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Bactericidal and virucidal mechanisms in the alkaline disinfection of compost using calcium lime and ash

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Abstract

In the present study, the bactericidal and virucidal mechanisms in the alkaline disinfection of compost with calcium lime and ash were investigated. Two indicator microorganisms, *Escherichia coli* and MS2 coliphage, were used as surrogates for enteric pathogens. The alkaline-treated compost with calcium oxide (CaO) or ash resulted primarily in damage to the outer membrane and enzyme activities of *E. coli*. The alkaline treatment of compost also led to the infectivity loss of the coliphage because of the partial capsid damage and RNA exteriorization due to a raised pH, which is proportional to the amount of alkaline agents added. These results indicate that the alkaline treatment of compost using calcium oxide and ash is effective and can contribute to the safe usage of compost from a mixing type dry toilet.

Keywords:

alkaline disinfection, ash, calcium oxide, compost, dry toilet, pathogen

1. INTRODUCTION

A mixing type composting toilet using an organic matrix is one of the established concepts of dry toilet. The mixture of human feces with organic matrix enhances aerobic degradation of feces with little odor, decreases the fecal volume by water evaporation, and contributes to internal heating generated by the activity of microorganisms. The dry toilet has been recognized as an improved sanitation facility (WHO and UNICEF, 2008) and has advantages for reducing the amount of water usage. It can be installed quickly and it saves public investment, since it does not require large-scale infrastructure such as a water distribution network and sewer piping system (Esrey, 1998; Lopez et al., 2002a). Furthermore, compost produced in a dry toilet promotes plant growth by providing nutrients and conditioning soil properties (Hijikata et al., 2011). These characteristics related to installability, usefulness, and nutrient usability are attractive for low-income countries, and rural areas, natural parks and emergency evacuation sites in developed countries, which often have a lack of improved sanitation facilities, incomplete water and sewer services, a restriction on wastewater discharge, and high demand for fertilizers.

From a hygienic perspective, feces should always be considered to contain pathogens that cause gastrointestinal infections. Although the composting process in dry toilets reduces concentrations of indicator microorganisms and pathogens to some extent (Sossou et al., 2011; Sossou et al., 2014), the compost recovered from a dry toilet always has the potential to

include pathogens derived from feces of infected persons, which pose infectious disease risks for users (Otaki et al., 2007). The highest infection risk most likely is posed during emptying the compost and exchanging the matrix (Nakagawa et al., 2006; Schönning et al., 2007). Potential hazard risks when the compost is removed from a composing toilet are caused by direct and/or indirect ingestion of enteric pathogens originating from human feces, which include bacteria, viruses, parasitic protozoa and helminthes (WHO, 2006). These pathogens may cause diarrhea, fever and cramps. According to the global burden of diseases study, diarrhea accounts for 89.5 million disability-adjusted life years (DALYs) and is still a large contributor to the burden, accounting for 3.6% of the global DALYs in 2010 (Murray et al., 2012). It is thus very critical to manage the infection risks of enteric pathogens associated with the use of composting toilets.

WHO (2006) and Schönning et al. (2007) have recommended storing mature fecal matter for 6-12 months to assure safety during handling. The recommendation mainly targets at pit-type dry toilets such as the urine-diversion dry toilet (UDDT), which has a double storage system. It may be thus difficult to apply this recommendation to the mixing type composting toilets as it is, because of their structure comprised of a single reactor. Kazama et al. (2011) showed that alkaline-treated compost with calcium lime was effective for the rapid inactivation of bacteria and virus indicators in sawdust used in a composting toilet. Lime and ash are often used in practical and scientific reports about the alkaline treatment of the UDDT,

and the potential for pathogen inactivation has been described (Nordin et al., 2009a; Niwagaba et al., 2009). However, bactericidal and virucidal mechanisms in the alkaline disinfection of compost from a mixing type composting toilet have not been well investigated.

In the present study, the inactivation mechanisms of surrogate microorganisms (*Escherichia coli* and MS2 coliphage) in compost treated with varied quantities of alkaline agents was investigated. Three types of growth medium were used for cultivating *E. coli* from the alkaline-treated compost, which allows us to identify the most affected physiological function of the surrogate for the bacterial pathogen. The plaque assay and the enzymatic treatment using Protease K and RNase A coupled with reverse-transcription quantitative PCR (ET-RTqPCR) were employed to identify the effect of the alkaline treatment on the infectivity of MS2 coliphage. Based on the results from these disinfection tests, the efficacy of alkaline disinfection of compost was discussed.

2. Materials and Methods

2.1. Compost preparation

Rice husks were used as a compost matrix and pig feces were used as a substitute for human feces since the characteristics of pig fecal matter are similar to those of human feces

(Lopez et al., 2002b). Fresh pig feces (500-600 grams) were mixed into 20 L (1429 g-dry, approximately a half volume of a general composting toilet reactor for one family) of the matrix in a composting machine (Hitachi, BGD-120). Fresh pig feces (500-600 grams) were put into the matrix in the composting machine everyday up to 38th day. At the end of the operation, the fecal load ratio (total input of feces [g-dry] per initial matrix [g-dry]) was 1.71, the fecal degradation (loss of the mass) ratio was 45.8%, and the water content was 48.5%, which is comparable with compost from a mixing type dry toilet. The obtained compost was stored in refrigerated room at 4 ˚C. The preparation process of the compost was depicted in Fig. 1S.

2.2. Test microorganisms

E. coli NBRC 3301 and MS2 coliphage were used as a surrogate for pathogenic bacteria and viruses, respectively. *E. coli* NBRC 3301 was incubated with 10 mL of 4% (w/w) of Tryptic Soy Broth (Difco Laboratory Inc., USA) in a shaking water bath at 37 ˚C for 4-6 h, and then the cultured *E. coli* was used as an inoculum for inactivation tests. *E. coli* NBRC 13965 was used as a host for MS2 coliphage. MS2 coliphage was co-incubated with precultured *E. coli* NBRC 139651 in 10 mL of liquid LB medium in the shaking water bath at 37 ˚C for 20–24 h. The culture was centrifuged at 6000 rpm for 10 min at 4 ˚C and the supernatant was filtered with a sterilized disposal filter (0.20 μm pore, DISMIC-25CS,

ADVANTEC). The filtered liquid was used as an MS2 coliphage inoculum for inactivation tests.

2.3. Inactivation test of indicator microorganisms in the composting toilet

Calcium lime (CaO, Wako chemical, Japan) was used as the calcium oxide reagent, according to the previous study (Kazama et al., 2011). Three types of ash, including wood ash, grass ash made from rice straws, and mixed ash (a mixture of wood grasses, crushed oyster shell and wood ash obtained from an Italian restaurant in Sapporo City, Japan) were also used as alkaline agents. The volatile solid [%, w/w] of these alkaline agents was obtained from a loss of weight by the incineration at 600 ˚C for 3 h. The chemical compositions of ash are indicated in Table 1.

Stored compost (20 g) was transferred to a glass bottle and autoclaved at 121 ˚C for 15 min, and then the water content was adjusted to 50% using sterilized deionized water to uniform the water content condition in the inactivation tests. After preincubation of the water content-adjusted compost at 37 ˚C for 1 h, an adequate amount of CaO or ash and 1 g of autoclaved pig feces were simultaneously added and mixed in the bottle, which can create a sterile but very similar condition with actual compost from a mixing type dry toilet. The pH value of compost mixture was measured by an electrode method using a suspension of compost mixture and pure water at a ratio of 1:20 (w:w), which was shaken for 30 min. The

pH of compost mixture was adjusted to pH 9.5, 10.0, 10.5 and 11 by adding the alkaline agents. *E. coli* inoculum (0.5 mL, approximately 10^8 CFU/mL) was spiked into the compost mixture and shaken by hand for 1 min, which gave the experimentally highest *E. coli* concentration, enabling us to follow the inactivation profile very efficiently. The compost mixture was incubated at 37 ˚C, the optimum temperature for the *E. coli* growth, which may attenuate the disinfection efficiency of alkaline treatments. One gram of the compost mixture was sampled in an appropriate time (0-8 h) and *E. coli* was extracted by suspending it in 40 mL of 3% (w/v) beef extraction solution adjusted to pH 9.5 with an NaOH solution (Otaki et al., 2002). The previous study reported that the total recovery efficiency using this method is 70-100% (Otaki et al., 2002). After dilution (10^1-10^4 folds) with an autoclaved phosphate buffer (pH 7.5 adjusted with the NaOH solution), 1 mL of each extract was separately inoculated on three types of agar media: Tryptic Soy Agar (TSA, Difco Laboratory Inc., USA), Desooxycholate Agar (DESO, Eiken Kagaku Inc., Japan), and Compact Dry EC (C-EC, Nissui Inc., Japan). TSA is a non-selective agar that contains two peptones (casein and soy) as nutrition for microorganisms. The agar can isolate and cultivate a wide variety of organisms that metabolize the peptones and grow. DESO is a selective agar that inhibits to grow gram-positive bacteria. The agar contains general growth requirements with sodium desoxycholate, sodium citrates, lactose, and neutral red. Sodium desoxycholate, a surfactant, lyses gram-positive bacteria because they do not have outer membrane unlike gram-negative

147 bacteria. Bacteria that ferment lactose produce acid and form red colonies in the presence of
148 neutral red. Bacteria that do not ferment lactose form colorless colonies. C-EC, a selective
149 agar for *E.coli* and coliform, includes general growth requirements with two chromogenic
150 substrates: X-GLUC and Magenta-GAL. The chromogenic substrates are hydrolyzed with
151 enzymes: ß-glucuronidase and ß-galactosidase that *E.coli* produces during fermenting lactose
152 and make colonies blue or blue purple color. These agar media were incubated at 37°C for 24
153 h and the colonies formed were counted.

154 The alkaline-treated compost identical with that used in the *E .coli* inactivation test
155 was also prepared for the inactivation test of MS2 coliphage. Five milliliters of MS2
156 coliphage inoculum were spiked into 20 g of the compost and incubated at 37°C. One gram of
157 the incubated compost mixture was sampled in an appropriate time (0-8 h) and suspended in
158 40 mL of the autoclaved phosphate buffer for 3 min. The suspension was diluted 10^1-10^4 fold
159 with autoclaved liquid LB culture. The diluted liquid was used for the plaque assay method
160 with a two-layer LB agar media (Adams 1985) and for enzyme treatment qPCR (ET-qPCR)
161 detection with proteinase K and RNase A (Pecson et al., 2009), respectively. In the plaque
162 assay, 25 ml of the diluted liquid were inoculated on the bottom layer agar. A top layer
163 medium (10-20 mL) including host *E. coli* cells (approximately 10^8 CFU per an agar plate)
164 was pulled on the bottom agar. The two-layer agar was incubated at 37 ˚C for 20-24 h and the
165 plaques formed were counted. In ET-qPCR detection, the 10-fold diluted liquid was applied to

166 the enzyme pretreatments, in which only RNase A (ET1-qPCR), a combination of proteinase
167 K with RNase A (ET2-qPCR), or no enzyme treatment (qPCR) was employed. These enzyme
168 treatments were performed according to Nuanualsuwan and Cliver (2003) with a slight
169 modification. For the ET2-qPCR reaction, 33 μL of proteinase K (20 U/min) and 7 μL of
170 Tris-EDTA buffer (TE buffer) were mixed into 250 μL of the diluted liquid. The mixture was
171 reacted in a shaking incubator at 37°C for 1 h. Then, 10μL of 10 ng/μL RNase A were added
172 to the mixture and incubated at 37 ˚C for 1 h. For the ET1-qPCR, 40 μL of the TE buffer were
173 mixed in the diluted liquid as a substitute for the proteinase K solution, and then the same
174 operation with ET2-qPCR was conducted. For the qPCR, 40 μL of TE buffer and 10 μL of TE
175 buffer were used as a substitute for the proteinase K solution and RNase A solution,
176 respectively. After the enzyme pretreatment, viral RNA was extracted with QIAamp Viral
177 RNA mini Kit (QIAGEN, Japan) according to the manufacturer's protocol. The extracted
178 RNA was applied to a reverse transcription reaction (RT) with PrimeScript RT reagent Kit
179 (Takara Bio, Japan) to synthesize complementary DNA (cDNA). For the quantitative PCR, a
180 25 μL reaction mixture, containing 5 μL cDNA, 12.5 μL Premix Ex Taq, 1 μL of forward and
181 reverse primer, 0.5 μL Taqman probe, 0.5 μL Rox Reference Dye and 4.5 μL sterilized pure
182 water, was prepared. The PCR thermal cycling conditions were 15 min at 55 ˚C for 15 min
183 and 95 ˚C for 15 sec at the beginning; 95 ˚C for 15 min and 60 ˚C for 1 min repeated for 45
184 cycles; and 60 ˚C for 1 min at the end, using the ABI 7500 Real Time PCR system (Applied

185 Biosystemes, USA). The sequence of PCR primers (Blaise-Boisseau et al., 2010) and the
186 probe are listed in Table S1.
187
188 2.4. Inactivation rate constant
189 The inactivation rate constant was calculated using the following Chick model:
190
191 $$\mathrm{Log}\ (N / N_0) = - k\ t \qquad (1),$$
192
193 where N is a concentration of the microorganism at a time t [number/g-compost mixture], N_0
194 is an initial concentration of the microorganism [number/g-compost mixture], k is an
195 inactivation rate constant expressed by a common logarithm [1/h] and t is the incubation time
196 in the compost mixture [h]. The microorganism concentration (N) corresponded to
197 CFU/g-compost mixture for the *E. coli*, PFU/g-compost mixture for the plaque assay of the
198 MS2 coliphage and cDNA copy number/g-compost mixture for the ETs-qPCR of the MS2
199 coliphage.
200
201 2.5. Statistical analysis
202 All statistical and regression tests were performed using IBM SPSS Statistics 21
203 software.

204

205

206 **3. Results**

207

208 3.1. Alkaline treatment of compost with CaO and ash

209 The pH of compost mixture was increased as the addition of CaO (Fig. 1A) and ash

210 (Fig. 1B). The regression lines for each alkaline agent were obtained as follows:

211

212 $$pH_{CaO} = 76.03\ W_{CaO} + 8.89\ (R^2 = 0.902) \quad (2),$$

213 $$pH_{mixed\ ash} = 0.32\ W_{mixed\ ash} + 9.59\ (R^2 = 0.855) \quad (3),$$

214 $$pH_{wood\ ash} = 0.80\ W_{wood\ ash} + 9.80\ (R^2 = 0.906) \quad (4),$$

215 $$pH_{grass\ ash} = 0.41\ W_{grass\ ash} + 9.71\ (R^2 = 0.852) \quad (5),$$

216

217 where pH_{agents} is the pH of compost mixture adjusted by each alkaline agent and W_{agents} is the

218 weight of the alkaline agent added [g/g-dry compost mixture]. The slope values of the

219 regression lines indicated that the pH increase with ash was more moderate than that with

220 CaO. Among three ash types, the alkalinization capacity of wood ash was higher than those of

221 the other two ahs types.

222

223 3.2. Alkaline-treated compost and inactivation rate of indicator microorganisms

224 Time-dependent changes of *E. coli* on the TSA media and MS2 coliphage on the
225 plaque assay in the alkaline-treated compost are shown in Fig. 2. The actual pH of compost
226 mixture was measured after the time-dependent test and the value variation was within 0.1.
227 The inactivation rate values of *E. coli* (Fig. 2A) and MS2 coliphage (Fig. 2B) were influenced
228 by the pH of compost mixture. The type of alkaline agent also affected the inactivation rate at
229 the identical pH value. The mixed ash gave higher inactivation rate of *E. coli* than CaO at pH
230 10 (Fig. 2A), while the addition of CaO led to the higher inactivation of MS2 coliphage than
231 the mixed ash at the identical pH value (Fig. 2B). The relationship between the inactivation
232 rate constant (k) and the pH of compost mixture was well fitted with a quadratic curve model
233 in the present experimental conditions ($p < 0.05$) for both *E. coli* on TSA (Fig. 3A) and MS2
234 coliphage on the plaque assay (Fig. 3B). The model gave the following equations:

235

236 $$k_{E.coli} = 1.444\ pH_{CaO}^{\ 2} - 26.384\ pH_{CaO} + 120.76\ (R^2 = 0.961) \quad (6),$$

237 $$k_{E.coli} = 1.673\ pH_{mixed\ ash}^{\ 2} - 27.97\ pH_{mixed\ ash} + 115.84\ (R^2 = 0.92) \quad (7),$$

238 $$k_{MS2} = 0.655\ pH_{CaO}^{\ 2} - 11.894\ pH_{CaO} + 53.96\ (R^2 = 0.981) \quad (8),$$

239 $$k_{MS2} = 0.195\ pH_{mixed\ ash}^{\ 2} - 3.5743\ pH_{mixed\ ash} + 16.41\ (R^2 = 0.862) \quad (9),$$

240

241 where $k_{E.coli}$ and k_{MS2} are the inactivation rate constant for *E. coli* [1/h] and MS2 coliphage

242 [1/h], respectively. These equations obviously show that the alkaline treatment of compost
243 with the mixed ash causes a faster inactivation of *E. coli* than with CaO at the identical pH of
244 compost mixture (Fig. 3A), but the opposite result (faster inactivation with CaO than mixed
245 ash) was obtained for MS2 coliphage (Fig. 3B).
246
247 3.3. Inactivation mechanism of indicator microorganisms
248 The inactivation rate constants of *E. coli* in three cultivation media were compared
249 (Fig. 4A). The value on TSA was lower than those on DESO and C-EC which was very clear
250 at pH 11. There was no significant difference between the values of inactivation rate constant
251 on DESO and C-EC at the compost pH of 10 to 11. These results suggests that the alkaline
252 treatment of compost with CaO led to the damage on the outer membrane and specific
253 enzymes before damaging the nucleic acid and/or metabolism of *E coli*. Very similar tendency
254 of *E. coli* inactivation was observed in the case of alkaline-treated compost with ash, which
255 suggested that the similar inactivation mechanism was exerted on *E. coli* when ash were
256 mixed with compost.
257 In the case of MS2 coliphage (Fig. 4B), the inactivation rate measured with the
258 plaque assay was 10-13 times higher than that measured with ET-PCR in all CaO-treated
259 compost. In the compost mixture at pH 10 and 10.5, the inactivation rate measured with
260 ET2-PCR and ET1-PCR were 1.9-2.1 times higher than that with qPCR. These results suggest

that the alkaline treatment of compost led MS2 primarily to reduce the infectivity, and then caused capsid damage and partial RNA exteriorization accompanied by a pH increase. At pH 11, on the other hand, there was no significant difference in the inactivation rate among ET2-PCR, ET1-PCR and qPCR, which implies that the capsid damage and the degradation of the exteriorized RNA progressed rapidly at this pH. The pattern of the inactivation rate constants in CaO and mixed ash was the same in the compost mixture at pH 10, meaning that there was no clear difference in the inactivation mechanism between alkaline agents at the identical pH of compost mixture.

4. Discussion

In the present study, the inactivation rate constant values of *E. coli* and MS2 coliphage in compost from a mixing type dry toilet, which was treated by CaO or ash, were obtained. The alkaline-treated compost mixture with CaO or ash resulted primarily in damage to the outer membrane and enzyme activities of *E. coli*. The alkaline treatment of compost also led to the infectivity loss of the coliphage because of the partial capsid damage and RNA exteriorization due to a raised pH, which is proportional to the amount of alkaline agents added.

Among the three types of ash used in this study, the wood ash efficiently raised the

compost pH (Fig. 2B). The increase of pH is considered to be dependent on the burning temperature and chemical composition of the ash, mainly the content of carbonates and hydroxides. According to Etiegni and Campbell (1991), carbonates and bicarbonates predominate when the combustion temperature is below 500 ˚C whereas oxides become more prevalent with temperatures over 1000 ˚C. Cations play the role of counter ion for these bases and determine the dissociation constant. In the present study, the lowest volatile solid concentration of wood ash (Table 1) possibly indicated that the ash had been burned at a high temperature, which might imply a relatively higher content of oxides. The commercial mixed ash used in this study contained crushed oyster shell to supply mineral divalent cations and moderate the pH increase for agricultural purposes, according to the manufacturer's instructions. The addition of crushed oyster shell must be the reason for the lower alkalinity of the mixed ash. It is important to analyze chemical compositions of ash prior to the usage for the alkaline treatment of compost.

Damaged components of *E. coli* in the alkalinized compost were estimated by a comparison of inactivation rate constants (*k*) in the three media (Kazama et al., 2011; Sossou et al., 2014). TSA is a nonselective agar that contains digested proteins and can detect bacteria that can metabolize these proteins. The higher *k* value on the TSA indicates that there were damages on the nucleic acid and/or enzymes involved in the protein metabolism. DESO is a selective agar and can detect bacteria that can metabolize lactose and peptone in the presence

of desoxycholic acid. Gram-negative bacteria with an incomplete outer membrane and gram-positive bacteria are unable to grow in the presence of desoxycholic acid due to its surface-active effect. The higher *k* value in the DESO indicates that there were damages in the outer membrane structure. C-EC is a selective agar for *E. coli* and coliform. The agar can detect bacteria that can produce ß-glucuronidase and ß-galactosidase and metabolize the carbon sources. The higher *k* value in the C-EC indicates that there were damages in specific enzymatic activities. These results suggest that alkaline-treated compost with CaO and ash result primarily in damages on the outer membrane and the enzyme activities of *E. coli* and then on the nucleic acid and/or metabolism. This result seems to be inconsistent with results reported by Kazama et al. (2011), who also observed the damage mechanism in alkaline-treated sawdust compost with CaO by the same method. This may be related to the type of matrix (Sossou et al., 2014) or the ammonium concentrations in the compost mixture. Low pH sawdust compost (6.96-7.51), in which ammonium was probably nitrified or volatilized, was used in Kazama et al. (2011). Several reports have revealed a positive correlation between the ammonium concentration and the inactivation rate of pathogenic bacteria (Vinneras et al., 2003; Nordin, 2009b). The *E. coli* disinfection efficiency is affected by the composting process; particularly, the ammonium ion generated through the composting process is supposed to cause the damage on the outer membrane and enzyme activities (Sossou et al., 2014).

318 Damaged components of the MS2 coliphage were also estimated by a comparison of
319 results from the plaque assay, ET-qPCR and qPCR as a model of enteric viruses, according to
320 Pecson et al. (2009). The *k* value in the infectivity titer indicates the total inactivation
321 efficiency, whereas the higher *k* value obtained by ETs-qPCR using both the protease and
322 RNase (ET2-qPCR) may show the extent of denaturation of the coliphage capsid, since
323 proteinase K digests the damaged capsid (Nuanualsuwan and Cliver, 2002) but is unable to
324 digest the intact capsid (Nuanualsuwan and Cliver, 2003). The *k* value obtained by ETs-qPCR
325 using only RNase (ET1-qPCR) reflects the level of RNA exteriorization. The infectivity of
326 enteric viruses requires the functional integrity of both viral RNA and the capsid. The capsid
327 must be sufficiently intact in terms of (i) protecting the genome from degradation and (ii)
328 interacting with host cell receptors (Pecson et al., 2009). At the protein structural level,
329 conformational changes in the capsid, which may affect viral stability and attachment
330 efficiency to the host cell receptor, directly leads to the loss of viral infectivity (Nuanualsuwan
331 and Cliver 2003). MS2 coliphage has a positive-sense, ssRNA genome of 3569 nt that
332 encodes just 4 proteins: maturation, coat, lysis and replicase. Its capsid comprises 90 coat
333 protein dimers arranged in a $T = 3$ icosahedral lattice (Toropova et al., 2011). Each virion also
334 incorporates a single copy of the maturation protein, which binds the virion to the side of the
335 bacterial F-pilus, as a receptor of host *E. coli*, and is therefore an essential protein for
336 infectivity (Toropova et al., 2011). Therefore, it was estimated based on the obtained results

that (i) the alkaline-treated compost primarily induced conformational change of the maturation protein on the capsid surface, which led to the reduction of infectivity; and (ii) an increase of the compost pH to 10-10.5 by the alkaline agents then accelerated the conformation change of the coat protein, that caused the partial capsid damage and RNA exteriorization; and (iii) 90 coat protein dimers were deformed and genomic RNA was exteriorized and degraded by bulk RNase in the compost mixture.

Multiple parameters of compost characteristics, including temperature and water content, affect the fate of pathogens in compost and also the efficacy of the alkaline disinfection. In this sense, this study represents the disinfection efficiency of surrogate microorganisms (*E. coli* and MS2 coliphage) under a limited but possible condition of compost. It is important in the further study to assess the efficacy of alkaline disinfection under varied environmental conditions to ensure the safe use of compost from a mixing type dry toilet. The effect of microflora on the fate of pathogens in compost is also not negligible. Under the real situation, there is a need to consider the microbial competitive exclusion processes in small bioreactors, where non-pathogenic environmental bacteria are predominant (Evans et al., 2009; Spinks et al., 2006). In this study, the compost mixture before the inoculation of indicator microorganisms was sterile, which means that the effect of microflora in compost on the disinfection efficiency was excluded. This means that an additional effect of the microbial competitive exclusion with the alkaline treatment on the pathogen reduction

in compost may be expected in the real operation of a mixing type dry toilet.

Indicator microorganisms have pros and cons as surrogates for pathogens (Wu et al., 2011). We employed *E. coli* and MS2 coliphage in this study, which are commonly the first choice as reduction indicators in disinfection practices (Chren et al., 2014; Mondal et al., 2015). However, the disinfection efficacy may depend on bacterial and viral species, particularly when some microbial species have a capability to specifically bind with biosolids (Sano et al., 2004). The comparison of the efficacy of alkaline disinfection on multiple microorganisms is a next research interest for the safe use of compost from a mixing type dry toilet.

5. Conclusions

The alkaline treatment of compost with CaO and ash resulted primarily in damage to the outer membrane and enzyme activities of *E. coli* and thus damage to the nucleic acid and/or metabolism. The alkaline treatment also led MS2 coliphage primarily to reduce the infectivity and secondarily to cause partial capsid damage and RNA exteriorization. These results indicate that the alkaline treatment can effectively contribute to the disinfection and safe use of compost from a mixing type dry toilet.

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Figure captions

Fig. 1. Compost pH response to the addition of alkaline agents. A, CaO; B, ash type.

Fig. 2. Inactivation profiles of surrogate microorganisms in alkaline-treated compost. A, *Escherichia coli*; B, MS2 coliphage.

Fig. 3. The relationship between inactivation rate constant and compost pH. A, *Escherichia coli*; B, MS2 coliphage.

Fig. 4. Inactivation rate constant of surrogate microorganisms in the alkaline treatment of compost. A, *Escherichia coli*; B, MS2 coliphage.

480 Table 1. Chemical composition of ash (average in triplicates ± standard deviation)

	Volatile solids [%]	Cations [mg/g-ash] K	Ca	Mg	Fe	Mn	Zn
mixed ash	1.7 ± 0.0	173.7 ± 13.1	80.7 ± 5.2	49.5 ± 2.4	10.6 ± 1.0	1.2 ± 0.1	0.5 ± 0.1
wood ash	0.2 ± 0.0	112.2 ± 3.1	294.7 ± 17.5	9.7 ± 0.3	1.5 ± 0.1	1.1 ± 0.0	0.1 ± 0.0
grass ash	5.8 ± 0.0	101.1 ± 4.9	14.3 ± 1.1	10.3 ± 0.7	8.7 ± 0.7	1.0 ± 0.1	0.1 ± 0.1

481

Fig. 1

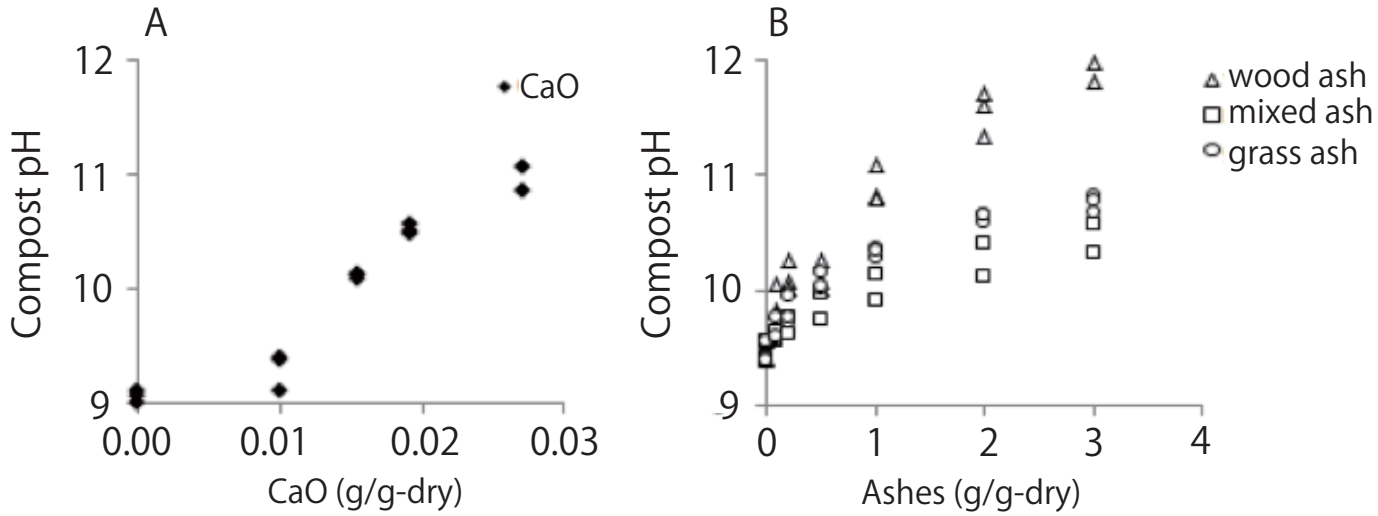

A
12
11
10
9
Compost pH
CaO
0.00
0.01
0.02
0.03
CaO (g/g-dry)
B
12
11
10
9
Compost pH
0
1
2
3
4
Ashes (g/g-dry)
wood ash
mixed ash
grass ash

Fig. 2

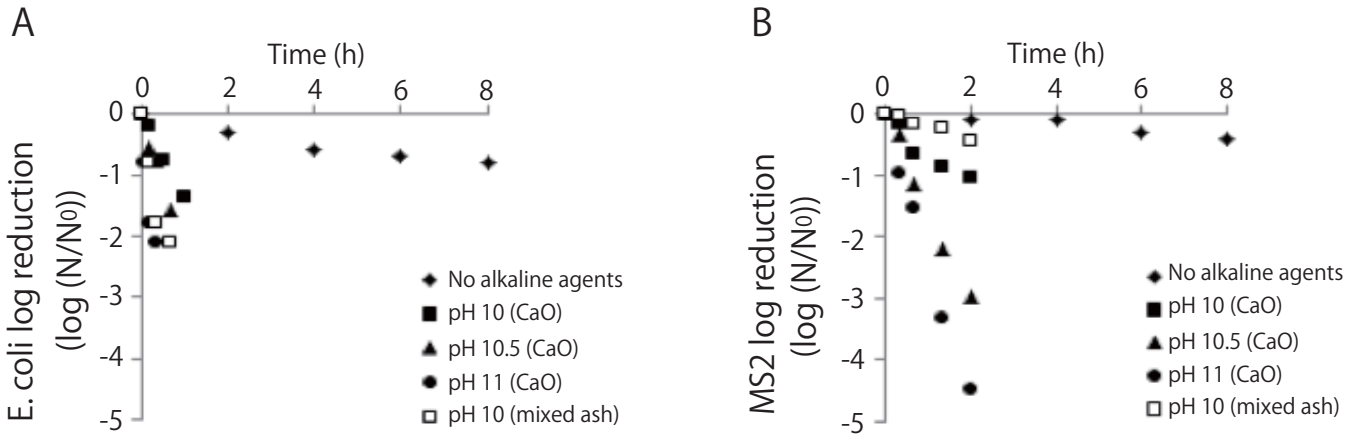

A
Time (h)
0
2
4
6
8
0
-1
-2
-3
-4
-5
E. coli log reduction
(log (N/N0))
No alkaline agents
pH 10 (CaO)
pH 10.5 (CaO)
pH 11 (CaO)
pH 10 (mixed ash)
B
Time (h)
0
2
4
6
8
0
-1
-2
-3
-4
-5
MS2 log reduction
(log (N/N0))
No alkaline agents
pH 10 (CaO)
pH 10.5 (CaO)
pH 11 (CaO)
pH 10 (mixed ash)

Fig. 3

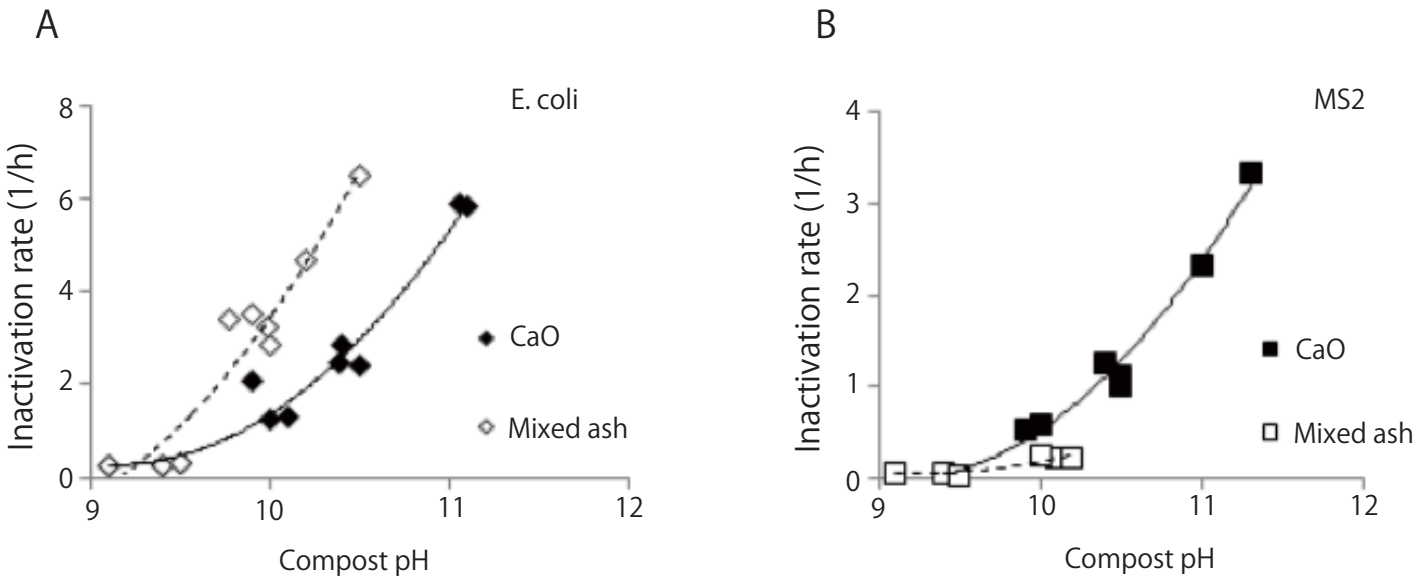
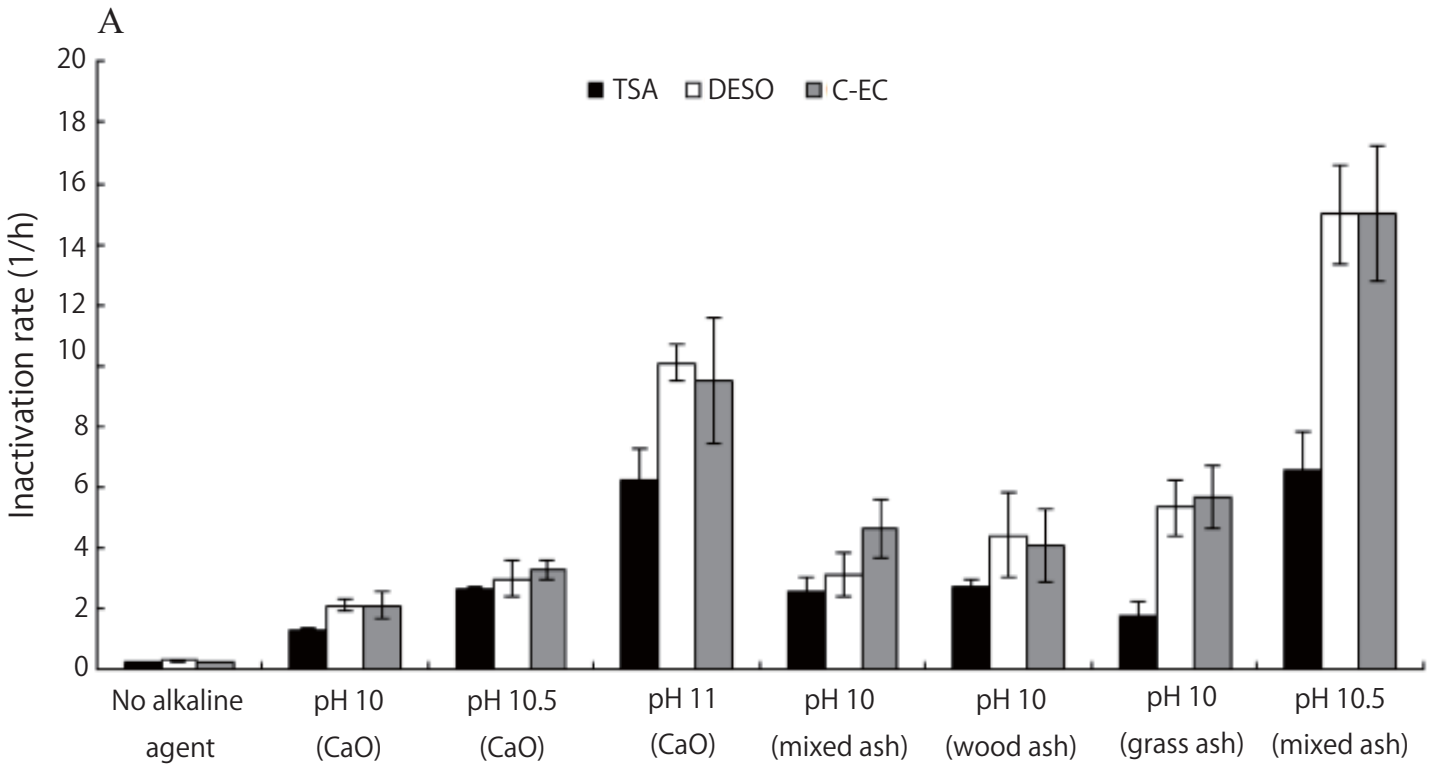
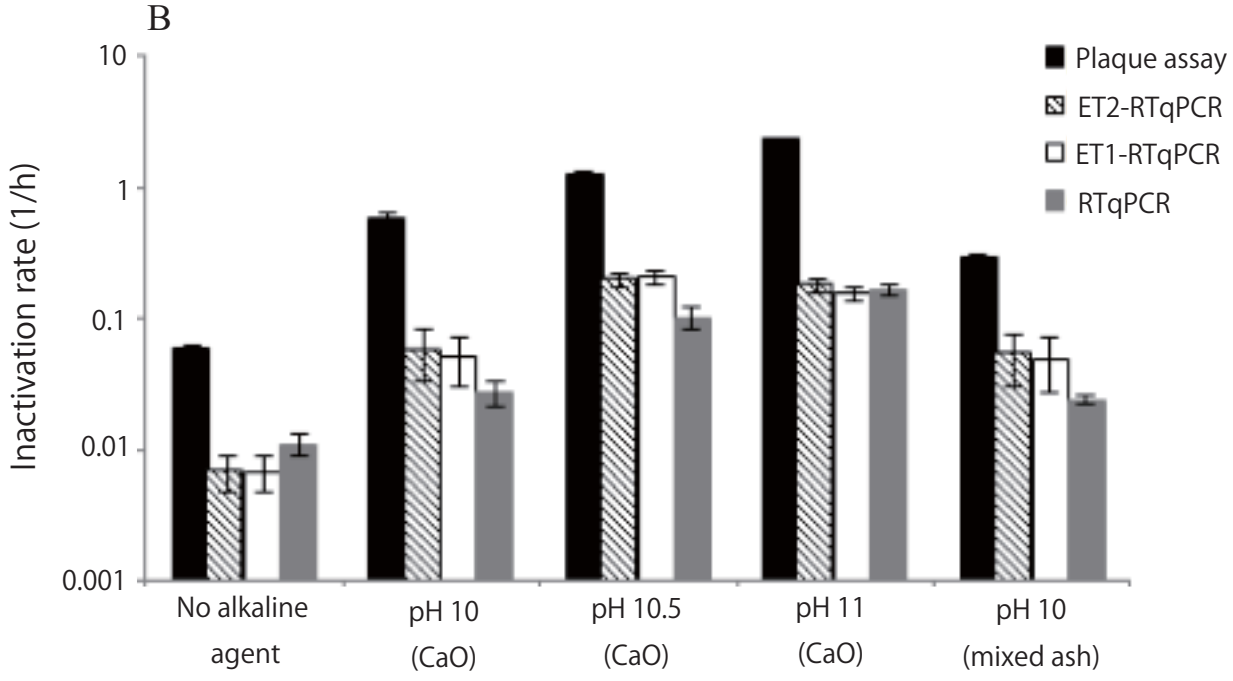

A
E. coli
Inactivation rate (1/h)
8
6
4
2
0
9
10
11
12
Compost pH
CaO
Mixed ash
B
MS2
Inactivation rate (1/h)
4
3
2
1
0
9
10
11
12
Compost pH
CaO
Mixed ash

Fig. 4


A
TSA
DESO
C-EC
Inactivation rate (1/h)
20
18
16
14
12
10
8
6
4
2
0
No alkaline agent
pH 10 (CaO)
pH 10.5 (CaO)
pH 11 (CaO)
pH 10 (mixed ash)
pH 10 (wood ash)
pH 10 (grass ash)
pH 10.5 (mixed ash)


B
Plaque assay
ET2-RTqPCR
ET1-RTqPCR
RTqPCR
Inactivation rate (1/h)
10
1
0.1
0.01
0.001
No alkaline agent
pH 10 (CaO)
pH 10.5 (CaO)
pH 11 (CaO)
pH 10 (mixed ash)