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ORIGINAL ARTICLE

Mobility and survival of *Salmonella* Typhimurium and human adenovirus from spiked sewage sludge applied to soil columns

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Abstract

Aims: This study investigated the survival and transport of sewage sludge-borne pathogenic organisms in soils.
Methods and Results: Undisturbed soil cores were treated with *Salmonella enterica* ssp. *enterica* serovar Typhimurium-*lux* (STM-*lux*) and human adenovirus (HAdV)-spiked sewage sludge. Following an artificial rainfall event, these pathogens were analysed in the leachate and soil sampled from different depths (0–5 cm, 5–10 cm and 10–20 cm) after 24 h, 1 and 2 months. Significantly more STM-*lux* and HAdV leached through the soil cores when sewage sludge was present. Significantly more STM-*lux* were found at all soil depths, at all time periods in the sewage sludge treatments, compared to the controls. The rate of decline of STM-*lux* in the controls was more rapid than in the sewage sludge treatments. Survival and transport of HAdV were minimal.
Conclusions: The presence of sewage sludge can significantly influence the transport and survival of bacterial pathogens in soils, probably because of the presence of organic matter. Environmental contamination by virus is unlikely because of strong soil adsorption.
Significance and Impact of the Study: This study suggests that groundwater contamination from vertical movement of pathogens is a potential risk and that it highlights the importance of the treatment requirements for biosolids prior to their application to land.

Introduction

Sewage treatment and disposal serve to isolate sewage from the community, thereby affording protection from pathogens capable of causing infectious diseases. However, sewage sludge is a valuable resource, containing high concentrations of plant nutrients that can be directly utilized by land application. Although it is accepted that pathogen numbers are reduced during sludge processing (e.g. anaerobic digestion), it is unlikely that they can be completely eliminated and this may be of potential significance for transmission of diseases in humans. One of the primary concerns with pathogenic enteric organisms is their transport and survival in soil after sewage sludge application.

The amount and rates of sewage sludge land-application are currently controlled via guidelines and regulations that set criteria for levels of pathogens, which are protective of the environment and human health (e.g. UK DoE 1989; Wolstenholme *et al.* 1992; US EPA 1993; NSW EPA 1998; NZWWA 2003). The guidelines require the sewage sludge be treated to such a level that pathogens

are reduced to below detection limits before land application (Class or Grade A). Alternatively, the sewage sludge can be treated to a lesser degree (Class or Grade B), but adequate time must be allowed for the sewage sludge to remain in or on the land for natural attenuation to further reduce the pathogen numbers before use of the land for cropping or public access. Class or Grade A and B indicator organisms and/or pathogen limit concentrations are shown in Table 1. Faecal coliform counts in Grade or Class B sludges can be high, up to 10^5 g^{-1} dry sewage sludge (Vasseur *et al.* 1996; Davies *et al.* 1999; LeClerc *et al.* 2001; Zaleski *et al.* 2005). In addition to run-off, bypass flow through soils has potential to contaminate surface and groundwater and is a potential public health risk.

Contamination of food and water by micro-organisms from animal manures has become a topic of concern after recent food-poisoning outbreaks (Islam *et al.* 2005; Johannessen *et al.* 2005; Cooley *et al.* 2007). Point sources of animal manure contamination can include animal feedlots, animal housing facilities, manure storage areas and fields where effluent or manures are spread. Rainfall may result in nonpoint-source pathogen contamination by carrying pathogens into nearby fields or surface waters by runoff or by leaching them through the soil into groundwater (Gagliardi and Karns 2000). Factors controlling survival, transport and regrowth include soil type (Mubiru *et al.* 2000; Aislabie *et al.* 2001; Cools *et al.* 2001; Trevisan *et al.* 2002), agricultural practice (Hutchinson *et al.* 2004), method of pathogen delivery (i.e. type of waste) (Avery *et al.* 2004, 2005; Sun *et al.* 2006) and environmental factors such as rainfall, temperature and humidity (Yeager and Ward 1981; Palacios *et al.* 2001; Hepburn *et al.* 2002; Jenkins *et al.* 2002; Zaleski *et al.* 2005). It has been shown that if transported below the top layers of the soil, pathogens can survive for extended periods of time after the initial application (Gagliardi and Karns 2000). Some studies with animal waste have shown that the presence of manure can enhance survival of pathogens such as *Escherichia coli* O157:H7, possibly because of enhanced microsite habitat and the addition of nitrogen (Gagliardi and Karns 2000; Oliver *et al.* 2006). Sewage sludge storage and application areas could constitute a point source of pathogen contamination, especially where lower grades of sewage sludge are concerned (e.g. Class or Grade B) and where pathogens could still be present.

It is not practicable to monitor sewage sludge for the presence of the wide variety of pathogenic and potentially pathogenic organisms that could be found. A more reasonable approach is to search for indicator organisms and representatives of known pathogenic species to represent the larger set of pathogenic organisms (e.g. *Salmonella* ssp.; enteric viruses and helminth ova.). Two important human pathogens are *Salmonella enterica* ssp. *enterica* serovar Typhimurium-*lux* (STM-*lux*) and human adenovirus (HAdV). *Salmonella* Typhimurium is one of the most common enteric infections in New Zealand [reported incidence rates are 32·2 cases per 100 000 persons (New Zealand Public Health Surveillance Report 2008)] and is also able to grow in the environment (Zaleski *et al.* 2005; Sinton *et al.* 2007). HAdVs are DNA viruses that cause a wide range of symptoms, are shed in large numbers in faeces and are frequently present at high levels in sewage (Irving and Smith 1981). HAdV may be useful as enteric virus indicators because of their prevalence and apparent environmental stability in environmental samples including wastewaters, sewage sludge and surface waters (Irving and Smith 1981; Enriquez *et al.* 1995; Pina *et al.* 1998). In addition, compared to other enteric viruses, HAdVs are reported to be UV light and heat resistant (Meng and Gerba 1996; Gerba *et al.* 2002). HAdV types 40 and 41 (that cause gastroenteritis) have shown to be more stable in the environment than other enteric viruses (Enriquez *et al.* 1995).

Detection and quantification of microbes in sewage sludge have inherent difficulties including the following: (i) many tests were developed for clinical material where the pathogen is likely to be present in large numbers relative to other organisms; (ii) techniques can have low

Table 1 Pathogen density limits adapted from United States Environmental Protection Agency (US EPA 1993); New South Wales Environmental Protection Agency (NSW EPA 1998) and New Zealand Waste Water Association (NZWWA 2003)

	USA		New Zealand*	New South Wales*
Micro-organisms	Class A	Class B	Grade A	Class A
Escherichia coli	NA	NA	<100 MPN per gram	NA
Faecal coliforms	<1000 MPN per gram	<2 000 000 MPN per gram	NA	<1000 MPN per gram
Salmonellae	<3 MPN per 4 g		<1 per 25 g	Not detected/50 g
Enteric viruses	<1 PFU per 4 g		<1 PFU per 4 g	<1 PFU per 4 g
Helminth ova	<1 per 4 g		<1 per 4 g	<1 per 4 g

NA, no limits; PFU, plaque-forming unit; MPN, most probable number.
*New Zealand and New South Wales Grade/Class B sludges have no limits for micro-organisms.

recoveries and be inaccurate; (iii) the waste stream is not an 'optimal' environment for many pathogenic organisms which will be damaged and, therefore, will not grow well in laboratory media (Cooper and Riggs 1994; EC 2001). To try to overcome these difficulties, we spiked sewage sludge with a STM-*lux*, a *Salm.* Typhimurium strain containing the bioluminescence gene set '*lux*' from the marine bacterium *Vibrio fisheri* (Redshaw *et al.* 2007). The reporter system is plasmid based and allows for easy identification of the bacterial cells by their bioluminescent or fluorescent phenotypes, without need for further identification. HAdV type 2 was chosen because of its relative ease of culture compared to HAdV type 40 or 41, which does not readily culture to high titres and develops cytopathic effects (CPE) relatively slowly. In addition, until recently, there were no reports of a reliable quantitative infectivity assay for types 40 and 41 (Cromeans *et al.* 2008). In addition to a most probable number (MPN) culture method for infectivity, PCR was also used for the detection of HAdV.

To investigate the survival and transport of sewage sludge-borne pathogenic organisms, sewage sludges were spiked with STM-*lux* and HAdV type 2 and were applied to the surface of undisturbed soil columns at agronomic rates. The soil columns were irrigated and the leachates collected; the soils were then monitored over a 2-month period for the two pathogens.

Materials and methods

Preparation of bacterial and virus cultures

A culture of STM-*lux* (Redshaw *et al.* 2007) was grown in 10 ml of Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 l distilled water) supplemented with 50 μg ml^{-1} of kanamycin (PS; Gibco, Invitrogen Corp.) and 12·5 μg ml^{-1} of tetracyclin (PS; Gibco, Invitrogen Corp.) in a 20-ml thin-walled universal bottle shaken at 180 rev min^{-1} at 25°C until a cell concentration of 10^8 colony-forming units (CFU) ml^{-1} was obtained (OD of 0·8, 550 nm). The cultures were then centrifuged at 7550 ***g*** for 1 min, washed twice in 1/4 – strength Ringer's solutions (Oxoid Ltd, Basingstoke Hampshire, UK) and concentrated threefold by re-suspension in 3 ml Ringer's. The STM-*lux* was quantified (CFU) on LB agar supplemented with 50 μg ml^{-1} kanamycin and 12·5 μg ml^{-1} tetracyclin. The bacterial cultures were then stored at 5°C until use.

HAdV type 2 [American Type Tissue Culture Collection (ATCC) VR-846] and A549 (human epithelial lung carcinoma cells, ATCC CCL-185) cells were obtained from ATTC (Virginia, USA). The A549 cells (passage 85–95) were propagated in 8% (v/v) foetal calf serum (FCS) in medium 199 (Gibco, Invitrogen Corp.), supplemented with 100 units of penicillin G sulfate and 100 μg ml^{-1} of streptomycin sulfate (PS; Gibco, Invitrogen Corp.). HAdV was prepared from infected A549 monolayers by freeze–thaw lysis followed by sonication for 3 min in an ultrasonic cleaner (Model FX10; Unisonics Pty, Ltd., Sydney, Australia). Lysates were extracted by the addition of an equal volume of chloroform, clarified by low-speed centrifugation (400 ***g***, 5 min) and then filtered through a 0·2-μm membrane filter (pretreated with FCS) to remove viral aggregates. The preparation was aliquoted and stored at −70°C. The infectivity titres of the HAdV stock preparation were determined using serial tenfold dilutions in triplicate 96-well microtitre tissue culture plates. The A549 cells were plated at a concentration of 10^4 cells per well in 2% (v/v) FCS in medium 199, followed immediately by the addition of serial dilutions of virus diluted in 2% (v/v) FCS in medium 199 per well. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere and examined regularly for CPE characteristic of HAdV. Results were expressed as the MPN ml^{-1}.

Preparation of soils and sewage sludge

Soil cores

Twenty-seven undisturbed soil cores were obtained by driving plastic pipes into the soil. The soil selected for the study was a silty clay topsoil overlying clay subsoil under Monterey pines, *Pinus radiata*, at Tahekeroa Forest, *c.* 10 km northwest of Silverdale, North Island, New Zealand (NZMS 260, Q10 472 163). The lysimeters were 100 mm diameter × 200 mm tall and had a 10 mm internal annulus filled with petroleum jelly to prevent water preferentially flowing at the soil–casing interface (Cameron *et al.* 1992). Lysimeters were irrigated with tap water for 1 day to bring the cores to field capacity with leachate emanating from the bottom and then allowed to drain for 2 days before application of the sewage sludge (McLeod *et al.* 2001).

Sewage sludge

Fresh anaerobically mesophillically digested, dewatered sewage sludge (*c.* 18% solids) was obtained from North Shore City (Rosedale) Wastewater Treatment Plant (Auckland, New Zealand) (Grade B). HAdV and STM-*lux* were added to sewage sludge to give final concentrations of 6·5 × 10^6 CFU g^{-1} dry weight STM-*lux* and 10^6 MPN g^{-1} dry weight HAdV. For the control lysimeters that did not receive inoculated sewage sludge, STM-*lux* and HAdV were added to soil to give final concentrations of 9·1 × 10^5 CFU g^{-1} dry weight and 10^6 g^{-1} dry weight STM-*lux* and HAdV respectively. The inoculated sludge and soil were

incubated at 16°C in the dark for 3 days to allow time for the bacteria and viruses to adsorb to the sludges and soils. An incubation temperature of 16°C was chosen to allow optimal survival of virus and minimum growth of bacteria. After incubation, the sewage sludge was applied to the surface of 18 replicate lysimeters at two rates: the New Zealand guideline agronomic rate of 200 kg N ha^{-1} (New Zealand Waste Water Association (NZWWA) 2003) and 600 kg N ha^{-1} [3 × the guideline limit (New Zealand Waste Water Association (NZWWA) 2003)]. This equated to 2·7 and 8·1 g (dry weight) of inoculated sewage sludge per lysimeter. For the controls, 12·1 g (dry weight) of inoculated soil was placed on the surface of nine replicate lysimeters; this equated to the same volume of wet weight sewage sludge at the 200 kg N ha^{-1} rate.

Leaching experiments

An artificial rainfall event was simulated by irrigating the lysimeters continuously with tap water at a rate of 5 mm h^{-1} (for 6 h, *c.* 1 l of water) using a drip-type rainfall simulator with drippers *c.* 100 mm above the soil surface, immediately after sewage sludge or soil application. Lysimeters were left to drain overnight and leachate samples collected in sterile 1-l glass bottles and subsampled for STM-*lux* and HAdV analyses. Determination of any ponding during irrigation was by visual observation. Recovery percentages of STM-*lux* and HAdV were calculated in the leachate. Following the artificial rainfall event, the lysimeters were weighed and transferred to a light incubator maintained at 16°C and 18 h photoperiod to emulate climatic conditions in Spring/Summer in New Zealand. The soil moisture was maintained by misting weekly with distilled tap water.

Analysis of leachate

STM-lux

The method for enumerating STM-*lux* in leachate was adapted from Part 9222.B, *Standard Methods for the Examination of Water and Wastewater* (APHA 2006). Leachate samples (1 ml) were diluted in phosphate buffer (pH 7·0) as required and then filtered through cellulose-ester membrane filters (0·45-μm pore size; Sartorius) according to standard procedures (APHA 2006). The filters were placed in duplicate on plates of LB agar supplemented with 50 μg ml^{-1} kanamycin and 12·5 μg ml^{-1} tetracyclin. After overnight incubation at 32°C, the plates were examined for growth of luminescent colonies. Total STM-*lux* in the leachate was determined by multiplying CFU ml^{-1} by leachate volume for each individual lysimeter and was expressed as percentage of total STM-*lux* added.

HAdV

Viruses from leachates were concentrated by polyethylene glycol (PEG) 6000 precipitation following elution from solids using beef extract solution (Oxoid). Briefly, 200 ml leachate was centrifuged at 10 000 ***g*** for 15 min and supernatant (SN1) recovered. The pellet was resuspended in 10 ml of 10% (w/v) beef extract solution and pH adjusted to 9. Following mixing for 30 min at room temperature, the solution was centrifuged at 10 000 ***g*** for 10 min and the supernatant (SN2) added to SN1. The pH was then adjusted to 7·2, followed by the addition of PEG 6000 (10% w/v) and NaCl (2% w/v). The solution was then mixed for a minimum of 2 h at 4°C and centrifuged at 10 000 ***g*** for 20 min. The pellet was resuspended in 2 ml phosphate-buffered saline (PBS) (pH 7·0) and 0·5 ml chloroform was added, mixed for 5 min and centrifuged at 10 000 ***g*** for 15 min. The supernatant was then collected and stored at −70°C until analysis. Total HAdV in the leachate was determined by multiplying PFU ml^{-1} by leachate volume for each individual lysimeter and were expressed as percentage of total HAdV added.

The method for enumerating HAdV by culture in leachates was the same as for the HAdV stock preparations except that up to two dilutions per sample were plated out (1 μl and/or 5 μl) in A549 cells with the MPN ml^{-1} calculated from 48 wells. In addition to the HAdV culture assays, its presence was also determined by HAdV-specific PCR. Viral DNA was extracted (200 μl sample) using the High Pure Viral Nucleic Acid kit (Roche Molecular Biochemicals, Ltd., Mannheim, Germany) and resuspended in 50 μl elution buffer as per the manufacturer's instructions. HAdV detection was performed using real-time PCR (Heim *et al.* 2003) with Platinum Quantitative PCR Supermix-UDG One Step System (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) on a Rotor-Gene 3000 real-time thermocycler (Corbett Research Ltd., Sydney, Australia). PCR primers and probes were as described by Heim *et al.* (2003). Each 25 μl reaction contained 2·5 μl of DNA, 12·5 μl of Platinum Quantitative PCR Supermix-UDG, 0·25 μmol l^{-1} probe and 0·6 μmol l^{-1} primers (Heim *et al.* 2003). Following an initial denaturation step for 3 min at 95°C, a three-step cycling procedure of denaturation at 95°C for 20 s with annealing step of 55°C for 15 s and extension step at 62°C for 1 min over 45 cycles was used. Anticontamination procedures were followed for all DNA extraction and PCR procedures. Controls included positive and negative controls, an extraction blank and a HAdV plasmid standard. HAdV quantification by PCR was not determined because quantities detected in the soil samples were often below the limits of quantification. The detection limit for HAdV by PCR was calculated as 1 PCR

unit ml^{-1} leachate. Raw data were analysed using Rotor-Gene software (Corbett Research Ltd.).

Analysis of soil

Destructive harvests took place at 24 h (T_0), 1 month (T_1) and 2 months (T_2) after the simulated rainfall event. At each harvest, three replicate lysimeters from each treatment were removed and soil sampled from different depths (0–5 cm, 5–10 cm and 10–20 cm). The samples were homogenized in sterile plastic bags and subsamples taken for determining the moisture content.

Moisture contents
Moisture contents were determined by drying three 10 g wet samples at 105°C for 24 h. An average value for the three replicates was determined and was used to calculate the moisture contents on a dry-weight basis.

STM-lux
The method for enumerating STM-*lux* in soils was a five-tube MPN method adapted from Part 9260D, *Standard Methods for the Examination of Water and Wastewater* (APHA 2006). At each harvest, 10 g of soil was vortexed briefly with 900 ml of buffered peptone water (BPW) (Difco Co., NZ) (1 in 100 dilution) and then shaken for 15 min on a reciprocal shaker at 200 rev min^{-1}. Following shaking, further dilutions were obtained through serial diluting into BPW, to allow 'quantitative MPN'. An initial nonselective pre-enrichment step in BPW [16–20 h at 35°C (Mills and Barea 2003)] was then carried out on the diluted samples to allow the recovery of cells that might have been sublethally damaged by physical changes in the environment. After preincubation, 1 ml aliquots of the BPW diluents was transferred into five tubes of 10 ml Rappaport Vassilidiales medium (Difco Co.) (prewarmed to 42°C) followed by incubation at 42°C for 24 h. Following incubation, each enrichment tube was streaked onto plates of LB agar supplemented with 50 $\mu g\ ml^{-1}$ kanamycin and 12·5 $\mu g\ ml^{-1}$ tetracyclin. After overnight incubation at 32°C, the plates were examined for the growth of luminescent colonies. Final confirmation of colonies was determined using polyvalent 'O' and 'H' serological tests (Remel Europe Ltd, Dartford, Kent, UK). The detection limit was 1 MPN STM-*lux* 10 g^{-1} (wet weight sewage sludge).

HAdV
Viruses from soil samples were concentrated by PEG 6000 precipitation following virus elution using beef extract (Oxoid Ltd.). Briefly, 150 ml beef extract (10% w/v) was added to 15 g soil and the pH adjusted to 9. The mixture was vigorously shaken for 30 min and then centrifuged at 10 000 ***g*** for 30 min. The pH was adjusted to 7·2, followed by the addition of PEG 6000 (10% w/v). The solution was then mixed for a minimum of 2 h at 4°C, centrifuged at 10 000 ***g*** for 20 min and the pellet resuspended in 5 ml PBS. Finally, 2 ml chloroform was added to the concentrate, mixed for 5 min and centrifuged at 10 000 ***g*** for 20 min. The supernatant was stored at −70°C until required. Detection of HAdV in soil was the same as for leachates; however, the detection limit for HAdV by PCR was calculated as 33 PCR units $gram^{-1}$ wet weight soil.

Statistical analysis

Leachate
Leachate data were transformed to percentage of total bacteria applied to each lysimeter, and percentages were routinely arcsine (square-root) transformed prior to ANOVA analysis. Mean values are presented as back-transformed means, and 95% confidence intervals were used as a measure of variability within the data set.

MPN analysis
The confidence limits for MPN population estimates for STM-*lux* were established by Cochran (1950) and can be calculated from prepared tables (Alexander 1982; Woomer 1994). A compilation of factors based on the rate of dilution and number of tubes per dilution are used in the calculation of the confidence factor. Two population estimates differ significantly ($P = 0{\cdot}05$) when the upper limit of the lesser estimate does not overlap with the lower limit of the greater estimate (Woomer 1994).

Pathogen die-off (decimal reduction time)
Decimal reduction time (D values), which is the number of days required to cause one $\log_{10}$ or 90% reduction in the initial population (Hutchinson *et al.* 2004), was calculated from the MPN STM-*lux* data collected over the three time periods (T_0, T_1 and T_2) at the three depths. The D-values were determined from the exponential decay curves for each depth sampled and were given by the equation: $N = N_0 e^{kx}$, where N is the number of bacteria; N_0, number of bacteria at time zero harvest; k, constant from equation and x, time in days. $D -$ value $= \ln(0.1)/k$. There were insufficient data points to calculate D values for the HAdV. We compared D values by using two-way analysis of variance (ANOVA) and least significance difference testing.

Results

Leachate

Both STM-*lux* and HAdV were detected in leachates from all the lysimeters. HAdV was detected by both culture

Table 2 Percentage of total *Salmonella enterica* ssp. *enterica* serovar Typhimurium-*lux* (STM-*lux*) and human adenovirus (HAdV) (95% confidence interval) in leachate. Arithmetic means of nine replicates followed by the same letter are not significantly different (P = 0·05)

Treatment			
Organism	Control	Sewage sludge (200 kg N ha^{-1})	Sewage sludge (600 kg N ha^{-1})
STM-*lux*	0·55% (0·25–0·95)^{b}	30·00% (13·88–52·26)^{a}	27·94% (9·89–55·16)^{a}
HAdV	0·0002% (0·0002–0·0003)^{e}	0·0774% (0·0432–0·1214)^{c}	0·0454% (0·0287–0·0659)^{d}

and PCR. Significantly more STM-*lux* and HAdV leached through the soil cores when sewage sludge was present (Table 2). In the control lysimeters, <1% of the applied STM-*lux* leached, compared to over 25% in the sewage sludge treatments. For STM-*lux*, the amount of sewage sludge (200 kg N ha^{-1} or 600 kg N ha^{-1}) applied did not affect leaching (Table 2). For HAdV, although only a very small amount of viable virus was detected in the leachates, significantly fewer viruses were found in the control lysimeters compared to the sewage sludge lysimeters. Significantly more viruses leached in the 200 kg N ha^{-1} than in the 600 kg N ha^{-1} treatment.

Soils

In general, the soils in the sewage sludge-treated lysimeters had greater moisture contents than the control soils (Fig. 1); this was significant in the top 5 cm of soil for all the sampling dates except the last sampling in the 600 kg N ha^{-1} treatment.

Immediately following the simulated rainfall event (T_0), STM-*lux* could be detected at all soil depths, in all treatments (Fig. 2). Highest numbers of STM-*lux* were found in the top 0–5 cm of the soils in both the control and sewage sludge treated lysimeters, and there was a steady

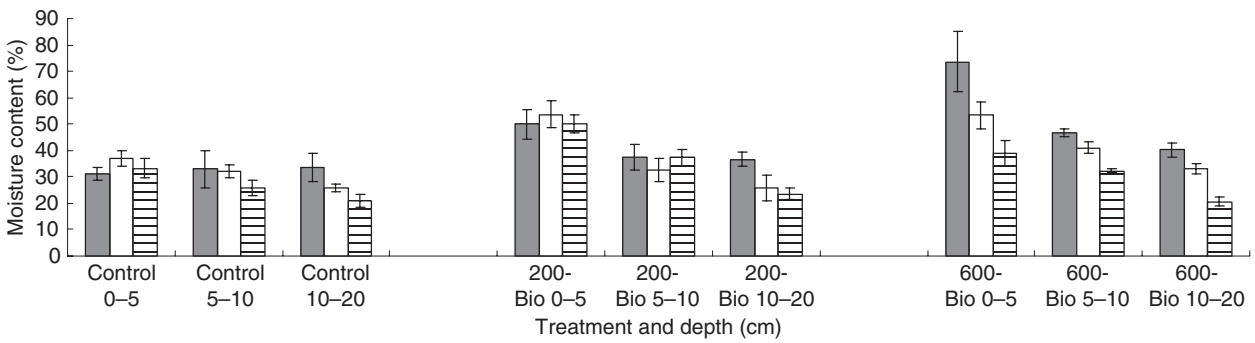

Figure 1 Soil moisture content in cores to which either pathogen contaminated control soil (control); 200 kg N ha^{-1} or 600 kg N ha^{-1} sewage sludge (200-Bio; 600-Bio) has been surface applied at different depths (0–5 cm, 5–10 cm and 10–20 cm). Error bars represent 95% confidence intervals. (■) T_0; (□) T_1 and (▤) T_2.

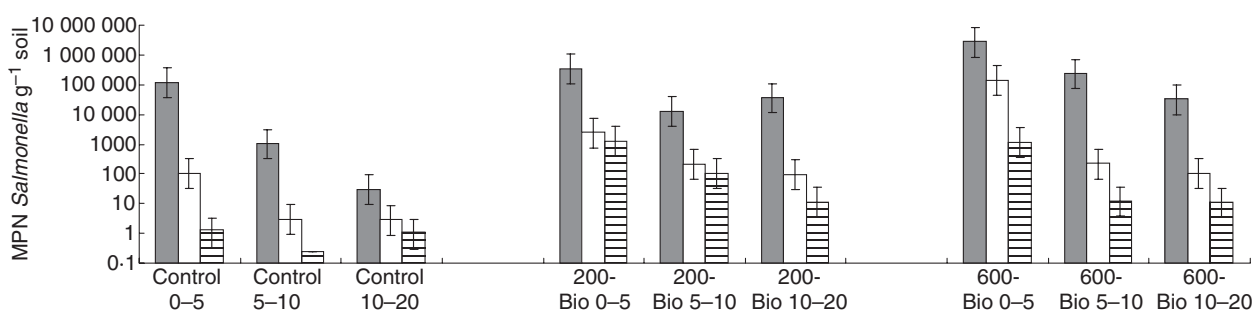

Figure 2 Survival of *Salmonella enterica* ssp. *enterica* serovar Typhimurium-*lux* (STM-*lux*) in soil cores to which either pathogen contaminated control soil (control); 200 kg N ha^{-1} or 600 kg N ha^{-1} sewage sludge (200-Bio; 600-Bio) has been surface applied. Data points represent most probable number estimates of STM-*lux* in soil at different depths (0–5 cm, 5–10 cm and 10–20 cm). Error bars represent 95% confidence intervals. (■) T_0; (□) T_1 and (▤) T_2.

Table 3 Number of days required for a 1-log decrease in *Salmonella enterica* ssp. *enterica* serovar Typhimurium-*lux* (STM-*lux*) numbers (*D* value) in soil cores at different depths (0–5 cm, 5–10 cm and 10–20 cm). Arithmetic means of nine replicates (±SE) followed by the same letter are not significantly different ($P = 0{\cdot}05$)

D values (days) for each depth			
Treatment	0–5 cm	5–10 cm	10–20 cm
Control	$4{\cdot}1 \pm 0{\cdot}07^{de}$	$4{\cdot}2 \pm 1{\cdot}8^{de}$	$3{\cdot}1 \pm 0{\cdot}6^{e}$
Sewage sludge (200 kg N ha^{-1})	$12{\cdot}7 \pm 2{\cdot}5^{abc}$	$10{\cdot}3 \pm 4{\cdot}3^{bcd}$	$5{\cdot}4 \pm 3{\cdot}4^{de}$
Sewage sludge (600 kg N ha^{-1})	$15 \pm 2{\cdot}7^{ab}$	$7{\cdot}8 \pm 0{\cdot}1^{cde}$	$19{\cdot}1 \pm 1{\cdot}2^{a}$

decline in numbers with each subsequent depth. In general, significantly more STM-*lux* were found at all depths, at all time periods in the sewage sludge treated lysimeters, compared to the controls (Fig. 2). In the control lysimeters, at T_2, only a small proportion of the original viable cells remained in the cores (0·01% in the 0–5 cm depth) (Fig. 2). High numbers of STM-*lux* persisted throughout the experiment in the top 0–5 cm of the soils in the sewage sludge-treated lysimeters, indeed at T_2, after 2 months, there were still $>10^3$ STM-*lux* present (g^{-1} dry soil) in the top 5 cm of the soils.

The *D* values calculated from the decline in STM-*lux* numbers are shown in Table 3. Significance testing [$P < 0{\cdot}05$ (ANOVA)] revealed that rates of decline for STM-*lux* in the control lysimeters were more rapid than in the sewage sludge-treated lysimeters. There was no significant difference in STM-*lux* *D* values between the 200 kg N ha^{-1} and 600 kg N ha^{-1} sewage sludge treatments.

No culturable HAdV was detected in any of the soil depths at any of the sampling periods in the control lysimeter, although HAdV was detected by PCR (two out of three replicates at each soil depth, 0–5 cm, 5–10 cm and 10–20 cm). Viable HAdVs were detected in the 200 kg N ha^{-1} and 600 kg N ha^{-1} sewage sludge treatments immediately following the simulated rainfall (T_0), but only in the 0–5 and 5–10 cm soil depths (Table 4). Significantly more culturable HAdVs were found in the 600 kg N ha^{-1} than in the 200 kg N ha^{-1} biosolid treatment in the 0–5 cm depth only (T_0). No HAdV was detected at soil depth 10–20 cm for either sewage sludge treatments.

Although no culturable HAdV was detected at T_1 or T_2 in any lysimeter soil, HAdV was detected by PCR only in the 600 kg N ha^{-1} sewage sludge treatment at T_1 in soil at 0–5 cm (three out of three replicate lysimeters) and at 5–10 cm (one out of three replicate lysimeters) (Table 4). All other samples were negative by PCR at T_1. Samples taken at T_2 were not analysed by PCR.

Discussion

The bioluminescence reporter system used in the STM-*lux* was selected for its utility in visualizing bacteria in biological systems and because cells can be easily identified by their bioluminescent phenotype, without need for further identification. The STM-*lux* proved very easy to culture and identify with very little background interference. An additional benefit of the *lux*-based system is the antibiotic-resistance genes carried on the *lux* plasmid which allows a further selection criterion.

Microbial movement in soils are mainly influenced by soil type and degree of water saturation. Soil type affects adsorption of the microbes to the soil particles (i.e. the degree of filtration potential of the soils); clay and organic matter are the major components affecting adsorption (Sobsey *et al.* 1980; Aislabie *et al.* 2001). The presence of macropores and soil structural cracks can increase bypass flow and increase microbial leaching (Gagliardi and Karns 2000; Aislabie *et al.* 2001). In this study, undisturbed soil columns were used where the natural soil structure remained intact and saturated flow was simulated with a

Table 4 Human adenovirus (HAdV) recovered (MPN 100 g^{-1} ± SE) in soil cores at different depths (0–5 cm, 5–10 cm and 10–20 cm). HAdV was added to either soil or sewage sludge, surface applied to the soil and analysed at T_0, T_1 and T_2 (results not shown for T_2). Numbers of replicate lysimeters ($n = 3$) that were positive by PCR are shown in brackets

	HAdV detected (number of lysimeter replicates PCR positive $n = 3$)					
Time	T_0			T_1		
Treatment	0–5 cm	5–10 cm	10–20 cm	0–5 cm	5–10 cm	10–20 cm
Control	ND* (2/3)	ND* (2/3)	ND* (2/3)	ND* (0/3)	ND* (0/3)	ND* (0/3)
Sewage sludge (200 kg N ha^{-1})	$36 \pm 11{\cdot}2^{a}$ (3/3)	ND* (2/3)	ND* (1/3)	$2 \pm 1{\cdot}1^{a}$ (0/3)	ND* (0/3)	ND* (0/3)
Sewage sludge (600 kg N ha^{-1})	$115 \pm 49{\cdot}8^{b}$ (3/3)	ND* (2/3)	ND* (1/3)	$2 \pm 1{\cdot}1^{a}$ (3/3)	ND* (2/3)	ND* (1/3)

*ND, not detected: below the detection limit of <140 MPN per 100 g (wet weight).

heavy rainfall event. Under these conditions, it would be expected that bacteria and viruses should move rapidly through the soils, but in the control lysimeters, <1% of the applied bacteria and viruses were recovered in the leachate. For these soils, it is likely that adsorption processes limited microbial leaching, and that the microbes were effectively filtered by the soils. For the sewage-sludge treated soils, the results in this study clearly indicate that the presence of sewage sludge can significantly influence the transport of bacteria and viruses in waste-amended soils. Previous studies have shown that animal manures increased total numbers of *E. coli* O157:H7 in leachate from intact cores (Gagliardi and Karns 2000); however, it is unclear how the presence of manure aids movement of the microbes. Gagliardi and Karns (2000) determined that *E. coli* O157:H7 was not attached to leaching particles, although Shelton *et al.* (2003) did determine a relationship between faecal coliform release and turbidity in studies with dairy manure. Bardford *et al.* (2006) looked at the transport of *Giardia* and manure suspensions in a saturated porous media and found that small pores in the soils became filled with manure particles confining both manure particles and *Giardia* transport to the more larger conductive pore; thus *Giardia* transport was increased in the presence of manure. Organic matter complicates viral adsorption by adding binding sites to soil (Bixby and O'Brian 1979; Powelson *et al.* 1991). In this soil type, it is unclear which is the dominant mechanism of increased microbial transport; however, pooling of water was visible on the surface of the sludge-treated lysimeters during the simulated rainfall event suggesting that some pore blockage was occurring. It is thus probable that the filtering of microbes by the soil is inhibited by the presence of sewage sludge which blocks the smaller pores and promotes bypass flow and increased leaching of microbes. It is clear from the results shown in Fig. 2 that bacteria applied to the surface were mainly harboured within the top layer of the soil in both the control and sewage sludge-treated lysimeters; thus, it is probable that straining or sieving at the soil surface, along with adsorption, concentrated bacteria in the top 5 cm of the soils (Sorber and Moore 1987). In a study investigating the survival of *E. coli* O157:H7 following the surface and subsurface application of contaminated organic waste, Avery *et al.* (2004) found that bacteria were also concentrated in the top layer of soils.

When sewage sludge was present, the recovery of STM-*lux* was much greater at all soil depths and all time periods than in control lysimeters with no sewage sludge (Fig. 2). It is possible that the sewage sludge particles provided the bacteria with additional nutrients, and/or that the presence of additional organic matter provided the bacteria with microsites to avoid predation by predators. In a comparative study, of the decline of *E. coli* in water, cattle manure and soil incorporated cattle manure, Oliver *et al.* (2006) found that the most rapid die-off was observed for those cells introduced via sterile water; this may be because the cells had no faecal substrate from which to obtain nutrients nor colloids with which to associate for protection from predators. In control lysimeters, the die-off (*D* value, Table 3) was increased as compared to the sewage-sludge amended lysimeters; this may have been because STM-*lux* had to compete more with the soil microflora for available nutrients that were in short supply (Gagliardi and Karns 2000). Although attempts were made to maintain a constant soil moisture in the lysimeters, soils from the control lysimeters were less moist than the sewage sludge-treated soils (Fig. 1). Numerous studies have demonstrated that increased soil moisture increases survival rates of pathogens in soil (Yeager and Ward 1981; Vasseur *et al.* 1996; Zaleski *et al.* 2005). It has also been shown that certain conditions, such as increased moisture and carbon availability, can cause re-growth of *Salmonella* ssp. in biosolids (Soares *et al.* 1995; Eamens *et al.* 2006). Therefore, it is possible that a variety of factors including increased moisture, additional nutrients and protection from predation and competition may have increased *D* values of STM-*lux* in the sewage sludge-treated lysimeters. Heavy application rates of wastes to soil have been shown to increase soil saturation, which can also increase pathogen mobility and decrease moisture loss, and these can increase survival times (Carrington 2001). In this study, for STM-*lux*, application rate did not affect numbers in leachate or *D* values.

The amount of virus recovered in the leachates (control and sludge amended) was extremely low compared to the levels of bacteria observed. Previous studies have demonstrated that viruses may adsorb readily to soils, particularly clay soils and sewage sludge, resulting in a large reduction in virus in the leachate (Bitton *et al.* 1984; Chetochine *et al.* 2006). It is probable that the vast majority of viruses added to the sludge became effectively irreversibly bound within sludge particles and/or soil and those that leached from the lysimeter were 'free' virus particles in the aqueous phase. In addition, because of the methods used to extract viruses from soil, it is also possible that the strong adsorption of virus particles to soil particles would result in low recovery during the extraction procedures which would affect the detection sensitivity. If adsorption is the dominant mechanism controlling transport of virus in the environment, then this may explain why significantly more virus leached in the 200 kg N ha^{-1} than in the 600 kg N ha^{-1} treatment as increased organic matter may interfere with virus adsorption (Bixby and O'Brian 1979).

It was interesting to note that viruses were detected by PCR in several cases in soil where no culturable viruses were detected. For example, HAdV DNA was detected in two out of three replicates at T_0 control lysimeters showing their presence (albeit low levels) at all soil depths despite the failure to grow. One reason may be that PCR will detect viable and nonviable viruses resulting in greater sensitivity. The PCR detected viral DNA at T_1 at the higher sludge rate (600 kg N ha^{-1}) despite failing to detect any viruses by culture. This may be explained that over time, factors such as microflora presence and temperature can have an effect on virus viability.

It is difficult to assess the survival of HAdV in the lysimeters because of limitations of the methodology; however, the results do suggest that the soil in this experiment is an effective filter for virus particles and that sewage sludge is an unlikely nonpoint source of environmental virus contamination.

The survival, fate and transport of microbial contaminants in sewage sludge applied to land is an important issue with regard to the risk of contamination of the surrounding land and water. The application rate and duration of the rainfall event used in this study is not unusual for a New Zealand winter [New Zealand National Climate Survey, (NIWA 2006)] and would likely cause surface flooding and overtopping of small streams. Although this study indicates a potential risk of environmental contamination from horizontal and vertical movements of sewage sludge-borne bacteria, the limitations must be recognized. Because sewage sludge that is land applied must be treated to provide a significant reduction in pathogens; results from this study may overstate any potential risks as levels of STM-*lux* and HAdV in spiked samples were significantly greater than what may be expected in treated sewage sludge [e.g. 10^0–10^3 MPN g^{-1} (Hon 2003; Horswell *et al.* 2007) and 10^2–10^4 MPN g^{-1} (Ward *et al.* 1984) for *Salmonella* ssp. and HAdV respectively]. However, this study highlights the importance of the pathogen treatment requirements for land-applied sewage sludge as well as the importance of selecting sites with adequate buffers to the receiving environment (e.g. surface water and groundwater).

Once applied to land, natural attenuation can reduce pathogens in the land-applied wastes. However, natural attenuation relies on environmental factors such as temperature, UV exposure and indigenous microbial competition; these will vary from site-to-site and cannot be controlled or predicted. Land management options to avoid scenarios likely to enhance transport and prolong survival (e.g. incorporation of sewage sludge with soil and not applying large inputs of waste) could be a viable approach to minimize nonpoint-source contamination and ensure the least public health risk.

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