Research Paper

Inactivation kinetics of indicator microorganisms during urea treatment for sanitizing finished compost from composting toilet

Seyram K. Sossou, Mariam Sou/Dakoure, Yacouba Konate, Amadou H. Maiga and Naoyuki Funamizu

ABSTRACT

This study aimed at estimating the sanitizing effectiveness of urea treatment by studying the inactivation kinetics of selected indicator microorganisms. Finished composts from a composting toilet were inoculated with indicator microorganisms and subjected to different urea concentrations (0.5–2% w/w) and temperatures (22, 32 and 42 °C). The inactivation kinetics parameters were determined in relation to pH, ammonia content and temperature during treatment time. The results show that urea addition to compost enhanced inactivation of microorganisms. The decline in number of *E. coli* and *Enterococus* followed a linear reduction, while that of *Ascaris lumbricoides* eggs followed a linear reduction plus shoulder. The inactivation rate constants of all microorganisms tested were positively correlated to the increase of NH_3(aq) concentration and temperature. The relationship between the inactivation rate of microorganisms, ammonia through urea concentration and temperature were established. Therefore, the best decimal decay of *E. coli, Enterococus* and *A. lumbricoides* eggs occurred with 2% w/w urea concentration at 42 °C within 0.9, 1.1 and 1.4 days, respectively. *E. coli* was the most sensitive microorganism to urea treatment, while *Enterococcus* and *A. lumbricoides* eggs showed resistance, especially at lower temperatures. Urea treatment has proved to be an efficient option for safe reuse of compost from composting toilets.

Key words | composting toilet, finished compost, inactivation kinetics, indicator microorganism, urea treatment

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INTRODUCTION

On-site sanitation systems have gained much interest in recent years and are still the main solution for the majority of the world's population, mainly in lower- and middle-income countries (WHO/UNICEF 2015). However, excreta management remains a thorny issue in these countries where some part of this fecal matter is used as fertilizer without treatment (Ogunyoku *et al.* 2015). Indeed, excreta obtained from household toilets contain the major proportion of nutrients (Zeng *et al.* 2013) with a high risk of pathogen content, and have to be disinfected before reuse as fertilizer or soil conditioner.

Common technologies applied in on-site sanitation include the use of different types of latrines and dry/or composting toilets (Magri *et al.* 2013). The most common treatment for sanitizing human faeces is composting, where the main inactivation of pathogens is due to increased temperature (Sunar *et al.* 2014). During a composting process, it is assumed that pathogens in the faeces are

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inactivated for protection of human health (Sossou *et al.* 2014a). Indeed, to ensure inactivation in composting processes, temperatures of around 55–65 °C are needed to kill all types of pathogens within hours (Sunar *et al.* 2014). However, in most cases, the temperature in composting toilets in some rural areas does not get hot enough to destroy all pathogens (Sossou *et al.* 2014a). Therefore, the compost from a composting toilet is hygienically unsafe.

Storage of fecal material requires a treatment time exceeding a year, and even then the product cannot be considered as pathogen free. The practice of adding alkalining agents can ensure the safe reuse of compost. One of the most widely used nitrogen fertilizers in the world is urea. When applied to soil it is hydrolyzed and converted to ammonia and carbon dioxide by the enzyme urease. The same effect should occur when urea is added to compost. One advantage with ammonia-based sanitation is that the ammonia is not consumed during the treatment, but remains in the treated material and increases the fertilizer value as an excellent source of nitrogen for crop production (Ogunyoku *et al.* 2015).

Several studies have reported inactivation based on intrinsic ammonia or added ammonia amendments as urea to human faeces (Nordin *et al.* 2009), manure (Vinnerås 2007), fecal sludge (Fidjeland *et al.* 2013) and sewage sludge (Nordin *et al.* 2015). To our knowledge, the effectiveness of these treatments on finished compost has not yet been studied. The lack of information about urea treatment in hot sahelian climates indicates the necessity for further studies to provide measurable factors on microbial die-off. This study aimed at estimating the sanitizing effectiveness of urea addition on compost as an alkaline treatment.

METHODS

Compost collection

Finished composts used for the experiments were collected from a urine-diverting composting toilet prototype installed in a pilot household. This toilet was coupled with an aerobic composting reactor and produced a total compost of 8 kg for approximately 2 months. The matrix used as bulking agent was sawdust, and the conditions of producing the compost are similar to those described in our previous paper (Sossou *et al.* 2014a). Composts collected were sent to laboratory within 5 h and stored at 4 °C before setting up the experiment. According to our recent paper (Sossou *et al.* 2016), the physicochemical properties of the finished composts used are as follows: pH: 7.64 ± 0.05; free ammonium (mg/l): 190 ± 7.01; moisture content (%): 20 ± 0.91 and ash content (%): 12 ± 0.08.

Microbial suspension preparation

The bacteria strains, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212, used in this study were purchased from the American Type Culture Collection (ATCC, USA). For suspension preparation, each bacteria, *E. coli* and *E. faecalis*, was cultured in a growth medium Nutrient Broth (BD, Franklin Lakes, USA) and incubated in a shaking water bath at 37 °C overnight. The bacteria suspensions were used as a source of inoculums. The parasite eggs, *Ascaris lumbricoides* eggs, were extracted from human faeces collected from patients at a hospital in Ouagadougou (Burkina Faso). In brief, the faeces collected were washed through a series of sieves ranging from 425 to 38 μm. Eggs were finally collected on the 38 μm sieve and stored in a 4 °C refrigerator in reverse osmosis water and 10% formaldehyde to prevent mould growths. Upon use, a portion of the egg solution was collected as a source of inoculums.

Urea treatment

Compost was subsequently treated with urea (Rosier SA, Belgium) at different concentrations from 0% to 2% (w/w), where the 0% control represented closed storage of untreated compost. Urea additions of 0.5–2% (w/w) were made based on wet weight, and the moisture content of the compost was adjusted to 20% (w/w) with sterilized deionized water. Approximately 300 g of compost, with or without urea, was transferred to 500 ml plastic bottles. For the inoculation of compost samples, an adequate aliquot of *E. coli* and *E. faecalis* (0.3 mL about 10^6 CFU/mL), and *A. lumbricoides* eggs (1 mL, about 10^3 eggs) was added. The bottle was only partially closed with a lid, allowing some ventilation. Subjected to different urea concentrations 0.5–2%, compost treatments were performed at the same incubation temperature (32 °C). Subjected to the same urea concentration (2%), compost treatments were performed at different incubation temperatures

(22, 32 and 42 °C), representing the range of average ambient temperature in Burkina Faso during the year.

Measurements of pH and aqueous ammonia (NH_3)

The pH was determined with a Hanna Digital Compo Meter (HI991405, Hanna, UK) in a compost suspension in de-ionized water at the ratio of 1:5 (w:v) after 30 min shaking at room temperature. Total ammonium was measured with a spectrophotometer Thermo Aquamate (Thermo Electron Co., USA) using the indophenol blue method (Merck; Whitehouse Station, NJ). The concentration of aqueous ammonia (NH_3) was calculated from the measured total ammonium, pH and temperature.

Measurements of indicator microorganisms

For bacteria extraction, a 3% (w/v) beef extraction solution was used. In sterilized tubes, ten (10) g of compost sample was added to a 90 ml volume of extracted solution and agitated for 3 minutes to extract bacteria. After adequate dilution (4 to 7 fold) with phosphate buffer, each extract was inoculated by the double layer method in chromocult coliform agar ES (BD, Franklin Lakes, USA) for *E. coli* and Slanetz-Bartley agar (BD, Franklin Lakes, USA) for *Enterococcus* spp., including *E. faecalis* added. These inoculated media were incubated at 37 °C for 24 h for *E. coli* colonies count and at 37 °C for 48 h for *Enterococci* colonies count. The detection limits of bacteria were 10 CFU/g and the concentration of bacteria count was expressed in CFU/g of dry compost.

For parasite analysis, 10 g of compost sample was homogenized with 90 mL of 0.1% Tween 80 for 1 min using a blender and screened through 4 layers of folded wet gauze. The filtrate was collected in round bottomed flasks and allowed to settle for 3 hours. The number of *A. lumbricoides* eggs was determined by the concentration method followed by a direct count under optical microscope (B-350, OPTIKA, Italy), the method described by Sossou *et al.* (2014a). The viability of *A. lumbricoides* eggs was assessed both by morphological analysis for cellular structure integrity and by a staining exclusion dyeing method using Safranin O. The detection limits of *A. lumbricoides* eggs were one egg/g and the number of count was expressed in number of eggs/1,000 g of dry compost.

Data analysis

Log10 transformed microorganisms' counts were subjected to microbial inactivation rate kinetics by using the Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFiT) (Geeraerd *et al.* 2005). The best-fitting models were the log-linear regression (Equation (1)) for *E. coli* and *Enterococci*, and the log-linear regression plus shoulder (Equation (2)) for *A. lumbricoides* eggs.

$$N(t) = N(0).e^{-kmax.t} \quad (1)$$

$$N(t) = N(0).e^{-kmax.t}.\frac{e^{kmax.Sl}}{1+(e^{kmax.Sl}-1).e^{-kmax.t}} \quad (2)$$

where, N(t) and N(0) expressed in CFU or number of eggs/g of dry compost, are the concentration of microorganisms in the compost at time t and 0, respectively; t is the reaction time (h). The parameters used for the estimation by GInaFiT were Sl (shoulder length, i.e., the length of the lag phase) (h), which represents the degrees of freedom. The estimated maximum inactivation rate constant *kmax* (h^{-1}) and the decimal decay (T90) were calculated using the best fitting model in GInaFiT. The *kmax* values and standard deviation were calculated directly by the GInaFiT program.

Treatment means were compared with at least corresponding significant p value. These statistical analyses were performed using the StatView software version 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

pH and ammonia in the compost during urea treatment at different concentrations

The change of pH and ammonia concentration in the compost during urea treatment at 32 °C are presented in Figures 1 and 2, respectively. The pH in the untreated compost, 0% urea, was around 7.68, and urea addition of 0.5, 1, 1.5 and 2% increased the pH to 8.8, 9.2, 9.5 and 9.7, respectively. After each urea addition, from 0.5 to 2%, the value of pH increased by 0.82 to 1.22 units and there was no

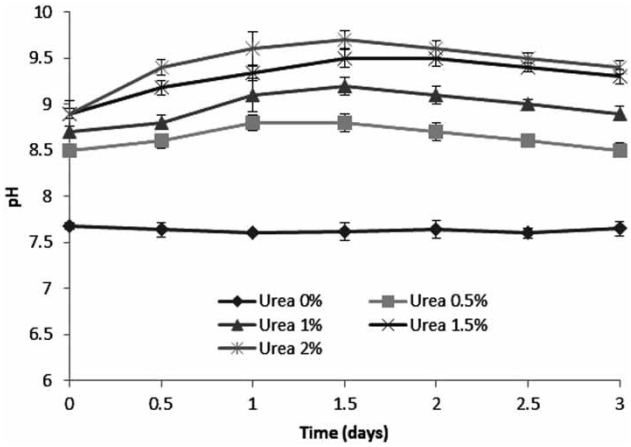

Figure 1 | Change of pH values in compost during urea treatment at 32 °C.

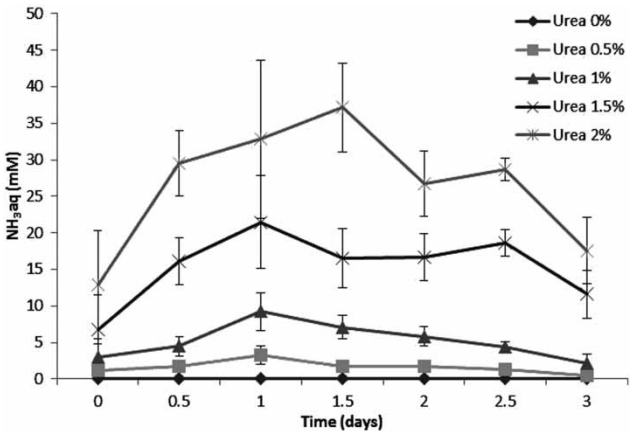

Figure 2 | Change of ammonia concentrations in compost during urea treatment at -32 °C.

significant variation ($p < 0.05$) during the treatment. However, the peak of pH changes ($\Delta pH = 1.2$ units) were obtained by applying the first urea dose of 0.5%. In the untreated compost, the NH_3 concentration remained very low (between 0.07–0.09 mM) throughout the 3 day study period. However, the NH_3 concentration in the urea-treated compost increased and then decreased during the treatment.

pH and ammonia in the compost during urea treatment at different temperatures

Subjected to the same urea treatment (2%), the pH in urea-treated compost at different temperatures 22, 32, 42 °C increased but there was no significant variation ($p < 0.05$) during the treatment. In addition, the different incubation temperatures did not result in a significant difference ($p < 0.05$) in the pH values after urea addition. However, the NH_3 concentrations in the urea-treated compost increased and then decreased during the treatment. The peak was higher ($p < 0.05$) at higher temperatures (32 and 42 °C) and remained fairly stable at lower temperature (22 °C).

Survival of indicator microorganisms in compost during urea treatment at different urea concentrations

The change in the number of *E. coli, Enterococci* and *A. lumbricoides* eggs during urea treatment at different concentrations is presented in Figures 3–5, respectively. The change of *E. coli* and *Enterococci* populations in urea-treated compost showed a linear curve of regression. The linear regression analysis indicated that there was a

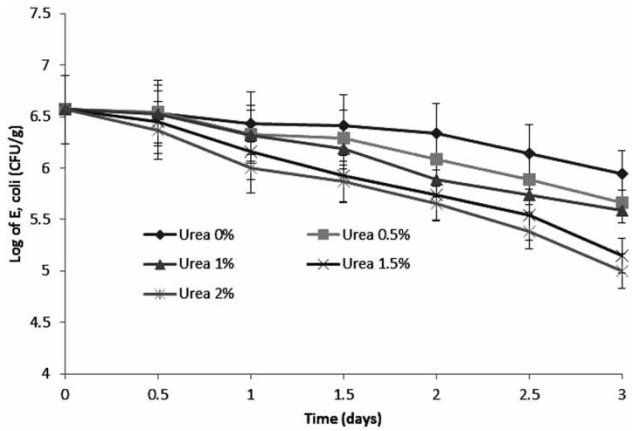

Figure 3 | Change of *E. coli* in compost during urea treatment at 32 °C.

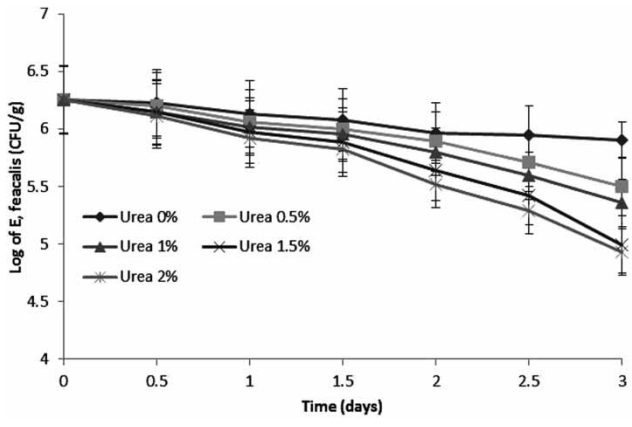

Figure 4 | Change of *Enterococci* in compost during urea treatment at 32 °C.

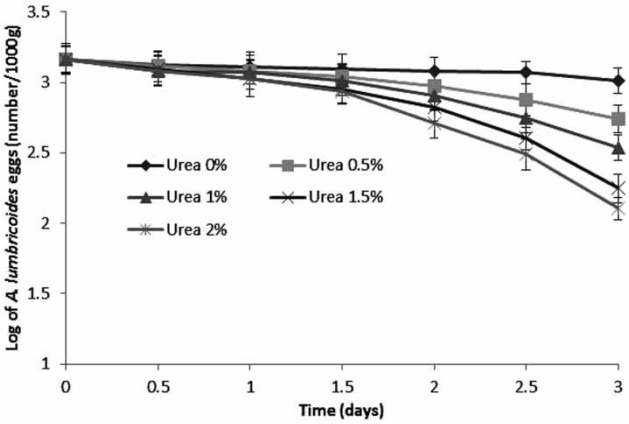

Figure 5 | Change of *A. lumbricoides* eggs in compost during urea treatment at 32 °C.

significant decay ($p < 0.001$) in the log number of *E. coli* and *Enterococci* during urea treatment in relation to incubation time. The inactivation curves of *A. lumbricoides* eggs showed two phases: a lag phase as an initial slight decrease followed by a rapid decrease corresponding to log-linear and shoulder decay. The non-linear regression analysis of *A. lumbricoides* eggs indicated that there was a significant decay ($p < 0.001$) in the log number during urea treatment in relation to incubation time.

Survival of indicator microorganisms in compost during urea treatment at different temperatures

The reduction of *E. coli*, *Enterococci* and *A. lumbricoides* eggs during urea treatment at different temperatures (22, 32 and 42 °C) varied. For all microorganisms tested, the log reduction during urea treatment was positively correlated with incubation temperatures.

Inactivation rate and decay of microorganisms in compost during urea treatment

The *kmax* and the T90 of indicator microorganisms during urea treatment are presented in Table 1. Subjected to different urea concentrations, the *kmax* values showed that the inactivation rate of microorganisms tested was fast in high urea-treated compost (1.5–2%) and slow in low urea-treated compost (0–1%). Subjected to the same urea treatment (2%), the *kmax* values showed that the inactivation rate of microorganisms tested was fast in urea-treated compost incubated at high temperatures (32 and 42 °C) and slow in urea-treated compost incubated at low temperature (22 °C). For all microorganisms tested, the T90 occurred rapidly in high urea-treated compost (1.5–2%) at high temperatures (32 and 42 °C) while it was prolonged in low urea-treated compost (0–1%) at low temperature (22 °C).

DISCUSSION

The addition of urea to the compost increased the pH, which may be due to enzymatic conversion of the added urea to ammonium (NH_4^+) by urease secreted by indigenous bacteria present in the compost (Ogunyoku *et al.* 2015). During the urea treatment, the pH increased and tended to stabilize; this is an important prerequisite for the formation of both NH_3 and CO_3^{2-}. However, a slight decrease was observed during the treatment. This variation of pH is likely a main factor behind the observed

Table 1 | Maximum *kmax* and T90 of regression lines for microorganisms in compost during urea treatment

		kmax ± **RMSE (day^{-1})**			**T90 (days)**		
Urea treatments (%)	**Temperatures (°C)**	***E. coli***	***Enterococci***	***A. lumbricoides* eggs**	***E. coli***	***Enterococci***	***A. lumbricoides* eggs**
0	32	$0.13^a \pm 0.07$	$0.1^a \pm 0.02$	$0.05^a \pm 0.01$	7.6	8.8	19.2
0.5		$0.21^a \pm 0.07$	$0.18^a \pm 0.05$	$0.10^a \pm 0.01$	4.6	5.4	9.7
1		$0.26^a \pm 0.06$	$0.24^a \pm 0.06$	$0.14^a \pm 0.02$	3.7	4.1	6.9
1.5		$0.39^b \pm 0.06$	$0.30^b \pm 0.11$	$0.19^a \pm 0.03$	2.5	3.3	5.4
2	22	$0.14^a \pm 0.07$	$0.09^a \pm 0.03$	$0.04^a \pm 0.01$	7.1	11.1	25
	32	$0.48^b \pm 0.06$	$0.35^b \pm 0.09$	$0.22^b \pm 0.03$	2.1	2.8	4.6
	42	$1.08^b \pm 0.06^b$	$0.89^b \pm 0.08^b$	$0.71^b \pm 0.03$	0.9	1.1	1.4

*Values followed by different alphabetical letter ('a', 'b') are significantly different for $p < 0.05$.

differences in ammonia (NH_3) concentration between the different urea concentrations added. Total ammonium will be mostly in NH_4^+ form, whereas the increase in pH dramatically increases the conversion of NH_4^+ to NH_3 (Ogunyoku *et al.* 2015). In this study, the experiment was performed in 3 days because this time corresponded to the peak dissociation of urea to ammonia in fecal material (Nordin *et al.* 2009).

Subjected to the same urea treatment (2%), the pH value increased with temperatures. However, the incubation temperatures did not result in a significant difference in pH values in the compost. The increase in NH_3 concentration with temperatures might be due to a temperature mediated shift in the equilibrium between NH_4^+ and NH_3 towards NH_3 (Fidjeland *et al.* 2015). The decrease in the level of NH_3 over time at 32–42 °C might be due to volatilization of NH_3, as well as the partial closure of the plastic bottles used. The slight increase in NH_3 levels over time at 22 °C might be due to a slow degradation of organic nitrogen during incubation (Zeng *et al.* 2013). No significant difference in the pH of compost at different temperatures (22, 32 and 42 °C) was observed for the same urea treatment (2%), but concentrations of NH_3 varied widely. Thus, small variations in pH can result in highly different NH_3 concentrations, especially for a combination of high temperature and slight alkalinity (Nordin *et al.* 2009).

For all microorganisms tested, the log reduction of microorganisms increased with ammonia concentration and temperature. These results concurred with those obtained by Nordin *et al.* (2009) with human faeces, by Vinnerås (2007) with manure, and by Fidjeland *et al.* (2013) with sludge. The ammonia is microbicidal for microorganisms and responsible for their destruction during the urea treatment. Previous studies have shown the effects of alkaline pH and ammonia on pathogen destruction in fecal material (Nordin *et al.* 2009; Katakam *et al.* 2014; Ogunyoku *et al.* 2015). Though the exact mechanism of action is not known, ammonia has been shown to have a toxic effect on many microorganisms (Anderson *et al.* 2015). For bacteria, the effects of alkaline pH are mainly the inactivation of the protein membrane or the inactivation of the nucleic acid strand (Sossou *et al.* 2014b). For parasite eggs, the primary inactivation mechanism could be protein capsid denaturation (Katakam *et al.* 2014).

The number of microorganisms detected in urea-treated compost declined more rapidly in compost treated with a high urea concentration (1.5–2%) and at high temperatures (32 and 42 °C) than with a low urea concentration (0–1%) and at low temperature (22 °C). Furthermore, the inactivation rate of microorganisms increased with ammonia concentration. Though there was a large increase in ammonia concentrations in urea-treated compost, the levels were apparently too low to inactivate the microorganisms at low temperature (22 °C). This indicates that temperature was the main contributing factor to microbial destruction, whereas the addition of urea helped increase NH_3 concentrations. Therefore, the inactivation rate of microorganisms was positively correlated to the ammonia concentration and temperature (Anderson *et al.* 2015). The present results confirm earlier studies that the increase of pH, NH_3 concentration and temperature may increase the inactivation of microorganisms (Nordin *et al.* 2009; Katakam *et al.* 2014).

The die-off time T90 of all microorganisms in urea-treated compost was longer at low urea concentration (0–1%) and low temperature (22 °C) than that at higher concentration (1.5–2%) and high temperatures (32 and 42 °C). For all microorganisms tested, the *kmax* was reduced while T90 increased. The *kmax* and T90 values obtained in this study with urea treatment of compost are similar to those obtained by Nordin *et al.* (2009) with faeces, by Fidjeland *et al.* (2013) with fecal sludge and by Nordin *et al.* (2015) with sewage sludge.

Comparing the log reduction, *kmax* and T90 values, *E. coli* was much more sensitive to urea treatment than *Enterococci* and *A. lumbricoides* eggs. Using fecal *Enterococci* as an indicator of bacterial survival in ammonia treatment will lead to an overestimation of the risk associated with the reuse of compost. Fidjeland *et al.* (2013) also reported that the inactivation of *Enterococci* was somewhat slower. In the same way, *A. lumbricoides* eggs were the most persistent of the organisms tested and showed little reduction in urea-treated compost. The resistance of *A. lumbricoides* eggs might be due to the structural characteristics of the egg shells. Indeed, the *A. lumbricoides* egg shell contains some surrounding layers with a special arrangement of chemical compounds inside the membrane layers (Fidjeland *et al.* 2015). This may make it more tolerant and resistant to other unfavorable conditions compared to bacteria (Katakam *et al.* 2014).

CONCLUSIONS

The conditions created in this study with the urea addition to compost and the temperature control around 42 °C have destroyed, with an elevated rate of success, *E. coli*, Enterococcus and *A. lumbricoides* eggs. These results proved the viability of generating proper conditions to destroy pathogenic microorganisms in finished compost through a fairly simple and low cost treatment suitable to regions with a hot climate.

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