Mechanisms for the Inactivation of Bacteria and Viruses in Sawdust Used in Composting Toilet

Shinobu KAZAMA, Masahiro OTAKI

Department of Human Environmental Science, Ochanomizu University, Tokyo 112-8610, Japan

ABSTRACT
A composting toilet, using sawdust as a matrix, has the potential to trap pathogens that might occasionally be contained in human feces. Therefore, care must be taken when handling the sawdust. High temperature and low water content conditions are effective for reducing pathogen content. However, it is not clear whether these effective conditions can cause lethal damage to these pathogens or not. Therefore, the present study investigates the inactivation mechanisms of pathogens in a composting toilet, using E. coli as a model of pathogenic bacteria and Qß as a model of pathogenic viruses. Escherichia coli was rapidly and lethally inactivated under high pH conditions. In dry conditions, they were inactivated lethally but not rapidly. High temperature treatment was unable to inactivate them lethally. For Qß, the capsid was mainly damaged under high temperature and dry conditions, whereas high pH conditions primarily damaged nucleic acids. Therefore, raising the pH was an effective way to rapidly and lethally reduce both pathogenic bacteria and viruses.

Keywords: composting toilet, inactivation mechanism, pathogen

INTRODUCTION
A composting toilet using sawdust as a matrix enhances the decomposition of feces under aerobic conditions and decreases the volume by evaporation of water. This mechanism can realize efficient organic decomposition of feces with little odor. This treatment requires no water or drainage, and the used sawdust can be recycled as fertilizer. Therefore, compared with a flush toilet, the composting toilet has many advantages from the viewpoint of preserving water resources and allowing nutrient recycling (Winblad et al., 2004). A composting toilet is a useful system especially for the improvement of sanitation in developing countries, because it does not require infrastructure and is inexpensive to introduce (Zavala et al., 2004). However, the sawdust used as matrix has the potential to trap pathogens derived from infected persons, which raises the possibility for other users to become infected (Otaki et al., 2006). Therefore, care must be taken when handling the used sawdust. Furthermore, it has to be also noted that pathogenic viruses have stronger tolerance than pathogenic bacteria. Although high temperature and low moisture conditions are known to reduce their numbers, and also, high pH condition by adding CaO just before using the composted sawdust as fertilizer (Kazama et al., 2010), it is not yet clear whether these effective conditions result in lethal damage to pathogens or not. Therefore, in order to propose an effective condition to reduce pathogen risk, the present study examined the inactivation mechanisms of pathogens under several conditions, focusing on which parts and/or functions were damaged.

To investigate the inactivation mechanisms, we compared the results obtained from multiple detection methods under several conditions. For the investigation of bacteria, E. coli was used as an indicator of fecal pathogenic bacteria. Three types of media
commonly used to detect *E. coli* were simultaneously studied to estimate the inactivation mechanism of *E. coli*.

For investigation of viruses, Qß, an F + RNA coliphage, was used as a model of fecal pathogenic viruses. Coliphages, especially the F + RNA type, have been used as model viruses in environmental and water treatment processes to estimate the fate of fecal pathogenic viruses, because their characteristics are similar to most fecal pathogenic viruses, which tend to be RNA viruses with round shapes (Sobsey *et al*., 1995). A virus consists of capsid and nucleic acid. The capsid is a protein shell that allows host cell infection and that protects the viral nucleic acid. Therefore, the mechanisms for inactivation of viruses are limited to the damage of the capsid and/or the nucleic acid (Black, 2003). In this study, the fates of Qß detected by a plaque assay and by a real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) method were compared in order to estimate the damage of Qß under composting toilet sawdust conditions.

**MATERIALS AND METHODS**

**Microorganisms (bacteria and virus)**

Microorganisms were purchased from NBRC (National Institute of Technology and Evaluation Biological Resource Center, Japan). *Escherichia coli* (NBRC3301) was used as a model microorganism of pathogenic bacteria. Coliphage Qß was used as a model virus of pathogenic viruses, and *E. coli* (NBRC13965) was also used as a host microorganism for multiplication of Qß. This host *E. coli* was NBRC13965 and different from previous one which was used as model bacteria.

Tryptic Soy Broth (Difco) was used as a growth medium for Qß and *E. coli*. Coliphage Qß was incubated in a growth medium with host *E. coli* (NBRC13965) in a shaking water bath at 37°C overnight. The medium was centrifuged at 6000 g for 10min and then filtered using a sterilized membrane filter (pore size 0.45 μm, DISMIC-25CS, Toyoroshi Co., Japan). This filtrated medium was used as a Qß stock solution. *Escherichia coli* cultures were incubated in a shaking water bath, at 37°C for 3 - 4 hr, for each experiment.

**Extraction of microorganisms from sawdust**

The microorganisms were extracted from the composting toilet sawdust using a 3% (w/v) beef extraction solution (Otaki *et al*., 2002). Beef extract (MP Biomedicals) was dissolved in deionized water, adjusted to pH9.6 with NaOH, and then sterilized. A weighed sample of sawdust (0.4 g) was added to a 20 mL volume of extraction solution and agitated for 3 minutes to extract microorganisms. Phosphate buffer (PBS) was used to dilute the extract to a suitable concentration for measuring microorganisms. For Qß, the extract was filtered using a sterilized membrane filter (pore size 0.45 μm, DISMIC-25CS, Toyoroshi Co., Japan) before dilution. It was reported that the recoveries of *E. coli* and Qß using this method were 70 - 100% and 40 - 80%, respectively (Otaki *et al*., 2002). In the present study, the recoveries of *E. coli* and Qß were approximately 70% and 50%, respectively, and stable at every experiment.
Measurement of microorganisms
The numbers of *E. coli* were measured by a double agar layer method using Tryptic Soy Agar (TSA) (Difco) and Desoxycholate Agar (DESO) (Eiken Chemical Co., Japan), and by X-Gluc and Magenta-GAL (C-EC) (Compact Dry EC, Nissui pharmaceutical Co., Japan).

For measuring Qß numbers, a plaque assay and a qRT-PCR method were used. The plaque assay used a double agar layer method (APHA/AWWA/WEF, 1998) using TSA (Difco). For the qRT-PCR method, Qß RNA was extracted using a QIAamp Viral RNA mini kit (Qiagen). Reverse transcription was carried out using a high capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA) to produce a cDNA. For real time PCR, the 25 μL reaction mixture contained 5 μL of cDNA, 12.5 μL of 2 × TaqMan Gene Expression Master Mix (Applied Biosystems, USA), forward and reverse primer (each with a final concentration of 400 mM, Invitrogen), TaqMan MGB Probe (with a final concentration of 300 nM) and distilled water. The PCR cycling conditions were: 2 min at 50°C and 10 min at 95°C; 50 cycles of 15 sec at 95°C and 1 min at 60°C, using an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The PCR primers and probe are listed in Table 1.

Composting toilet
In this study, sawdust was obtained from an actual operating composting toilet at a household in Chichibu City (Saitama Prefecture, Japan). The schematic diagram of this composting toilet is shown in Fig. 1. The specifications are listed in Table 2. Cedar wood sawdust was used as a matrix.

<table>
<thead>
<tr>
<th>Sequence (5'-3')</th>
<th>Sense</th>
<th>Location</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>GYG GTG CYA CAA CRA CGA AT</td>
<td>+</td>
<td>2778-2797</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>GWG GSG TAC ACK CTT GCG</td>
<td>-</td>
<td>2838-2855</td>
</tr>
<tr>
<td>Prove</td>
<td>TAC GGY CAT CCG TTC TTC AAG TTT G</td>
<td>+</td>
<td>2807-2831</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reactor specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
</tr>
<tr>
<td>Width (mm)</td>
</tr>
<tr>
<td>Depth (mm)</td>
</tr>
<tr>
<td>Height (mm)</td>
</tr>
<tr>
<td>Capacity (m³)</td>
</tr>
<tr>
<td>Electric power of heater</td>
</tr>
<tr>
<td>Electric power of motor (W)</td>
</tr>
<tr>
<td>Use a day (times)</td>
</tr>
<tr>
<td>Target number of users (person)</td>
</tr>
</tbody>
</table>

*Seiwa-denko co., Japan*
Sawdust matrix conditions

*Escherichia coli* and Qβ were inactivated under several different conditions. As shown in Table 3, the temperature was set to 37°C which is similar to the actual operating temperature of the composting toilet (Kazama et al., 2010) and 50°C was used as a high temperature test. Water content was set to 50% similar to the operating composting toilet and 30% was used as a low water content test. The pH was adjusted by adding CaO or HCl (only Qβ case) because high pH condition was effective to reduce *E. coli* and Qβ according to the previous study (Kazama et al., 2010).

The sawdust was derived from an operating composting toilet (sawdust used for 100 days.). At first, its water content was decreased by heating at 70°C for 3 hours and then adjusted to 30% or 50% with deionized water. Ten grams of sawdust was transferred to a sterilized glass bottle with a cotton plug and kept at 37°C or 50°C in an incubator. A 0.1 mL volume of *E. coli* (about 10⁹ CFU/mL) or Qβ (about 10⁹ PFU/mL) stock solution was added and the sawdust was agitated for 1 minute. An aliquot of sawdust was sampled and the target microorganism was extracted following the previously described methods. The concentration of microorganism in the sawdust was measured by extraction at several time periods.

For pH analysis, the CaO or HCl was added first, and then the microorganisms were added. This allowed any heat released by chemical addition due to an exothermic reaction to be dissipated before the microorganisms were added. Thus, the effect of the change in pH without heat was investigated. Calcium oxide was added as a solution in deionized water and the water content of the sawdust was adjusted to account for this addition.

For experimental periods over 24 hr, the water content of the sawdust was maintained by adding deionized water to replace the amount of water lost by evaporation. Moreover, in order to compare the fate of Qβ in the presence and absence of sawdust, the fate of Qβ was investigated in PBS. A volume of 9.9 mL PBS was kept at 37°C or 50°C in an incubator. A 0.1 mL volume of Qβ solution (about 10⁷ PFU/mL) was added
Table 3 - Experimental conditions for the inactivation of microorganisms in the sawdust or in PBS

<table>
<thead>
<tr>
<th>Target microorganisms</th>
<th>Temperature [°C]</th>
<th>Water content [%]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>37</td>
<td>30</td>
<td>7.51</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.96</td>
<td>9.25, 10.01 (with CaO)</td>
</tr>
<tr>
<td>Qß</td>
<td>50</td>
<td>30</td>
<td>7.51</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6.65-6.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.65-6.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>9.64, 10.20 (with CaO)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.76, 4.81 (with HCl)</td>
<td></td>
</tr>
<tr>
<td>100 (in PBS)</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>6.50-6.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.50-5.70</td>
<td></td>
</tr>
<tr>
<td>100 (in PBS)</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

into the PBS and agitated well. The concentration of Qß was then measured using the methods previously mentioned. This was used as the initial concentration of Qß in PBS. The temperature of the culture was maintained in an incubator and the concentration of Qß was measured at several time periods.

For measuring Qß by qRT-PCR, the Qß extraction solution was diluted $10^2$ times by PBS, also in the case of Qß inactivation in PBS. Each experiment was replicated twice or three times, except in cases where CaO and HCl were added.

**Estimation of the damage to E. coli**

In order to estimate the inactivation mechanism of E. coli, 3 types of media were used. According to their detection principles, the damage to E. coli can be assumed as shown in Table 4 (Kubo et al., 2005, 2006; Wang and Otaki, 2007; Mizozoe et al., 2010).

Using TSA (a non selective agar), the E. coli which can metabolize proteins (casein and soybean) and grow, can be detected. Therefore, when the E. coli growth cannot be detected on TSA, it is assumed that its nucleic acid and/or its metabolic function has been damaged. DESO (a selective agar) selects for E. coli that can grow by metabolizing lactose in the presence of desoxycholic acid. Gram-positive bacteria are unable to grow in the presence of desoxycholic acid because they lack an outer membrane and their growth is inhibited by its surface-active effects. Therefore, when E. coli cannot be detected on DESO, this indicates that its outer membrane and/or, its
nucleic acid and/or its metabolic function has been damaged. C-EC (a selective agar) selects for *E. coli* that can produce ß-glucuronidase (the enzyme involved in the metabolism of peptone, pyruvic acid and lactose). Therefore, when *E. coli* cannot be detected on C-EC, it is assumed that its enzyme activity and/or, its nucleic acid and/or its metabolic function has been damaged. By comparing the degree of inactivation on each medium, the damage to *E. coli* could be estimated.

**Estimation of the damage to Qß**

In this study, the purpose of using PCR is for detecting non-damaged RNA strands. However, the PCR target regions of Qß RNA are only 77 bases compared with the total 4215 bases (NCBI No.001890). If there is no damage to the PCR target region, but damage to other regions, Qß will still be detected by the PCR assays. Thus, detection by qRT-PCR would be different from that obtained with the plaque assay, even though Qß inactivation was only due to RNA damage. Therefore, we presented the reference ratio of qRT-PCR to plaque assay to account for RNA damage without capsid damage. In this study, UV inactivation was used to identify the ideal condition.

As UV treatment is known to damage nucleic acids, but not the capsid (Kaneko, 1996), UV treatment is a useful monitor of Qß inactivation. A 15 mL sample of Qß solution (10^3 PFU/mL in PBS) in a petri dish (diameter: 5.2 cm, height: 1.2 cm) was irradiated with a low pressure UV lamp (STANLEY infection lamp GL 20W, Toshiba Co., Japan) for 0 - 120 sec. During this experiment, the Qß solution was agitated with a magnetic stirrer. The concentrations of Qß were measured by plaque assay and by qRT-PCR at several different time periods.

**RESULTS AND DISCUSSION**

**Damage to *E. coli***

Fig. 2 shows the change in the concentration of *E. coli* in the sawdust, as determined by its growth on three different media.
Fig. 2 - Change in concentration of *E. coli* in the sawdust measured by three different media
(a) at 37°C and 50°C with 30% water content; (b) at 37°C and 50°C with 50% water content; (c) at 37°C with 50% water content with CaO; *N*: concentration of microorganisms at time *t*; *N₀*: concentration of microorganisms at time 0

According to previous studies (Nakagawa *et al.*. 2006, Otaki *et al.*., 2007), the inactivation of microorganisms followed a first order reaction, expressed as follows:

$$\ln \left( \frac{N}{N_0} \right) = -kt$$  \hspace{1cm} (1)

*N*: concentration of microorganisms at time *t*;

*N₀*: concentration of microorganisms at time 0;

*k*: inactivation rate constant;

*t*: retention time.
In order to compare these results, the inactivation rate constants were calculated using the above equation (1), and the data shown in Fig. 3. However, the plots at 4 hr and 8 hr in Fig. 2 (a), and at 8 hr and 24 hr in Fig. 2 (b) were excluded from calculating the inactivation rate constants, because their positions were apparently apart from the respective linear lines. Table 4 shows the relationship between the detection by three media and the estimated damage to \textit{E. coli}. The damages were estimated using both the differences of the inactivation rate constants by each medium in Fig. 3, and Table 5.

No significant differences were observed in the inactivation rate constants by any of the three media at 37°C with 30% and 50% water content, including that with high pH (CaO addition). Now, as previously described, the inactivation rate constants at 37°C with 30% and 50% water content were calculated with the exception of some plots. However, as shown in Fig. 2 (a) and (b), no significant differences were observed in these plots by any of the three media, too. Therefore, because \textit{E. coli} could not be detected by any of

![Fig. 3 - Inactivation rate constants by each medium under several temperature, water content and pH conditions](image)

**Table 5 - Estimated parts of \textit{E. coli} damaged according to the detection differences among the three media**

<table>
<thead>
<tr>
<th>Media</th>
<th>Estimated parts which were damaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA</td>
<td><img src="image" alt="image" /></td>
</tr>
<tr>
<td>DESO</td>
<td><img src="image" alt="image" /></td>
</tr>
<tr>
<td>C-EC</td>
<td><img src="image" alt="image" /></td>
</tr>
</tbody>
</table>

○ : detected  
× : not-detected
the three media, inactivation is mainly due to damage to nucleic acids and/or metabolic function as shown in Table 5. In other words, these conditions could lethally reduce *E. coli*. At 50°C with 30% and 50% water content, the increase of temperature rapidly caused damages to their metabolic functions (nucleic acids and/or metabolic, enzyme activity, membrane) because the inactivation rate constants by each medium were significantly high compared with that at 37°C with 30% and 50% water content. However, the inactivation rate constants by TSA were smaller than those by the other two media, which indicated that temperature increase caused a significant damage on enzyme activity and/or membrane function more than nucleic acids and/or metabolic activity. In short, high temperature conditions resulted primarily in damage to enzyme activity and/or membrane function.

**Damage to Qß**

Fig. 4 shows the changes in the concentration of Qß detected by the plaque assay and qRT-PCR. The inactivation rate constants calculated using equation (1) are shown in Fig. 5.

According to the plaque assay results, higher temperature and lower water content condition were effective in reducing infectivity of Qß. On the other hand, with qRT-PCR, when comparing results in sawdust to that in PBS (100% water content), water content less than 50% is effective in decreasing Qß RNA, but there was not much difference between 50% and 30% water content. At changing pH, the inactivation rate measured by qRT-PCR was faster for other cases. Moreover, in the case of alkaline pH (CaO addition), the inactivation rate measured by qRT-PCR was faster, even though the rate determined by the plaque assay was not much different.

![Fig. 4](image_url)

**Fig. 4** - Inactivation rates of Qß measured by plaque assay and qRT-PCR under several conditions (a) 37°C; (b) 50°C; (c) 37°C with CaO (pH10.01, 9.64) or HCl (pH4.81, 3.76)
Fig. 5 - Inactivation rate constants of Qβ measured by plaque assay and qRT-PCR under several temperature, water content and pH conditions. *95% confidential intervals from 3 independent experiments, **95% confidential intervals from 2 independent experiments.

Fig. 6 shows the UV inactivation of Qβ using plaque assay and qRT-PCR. With these results, it was calculated that the inactivation rates by plaque assay and qRT-PCR were 0.071 and 0.012 respectively. In order to use as the reference, the ratio between the inactivation rates by plaque assay and that by qRT-PCR was calculated as follows.

\[ R_{\text{plaque/qRT-PCR}} = \frac{\text{Inactivation rate by plaque assay}}{\text{Inactivation rate by qRT-PCR}} \] (2)

\( R_{\text{plaque/qRT-PCR}} \) for UV treated viruses was calculated at a value about 6. Therefore, when damage has occurred only to Qβ RNA, the \( R_{\text{plaque/qRT-PCR}} \) value of 6 can be used as a reference value. In other words, if the \( R_{\text{plaque/qRT-PCR}} \) is larger than 6, some damage to the capsid can also be assumed.

Fig. 7 shows the \( R_{\text{plaque/qRT-PCR}} \) values for viruses from sawdust and from PBS. Compared to the UV value, the \( R_{\text{plaque/qRT-PCR}} \) values for viruses treated at 37°C and 30% water content, 50°C, and at low pH were large. Therefore, capsid destruction was assumed to be rapid in these cases. In the other cases, the \( R_{\text{plaque/qRT-PCR}} \) values were similar to the UV value, indicating only RNA damage.

At 37°C, it was assumed that capsid was damaged when water content decreased under
Fig. 6 - Inactivation rates of Qβ by UV irradiation measured by plaque assay and qRT-PCR

In this study, the damage to Qβ was estimated by comparing the value of $R_{\text{plaque/qRT-PCR}}$ to the value obtained for the UV-treated reference case. The difference between the inactivation rate using the plaque assay and that using qRT-PCR was considered to be about 50%. At 50°C, it was also assumed that capsid was damaged even at high water content. High pH conditions were assumed to damage Qβ RNA, which may affect the ribose in the RNA structure. Ribose contains a hydroxyl base at carbon C2’, which makes it prone to hydrolysis under alkaline conditions. At low pH, capsid destruction was considered to be rapid due to the pH change.

In this study, the damage to Qβ was estimated by comparing the value of $R_{\text{plaque/qRT-PCR}}$ to the value obtained for the UV-treated reference case. The difference between the inactivation rate using the plaque assay and that using qRT-PCR was considered to be...
the detection probability among each method. The theoretical detection probability by
PCR could then be estimated for the case where Qβ was inactivated only by RNA. The
theoretical rate of damage occurring in the target region with damage occurring in
whole region was estimated using Poisson distribution and Binominal distribution. At
the same time, the lethal region on Qβ RNA was considered. The template activity of
Qβ RNA has been reported (Miranda et al., 1997; Schuppli et al., 1998) and more than
2405 bases out of a total of 4215 bases are not essential to the template Qβ RNA. The
theoretical $R_{\text{plaque/qRT-PCR}}$ value was estimated at about 24. The difference between this
value of 24 and the value of 6 (UV case) was considered to reflect the fact that there is a
greater region unassociated with the template (Klovins et al., 1998, 1999; Schuppli et
al., 1998) and the PCR target region is included in the lethal region.

**Effective conditions for reducing pathogen risks**

For bacteria, high temperature conditions rapidly reduced them lethally, but if the initial
concentration in the sawdust was high, the treatment must be of sufficient duration or
there will be a potential for viable pathogenic bacteria to persist. The low water content
condition lethally reduced them even under low temperature, but needed a prolonged
treatment period. Moreover, some additional energy may be needed to set the high
temperature and/or low water content condition. In this experiment, a small amount of
CaO, about 100 - 150 mg, was used to change the pH of 10 g of sawdust. Therefore,
from the handling viewpoint, CaO addition is an easy method to reduce them lethally
and rapidly.

For viruses, high temperature conditions and low water condition were considered to
result primarily in capsid damage. Virus capsids consist of proteins that may vary
among pathogenic viruses. Therefore, regarding viruses that show stronger tolerance, it
is considered that treatments which can damage RNA may be required. Hence, the best
treatment ensuring the rapid inactivation of both pathogenic bacteria and viruses
appeared to be high pH.

**CONCLUSIONS**

This study was focused on the damaged part or function of the pathogen, and
investigation was also done on whether some effective conditions could give fatal
damage to these pathogens or not.

For bacteria, it was assumed that high temperature apparently damaged the enzyme
activity and/or membrane structure, but the potential for growth persisted. Conditions
that combined low water content and high pH were lethal to bacteria.

For viruses, high temperatures or a combination of low temperature and low water
content was assumed to result in capsid damage. In order to ensure the rapid
inactivation of both pathogenic bacteria and viruses, it was considered that a high pH
condition resulting from CaO addition was effective.

**ACKNOWLEDGEMENT**

This study was supported by Grants-in-Aid for Scientific Research (S) project of JSPS
REFERENCES


