Proportion of sewage sludge to soil influences the survival of *Salmonella* Dublin, and *Escherichia coli*

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Abstract

The survival of enteric pathogens in sewage sludge could lead to their transferral into the soil environment and subsequent contamination of crops and water courses. This, in turn, could increase the potential spread of gastrointestinal disease. This work aimed to determine the persistence of several microorganisms, co-introduced with sewage sludge, when exposed to varying proportions of sewage sludge to soil. Three microcosm-based studies were established, inoculated with *Salmonella* Dublin or an environmentally-persistent strain of *Escherichia coli* (quantified periodically over a period of 42 days), or indigenous sewage sludge *E. coli* (quantified over a period of 56 days). Treatments consisted of a mixture containing: 0, 15, 25, 50, 75 and 100% soil or sludge, depending upon the experiment. Each introduced microorganism declined significantly over time, with greater quantities of soil generally instigating greater die-off particularly in the cases of environmentally-persistent *E. coli* and *S. Dublin*. However, this relationship was not proportionally related as sludge/soil mixtures showed greater declines than pure soil treatments. In contrast, indigenous sewage sludge *E. coli* had a more consistent decline across all treatments. This may indicate that indigenous strains are more resilient and may be indicative of natural behaviour. Moreover, the effects of soil-borne factors on pathogen attenuation were context dependent and non-linear, possibly arising from the relative spatial distribution of introduced sludge and attendant microbes in soil.

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Abbreviations: **ANOVA**, analysis of variance; **CFU**, colony-forming units; **DS**, dry solids; **MLGA**, membrane lactose glucuronide agar; **MRD**, maximum recovery diluent; **OM**, organic matter; **WWTF**, wastewater treatment facilities; **XLD**, xylose-lysine-desoxycholate

1. Introduction

Sewage sludge can confer improved structure and fertility to soil through the addition of organic matter (OM) and nutrients [1]. However, sewage sludge can harbour enteric pathogens, shed in the faeces of infected individuals and accumulate at wastewater treatment facilities (WWTFs) [2]. Furthermore, these pathogens are capable of surviving the treatment processes implemented at WWTFs [3, 4]. Therefore, their survival in sewage sludge and the subsequent use of this product as an agricultural fertiliser and soil conditioner could lead to the transfer of enteric pathogens into the soil environment. This could subsequently contaminate crops and water courses, increasing the potential for gastrointestinal disease outbreaks [5--7]. Not only do such outbreaks pose a risk to individuals, but they can also place a heavy economic burden on society [8, 9].

Well-publicised outbreaks can also elicit strong, negative responses from the general public, leading to a drop in sales of related produce and a decrease in trust in relation to shops and their suppliers, as well as monetary fines. Any association between these opinions, monetary losses and hazards with the practice of sewage sludge application to agricultural land could therefore prove to be detrimental to its continued use as a fertiliser. Additionally, when considering the large quantities of sewage sludge produced annually across the UK (1.4 million tonnes as of 2008) and Europe (10.13 million tonnes) [10, 11], the need to dispose of this material in a sustainable manner is paramount to developing and maintaining the goal of a more sustainable society.

Furthermore, the persistence of enteric pathogens in the soil environment can vary significantly. *Escherichia coli* have been shown to persist for 29 days in slurry when applied to arable and grass plots [12], 231 days in manure-amended autoclaved/un-autoclaved soil [13], with environmental strains of *E. coli* persisting for upwards of nine years in soil [14]. Similarly, *Salmonella* species have been shown to survive for approximately 200 to 400 days in soil when co-introduced with manure, irrigation water or slurry [15, 16]. Additionally, Avery et al. [17] found that *E. coli* populations originating from livestock faeces persisted for approximately 180 days in a grassland pasture where livestock had been penned. It is therefore important to elucidate what factors may be of greatest influence. For example, persistence can be attributed to variation in nutrient availability and microbial diversity [18, 19].

It is theorised that the application of sewage sludge to land could lead to a hot spot of activity, where there is enhanced activity within the local microbial community triggered by the increased availability of carbonaceous substrate and nutrient elements [20, 21]. This hot spot of activity could drive the exclusion or greater decline of enteric pathogens when co-introduced with sewage sludge, via greater competition for local resources. Moreover, previous work has shown that hot spots of activity after an input of assimilable carbon/nutrients can occur in the presence of animal manure, accumulated OM or the rhizosphere [22--24].

Therefore, this study aimed to assess the persistence of several model/indigenous microorganisms, co-introduced with sewage sludge, in relation to loading rates of sewage sludge in soil. We hypothesised that there was a positive correlation between increased proportions of sludge to soil and the survival of

model/indigenous microorganisms. To investigate this, two microcosm-based studies were established, using an environmentally-persistent strain of *E. coli* or model microorganism *Salmonella* Dublin, within microcosms containing varying proportions of soil to sewage sludge. Following this, a further microcosm study determined the effects of loading rate of sewage sludge on the persistence of indigenous, sewage sludge *E. coli* in soil.

2. Materials and methods

2.1. Experiment 1

Soil A, a loamy, brown earth soil, was collected from a cattle-grazed pasture comprised of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.), located at Teagasc Environment Research Centre, Johnstown Castle, Wexford, Ireland (52.3342°N, −6.4575°W) (http://met.ie/climate/rainfall.asp; http://met.ie/climate-ireland/surface-temperature.asp). Three top-soil samples (0--10 cm) were collected from random points within the pasture, within one month of the onset of each experiment. The samples were then sieved to approximately 5 mm and coned/quartered to produce a homogeneous composite sample [25]. Sludge A, an anaerobically digested and dewatered sewage sludge cake, was provided by United Utilities, Ellesmere Port, UK. The sewage sludge was air-dried to a fixed moisture content of 60% and pasteurised (70°C) for 24 h to increase friability and reduce the concentration of indigenous microorganisms. It was then sieved to approximately 5 mm. Soil and sewage sludge composites were assessed, in triplicate, for levels of indigenous microorganisms (*E. coli* and a viable bacterial count). Following a modified protocol by Troxler et al. [26], sterile quarter strength Ringer's solution (50 mL) was added to each sample and shaken gently by end-over-end rotation (100 rpm) for 30 min and vortexed for 10 s. A tenfold dilution (100 µL sample to 900 µL sterile quarter strength Ringer's solution) was then made up. A selection of the dilution series was then spread plated onto Sorbitol MacConkey and Standard Plate Count Agar, to analyse *E. coli* and viable bacterial cells respectively. The plates were then incubated at 37°C for 24 h. Physico-chemical analyses were also performed within one month of collection and are summarised in Table 1 for soil and Tables 2 and 3 for sludge, respectively.

A set of microcosms (sterile 100 mL plastic containers) containing soil and sewage sludge was established (Fig. 1). The weights for each treatment were prescribed on a volume to volume ratio, with a standardised volume of 16 mL per sample used throughout (Table 4). The microcosms were inoculated with an environmentally persistent strain *E. coli* Lys 9 [27]. Initially, aliquots of the environmentally-persistent *E. coli* were grown for 24 h in Luria-Bertani broth at 37°C on an orbital shaker (120 rev min^{-1}). Aliquots of the culture, taken at peak cell propagation, were then transferred to another vial containing 50 mL of fresh LB broth and incubated following the same procedure. The culture was then centrifuged (4500 rpm) and re-suspended in sterile quarter strength Ringer's solution three times. Aliquots (100 µL) of the cell suspension were then inoculated into the containers holding sewage sludge. A tenfold dilution series (100 µL of culture to 900 µL sterile quarter strength Ringer's solution) was also created from the culture and used to assess absorbency and cell count. The initial quantity of environmentally-persistent *E. coli* added to relevant treatments was approximately 2.3×10^8 colony -orming units (CFU) mL^{-1}. The un-inoculated soil was then added to the spiked sewage sludge.

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The resulting microcosms were then gently shaken by end-over-end rotation for 60 s. Where there were treatments without sludge, bacteria were added directly to the soil. The two control treatments for soil and sludge were inoculated with sterile quarter strength Ringer's solution, allowing for the analysis of background levels of microorganisms. The microcosms were then incubated at 10°C. At each time point (0, 1, 3, 7, 14, 28, 42 days), one full cohort of treatments ($n = 3$) was removed and *E. coli* extracted/enumerated as described above.

2.2. Experiment 2

Experiment 2 was run concurrently alongside Experiment 1 (Section 2.1), using the same matrices (soil A and sludge A) and microcosm set-up (Tab. 4, Fig. 1). These matrices were then assessed for presumptive *Salmonella* and *Salmonella* Dublin (NCTC 9676) substituted as the inoculant. The initial quantity of *S.* Dublin was approximately 1.0×10^9 CFU mL^{-1}. Again, the microcosms were incubated at 10°C and removed at the same time points ($n = 3$). *Salmonella* Dublin was then extracted and spread plated onto xylose-lysine-desoxycholate (XLD) agar, as described above in Section 2.1.

2.3. Experiment 3

Soil and sludge matrices were sourced from the same locations and analysed using the same protocols as outlined in Section 2.1, but are hereafter denoted soil B and sludge B to emphasise that they represent different aliquots of these materials. The matrices were then assessed for general coliforms and *E. coli*. The sludge was manually crumbled to an aggregate size of approximately 5 mm to ensure homogeneity and its moisture content remained unaltered. Background levels of indigenous sewage sludge microorganisms were extracted in triplicate as described in Section 2.1, substituting sterile maximum recovery diluent (MRD) (Oxoid) instead of ¼ Ringer's. Aliquots of each extract were diluted, 1 mL extract to 5 mL MRD to aid dispersion, then filtered and enumerated using membrane filtration and membrane lactose glucuronide agar (MLGA) [28]. The sewage sludge contained approximately 1.1×10^5 g^{-1} dry solids (DS) indigenous sewage sludge *E. coli*, sufficient to study population decline without the need for additional inoculation.

One set of microcosms containing soil and sewage sludge was established (Table 5 and Fig. 2). The initial cell count was taken from the first reading (day 0) of the control sludge treatment, 1.13×10^5 CFU g^{-1} DS. The weights for each treatment were prescribed on a dry weight ratio, with a consistent weight of sewage sludge used throughout. This ensured a consistent concentration of indigenous *E. coli*. For mixed treatments, soil and sewage sludge were weighed out separately into sterile 100 mL, plastic, screw-cap containers. The soil was then incorporated into the containers holding the sewage sludge, through manual end-over-end rotation for 60 s. These microcosms were then incubated at 10°C. At each time point (0, 1, 3, 7, 14, 28, 42, 56 days), one full cohort of treatments ($n = 3$) was removed from the incubator. Indigenous sewage sludge *E. coli* was then extracted as described in Section 2.1, using MRD. The extract was then filtered and enumerated using membrane filtration and MLGA.

2.4. Statistical analysis

A two-way factorial analysis of variance (ANOVA) was implemented to analyse treatment and time

effects on microbial survival, using R version 3.2.3 for Windows (http://R-project.org). The Bonferroni correction was used to assess means for homogeneity with a significance level of 95%. A one-way ANOVA was also performed on the final time point for each experiment and a Tukey multiple comparisons of means implemented, to a 95% family-wise confidence level.

3. Results and discussion

The un-inoculated control soil treatments across all three experiments did not contain detectable concentrations of *E. coli* or *Salmonella*. There were also no detectable concentrations of *Salmonella* Dublin within the un-inoculated control sludge for the second experiment. They were therefore omitted from further statistical analysis and associated figures. Indigenous *E. coli* within the first experiment prevailed in the un-inoculated control sludge indicating that pasteurisation had not eradicated them. However, as they were detectable using the agar medium the *E. coli* count was considered to have included both the environmentally-persistent and indigenous *E. coli*.

A one-way ANOVA comparing the quantity of environmentally-persistent *E. coli* between treatments, at the final time point (day 42), revealed three homogeneous groupings (Fig. 3). The first group, containing 100% sludge and control sludge treatments, maintained the highest levels of *E. coli*. Whilst the *E. coli* in the second group, which contained 75% sludge, 50% sludge and 100% soil, declined to a greater extent. The third group containing only 25% sludge showed the greatest attenuation of *E. coli*. It is also pertinent to note the marked and sustained effect that 25% sludge treatment has on attenuation, which became apparent from day 14 onwards (Fig. 3). Similarly, a one-way ANOVA comparing the levels of *Salmonella* Dublin between treatments on day 42 resulted in essentially the same groupings of treatments, discounting control sludge (Fig. 4). Here, each treatment declined at a similar rate that was more linear than in the previous experiment for environmentally-persistent *E. coli* (Fig. 5). Additionally, a one-way ANOVA comparing the quantity of indigenous sewage sludge *E. coli* between treatments at the final time point (day 56), again showed three homogeneous groups. Here the first group contained the control sludge treatment and again maintained the highest concentrations of *E. coli*. The second group, containing 75% sludge, 25% sludge and 15% sludge, showed a slight decline in *E. coli* levels. The third group contained only 50% sludge and showed the greatest attenuation of *E. coli*. There was an apparent sharp decline and subsequent increase in indigenous sewage sludge *E. coli* within the first week. This sharp decline is unusual for microbial propagation patterns and difficult to explain. This could have occurred due to the change in temperature conditions the microorganisms within the sludge underwent from the outset of the experiment. Prior to the start of the experiment the sludge was stored at 4 °C. The experiment itself was maintained at 10 °C. This change could have led to a crash and re-establishment of the indigenous sewage sludge *E. coli*.

These findings show that the greatest declines for both strains of *E. coli* and *Salmonella* Dublin were observed within the treatments containing 25–50 % sludge, with soil/sludge mixtures generally showing greater declines than in the pure sludge treatments. These ratios could have instigated the greatest decline due to a balance of physicochemical and biological factors being reached. For example, the quantity of sludge could have been optimal for nutrient provision, whilst the potential toxicity could have been minimal due to the lesser quantity added. Additionally, these ratios of sludge to soil may

have favoured the soil microbial community potentially leading to more antagonistic interactions with the introduced microorganisms. Furthermore, this indicates that the presence of any quantity of soil can induce greater attenuation of *E. coli* or *Salmonella* Dublin, with greater quantities of soil generally instigating greater die-off. However, rather than pure soil treatments initiating greatest die-off, treatments containing sludge/soil mixtures showed greater declines. Therefore, the initial hypothesis stating that there would be a positive correlation between increased proportions of sludge to soil and the survival of model/indigenous microorganisms is accepted, with the qualification that this is in the context of some proportion of sludge.

Furthermore, these findings also indicate that soil and its constituents have some form of detrimental effect on the survival of introduced pathogens, but the effects are not directly proportional to the ratio. They also suggest that there may be an optimal ratio of sludge to soil that would provide a balance of factors which induce greatest attenuation of introduced microbes. For example, Jiang et al. [13] found that the greatest ratio of manure to soil, 1:10 manure to soil versus 1:25, 1:50 or 1:100, resulted in a greater decline in *E. coli* O157:H7. It was suggested that such an outcome may have been related to antagonistic interactions between the soil microbial community and *E. coli* O157:H7. Schwarz et al. [29] also demonstrated a greater decline in *E. coli* and *S. enterica* in anaerobically-digested dewatered biosolids applied to soils, in comparison to un-amended soils. They postulated that the greater decline in *E. coli* and *S. enterica* in amended soils could have been caused by enhanced antagonistic activity of the indigenous microbial populations in relation to microbially-available substrate and improved moisture content. Additionally, Moynihan et al. [30] monitored the persistence of *Salmonella* Dublin, *Listeria monocytogenes,* non-toxigenic *E. coli* O157 and environmentally-persistent *E. coli* over 110 days across 12 different soils with contrasting land-management practices. They found that there was a wide variety of factors which influenced the survival of *Salmonella* Dublin, *L. monocytogenes* and non-toxigenic *E. coli* O157, which indicated that whole-scale community interactions played a significant part in their survival and were context specific. However, these factors did not appear to affect the environmentally-persistent strain of *E. coli*. As such, it can be postulated that the local microbial community within the soil could act as attenuating agents to introduced pathogens, and further argued that access to these agents would be important. Hence the intrinsic frequency of contact between these two microbial communities and their respective matrices (soil and sludge) would be a factor in the attenuation of pathogens. It follows that a greater proportion of soil/sludge will increase the likelihood of such interactions. Therefore there would be a greater possibility for microorganisms to move between these matrices, due to shorter path lengths between these phases in the pore network, and greater connectivity between pore space, modulated by water content. This shortening of path length would arise inevitably from there being a greater concentration of soil and hence average distance between soil/sludge particles (or zones). There would also be an increased likelihood of interaction between the soil microbial community and the introduced pathogens due to a greater dispersal of both throughout the matrices. The nature and extent of such phenomena would also be influenced by the degree of mixing of the two phases, which was experimentally controlled here to be consistent between treatments.

Moreover, the influence of soil on persistence was more apparent with the environmentally-persistent

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E. coli and *S.* Dublin than the indigenous sewage sludge *E. coli*, which had a more consistent linear decline across all treatments. This may indicate that indigenous strains are more resilient and indicative of natural behaviour. Further evidence of this has been shown by Franz et al. [31] who studied the persistence of 18 *E. coli* O157 strains; eight animal, one food and nine human isolates, in manure-amended sandy soil. They found a high degree of variation in survival across all strains, with human isolates generally surviving for significantly greater periods (median 211 days) when compared to animal isolates (median 70 days). Alternately, the location of the model and indigenous microorganisms within or on the soil/sludge matrices may have influenced these apparent differences in attenuation. For instance, the model microorganisms where inoculated onto soil and sludge matrices, whilst the indigenous *E. coli* were already present and established within sludge aggregates. Therefore, this potential difference in location could affect the degree of exposure or interaction between such microorganisms and the soil microbial community, as well as other physicochemical characteristics.

4. Concluding remarks

The presence of any quantity of soil induced greater attenuation of introduced microorganisms, with greater quantities of soil generally instigating greater die-off. In general, these results indicate that the loading rate of sewage sludge in agricultural soils is a significant factor in their persistence. However, other factors such as location of microorganisms within the profile, the nature of the introduced species, substrate content and mixing between matrices may be of importance. There appears to be no optimal ratio of sludge/soil, rather this is content dependent.

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Table 1. Physicochemical and biological characteristics of soil (Mean ± SEM, n=3). [a]

Analysis	Soil A	Soil B
pH (H_2O) 1:1	6.37 ± 0.11	6.1 ± 0.06
Total exchange capacity	12.3 ± 0.23	9.34 ± 0.27

(mEq 100 g^{-1})		
Moisture content (%)	31.8 ± 0.3	24.5 ± 0.04
Organic matter (Loss on ignition) %	6.92 ± 0.18	4.95 ± 0.07
Bray I phosphorus (P) (mg L^{-1})	111 ± 0.7	64.3 ± 1.2
Nitrogen (N) (mg L^{-1})		
Nitrate (NO$_3$)	49.6 ± 0.51	20.1 ± 1.19
Ammonium (NH$_4$)	6.07 ± 0.25	1.13 ± 0.09
Mehlich III extractable (mg L^{-1})		
Phosphorus (P)	108 ± 0.77	66.7 ± 0.25
Potassium (K)	228 ± 0.89	96.3 ± 0.67
Bacterial count (CFU g DS^{-1})		
Viable bacterial count	$4.50 \times 10^4 \pm 4.20 \times 10^1$	[b)
E. coli O157	$2.83 \times 10^2 \pm 6.00 \times 10^0$	[b)
Presumptive *Salmonella*	$2.33 \times 10^2 \pm 7.00 \times 10^0$	[b)
E. coli	[b)	0 ± 0
General coliforms	[b)	$4.43 \times 10^3 \pm 5.70 \times 10^2$

a) [32--39]; performed by Brookside Laboratories, New Knoxville, Ohio, US.

b) Not applicable to given experiment

Table 2. Physicochemical and biological characteristics of sewage sludge (Mean ± SEM, *n* = 3). [a)

Analysis	Sludge A	Sludge B
pH (H$_2$0) 1:1	7.54 ± 0.8	7.39 ± 0.17
Total solids (g kg^{-1})	247 ± 0.56	245 ± 0.44
Moisture content (%)	64.8 ± 0.41	74.6 ± 0.04
Total phosphorus (g kg^{-1})	203 ± 6.17	5.68 ± 0.06
Phosphorus as P$_2$O$_5$ (g kg^{-1})	476 ± 9.43	13 ± 0.09
Potassium (g kg^{-1})	22.2 ± 2.04	195 ± 6.12
Potassium as K$_2$O (g kg^{-1})	26.8 ± 2.23	0.72 ± 0.04
Nitrogen (g kg^{-1})		
Total Kjeldahl	11.3 ± 0.13	9.73 ± 0.16
Organic	8.96 ± 0.16	9.58 ± 0.16
Nitrate	<0.1 ± 0.0	44.3 ± 2.35
Ammonia	2.35 ± 0.1	0.16 ± 0.03
Bacterial Count (CFU g^{-1} DS)		
Viable bacterial count	$7.00 \times 10^5 \pm 1.76 \times 10^2$	[b)
E. coli O157	$5.00 \times 10^3 \pm 24 \times 10^1$	[b)
Presumptive *Salmonella*	0 ± 0	[b)

E. coli	[b]	$6.57 \times 10^5 \pm 9.39 \times 10^4$
General coliforms	[b]	$1.13 \times 10^5 \pm 2.82 \times 10^4$

a) [35, 37, 40--46]; performed by Brookside Laboratories, New Knoxville, Ohio, US.

b) Not applicable to given experiment

Table 3. Heavy metal elemental composition of sewage sludge (Mean ± SEM, *n* = 3). [a]

Analysis	Fresh weight ($mg \, kg^{-1}$)	Dry weight ($mg \, kg^{-1}$)
Arsenic (As)	3.12 ± 0.11	13.4 ± 0.16
Cadmium (Cd)	0.53 ± 0.06	2.28 ± 0.08
Chromium (Cr)	8.28 ± 0.20	35.4 ± 0.30
Copper (Cu)	60.7 ± 0.42	259 ± 0.55
Lead (Pb)	34.0 ± 0.37	145 ± 0.55
Mercury (Hg)	0.10 ± 0.02	0.45 ± 0.07
Molybdenum (Mo)	1.42 ± 0.06	5.85 ± 0.18
Nickel (Ni)	31.1 ± 0.23	133 ± 0.20
Selenium (Se)	0.55 ± 0.00	2.32 ± 0.06
Zinc (Zn)	162 ± 0.46	691 ± 0.70

a) [46--49]; performed by Brookside Laboratories, New Knoxville, Ohio, US.

Table 4. Treatment outline (*n* = 3) based on volume basis (%), with equivalent weight of sewage sludge and soil (±0.05 g).

	Fresh weight (g)	
Content	Soil	Sludge
Un-inoculated soil	24.22	0
Soil	24.22	0
25% sludge	18.17	3.56
50% sludge	12.11	7.12
75% sludge	6.05	10.68
Un-inoculated sludge	0	14.24
Sludge	0	14.24

Table 5. Treatment outline (*n* = 3), with equivalent weight of sewage sludge and soil (±0.02 g).

	Fresh weight (g)	
Content	Soil	Sludge
Soil	1.35	0
15% sludge	7.63	4.00

25% sludge	4.04	4.00
50% sludge	1.35	4.00
75% sludge	0.45	4.00
Sludge	0	4.00

Figure legends:

Figure 1. Treatment outlines for Experiments 1 and 2. ☐ soil; ▨ sludge; ▦ inoculated with *E. coli* or *S. Dublin*.

Figure 2. Treatment outline for Experiment 3. ☐ soil; ☐ sludge, with indigenous sewage sludge *E. coli*.

Figure 3. Survival of *E. coli* within soil/sludge microcosms (*n* = 3). ──●── soil; ──○── 25% sludge; ──▲── 50% sludge; ──△── 75% sludge; ──■── sludge; ─▫─ un-inoculated sludge. Pooled standard error of log transformed data: 0.15. Letters denote homogeneous means at 5% significance level on day 42.

Figure 4. Survival of *S*. Dublin within soil/sludge microcosms (*n* = 3). ──●── soil; ──○── 25% sludge; ──▲── 50% sludge; ──△── 75% sludge; ──■── sludge. Pooled standard error of log transformed data: 0.11. Letters denote homogeneous means at 5% significance level on day 42.
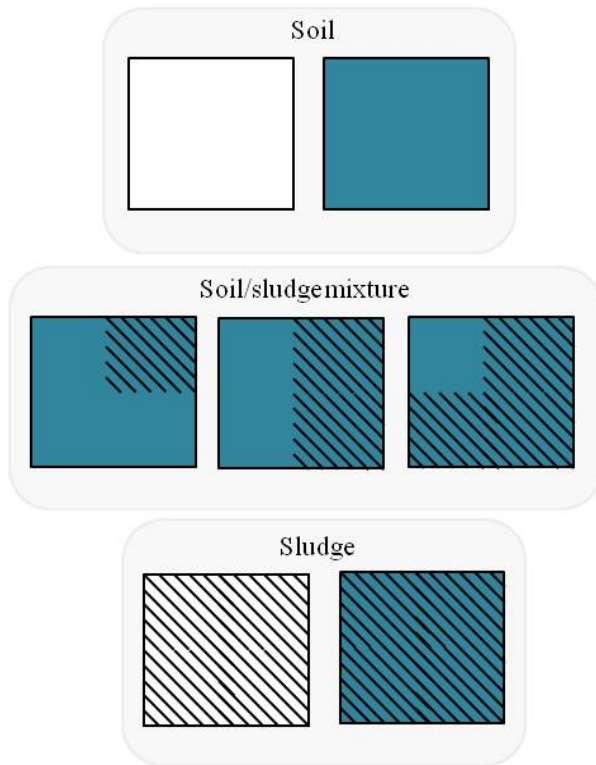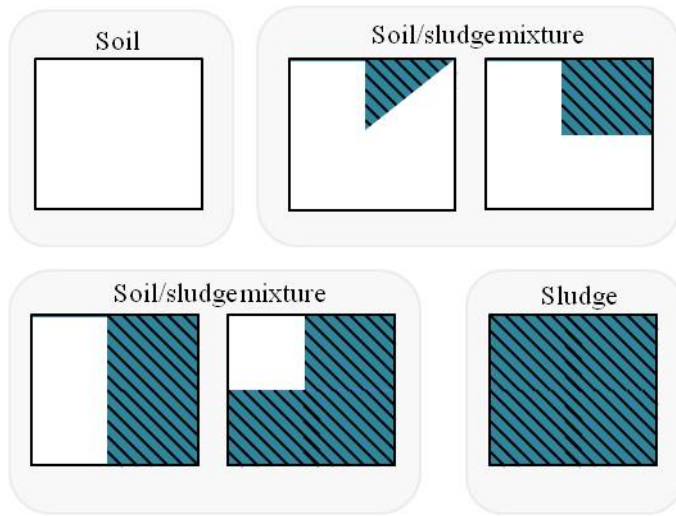
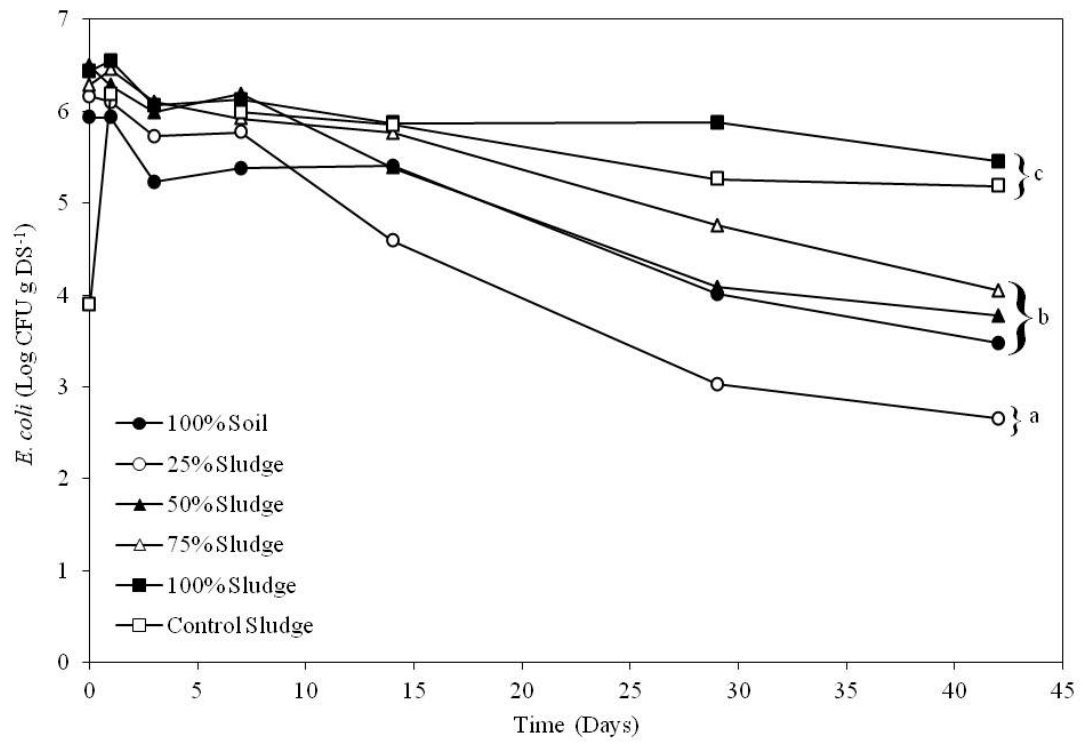Figure 5. Survival of indigenous sewage sludge *E. coli* soil/sludge microcosms (*n* = 3). ──●── 15% sludge; ──○── 25% sludge; ──▲── 50% sludge; ──△── 75% sludge; ──■── sludge. Pooled standard error of log transformed data: 0.12. Letters denote homogeneous means at 5% significance level on day 56.
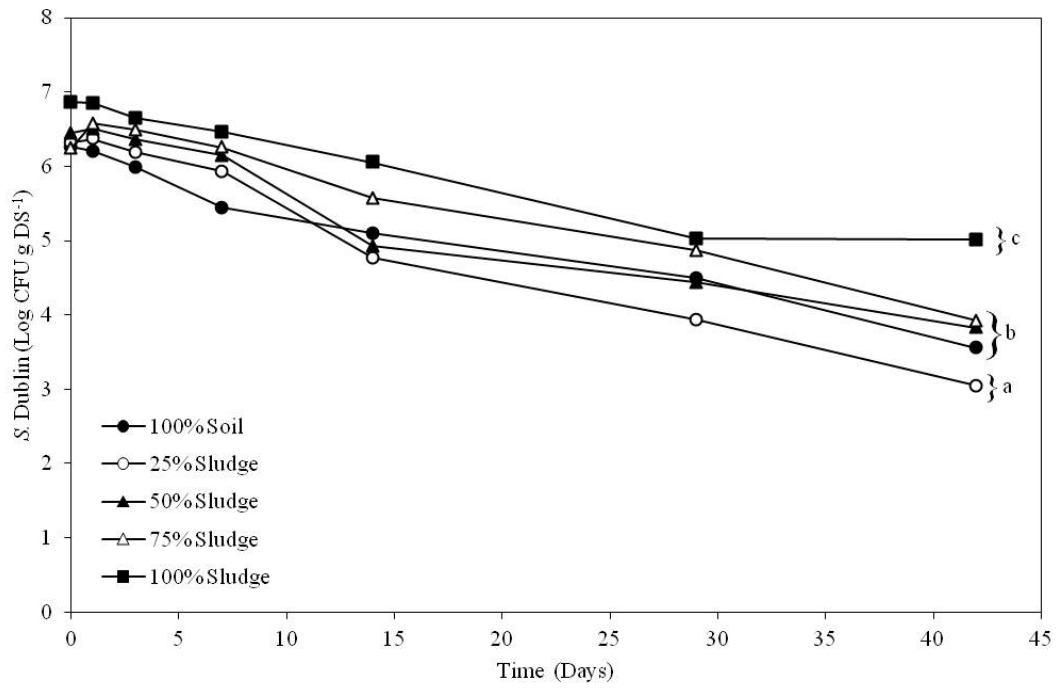
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.