Title: Fate and transport of viruses during sewage treatment in a mound system

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Abstract

Studies undertaken to assess the performance of filter materials to remove phosphorus in decentralised sewage systems have not reported on the broader performance of these systems. This study aimed to identify virus fate and transport mechanisms at the laboratory scale for comparison with field experiments on a mound system amended with blast furnace slag. Inactivation was a significant removal mechanism for MS2 bacteriophage, but not for PRD1 bacteriophage. Column studies identified rapid transport of PRD1. Laboratory studies predicted lower removal of PRD1 in a full scale system than was experienced in the field study, highlighting the importance of considering pH and flow rate in pathogen removal estimates. The results highlight the necessity for studying a range of organisms when assessing the potential for pathogen transport.

Keywords. Bacteriophage, mound, sewage, treatment, virus

Nomenclature

α	Fraction of viruses subject to higher inactivation in biphasic inactivation model
ARW	Artificial Rain Water
C	Virus concentration
C_O	Virus concentration at time = 0
DO	Dissolved Oxygen

EC	Electrical Conductivity
k_{att}	Attachment coefficient
k_{det}	Detachment coefficient
pfu	Plaque forming units
t	Time
SD	Standard deviation
μ	Inactivation rate
μ_1	Higher biphasic inactivation rate
μ_2	Lower biphasic inactivation rate

Introduction

A broad range of treatment options are available for decentralised sewage systems, but high rates of failure of traditional septic tanks (USEPA, 2002) are discouraging innovation in favour of centralised sewerage installation. Uptake of alternative technologies has also been limited where the ability to provide centralised management is limited by geographic or economic constraints. A further concern to regulatory authorities is the increasing potential for failure as the complexity of systems and treatment processes increases, the belief that householders will not maintain them, and the greater human and environmental risks arising from such failures.

Soil mound systems are one type of low-maintenance system, and can be enhanced with various materials to facilitate phosphorus removal. These materials facilitate ion

63 exchange and precipitation of phosphorus through provision of increased surface area,
64 pH and chemicals such as aluminium, iron and calcium compounds. Amendment
65 materials investigated for their nutrient removal capabilities have included red mud (Ho
66 *et al*., 1991), blast furnace slag (Johansson, 1999), fly ash (Cheung and Venkitachalam,
67 2006), zeolite (Sakadevan and Bavor, 1998) and lightweight expanded clay aggregate
68 (Ausland *et al*., 2002). However, limited research has been undertaken on how these
69 materials affect the fate and transport of other contaminants, including pathogens.

70 A previous study aimed to assess the performance of a sand mound amended with blast
71 furnace slag (Charles *et al*., 2004). Two sand mounds (138 m^2 x 1 m high) were
72 installed to treat septic tank effluent from four households and two public toilet blocks.
73 Effluent flowed horizontally out from a central trench to the edge of the impermeable
74 liner, with pH increasing with distance travelled due to the high pH of the slag. The
75 horizontal flow path resulted in saturated conditions within the mound. Bacteriophage,
76 viruses that infect bacteria, were used as models for human enteric virus behaviour in
77 the environment. They span the range of shapes, sizes, surface charges and persistence
78 exhibited by many human enteric viruses. Their physical characteristics and tolerance of
79 wastewater treatments make them ideal models to illustrate the behaviour of enteric
80 viruses in groundwater, soil or subsurface environments and for sewage treatment
81 efficacy evaluation. Routine sampling results indicated high removal of thermotolerant
82 coliforms (> 3.8 $\log_{10}$), however there was significant transport of somatic coliphages
83 present in the sewage with a mean concentration in effluent of 15 pfu (plaque forming
84 units).mL^{-1}, a reduction of 1.5 $\log_{10}$ units. Two bacteriophage were used for field tracer
85 experiments. MS2 is an icosahedral phage with a diameter of 27 nm and an isoelectric
86 point of 3.5 reported to have little or no adsorption in saturated sandy soils at pH 6 - 8

87 with low organic carbon content. PRD1 is an icosahedral phage with a diameter of 62
88 nm and an isoelectric point between 3 and 4. Field tracer experiments resulted in similar
89 transport of PRD1 bacteriophage (2.9 $\log_{10}$ removal) compared to somatic coliphages,
90 but over 5 $\log_{10}$ removal of MS2 bacteriophage.

91 Virus transport and fate in soils is predominantly a function of advection, inactivation,
92 sorption and desorption (Schijven and Hassanizadeh, 2000). While advection and
93 desorption are dependant on the flow conditions, adsorption and inactivation depend on
94 a range of additional factors. Virus adsorption is generally the most important process
95 for attenuation and depends on the pH, soil type, organic matter content, ionic strength
96 and flow rate. Inactivation and irreversible adsorption are required for virus removal.
97 Fate and transport characteristics vary between enteric viruses (Schijven and
98 Hassanizadeh, 2000). Virus inactivation is dependant on the physical characteristics of
99 the different viruses (e.g. surface chemistry and morphology) and environmental factors
100 (e.g. temperature, microbial activity, pH and ammonia) (Schijven and Hassanizadeh,
101 2000). Flow rate can affect the contact of viruses with the attachment sites, with
102 increasing velocities reducing contact time and therefore attachment (Schijven and
103 Hassanizadeh, 2000). High ionic strength, such as septic tank effluent, favour virus
104 adsorption; with low ionic strength waters, such as rainfall, able to remobilise attached
105 viruses (Schijven and Hassanizadeh, 2000).

106 It was hypothesised that inactivation was likely to be the main mechanism for virus
107 removal as the conditions within the mound were generally favourable for virus
108 transport (high pH), but unfavourable for virus survival (up to 25 °C and pH 11.0).
109 Furthermore, ammonia is known to be virucidal above pH 8 (Ward and Ashley, 1977).

110 This paper reports the results of laboratory experiments undertaken in collaboration
111 with the Sydney Catchment Authority to assess the mechanisms of virus transport in an
112 amended sand mound system. The first set of experiments aimed to investigate the role
113 of inactivation in removal of viruses in the field experiments, under the neutral pH
114 conditions in the influent and high pH conditions in the effluent. The second set of
115 experiments aimed to quantify the transport and fate of viruses in a laboratory column
116 under more adverse conditions for virus removal; specifically high flow rates and low
117 ionic strength.

118 Materials and Methods

119 *Bacteriophage Inactivation in Sewage and Effluent*

120 Ten glass containers (100 mL) were filled with wastewater from the mound, making
121 five influent-effluent pairs. Physico-chemical analyses (pH, temperature and DO
122 [dissolved oxygen]) were undertaken on two pairs one incubated in the dark at 4 °C, the
123 other at ambient temperature (22 °C). Of the remaining three pairs (Table 1), one pair
124 (SI22 and SE22) were sterilised by autoclaving at 121 °C for 15 minutes to reduce the
125 influence of microbial activity within the sample. Suspensions of MS2 and PRD1 (10^6
126 $pfu.mL^{-1}$) were added to each of the six containers. One unsterilised pair (I22, E22),
127 with the sterilised pair, were stored in the dark at 22 °C to replicate conditions within
128 the mound (Table 1). The remaining unsterilised pair (I4, E4) were stored at 4 °C
129 providing a temperature control to assist in the identification of the impact of the
130 amending material. The low temperature was also assumed to reduce the influence of
131 microbial activity. All containers were sampled over a 28 day period, which
132 corresponded with the time period of the previous field experiments.

Independent duplicate 1 mL aliquots were sampled from each container with separate dilution series for each sample. Three dilutions were analysed for each sample. On three occasions, duplicate analyses of the sample were undertaken. Results are reported as the average of duplicate plates, from the appropriate dilution. Bacteriophage analyses were undertaken by AMS Laboratories, Sydney. The spiked MS2 coliphage (ATCC 15597-B1) was analysed by the method of Havelaar and Hogeboom (1984) using host *E. coli* (Migula) (ATCC 15597) (phage and host supplied by the American Type Culture Collection, Manassas, USA). The spiked PRD1 bacteriophage was analysed by the method for somatic coliphages (ISO 10705-2, 2000) using host *Salmonella typhimurium* L29 (phage and host supplied by Prof. C. Gerba, University of Arizona, Tucson, Arizona). Background concentrations were measured prior to each spike.

PRD1 Laboratory transport study

Column experiments were undertaken using the amended soil with artificial rain water (ARW, per L of deionised water: 4.07 mg $NaNO_3$, 3.24 mg NaCl, 0.35 mg KCl, 1.65 mg $CaCl_2.2H_2O$, 2.98 mg $MgSO_4.7H_2O$, 3.41 mg $NH_4.2SO_4$) (Davies *et al*., 2004).. The column was constructed from 10.5 cm PVC (internal diameter), capped with a reducer and 5.0 cm internal diameter PVC cap, fitted with a brass nipple (2 cm long, 2 mm internal diameter). The inside of the column was spirally machine roughened to minimise soil-column interface flow and therefore limit edge effects. Silicone tubing (Masterflex HV-96410-16 Precision Silicone (platinum) Tubing, internal diameter 3.1 mm) was fitted to the brass tube. The base of the soil was supported with three layers of wire mesh (pore size 2 mm) overlaid with a 2 cm layer of 2 mm glass beads. Soil slurry was added to a depth of 50 cm during up-flow conditions in small amounts. Regular stirring and shaking aimed to achieve uniform packing. At the top of the column, the

157 interface of the soil and the column was sealed with silicone sealant and a ring of
158 aluminium foil to further limit the short-circuiting of water via the column edges. A
159 soil-free control column was used as a control with the supporting layers of wire mesh
160 and beads, but no soil.

161 Several pore volumes of ARW were fed (downwards) through the column prior to
162 experimentation to equilibrate the column. Saturated flow conditions were used as flow
163 in the field is horizontal, and therefore primarily saturated. A flow rate of 1.1 $m.d^{-1}$ was
164 used. The sodium chloride concentration of the ARW was increased by 2.9 $g.L^{-1}$ for
165 several pore volumes to enable determination of flow dispersion. The electrical
166 conductivity (EC) was analysed with a Lab Analyser 440 (TPS Pty Ltd, Australia).
167 After completion of the salt tracer, the ARW was inoculated with PRD1 (final
168 concentration 10^4 per mL). PRD1 was grown up in host cells of *Salmonella*
169 *typhimurium* strain LT2 (ATCC 19585). The column influent was inoculated with
170 PRD1, and the concentration in column effluent was quantified periodically during and
171 after the application of the inoculum to determine the removal of viruses by passage
172 through soil. A minimum of triplicate samples were collected to determine the
173 background and final effluent phage concentrations. Column experiments were
174 undertaken at ambient temperature (approximately 20 ºC). At the completion of
175 experiments, the column was destructively sampled to determine percent moisture
176 determination by drying in pre-weighed crucibles at 105 °C for 48 hours (APHA, 1998).

177 Inactivation in the column material and ARW was studied. Seventy portions sieved
178 amended mound soil (2 g) was weighed into 5 mL polyethylene vials, and an additional
179 70 vials used with only 2 mL of ARW. Soil was analysed using standard methods
180 (APHA, 1998) for pH, grain size distribution, carbon content, total and exchangeable

181 iron, bulk density and cation exchange capacity. Phosphorus sorption was analysed
182 using the method from Rayment and Higginson (1992). Stock suspensions of PRD1
183 were diluted in sterile deionised water such that 0.1 mL of the diluted bacteriophage
184 suspension could be added to achieve approximately 1 x 10^6 virions per vial. Sterilised
185 (autoclaved) ARW was added to the soil microcosms to saturate each of the soils as
186 equally as possible. Control vials of each soil type were left uninoculated to be used for
187 moisture determinations; 0.1 mL of sterile deionised water was added in place of the
188 inoculum. The vials were incubated at 20 °C in the dark. An ibutton™ miniature
189 temperature probe (Maxim/Dallas Semiconducter Corp., Dallas, Texas, USA) taped to
190 the inside of the jar was used to record the temperature inside the jar at 120 min
191 intervals throughout the experiment. The soil was then inoculated with PRD1 and
192 triplicate vials were randomly selected and withdrawn periodically from each sealed jar
193 for determination of infectious PRD1. Hence, the microcosms were sampled
194 destructively. In addition, duplicate vials were removed periodically from each jar for
195 percent moisture determination by drying in pre-weighed crucibles at 105 °C for 48
196 hours (APHA, 1998).

197 Each 2 g of inoculated soil from sampled vials was washed into a 50-mL Falcon tube
198 using 20 mL 3 % (w/v) beef extract solution (Straub *et al*., 1992) (pH 9). The soil slurry
199 was then vortexed for 2 mins and shaken for 30 mins. After further vortexing for a few
200 seconds the slurries were centrifuged for 15 mins at 2 500 g, after which 1 mL of the
201 supernatant was withdrawn by pipette. This was diluted serially in sterile deionised
202 water and assayed by the double agar layer technique (Adams, 1959). Concentrations of
203 phages were expressed as plaque-forming units per vial.

A recovery control was prepared for each soil type by freshly inoculating 2 g of soil with approximately 1 x 10^6 PRD1 from a stock suspension. The phage was allowed to adsorb to the soil by storing at room temperature for two hours, before being processed as described above. The titre of the stock suspension used to inoculate the recovery controls was also determined. Percent recovery for each soil type was determined as the concentration of phage recovered divided by the concentration inoculated into the soil x 100. Phage concentrations in ARW were measured by directly diluting the contents of the microcosm vials with deionised water, and assaying by the double agar layer technique as above.

Statistical analyses

For the inactivation studies, two inactivation models were applied: first-order and biphasic. First-order inactivation was modelled as $C = C_0.Exp(-\mu t)$ (Equation 1), where C was the concentration of phages, C_0 was the concentration of phages at time = 0, t was time, and the inactivation rate, μ, was considered to be a function of pH, temperature and/or microbial activity. Biphasic inactivation was modelled as $C = C_0 [\alpha.Exp(-\mu_1.t) + (1-\alpha).Exp(-\mu_2.t)]$ (Equation 2), α was the fraction of less stable viruses with a higher inactivation rate coefficient μ_1, and $(1- \alpha)$ is the fraction of more stable viruses with a lower inactivation rate coefficient μ_2 (Petterson *et al*., 2001) . The inactivation rate coefficients were derived from the experimental data by fitting the inactivation models with a log-likelihood method previously described by Schijven *et al*. (2002). Using the likelihood ratios test, the first-order and biphasic inactivation models were compared for each dataset, with significance defined by the χ^2 test at $p < 0.05$ level. Outliers were identified as the residuals from fitting of first-order and biphasic models that were statistical outliers based on boxplot results using SPSS (version 11.5.2.1, SPSS Inc.,

228 2003). They were defined as points where the residual from the fitted inactivation model
229 was more than 1.5 times the inter quartile range above the third quartile. Outliers were
230 excluded from final inactivation rate analyses to improve the statistical comparison of
231 different experimental conditions and prevent the overestimation of the inactivation
232 rate. Outliers only occurred in the initial two days of the studies, therefore the remaining
233 data provided information on the longer term behaviour of the viruses. A maximum of
234 one time point within the first two days of the study was excluded from five of the
235 twelve studies. Two time points (t = 0,1) were excluded from one study.

236 Modelling of breakthrough curves was undertaken for the field experiments (Charles *et*
237 *al*., 2004) and laboratory experiments. HYDRUS-1D (United States Salinity
238 Laboratory, Riverside) (Šimùnek *et al*., 1998) is commonly used in modelling micro-
239 organism transport in porous media (Schijven and Simunek, 2002; Charles, 2007;
240 Foppen *et al*., 2007). Conservative tracer breakthrough curves in the column and in the
241 mound were fitted to the convection-dispersion solute transport equation using
242 HYDRUS-1D to calculate porosity and dispersion within the columns. The fit was
243 optimised using the Levenberg – Marquardt non-linear minimisation algorithm for
244 least-squares solutions. The relative concentrations from the phage breakthrough curves
245 (C/C_0) were fitted (using log resident concentrations) to the one-site kinetic adsorption
246 equations using HYDRUS-1D. For modelling purposes, inactivation of unattached
247 viruses (μ_1) was assumed to equal the inactivation in ARW, and inactivation of attached
248 viruses (μ_s) was assumed to equal inactivation in the soil-water microcosm. Virus
249 transport coefficients were fitted from the column breakthrough curve to the one-site
250 kinetic adsorption equations, and were compared with the results from the field study
251 results.

Results

Survival in sewage and effluent

The sample chemistry was generally stable over the duration of the study. Samples had initial pH values of 8.1 and 10.6 for influent and effluent respectively. Final pH values (32 days) were 7.9 and 7.3 for influent at 4 °C and 22 °C respectively, and 10.8 and 10.5 for effluent at 4 °C and 22 °C respectively. An initial decrease in dissolved oxygen and slight pH variation was observed over the first 24 hours from sampling, with the sample stabilising after such time. Average initial concentrations, as quantified on day zero from the spiked sample containers, of 10^6 pfu.mL^{-1} of MS2 and PRD1 were achieved for all samples except unsterilised effluent at 22 °C where MS2 was 10^4 pfu.mL^{-1}. The average temperatures were 4.7 °C (± 1.7 SD) and 22.8 °C (± 1.3 SD).

MS2 bacteriophage was very sensitive to the conditions within the mound: high pH, 22 °C, high microbial activity (Figure 1). Unsterilised effluent exhibited the most rapid inactivation with no phage detectable (<1 pfu.10 mL^{-1}) after two days. PRD1 was more stable in the conditions in the mound (Table 2). Both MS2 and PRD1 exhibited an increased inactivation with increased temperature. Outliers were identified in six of the twelve studies which included t = 0 for PRD1 E4 and MS2 I4; t = 1 for PRD1 SI22 and I22; t = 2 for PRD1 SE22; and t = 0 and 1 for MS2 I22 (see Table 1 for sample codes). The biphasic inactivation model (Equation 2) was preferred for four of the studies (Table 2) based on the log-likelihood ratios test (significant at the χ^2 95th percentile). PRD1 had significantly ($p < 0.05$) lower rates of inactivation than MS2 and the first-order model was preferred in all except for the case with highest inactivation, E22 (Figure 2).

275 The first-order model inactivation coefficient, μ, for MS2 (Table 3) varied significantly
276 between each sample type, except for the sterilised influent/effluent pair. Inactivation
277 was significantly more rapid ($p < 0.05$) in unsterilised effluent than in unsterilised
278 influent, both at 4 °C and 22 °C. Temperature was a major factor for MS2 inactivation
279 in influent, with μ_{SI22} significantly ($p < 0.05$) greater than μ_{I4}. The MS2 biphasic models
280 (Table 3) for sterilised influent and effluent were not significantly different, with the
281 data able to be described with all common coefficients. However, both were
282 significantly different from MS2 I22. PRD1 was more robust under the conditions of
283 the study, and hence, comparison of the PRD1 first-order inactivation model
284 coefficients (Table 3) elicited fewer significant factors. Nonetheless, sample type was
285 significant ($p < 0.05$) for unsterilised samples with inactivation being more rapid in
286 effluent than in influent at 4 °C and 22 °C. Temperature and microbial activity,
287 separately, were only significant in effluent however combined were significant in
288 influent but not effluent.

289 *Survival in amended soil*

290 The amending material resulted in increased pH and phosphorus sorption capacity as
291 well as in calcium and cation exchange capacity (Table 4). The average temperature
292 within the microcosm jars was 19.5 °C (SD ± 0.77 ºC) for the duration of the
293 experiment (131 days). The moisture content was 17.7 ± 1.0 %. The efficiency of the
294 phage recovery method for the soil microcosms was 56 ± 25 %, and was not affected by
295 time. Percent recovery was not used to adjust the phage concentrations. PRD1
296 inactivation (Table 2; Figure 3) in the soil/water matrix was significantly lower than
297 inactivation in ARW alone. The biphasic model provided a better fit in the ARW only.

298 *Transport in columns*

299 Salt tracer recover was 100%, providing a good fit of the salt tracer breakthrough curve.
300 The porosity used in the model was based on the total porosity of the column (including
301 the pore space in the cap), with the range calculated from minimum to maximum water
302 content. Therefore the porosity in the model was higher than the water content of the
303 soil. The modelled porosity was 0.233 ± 0.021 (standard error), with a dispersivity
304 0.042 ± 0.015 m. The values of porosity and dispersivity were then used in the fitting of
305 a one-site kinetic model in HYDRUS-1D to the breakthrough curve data for PRD1, to
306 estimate the attachment and detachment coefficients, k_{att} and k_{det} respectively.
307 Dispersivity and μ_s were varied where acceptable fits were not possible fitting just k_{att}
308 and k_{det}.

309 PRD1 breakthrough was rapid, occurring after 0.34 pore volumes (Figure 4). Over the
310 50 cm of transport in the columns, there was a 0.36 $\log_{10}$ reduction in PRD1
311 concentration, calculated as the $\log_{10}$ of the maximum effluent concentration divided by
312 the maximum influent concentration. The total load recovered of inoculated phage after
313 transport through the column was 59 % (0.39 $\log_{10}$), which is comparable to the
314 reduction in concentration. Sampling was stopped prematurely due to difficulties with
315 the inocula. This resulted in limitations for fitting and high standard errors.

316 Modelling of the virus breakthrough curve resulted in a k_{att} of 11.1 ± 8.24 $.d^{-1}$, k_{det} 0.91 ±
317 2.72 $.d^{-1}$ and an R^2 of 0.910. The attached inactivation rate for the model was 0.831 ±
318 1.17 ln units per day. The standard errors for k_{att} and k_{det} were high, which needs to be
319 considered in the application of the results to the field scale. Inactivation was observed
320 in the phage inocula (Figure 4), and was included in the breakthrough curve modelling.

321 Inactivation in the inoculated ARW fed into the columns where full breakthrough
322 curves were obtained resulted in the overestimation of PRD1 removal (Table 2).

323 Extrapolation of the HYDRUS-1D model from the laboratory column experiments to
324 the field scale predicted a reduction in the concentration of PRD1 of 0.53 $\log_{10}$ over 1.4
325 m transport (0.73 $\log_{10}$ over 1.9 m transport), compared with the 2.9 $\log_{10}$ reduction
326 experienced in the field mound studied.

327 Discussion

328 *Inactivation*

329 The inactivation rates in this study were comparable to previously reported groundwater
330 studies (Logsdon, 1994; Schijven and Hassanizadeh, 2000). Temperature is considered
331 one of the most important factors in, and is negatively correlated with, virus survival. In
332 the current study, increasing temperature significantly increased MS2 inactivation in
333 influent, which was consistent with previous reports that MS2 is more stable at
334 temperatures of less than 10 °C (Schijven and Hassanizadeh, 2000; Schaper *et al*.,
335 2002). For PRD1, which has been reported to remain relatively stable at temperatures
336 up to ambient temperature (23 °C) (Schijven and Hassanizadeh, 2000), temperature was
337 only a significant factor in the more adverse high pH conditions of the effluent. Human
338 enteric virus survival has generally been considered to be greatest near neutral pH, yet
339 these viruses are stable within a range of pH 3-10 (Gerba *et al*., 1996).

340 Rapid inactivation of MS2 was experienced in unsterilised effluent (pH 10.6) in this
341 study, with 4.9 $\log_{10}$ and >3.6 $\log_{10}$ reduction in one day at 4 °C and 22 °C respectively,
342 compared with no detectable inactivation in unsterilised influent (pH 8.1). However, the
343 effluent samples also had the lowest initial concentrations (8.6×10^5 and 2.5×10^4

344 pfu.mL^{-1}), compared to a mean initial concentration in the other samples of 1.8 x 10^6
345 pfu.mL^{-1}, which may have resulted from rapid inactivation in the time (two hours)
346 between introducing the phage to the sample and sampling than could not be measured
347 by this experiment but assumed given that the same inoculum was used. Such rapid
348 rates of MS2 inactivation have been reported at high pH, with greater than 4 $\log_{10}$
349 reduction in 2 hours at pH 11 - 11.5 (Logsdon, 1994). While MS2 is more resistant to
350 ammonia than other F-RNA coliphages (Schaper *et al*., 2002), higher temperatures and
351 ammonia may have a synergistic effect resulting in an increased rate of inactivation.
352 The rapid inactivation of MS2 in the effluent sampled from the mound indicates that
353 inactivation was likely to be a significant removal mechanism, with a 6 $\log_{10}$ reduction
354 predicted in less than 48 hours based on the first-order inactivation rate.

355 PRD1 was more robust under the mound conditions than MS2, with inactivation
356 therefore predicted to account for only 0.6 $\log_{10}$ reduction over 21 days in the mound.
357 Rotavirus is considered to be one of the more resistant human enteric viruses (Höglund
358 *et al*., 2002). However, PRD1 was more resistant to high pH than has been reported for
359 simian rotavirus, SA11 (Estes *et al*., 1979), and reovirus (Ward and Ashley, 1977).
360 Inactivation at high pH in wastewater, however, is confounded by the presence of
361 ammonia and its greater virucidal effect on viruses (Ward and Ashley, 1977). For both
362 model viruses examined, pH was the most significant factor (Table 3), with the
363 inactivation rates in effluent consistently greater than those in influent regardless of
364 temperature or sterilisation. Therefore, the ability to modify pH within sewage treatment
365 systems, such as amended mounds, can potentially increase inactivation of human
366 enteric viruses. However, it is important to recognise that the efficacy of pH is
367 dependent on the type of virus.

368 While pH was assumed to be the main difference between the influent and effluent
369 samples, the transport of the influent through the high pH sand mound would have
370 resulted in changes to the composition of the wastewater that were not quantified here.
371 For example, the effect of microbial activity in effluent may have been less than for
372 influent: temperature was a significant factor in PRD1 survival in influent but not in
373 effluent. Ammonia may have contributed to the increased inactivation in effluent as it is
374 known to be virucidal above pH 8 as discussed above. While total nitrogen decreased in
375 the sewage system, the proportion of ammonia increased from 89 % to 92 %, and the
376 calculated concentration (at 25 °C) of free ammonia increased with pH from 5 $mg.L^{-1}$ in
377 influent (pH 8.1) to 53 $mg.L^{-1}$ in the effluent (pH 10.6).

378 The increased inactivation of MS2 in sterilised influent may indicate changes in
379 chemistry during the autoclaving process, as the elimination of microbial activity would
380 generally be expected to decrease the inactivation rate. Similarly the decreased
381 inactivation with increasing temperature experienced in sterilised effluent at 22 °C
382 compared to the unsterilised effluent at 4 °C, where no significant microbial activity
383 was assumed, indicated that autoclaving affected the matrix chemistry. Unfortunately,
384 due to low influent flow at the field site additional samples for chemical analyses were
385 not available.

386 Outliers were defined for this study by both the first-order and biphasic models.
387 Commonly in inactivation studies, when the second sample is taken shortly after the
388 first, there is an apparent increase in concentration (e.g. Yates *et al.*, 1985). It is
389 hypothesised that this is due to disaggregation in the inactivation media. Inclusion of
390 this first data point value in the determination of the inactivation rate coefficient will
391 underestimate the initial inactivation. Hence, sampling time points were included at one

392 and two days, providing a more reliable value of the actual virus load at the time of
393 inoculation.

394 Inactivation rates are quantified to allow the comparison and extrapolation of
395 experimental data sets. These are used for use in management, such as in risk
396 assessment, or in the calculation of the contribution of inactivation to the removal of
397 culturable viruses in experiments. Inactivation has typically been modelled using a first-
398 order model, which assumes a log-first-order decay. However, in many studies, the rate
399 of inactivation decreases over the duration of the study. In these cases, application of the
400 first-order model overestimates the long-term inactivation rate. Alternative inactivation
401 models have been developed to address this issue. Cerf (1977) developed the biphasic
402 model used here, where there are two distinct subpopulations with separate inactivation
403 rates. This model has been employed in the food industry (Geeraerd *et al*., 2005), and in
404 previous environmental survival of viruses (Petterson *et al*., 2001). First-order
405 inactivation was experienced under a variety of conditions including high temperature
406 and high pH, however in all cases first-order inactivation was preferred where $\mu <$
407 $0.05.d^{-1}$. Above this value, the biphasic model was the preferred model (where sufficient
408 data was available). For the four cases where the biphasic model was preferred, the
409 long-term inactivation rates varied from 0.00 to $0.26.d^{-1}$ for both phages. Where the
410 first-order model was preferred, a maximum of 0.6 $\log_{10}$ reduction was experienced
411 compared to a minimum 0.8 $\log_{10}$ reduction before the lower of the biphasic inactivation
412 rates dominated. It is therefore conceivable that the biphasic model would become
413 preferable in more scenarios with increased time and inactivation.

414 The implications of biphasic decay are that over time the inactivation rate decreases,
415 and this long term inactivation rate is therefore overestimated by a linear model. In the

416 mound investigated, the timescale for a virus to pass through the system was
417 approximately 20 – 30 days. Over this timescale, there was limited biphasic behaviour
418 exhibited, and due to the short duration and the nature of the project, there was not
419 sufficient confidence in the biphasic data to extrapolate the results. However, there was
420 evidence that there were small fractions of viruses, in this case 0.4 to 0.04 % MS2 and
421 12 % PRD1, that were considerably more robust in these conditions. It has been
422 theorised that these robust viruses may represent a more resistant subpopulation
423 (Petterson *et al*., 2001). It is not known is how these more robust viruses will behave in
424 the environment when they are released from the sand mound, and therefore how to
425 assess the risks of such enteric viruses as they are transported through the system and
426 potentially ingested.

427 *Column and field studies*

428 Virus inactivation is usually considered to be insignificant in soil column transport
429 experiments due to the small timescales involved. However, as breakthrough was
430 monitored for up to six weeks in the test columns, phage inactivation was considered to
431 be influential. Inactivation in soil-water microcosms was assumed to represent phage
432 inactivation in the attached phase, μ_s. However, the method does not differentiate
433 between attached and free viruses. The amended soil had a lower rate of removal
434 (attachment) within the column and therefore it is likely they had lower rates of
435 attachment within the microcosms. However, quartz sand has been reported to have a
436 virus sorption capacity of 2.2×10^{12} pfu.kg^{-1} (Moore *et al*., 1981) which far exceeds the
437 initial virus load applied (10^6 pfu to 1.3 g^{-1} soil dry weight).

438 Column experiments using repacked soil are commonly used to characterise virus
439 transport in soil (e.g. Sobsey *et al*., 1995), with results generally considered to be

440 representative of field scale. Column experiments with amended sand (Ho *et al*., 1991)
441 have demonstrated higher removal of *E. coli* and poliovirus in sand amended with red-
442 mud than unamended sands, with greater than 7 $\log_{10}$ removal of both organisms in 65
443 cm columns of amended sand. However, field verification has not been reported, nor
444 virus removal in other media used in decentralised sewage systems. Laboratory studies
445 aimed to provide quantitative data on virus fate and transport in a mound under
446 conditions anticipated to maximise transport: saturated, low retention time, rain water.
447 Previous studies of virus transport in sand have found similarly rapid transport of
448 viruses (McKay *et al*., 1993; Schijven *et al*., 2002). Physical heterogeneities, such as
449 roots, macropores and rock fractures, can provide preferential water flow paths (McKay
450 *et al*., 1993). Soil columns by their nature introduce paths for preferential transport
451 along the soil-column interface, however as repacked column experiments aim to
452 establish the adsorption capacity of the soil and its influence on virus transport and fate,
453 every attempt is made to minimise their influence. The use of rainwater was assumed to
454 provide a worst-case scenario for transport due to low ionic strength resulting in low
455 attachment. However, in sewage there is the potential for viruses to attach to colloids
456 which may decrease inactivation and facilitate transport (Jin and Flury, 2002). Organic
457 matter, including surfactants in the water have been reported to decrease adsorption due
458 to competition for attachment sites and are also reported to increase desorption of
459 viruses (Schijven and Hassanizadeh, 2000).

460 Removal within the amended sand column was greater than in natural sandy loams
461 (Charles, 2007) although inactivation in this soil was lower. The amended soil was not
462 preconditioned and therefore was expected to have little microbial activity, and hence
463 low inactivation (Jin and Flury, 2002). Inactivation of PRD1 in the soil-water matrix

464 without preconditioning was lower than in effluent from the soil matrix. Virus removal
465 within the sewage treatment mound system that utilises this soil was greater in the field
466 experiments (1.1 $\log_{10}$ in 0.7 m) than experienced in the column experiments carried out
467 in the present study (0.36 $\log_{10}$ in 0.5 m).

468 The differences between these column experiments and the onsite system scenario that
469 will affect the up-scaling of these results to the field situation are:

470 • Rainwater, as used in the columns, will result in decreased attachment compared
471 to sewage, however colloids/organics in the sewage may also facilitate virus
472 transport and survival;

473 • Heterogeneity in the field may increase travel velocity compared to the repacked
474 columns, thereby decreasing attachment, and may affect removal over the site;
475 and

476 • Lack of microbial activity in the microcosms, if extrapolated, may underestimate
477 inactivation in the field.

478 *Implications for household systems*

479 The mound system received flows averaging one third of the design flow during the
480 field study. This low hydraulic load was anticipated to result in an over estimation of the
481 performance of the system, with microorganism inactivation and attachment expected to
482 decrease during increased flows. For a single household system, this degree of under-
483 loading is equivalent to household occupancy rates of two to three people living in a
484 four to five bedroom house, assuming the system is conservatively designed for dual
485 occupancy of each bedroom. Therefore the higher loading rates that would be expected

at houses with higher occupancy rates may result in decreased performance of the system.

Extrapolation of the column study model to the field-scale provided a prediction of virus transport in a less well established system and under higher flow rates. At the reduced detention time modelled, seven days to maximum breakthrough compared to over 20 in the field, the removal was only 0.73 $\log_{10}$ over the 1.9 m width of the system. It is anticipated that this would generally be an underestimate. In an established, well constructed system, the development of a clogging layer would retard virus transport. The high degree of homogeneity in the systems would be expected to increase virus removal, although poor construction could lead to areas without sufficient amending material, and therefore variable pathogen and nutrient removal. However under other conditions, such as prior to clogging layer development and under high flow, this may be representative of transport.

The high pH environment within the mound strongly affected many of the indicator organisms studied. It is not known how long this virucidal high pH may persist, but as it is also important for phosphorus removal it is a key management parameter. Furthermore, high pH is possibly more important than the availability of sorption sites as calcium leaching is expected to result in a decrease in phosphorus sorption.

In the mound system studied, the laboratory estimate of the inactivation rate indicates that inactivation is an important removal mechanism. Therefore these results would not necessarily hold for a similar design with a lower pH. Overall, the amended material sand mound provided greater removal of enteric virus models from sewage than reported for other sewage treatment systems including septic tanks (0.6 $\log_{10}$), aerated

509 treatment units (1 $\log_{10}$) , and disinfection systems (1 - 1.8 $\log_{10}$ for MS2) (Charles,
510 2007).

511 Conclusions

512 • The primary mechanisms for virus removal varied significantly depending on
513 the susceptibility of the individual virus to the system chemistry. MS2 removal
514 as with thermotolerant coliforms, was rapid in the mound. Inactivation studies
515 for MS2 suggested that inactivation was the primary mechanism a 6 $\log_{10}$
516 reduction in less than 48 hours in effluent. Similarly, removal of PRD1 and
517 somatic coliphages was lower in the mound, and inactivation of PRD1 was
518 lower. These results highlight the necessity for studying a range of organisms
519 when assessing the potential for pathogen transport.

520 • The application of the amending material to mound systems at the individual
521 household scale requires considerations of the likely flow rates and pH of the
522 system. Considerations for implementing amended soil mound technology
523 should also include the life expectancy of the system and management protocols
524 for decommissioning and/or replacement.

525 *Acknowledgements*

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529 as part of their commitment to improve decentralised sewage management within
530 Sydney's drinking water supply catchments.

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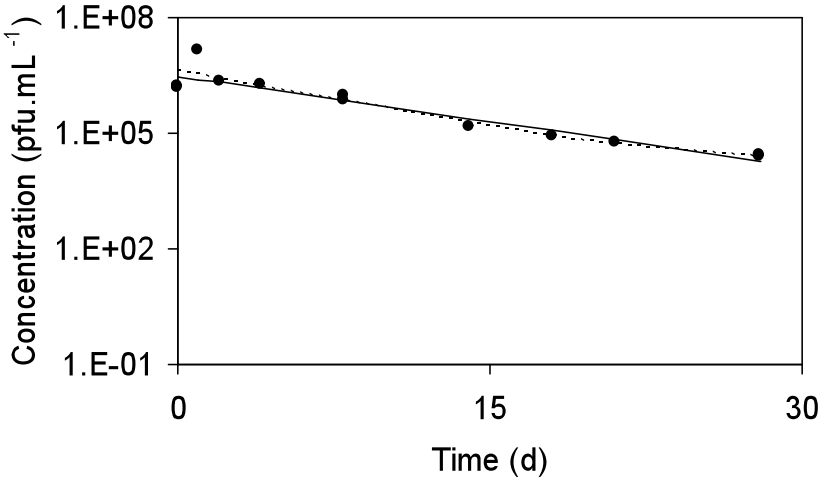
627

628 Tables and Figures

629 (a)



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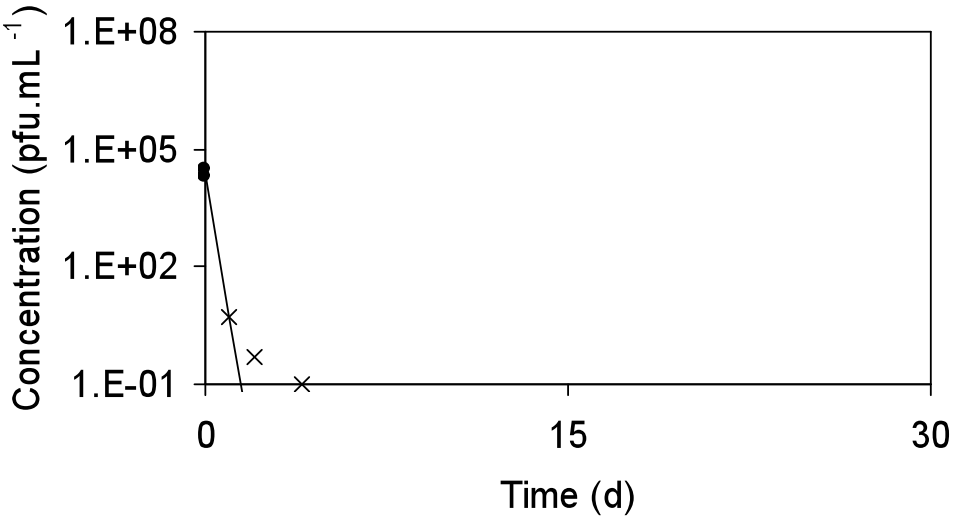
631 (b)



632

633 Figure 1 MS2 data (circles), first-order model (continuous line) and biphasic model

634 (dotted line) for (a) influent and (b) effluent at 22 °C

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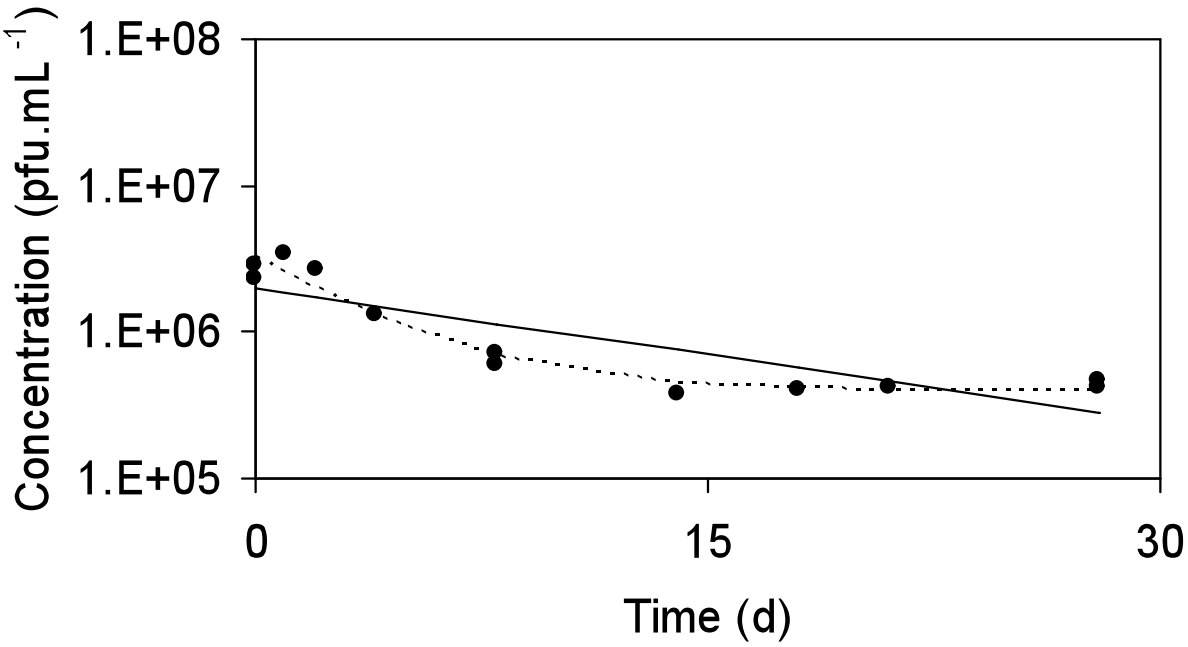
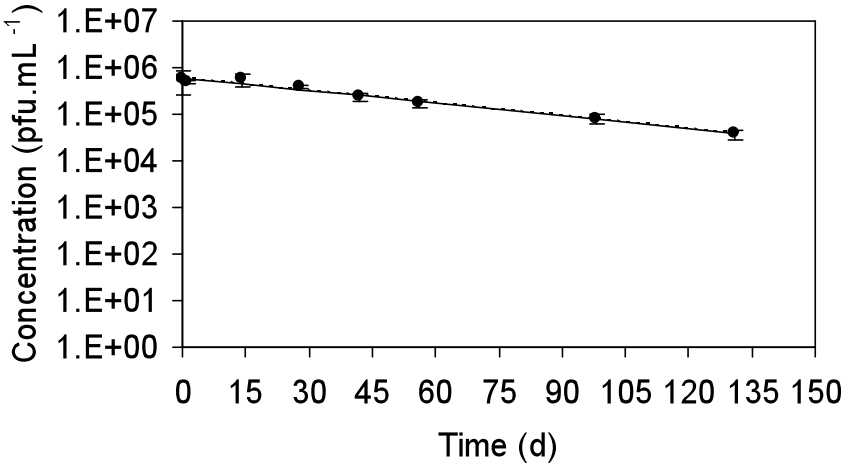


636
637 Figure 2 PRD1 data (circles), first-order model (continuous line) and biphasic model

638 (dotted line) effluent at 22 °C

639

639 (a)



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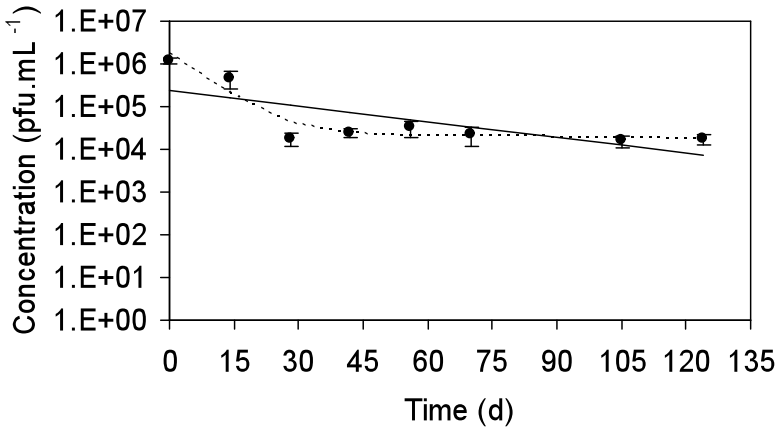
641 (b)



642

643 Figure 3 PRD1 inactivation data (circles) with error bars indicating standard deviation,
644 linear model (continuous line) and biphasic model (dotted line) for (a) Artificial rain
645 water; (b) amended soil

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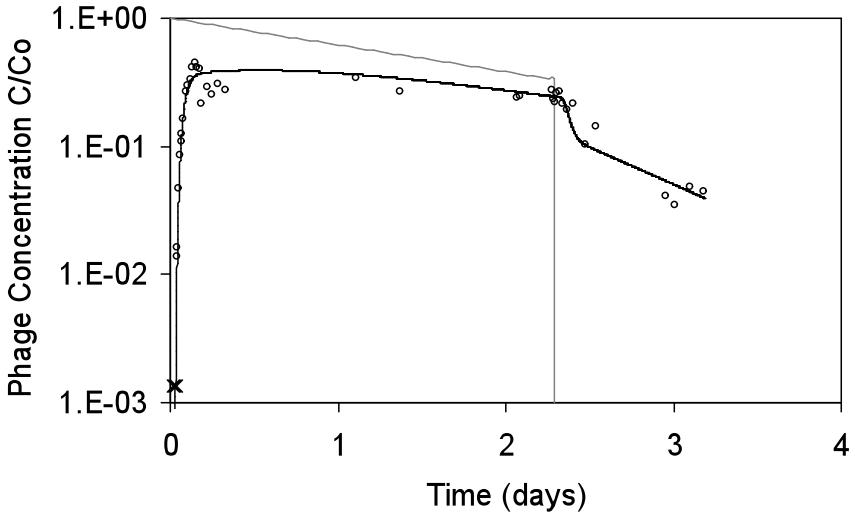

646

647 Figure 4 PRD1 bacteriophage breakthrough curve data (circles), model PRD1 input

648 (grey line) and the fitted one-site kinetic model (solid line)

649

649 Table 1 Experimental conditions for bacteriophage inactivation in influent and effluent

Sample Code	Sample	pH	Temperature	Sterilised
I4	Influent	8.1	4°C	No
I22	Influent	8.1	22°C	No
SI22	Influent	8.1	22°C	Yes
E4	Effluent	10.6	4°C	No
E22	Effluent	10.6	22°C	No
SE22	Effluent	10.6	22°C	Yes

650

650 Table 2 Bacteriophage inactivation rate coefficients and likelihood ratios in sewage,

651 ARW and ARW with amended soil

Sample	First-order model	Biphasic model			Likelihood ratio test ($\chi^2_{0.95}$)	
	μ (day^{-1})	α	μ_1 (day^{-1})	μ_2 (day^{-1})	Log likelihood ratio	Preferred model
MS2						
I4	0.028	0	2.1	0.028	-1.6×10^{-14}	First-order
I22	0.18	0.9956	0.23	0	11.4*	Biphasic
SI22	0.52	0.9996	1.23	0.26	40.8*	Biphasic
E4	11.3	Insufficient data			-	(First-order)
E22	> 8.48	Insufficient data			-	(First-order)
SE22	0.44	0.996	1.09	0.26	17.8*	Biphasic
PRD1						
I4	0.0012	0	31.7	0.0012	-4.4×10^{-12}	First-order
I22	0.018	0	15.4	0.018	-1.7×10^{-10}	First-order
SI22	0.015	0	3.85	0.015	2.8×10^{-9}	First-order

E4	0.049	1	0.049	3.3 x 10^{-5}	-1.6 x 10^{-6}	First-order
E22	0.071	0.877	0.287	0	22.3*	Biphasic
SE22	-0.00015	0	3.45	-2E-04	-4.9 x 10^{-8}	First-order
ARW	0.0283	0.014	0.161	0.0022	13.2*	Biphasic
ARW + soil	0.0210	0	0.238	0.021	-4.0 x 10^{-11}	First-order

652

652 Table 3 Comparisons of phage inactivation based on first-order inactivation model

Samples	Variable*	MS2 Likelihood ratios test ($\chi^2_{0.95}$)		PRD1 Likelihood ratios test ($\chi^2_{0.95}$)	
		Result	Common μ	Result	Common μ
I4 v I22	T, M	$\mu_{I22} > \mu_{I4}$	-	$\mu_{I22} > \mu_{I4}$	-
I4 v SI22	T	$\mu_{SI22} > \mu_{I4}$	-	-	0.00719
I4 v E4	S	$\mu_{E4} > \mu_{I4}$	-	$\mu_{E4} > \mu_{I4}$	-
I4 v E22	T, S, M	$\mu_{E22} > \mu_{I4}$	-	$\mu_{E22} > \mu_{I4}$	-
I4 v SE22	T, S	$\mu_{SE22} > \mu_{I4}$	-	-	0.000824
I22 v SI22	M	$\mu_{SI22} > \mu_{I22}$	-	-	0.0170
I22 v E4	T, S, M	$\mu_{E4} > \mu_{I22}$	-	$\mu_{E4} > \mu_{I22}$	-
I22 v E22	S	$\mu_{E22} > \mu_{I22}$	-	$\mu_{E22} > \mu_{I22}$	-
I22 v SE22	S, M	$\mu_{SE22} > \mu_{I22}$	-	$\mu_{SE22} > \mu_{I22}$	-
SI22 v E4	T, S	$\mu_{E4} > \mu_{SI22}$	-	$\mu_{E4} > \mu_{SI22}$	-
SI22 v E22	S, M	$\mu_{E22} > \mu_{SI22}$	-	$\mu_{E22} > \mu_{SI22}$	-
SI22 v SE22	S	-	0.470**	-	0.0100
E4 v E22	T, M	$(\mu_{E4} > \mu_{E22})^+$	-	-	0.0495
E4 v SE22	T	$\mu_{E4} > \mu_{SE22}$	-	$\mu_{SE22} > \mu_{E4}$	-
E22 v SE22	M	$\mu_{E22} > \mu_{SE22}$	-	$\mu_{SE22} > \mu_{E22}$	-

653 *T= temperature; M= microbial activity; S = sample; **common biphasic model also; +

654 limited data

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656

657

657 Table 4 Physicochemical properties of amended soil used for laboratory experiments

Parameter	Slag	Amended sand	Sand	Sieved Column material
Texture	Sand	Loamy Sand	Clayey sand	Clayey sand
d50 (mm)	0.63	0.43	0.38	0.50
pH	9.4	9.2	5.4	8.7
Bulk Density ($g.cm^{-3}$)	1.6	1.4	1.35	1.68
Electrical conductivity ($d.m^{-1}$) (1:5 water)	1.2	0.5	<0.1	-
Exchange Capacity ($cmol.kg^{-1}$)				
Aluminium	0	0.03	2.82	0
Calcium	41.55	13.99	0.67	18.4
Cation	42.36	14.59	4.29	18.9
Magnesium	0.36	0.28	0.59	0.3
Phosphorus sorption capacity	240	15 000	1 700	-

(kg/hectare)	0 0			
% Carbon (% dry weight)	0.4	0.4	0.4	0.3

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