Evaluating factors in the fate of pathogens during wastewater treatment

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ABSTRACT

Our previous studies showed that microbial indicators (*Escherichia coli* and coliphage) decrease to undetectable numbers before the end of the pan drying stage of sludge treatment. While there are many possible reasons for this decrease in numbers, we have examined two factors, salt and autoclaving on the fate of *E. coli, Enterococcus faecalis* and *Salmonella*. Autoclaving eliminates indigenous content of biosolids and may increase available nutrients. Biosolids samples consisted of pan samples collected during the filling phase (~4% DS). The growth of strains was examined in vitro with media containing levels of salts based on those reported for sludge; $CaCl_2$, $MgCl_2.6H_2O$, KCL, NaCl and ammonium acetate. Cultures were incubated at 37 ^oC overnight. All strains survived in salt mixtures ranging from 3% to 11% and grew in mixtures containing up to 9% salt. This result suggests that with the increase in salt level, the growth of organisms decreased, however, even salt levels of 9% salt did not totally inhibit growth. Since our previous field studies showed a decrease in the levels of *E. coli* during of the pan drying stage of sludge treatment, these results also suggest that factors other than salt levels are responsible for that die-off. Using the same biosolids samples, the effect of autoclaved biosolids on the growth of *E. coli*, *Enterococcus faecalis* and *Salmonella* was examined. Although there were differences between strains, all grew in pure biosolids, in media containing a mixture of biosolids and nutrient broth and in biosolids and water mixtures. As this increase in numbers of *E. coli* could be due to either depletion of indigenous flora or release of additional nutrients by autoclaving, additional experiments are underway to examine the fate of *Salmonella* and indicator organisms incubated in gamma-irradiated, autoclaved and un-autoclaved biosolids.

INTRODUCTION

Wastewater comprises liquid waste discharged by domestic residences, commercial properties, industry, and/or agriculture and can include a wide range of potential contaminants and concentrations. Wastewater treatment removes contaminants by physical, chemical and biological processes. The purification mechanisms result in the pollutant load transferring to sewage sludge. In a treated condition, this material is referred to as biosolids and is suitable for beneficial use, such as a fertilizer and soil improver in agriculture. Generally, sewage sludge is composed of organic compounds, macronutrients, a wide range of micronutrients, non-essential trace metals, organic micro pollutants and microorganisms (Kulling *et al*. 2001).

Typically, the different stages for wastewater treatment include grit removal, primary sedimentation and biological treatment (activated sludge process). In metropolitan Wastewater Treatment Plants in Victoria, these processes are followed by mesophilic anaerobic digestion of the sludge, air drying in pans and stockpiling. Biosolids are valuable nutrient sources for plants, however, before they are released for unrestricted use in agriculture, is essential that treatment processes reduce pathogen loads to levels that do not impact on health or the environment. (Rouch *et al*. 2008).

Previous work from our laboratory has shown that levels of indicators (coliphage and *Escherichia coli*) drop to undetectable levels after about nine months in drying pans. The factors responsible for that die-off are incompletely understood. Factors that have been proposed to cause die-off of pathogens during waste water treatment include temperature, microbial competition, pH, chemical interactions (Pike and Carrington, 1986), degree of dryness, predation by indigenous flora and UV exposure. Some research on the factors affecting die-off of pathogens was performed during anaerobic digestion of biowastes (Smith et al, 2005), but no work has been done on factors, such as salt content and indigenous flora, on the die-off of bacteria in drying pans. The aims of this project were to investigate the fate of indicator organisms and potential pathogens under conditions they are exposed to during the treatment of sludge in drying pans. Specifically the aims were to determine (a) the effect of different salt mixtures on the fate of field strains of *E. coli* and (b) the effect of autoclaved biosolids on the fate of *E. coli*, *Enterococcus faecalis* and *Salmonella.* We were particularly interested to determine whether field isolates of *E. coli* have adapted to environmental conditions to enhance their survival and growth in biosolids.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

For experiments to determine the effect of salt mixtures on the growth of *E. coli*, five field strains of *E. coli* (M34, M1-1, MOA-5, MOA2-1, and MOA2-5) and one reference strain, *E. coli* ATCC 25922, were

used. In addition *E. faecalis* ATCC 25922 was used as a control. To examine the effect of biosolids on bacterial growth, a field strain of *Salmonella* Birkenhead was also used. Field strains were isolated from drying pans (*E. coli*) or anaerobic digester output (*S*. Birkenhead). Isolates were stored in 90% glycerol at -20 ºC and working cultures were stored at 4 ºC. For inoculation into salt mixtures, overnight cultures were diluted in broth to the appropriate level. Luria Bertani (LB), a chemically defined medium, was used in the experiments involving salt mixtures, whereas nutrient broth (NB), a more nutritious medium, was used in biosolids-broth mixtures.

Preparation of salt mixtures

Salt mixtures were prepared in LB broth by the addition of KCl, CH_3COONH_4, $MgCl_2.6H_20$ and $CaCl_2$ to produce total salt concentrations ranging from 3% to 11% (Table 2). This range of salt concentrations was based on a publication by Park et al (2006), which reported levels for Na^+, K^+, NH_4^+, Mg^{2+} and Ca^{2+} in output from an anaerobic digester. Expected salt concentrations in pan and stockpile samples were extrapolated from these values taking into account the known dry solids (DS) content of pan samples at various stages of drying and of stockpiles. Based on these estimates, the total salt concentrations in drying pans would be expected to range from 0.4% for early pan samples (1% DS) to 7.2% for late pan samples (20% DS). The total salt concentration in stockpiles was estimated to be in excess of 11%.

Table 1 Salt concentrations (%) used in Luria broth

Total Salt	NaCl	KCl	CH_3COONH_4	$MgCl_2.6H_20$	$CaCl_2$
3	1.30	0.19	1.12	0.16	0.23
5	2.16	0.32	1.86	0.27	0.39
7	3.03	0.44	2.61	0.38	0.54
9	3.90	0.57	3.35	0.48	0.70
11	4.76	0.69	4.10	0.59	0.85

Preparation of biosolids mixtures

Drying pan samples (4% dry solid) were used for this experiment. Biosolids samples were autoclaved (121ºC for 15 mins) to destroy indigenous flora, which could affect growth of potential pathogens due to competition and predation. Four biosolids mixtures; pure biosolids (100%), biosolids and nutrient broth (50% biosolids + 50% nutrient broth), biosolids and sterile water (50% biosolids+50% RO water) and pure nutrient broth (100%) were prepared.

Fate of different strains in salt mixtures

100 µl of a 10^6 dilution of each bacterial culture was added to 10 mL of LB (final concentration 10^4 CFU/mL) containing different percentages of salt. LB without any salt was used as a control. Cultures were incubated overnight at 37 ºC. After incubation, the optical density (OD_{600}) was measured using an Eppendorf Biophotometer. Colony counts were performed using the spread plate method and CFU/mL was calculated.

Fate of different strains in biosolids mixtures

Bacteria were added to the biosolids mixtures to a final concentration of 10^5 CFU/mL and cultures were incubated overnight at 37 ºC. Colony counts were performed using spread plate count and CFU/mL was calculated.

RESULTS AND DISCUSSION

Effect of different salt mixtures on the fate of *E. coli*

No die-off of any strain was demonstrated, even in the presence of 11% salt. In contrast, all strains grew in broth with salt concentrations ranging from 3% to 9%, with increases in CFU/mL of ~5 log (in 3% salt) to 0.5 -1 log (9% salt). In the presence of 11% salt, no growth occurred, however the numbers of bacteria remained constant over the 24 hours in which the study was conducted. Field isolates and reference strains behaved in a similar manner. In contrast, *Enterococcus faecalis* was more resilient, showing a 2-log increase in growth even in the presence of 9% salt (Figure 1). These results suggest that the salt concentration in older drying pans (~7.2%) containing 20-% DS would not allow growth of *E. coli* or related bacteria such as *Salmonella*. spp. but high levels of salt alone are unlikely alone to be responsible for the die-off reported in our previous study. These experiments have examined the effect of salt mixtures in isolation and have not taken into account other factors likely to affect survival, die-off and growth of *E. coli*, such as the presence of organic matter and indigenous flora.

Effect of biosolids on the fate of different strains

In autoclaved biosolids, no die-off of pathogens was observed. In contrast, all field isolates and reference strains grew in all biosolids mixtures, although growth was better in mixtures containing nutrient broth and less in pure biosolids and biosolids-water mixtures. There were no major differences between the growth of field isolates and reference strains of *E. coli* or between species. Even in pure biosolids, there was a 2-log increase in the CFU/mL of most strains (Figure 2). The ability of autoclaved material from drying pans (4% DS) to support the growth of *E. coli*, *E. faecalis* and *S*. Birkenhead could be due to depletion of indigenous flora, release of nutrients through degradation of macromolecules or both. However, in another

study in our laboratory, *S*. Typhimurium failed to grow in non-autoclaved biosolids incubated at 35 ^{o}C and moreover died slowly over a period of four weeks (data not shown), suggesting that the indigenous flora could have played a role in the present study.

CONCLUSION AND FUTURE DIRECTIONS

The experiments described here suggest that the higher salt levels (~ 7%) together with predation by indigenous flora are important factors contributing to pathogen growth inhibition and die-off during pan drying stage of wastewater treatment. Further studies, using gamma-irradiated biosolids, are underway to separate the effects of nutrient release and predation by indigenous flora on the fate of field and reference strains of bacteria in the presence of biosolids. It is likely that other factors alone or in combination also contribute to the die-off of pathogens reported in our previous study. These include exposure time, UV irradiation and degree of dryness.

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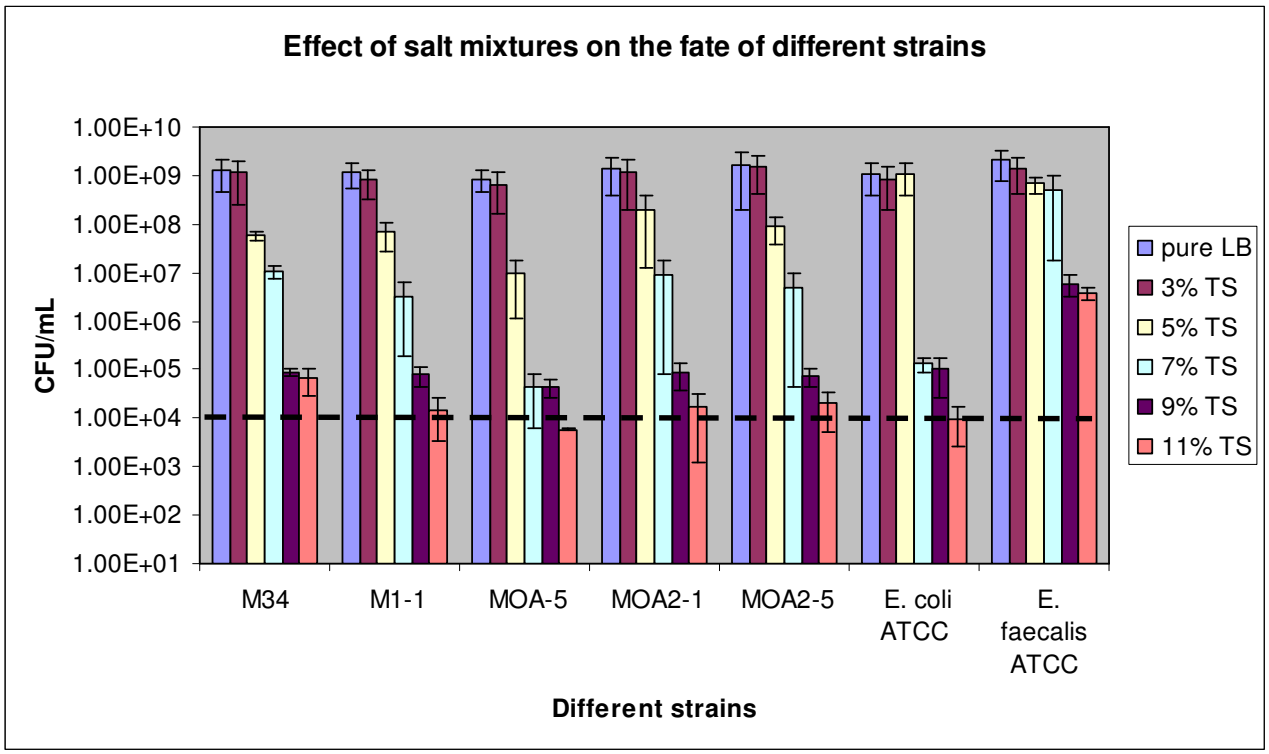

Figure 1: Growth of field isolates of E. coli and two reference strains, E. coli ATCC 25922 and E. faecalis 29212 in different of salt mixtures. LB containing the appropriate salt concentration was inoculated with ~10^4 CFU/mL bacteria. TS total salt (%). The results show means (SD) values obtained from three independent experiments.

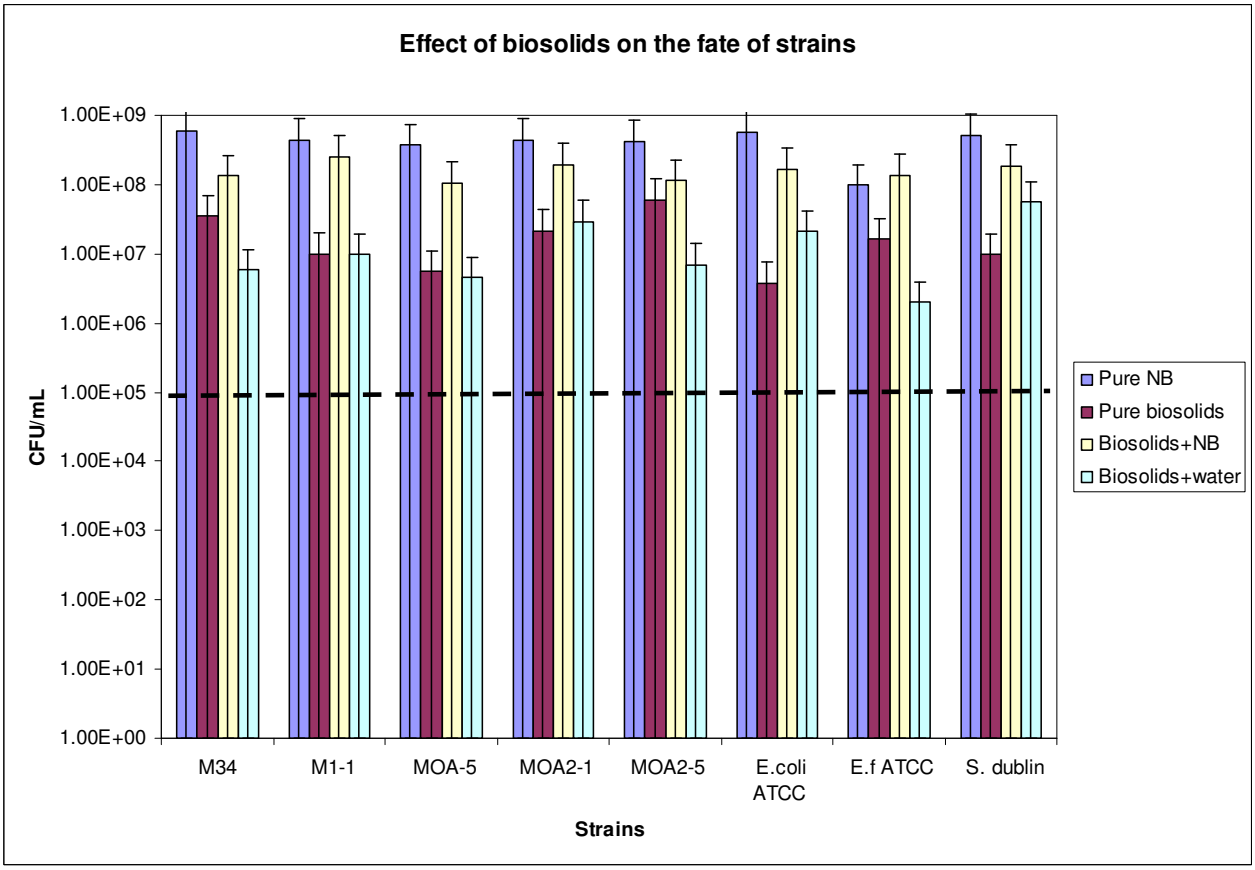

Figure 2: Growth of bacteria in autoclaved biosolids mixtures. Nutrient broth (NB) containing the appropriate salt concentration was inoculated with ~10^4 CFU/mL bacteria. The results show means (SD) values obtained from three independent experiments.