2. ***E. coli*** **RR (log_{10} *E. coli* 100 g^{-1}) Recovery from Sawdust at Three Levels of Contamination, Designated Low, Medium and High, after 28 Days Storage at 15°C**

	E. coli (log_{10} 100 g^{-1} Sawdust)		
Time	Low	Medium	High
Day of inoculation	4.36	6.54	8.59
After 28 days	1.36	3.34	4.54

A higher rate of bacterial adsorption would be expected in sawdust due to a larger surface area compared to bark but it is unlikely that this explained the low recovery of *E. coli* as the MPN test was done by direct addition of weighed portions of sawdust to microbiological enrichment broth. This procedure was adopted to allow any viable *E. coli* that were firmly attached to sawdust particles to grow during incubation. As the analysis of *E. coli* was by laboratory culture it is not known whether the unaccounted for *E. coli* cells had entered a non-culturable state [20] or were dead. In either event, it seems that *Pinus radiata* sawdust is an unfavourable environment for *E. coli* but that this does not apply to *P. radiata* bark.

There are conflicting reports on the antibacterial nature of wood products. For example Zehner *et al*. [13] found there was a rapid decline in three bacterial species associated with mastitis when paper and softwood sawdust were used as bedding materials but LeJeune and Kauffmann [15] reported a higher prevalence of *E. coli* O157:H7 in sawdust than in sand bedding. Milling *et al*. [16] reported that pine sawdust had substantially better hygienic performance than five other wood species and also plastic.

Escherichia coli RR grew rapidly in yeast extract at 15°C and survived over the 4 week experimental period. However, in a parallel experiment carried out in the presence of sawdust, there was a substantial decrease, of at least 3 logs, in *E. coli* RR numbers. This observed decline in sawdust was about 10-fold greater than that reported for soils [21, 22]. Analysis for *E. coli* (by both methods used in this study) is a two-stage process. In the first stage coliforms are recovered and in the second those coliforms that are *E. coli* are identified [19]. Large numbers of coliforms, most of which were found to be *Klebsiella oxytoca*, were identified in the samples of sawdust that were analysed for *E. coli*. *Klebsiella* species can fix nitrogen, grow well on carbohydrates and have a known association with timber processing waste streams [23]. *K. oxytoca* and *E. coli* are members of the same family (Enterobacteriaceae) and share a number of properties, so it is possible that *K. oxytoca* could have "masked" the presence of *E. coli*. If this were the case then the apparent loss of *E. coli* in sawdust would be an artefact of the analytical procedure. The use of an antibiotic resistant strain (*E. coli* RR) that could be measured without interference from closely related but antibiotic sensitive bacteria such as *Klebsiella* spp. confirmed that sawdust is not a favourable environment for *E. coli* and that the observed decline in numbers was in fact real.

Two of the three batches of *P. radiata* bark used over the course of this study were found to contain natural *E. coli*, presumably due to contamination with animal (e.g. bird) faeces during outdoor storage. Unlike sawdust we did not observe a significant decline in *E. coli* numbers in bark microcosms. No natural *E. coli* were found in the freshly milled sawdust and there was a significant reduction in *E. coli* in all experimental sawdust microcosms.

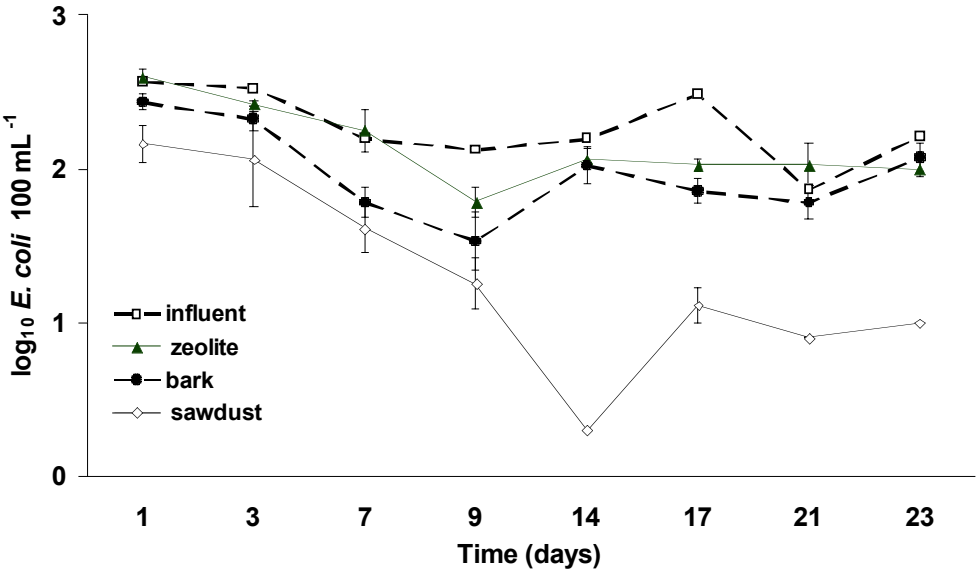

Fig. (1). Numbers of *E. coli* 100 mL^{-1} recovered in influent (stand-off pad drainage) applied to microcosms of sawdust, bark or zeolite and in the effluent from these microcosms. For each material data are the average (and SEM) for 4 microcosms and for the influent represent the number of *E. coli* measured on the day of analysis.

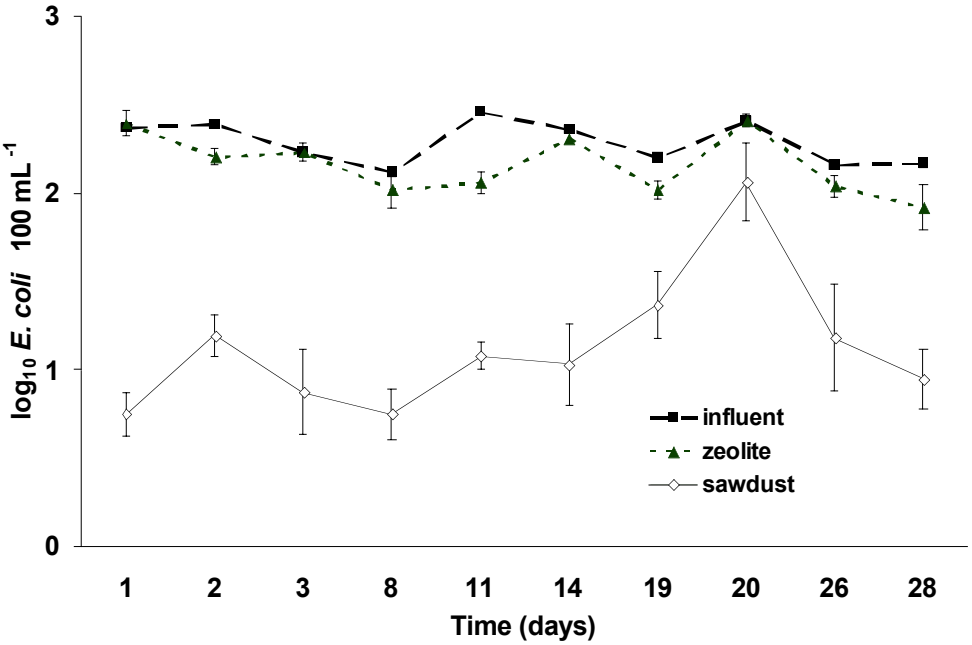

Fig. (2). Numbers of *E. coli* 100 mL^{-1} recovered from influent (stand-off pad drainage) applied to microcosms of sawdust or zeolite and in the effluent from these microcosms. For the two materials data are the average (and SEM) for 4 microcosms and for the influent represent the number of *E. coli* measured on the day of analysis.

There were differences in removal rates of *E. coli* from stand-off pad drainage in two microcosm studies indicating that further work is needed to optimise this procedure. At a full-scale stand-off pad on a dairy farm, a lower recovery of both *E. coli* and the pathogen *Campylobacter* was observed in sawdust than in bark drainage [17] suggesting that sawdust may be inhibitory to other bacteria as well as *E. coli*. Further work is needed to determine the fate of *E. coli*, the mechanism of it's inhibition by *P. radiata* sawdust and the range of bacteria that are inhibited.

CONCLUSIONS

When *E. coli* was washed through *P. radiata* sawdust, there was a substantial reduction in numbers suggesting that the use of this sawdust in stand-off pads would be a useful way of reducing transfer of faecal microorganism from dairy farms to the wider environment.

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