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Microbial Risk Assessment of Mature Compost from Human Excreta, Cattle Manure, Organic Waste, and Biochar

Katharina A. Werner [1], Daniela Castro-Herrera [2], Fantaw Yimer [3], Menfese Tadesse [3], Dong-Gill Kim [3], Katharina Prost [2], Nicolas Brüggemann [2] and Elisabeth Grohmann [1,*]

[1] Faculty of Life Sciences and Technology, Department of Microbiology, Berliner Hochschule für Technik, 13347 Berlin, Germany
[2] Institute of Bio- and Geosciences—Agrosphere (IBG-3), Forschungszentrum Jülich, 52425 Jülich, Germany
[3] Wondo Genet College of Forestry and Natural Resources, Hawassa University, Shashemene P.O. Box 128, Ethiopia
* Correspondence: egrohmann@bht-berlin.de; Tel.: +49-304504-3942

Abstract: Lack of sanitation is the underlying cause of many diarrheal infections and associated deaths. Improving sanitation through the set-up of ecological sanitation dry toilets, followed by the thermophilic composting of human excreta, could offer a solution. In addition, treating the excreta via thermophilic composting allows us to recycle the nutrients to be used as fertilizer for agriculture. However, for this purpose, the compost should be free of pathogens. We conducted a thermophilic composting trial over 204 to 256 days with human excreta, along with vegetable scraps and teff straw, with and without biochar. A sawdust–cattle manure mixture with the same supplements served as a control treatment. To evaluate the hygienic quality of the mature compost, the bacterial indicators *Escherichia coli* and *Salmonella* were assessed using the cultivation-based most probable number method. In addition, *Ascaris lumbricoides* eggs were quantified through light microscopy. The amount of detected *E. coli* was below the thresholds of German and European regulations for organic fertilizer. *Salmonella* and *Ascaris* eggs were not detected. No significant differences between the treatments were observed. Thus, the composting process was efficient in decreasing the number of potential human pathogens. The mature compost fulfilled the legal regulations on organic fertilizer regarding potential human pathogens.

Keywords: thermophilic composting; ecological sanitation; dry toilets; pathogens; *E. coli*; *Salmonella*; MPN; *Ascaris lumbricoides* eggs; Mini-FLOTAC; biochar

Citation: Werner, K.A.; Castro-Herrera, D.; Yimer, F.; Tadesse, M.; Kim, D.-G.; Prost, K.; Brüggemann, N.; Grohmann, E. Microbial Risk Assessment of Mature Compost from Human Excreta, Cattle Manure, Organic Waste, and Biochar. *Sustainability* **2023**, *15*, 4624. https://doi.org/10.3390/su15054624

Academic Editor: Konstantinos Ar. Kormas

Received: 1 February 2023
Revised: 1 March 2023
Accepted: 3 March 2023
Published: 5 March 2023

1. Introduction

Worldwide, 2.3 billion people do not have access to basic sanitation. With less than 50% of its population using basic sanitation, sub-Saharan Africa is one of the most affected regions in the world [1,2]. Lack of sanitation was attributed to be the underlying cause of an estimated 430,000 deaths of diarrheal infections in low- and middle-income countries in 2016, with more than half of them in Africa [3]. Ecological sanitation (EcoSan) aims to establish a closed-loop system between on-site sanitation and agriculture by recycling the nutrients contained in excreta [4]. Dry toilets, which are easy to establish, allow the collection of feces and urine for their subsequent use as fertilizer, thereby improving soil fertility and saving water [4–6]. However, various human pathogens are present, particularly in feces [7–10]. Pre-treatment of the excreta is necessary to prevent the entry of pathogens into the field and the food chain. *Campylobacter*, *E. coli*, *Salmonella*, *Providencia*, and *Listeria* spp. are examples for human pathogens that can be transmitted through, e.g., animal manures used for fertilization [11]. Enteric pathogens such as *E. coli* and *Salmonella*, moreover, can persist in soil and colonize plants, thus highlighting the importance of preventing entry routes to the food production sector [12–14]. Aside from bacterial pathogens, infection with viruses and parasites, such as the protozoan *Cryptosporidium* spp. (via oocyst stages) or the

tapeworm *Taenia* (via eggs), can occur through contaminated raw vegetables [15–17]. One option to sanitize human and animal dung is thermophilic composting, a controlled, aerobic, and exothermic process, transforming organic material into humus [18–20]. Biochar as an amendment to the compost may improve the process; for example, through increased aeration, an extended thermophilic phase, or higher temperatures [21,22].

In China, the use of human excreta (so-called "night soil") as a fertilizer has a long tradition [23] and is still practiced at household scale in some rural areas [24]. Similarly, latrine wastes, usually composted, are applied to soils in rural Vietnam [25]. In some African countries such as Senegal and Uganda, fecal sludge is used for fertilization, although other uses such as energy production are more prevalent [26]. In the US and the UK, over 50% and 80% of the biosolids from wastewater treatment plants are recycled for fertilization of soils, respectively [27,28]. The EU regulates the application of sewage sludge to soils in its sewage sludge directive of 1986 [29]. An average of 50% is used on agricultural soils with strong variations between the member states that follow their country-specific quality criteria [27,30]. However, this directive does not cover fertilizers derived from human excreta or human excreta that are not collected in wastewater treatment plants [28]. Globally, an estimated 11% of the produced wastewater is reused [31].

Regulations on organic fertilizer require quantification of different indicator organisms to ensure that the end-product is safe to use. Among the widely used indicators are *E. coli* as a measure for fecal contamination, and *Salmonella* as an indicator for pathogenic bacteria [7,32–35]. In addition, the eggs of the nematode *Ascaris* are used to assess the risk of more heat-resistant organisms [7]. Apart from its role as an indicator organism, the common roundworm *Ascaris lumbricoides* is the most prevalent helminth in humans worldwide, especially in developing countries as a consequence of insufficient sanitation [10]. Two recently published systematical reviews dealt with the prevalence of human intestinal helminth infections in Ethiopia during the last decade. Both studies found that around one third of the population suffered from helminth infections. With 11 and 18%, respectively, *A. lumbricoides* was identified as the predominant species [36,37].

We aimed to establish a closed-loop system between ecological sanitation and climate-smart agriculture via (biochar-)compost used as organic fertilizer. Thermophilic composting of dry toilet contents along with other organic wastes, with and without biochar, was applied as a treatment to sanitize the fecal material. In the present study, we assessed the effectiveness of this system regarding the elimination of human pathogenic microorganisms. Bacterial indicators *E. coli* and *Salmonella* spp. were quantified in the mature compost according to German regulations for organic fertilizers [32]. The potential parasitic burden was assessed through microscopy of *Ascaris* eggs.

2. Materials and Methods

2.1. Composting Trial and Sampling

The composting trial, properties of compost substrates, and physical–chemical properties of the compost, including nutrient contents (C, N, P, K, Ca, Mg, and micronutrients) during the composting process, were described in detail by Castro-Herrera et al. [38]. In brief, "humanure", i.e., feces and urine together with toilet paper and sawdust, was collected in dry toilets following the approach described in Jenkins [39] (Figure 1). Thermophilic composting was conducted at Wondo Genet College of Forestry and Natural Resources in southern Ethiopia between April and November 2019. The trial comprised four treatments with four replicates each: (i) humanure, vegetable scraps and teff (*Eragrostis tef* (Zuccagni) Trotter) straw (HM); (ii) the same ingredients as in (i) plus biochar (HM+BC); (iii) cattle manure–sawdust mixture, vegetable scraps, and teff straw (CM); and (iv) the same ingredients as in (iii) plus biochar (CM+BC). Cattle manure served as a control for the humanure treatments, and treatments without biochar served as controls for the biochar treatments. Thermophilic composting was conducted in boxes of 1.5 × 1.5 × 1.4 m (width × depth × height), insulated with teff straw (Figure 1).

Figure 1. An installed dry toilet (**A**); a toilet bucket filled with collected materials (**B**); and a compost box after experimental set-up with dimensions (**C**) (Photos: K. Prost and K. A. Werner).

The four composting replicates of each treatment were combined and distributed into two boxes (Figure 1) in September 2019. Three months after combining the replicates, samples were taken for pathogen analysis. The compost was mixed before sampling. Samples of 100 g each were taken randomly from different positions in the compost and stored at 4 °C until analysis. In total, 24 samples from the four treatments with six replicates for each treatment were analyzed. From compost set-up to sampling, the total composting duration was 204–256 days for the samples analyzed here. Since replicates started at intervals of 15–18 days, composting times differed for the four replicates. A scheme describing the experimental set-up is shown in Table 1.

2.2. Most Probable Number Method for the Detection of E. coli and Salmonella spp.

For the assessment of compost hygiene, the indicator organisms *Salmonella* and *E. coli* were detected by means of the most probable number technique (MPN) according to the protocol published by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety [32].

Compost samples were suspended 1:10 (*w*/*v*) in 0.9% sodium chloride solution and shaken at 150 rpm at 4 °C overnight. Specific cultivation was conducted from triplicates of a tenfold dilution series (dilution stages 10^{-2}–10^{-9}). *E. coli* and *Salmonella* analyses were conducted in parallel from the same dilutions.

For the detection of *E. coli*, 1 mL of each dilution was transferred to 9 mL MacConkey broth (Carl Roth GmbH, Karlsruhe, Germany) and vortexed briefly. After incubation at 37 °C for 24 h ± 1 h, samples were checked for a color change to yellow, indicating lactose fermentation. These samples were streaked on MacConkey agar (Carl Roth GmbH) to confirm *E. coli* growth.

Table 1. Set-up of the composting experiment with the four treatments HM, HM+BC, CM, and CM+BC.

Replicates *	Treatments	Start	Combining Replicates #	Sampling #	Composting Duration #	
Block 1	HM HM+BC CM CM+BC	02/04/2019	15/09/2019	14/12/2019	Block 1	256 d
Block 2	HM HM+BC CM CM+BC	19/04/2019			Block 2	239 d
Block 3	HM HM+BC CM CM+BC	08/05/2019			Block 3	220 d
Block 4	HM HM+BC CM CM+BC	24/05/2019			Block 4	204 d

HM = Human Manure (Humanure) plus sawdust, straw, and vegetable waste; CM = Cattle Manure plus sawdust, straw, and vegetable waste; HM+BC = Human Manure (Humanure) plus sawdust, straw, vegetable waste, and biochar; CM+BC = Human Manure (Humanure) plus sawdust, straw, vegetable waste, and biochar. * For each treatment four replicates were set up in a randomized complete block design at intervals of 15–18 days. The replicates are indicated as "Block 1", "Block 2", "Block 3", and "Block 4" for the replicates set up at the different dates, respectively. Each Block therefore consisted of all four treatments [38]. # The four replicates of each treatment were combined on 15 September 2019 [38]. On 14 December 2019, sampling for pathogen analysis was conducted and samples were stored at 4 °C until analysis. Due to the different starting days for the replicates, the composting duration ranged between 204–256 days.

For the detection of *Salmonella*, 1 mL of each dilution was pipetted to 9 mL buffered peptone water (Carl Roth GmbH), vortexed briefly, and incubated at 37 °C for 24 h ± 1 h. Afterwards, 100 µL were transferred into 10 mL Rappaport-Vassiliadis broth (Carl Roth GmbH), vortexed briefly, and incubated at 42 °C for 24 h ± 1 h. Brilliant green-lactose–sucrose agar (BPLS, Carl Roth GmbH) and xylose–lysine–deoxycholate agar (XLD, Carl Roth GmbH) were used for identification. One loopful of the liquid culture was streaked to each of the agars and incubated at 37 °C for 24 h ± 1 h. Putative positive colonies were transferred to standard-I-agar (15 g/L peptone ex casein, 6 g/L NaCl, 3 g/L yeast extract, 1 g/L D (+) Glucose, 12 g/L agar-agar). After incubation at 37 °C overnight, slide agglutination tests with the monoclonal test serum "Anti-Salmonella A-67 + Vi, omnivalent" (Sifin Diagnostics GmbH, Berlin, Germany) were conducted according to the manufacturer's instructions.

For both organisms, positive (+) and negative (-) replicates for each dilution step were documented. The highest three dilutions with positive results were used for the generation of an index number (number of positive results per dilution). Statistical estimates of MPNs of colony forming units (CFU) in the original samples were taken from the tables of De Man (1983) by means of the index numbers [40].

2.3. Microscopy Count of Ascaris Lumbricoides Eggs

Ascaris eggs were counted by means of the Mini-FLOTAC counting chamber [41] (Figure 2A) and a Primo Star light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The experimental procedure of the group of Samson-Himmelstjerna (FU Berlin) was used [42]: five grams of compost were mixed and stirred well with 45 mL of saturated sodium chloride solution. The mixture was filtered over a 0.25 mm mesh to retain the compost particles. The filtrate was filled into the chamber and incubated for 10 min for the eggs to float at the surface. To generate a layer thin enough for microscopy, the chamber

was turned to separate the surface layer containing the eggs from the remaining liquid. Two chambers of 1 mL volume each were counted for each sample. An example for *Ascaris* eggs is given in Figure 2, with panel B showing the non-larvated stage usually found in compost and panel C showing the larvated egg.

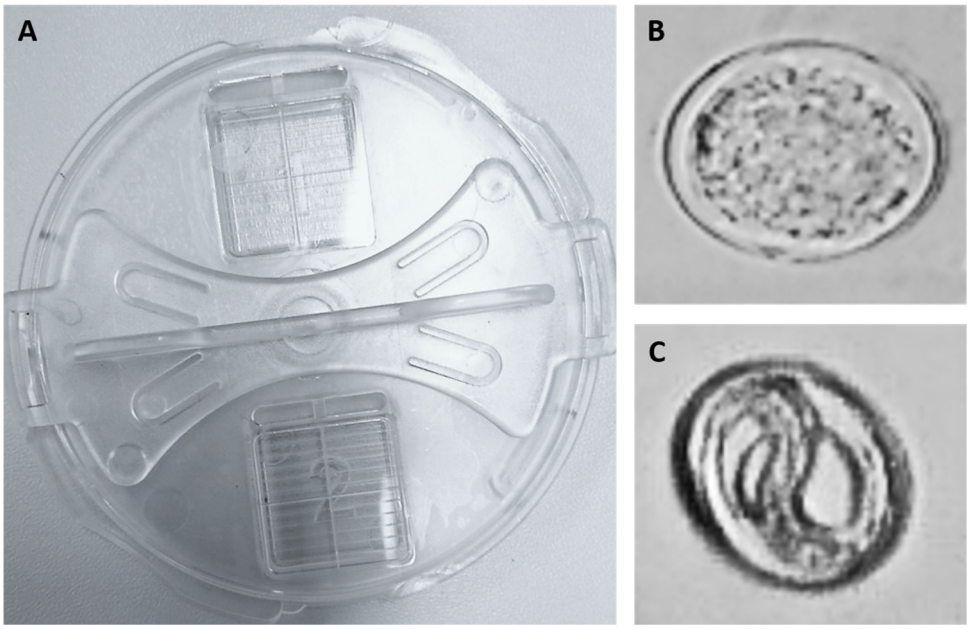

Figure 2. Mini-FLOTAC counting chamber (**A**); light microscopy of *Ascaris suum* egg before larvation, isolated from female worms (**B**); larvated *A. suum* egg after development in 0.1 M H_2SO_4 at room temperature for 28 days (**C**) (Photos: Khaliel El-Said).

2.4. Statistical Analysis

Statistical significance of indicator MPNs was determined using two-tailed, paired Student's *t*-test using Microsoft Excel (Microsoft Office Home and Student 2016, Version 2301). Significance is indicated by *p*-values below 0.05. Significance was tested to compare the treatments with and without biochar, both for humanure and cattle manure treatments separately (HM versus HM+BC, and CM versus CM+BC) and for all samples (HM/CM versus HM+BC/CM+BC). Moreover, humanure and cattle manure treatments were compared, both separately (HM versus CM, and HM+BC versus CM+BC) and for the complete set of samples (HM/HM+BC versus CM/CM+BC).

3. Results

3.1. Detection of Bacterial Indicators E. coli and Salmonella spp.

The mature compost was investigated for the presence of *E. coli* and *Salmonella* as indicators for fecal contamination and human pathogenic bacteria, respectively, using the cultivation-based MPN technique. *E. coli* was present in low numbers in treatments HM+BC, CM, and CM+BC, with slightly higher numbers in the cattle manure treatments (CM and CM+BC) which had served as our control treatments. Two-tailed student's *t*-tests did not reveal significant differences between HM and CM treatments or with and without BC. *Salmonella* was not detected in any sample. Detailed results are given in Table 2.

Table 2. Cultivation-based detection of *E. coli* and *Salmonella* spp. in the mature compost by means of the MPN method [40]. Results are depicted as colony-forming units per gram compost (CFU g^{-1}). Numbers below 30 mark the detection limit of the method. Six samples were analyzed for each of the four treatments (humanure (HM); cattle manure (CM); without and with biochar (HM+BC and CM+BC), respectively. Mean values for each treatment are given below the individual counts.

Treatment	Replicate *	*E. coli* [CFU g^{-1}]	*Salmonella* [CFU g^{-1}]
HM	HM_1	<30	<30
	HM_2	<30	<30
	HM_3	<30	<30
	HM_4	<30	<30
	HM_5	<30	<30
	HM_6	<30	<30
HM	Mean	<30	<30
HM+BC	HM+BC_1	<30	<30
	HM+BC_2	<30	<30
	HM+BC_3	<30	<30
	HM+BC_4	<30	<30
	HM+BC_5	<30	<30
	HM+BC_6	36	<30
HM+BC	Mean	<31	<30
CM	CM_1	61	<30
	CM_2	<30	<30
	CM_3	<30	<30
	CM_4	<30	<30
	CM_5	92	<30
	CM_6	<30	<30
CM	Mean	<45.5	<30
CM+BC	CM+BC_1	<30	<30
	CM+BC_2	<30	<30
	CM+BC_3	<30	<30
	CM+BC_4	<30	<30
	CM+BC_5	150	<30
	CM+BC_6	210	<30
CM+BC	Mean	<80	<30

* Internal label indicating the treatments (HM, HM+BC, CM, CM+BC) and sequence number of the replicates (1–6).

3.2. *Ascaris Lumbricoides* Eggs Microscopy Count

Light microscopy by means of the Mini-FLOTAC counting chamber was used to detect *Ascaris lumbricoides* eggs as indicators of parasitic contamination in the mature compost. No eggs were detected in any of the samples; hence, the number of eggs per gram of compost was zero in all replicates of all treatments (Table 3).

Table 3. *Ascaris* egg count by means of the Mini-FLOTAC counting chamber in the mature compost. Six samples were analyzed for each of the four treatments (humanure (HM); cattle manure (CM); without and with biochar (HM+BC and CM+BC)), respectively. Mean values for each treatment are given below the individual counts.

Treatment	Replicate *	*Ascaris* Egg Count/g Compost
HM	HM_1	0
	HM_2	0
	HM_3	0
	HM_4	0
	HM_5	0
	HM_6	0

Table 3. *Cont.*

Treatment	Replicate *	*Ascaris* Egg Count/g Compost
HM	Mean	0
HM+BC	HM+BC_1	0
	HM+BC_2	0
	HM+BC_3	0
	HM+BC_4	0
	HM+BC_5	0
	HM+BC_6	0
HM+BC	Mean	0
CM	CM_1	0
	CM_2	0
	CM_3	0
	CM_4	0
	CM_5	0
	CM_6	0
CM	Mean	0
CM+BC	CM+BC_1	0
	CM+BC_2	0
	CM+BC_3	0
	CM+BC_4	0
	CM+BC_5	0
	CM+BC_6	0
CM+BC	Mean	0

* Internal label indicating the treatments (HM, HM+BC, CM, CM+BC) and sequence number of the replicates (1–6).

4. Discussion

In the present study, pathogen analyses were conducted to assess the hygienic state of mature thermophilic compost from dry toilet contents and other organic wastes with and without biochar compared to cattle manure compost with and without biochar. These analyses complement an extensive study of physical and chemical parameters on the same composting trial [38].

In this study, *E. coli* as a typical indicator organism for fecal contamination revealed low MPNs in treatments HM+BC, CM, and CM+BC, respectively, that are far below the threshold of 1×10^3 CFU g^{-1} of regulations by the German Environment Agency and European Commission for sludge used as fertilizer [7,32]. *E. coli* MPN were below the detection limit of the method in the HM treatment. *Salmonella* as an indicator for human pathogens was not detected in any sample, which matches German and European thresholds for treated sludge used for land application (not detectable in 50 g fresh weight) [7,32–35]. Bacteria associated with humans or mammals are typically not adapted to elevated temperatures for longer times [43,44]. Peak temperature values of 62–66 °C were reached during the composting process, and mean temperatures above 60 °C for five to eight consecutive days [38]. For organic waste composting, temperatures of 55 °C for two weeks, 60 °C for six days, or 65 °C for three days are required to ensure the proper elimination of pathogens [35].

Significant decreases of *E. coli* MPNs were described earlier for sewage sludge composting [45] and an eco-sanitation static composting set-up using latrine wastes and sugarcane husks in Haiti [46]. In line with our results, the addition of biochar did not result in clear effects on the abundance of *E. coli* in a recent study on compost of dry toilet contents with and without biochar [47]. However, opposite results have also been published for the composting of feces, sawdust, rice husk, and rice husk charcoal [48]. The authors found the charcoal-treatment to be most effective regarding *E. coli* removal (not detectable after five weeks of composting). Considering that the samples in the present study were taken only from the mature compost after several months of composting, the time effects of

biochar on *E. coli* removal cannot be assessed. This leaves the possibility open for beneficial effects of biochar treatment regarding the sanitization of compost.

For the removal of *Salmonella*, a minimum peak temperature of 60 °C and moisture contents of 60–65% were found to be optimal [49]. Moisture contents in the present study were higher in the beginning (79–81%), but decreased to 60–63% in the mature compost [38]. The optimal combination was not obtained, since temperatures were lower at the time in which the moisture contents were in the optimum range (i.e., at the end of composting, when the compost had already cooled to ambient temperature). However, temperature alone is an important parameter affecting the survival of *Salmonella* [50,51]. It can thus be concluded that composting conditions were adequate for the removal of *Salmonella*. In a study by Chung et al. [52] on composting of chicken manure, sawdust, and rice husk without and with biochar, the treatments with 5 and 10% biochar showed slightly faster removal of *Salmonella*, as compared with treatments without and with 3% biochar. From 21 days until the end of composting after 50 days, *Salmonella* was not detected in any treatment [52].

Ascaris eggs are a typical indicator of parasitic contamination due to their high resistance to environmental conditions, such as increased temperature, desiccation, and alkaline or acidic conditions [53]. Eggs were not detected in any sample, thereby complying with the World Health Organization (WHO) guidelines of <1 egg g^{-1} total solids for feces and fecal sludge [9]. *Ascaris* eggs can be expected in the fecal material, since *Ascaris lumbricoides* is the most prevalent human helminth in Ethiopia [36,37]. Prevalence of *Ascaris* eggs in stool samples was also described for the region where the present study was conducted [54,55]. Moreover, pretests on the toilet contents used for the present study had revealed the presence of *Ascaris* eggs (K. A. Werner, unpublished data). We attribute the lack of eggs to the long duration of the composting trial, making it more likely that dead eggs were degraded. Decreasing *Ascaris* egg counts with increasing composting duration was previously described for dewatered sewage sludge compost [56,57]. The decline of viable *Ascaris* eggs is dependent on different parameters. Increasing ammonia concentrations, increased pH, and elevated temperatures promote egg inactivation in sewage sludge [58,59]. However, temperature proved to be the most important parameter [58–60]. Hence, in the present study, the absence of *Ascaris* eggs was probably mainly due to the high temperatures reached during composting. The elimination of viable *Ascaris* eggs through thermophilic composting of sewage sludge [61,62] and latrine wastes [46] was described before and is in line with our results.

In conclusion, the present study evaluated the hygienic state of mature compost from human excreta and cattle manure together with organic waste and teff straw, with and without biochar, through the detection of microbiological indicator organisms. *E. coli* MPNs were below the threshold for organic fertilizer without any significant differences between the two treatments. *Salmonella* and *Ascaris lumbricoides* eggs were not detected. The compost, therefore, fulfilled German and European regulations on human indicator pathogens for organic fertilizer. This suggests that thermophilic composting can be an efficient and hygienically safe treatment for human excreta; thus, it is a feasible option for producing organic fertilizer from ecological sanitation.

Author Contributions: Conceptualization, K.A.W., D.C.-H., K.P., N.B. and E.G.; methodology, K.A.W. and E.G.; validation, K.A.W. and E.G.; formal analysis, K.A.W.; investigation, K.A.W.; resources, E.G. and K.P.; writing—original draft preparation, K.A.W.; writing—review and editing, K.A.W., D.C.-H., F.Y., D.-G.K., K.P., N.B. and E.G.; visualization, K.A.W.; supervision, E.G., K.P. and N.B.; project administration, E.G., F.Y., M.T., D.-G.K., K.P. and N.B.; funding acquisition, E.G., D.-G.K., K.P. and N.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by BMBF (Federal Ministry of Education and Research), grant number 01DG18005, to E.G. (ClimEtSan-Pathogene, being part of the ClimEtSan-Project, funded by BMBF grant 01DG17010 and the German academic exchange service, DAAD, grant 57354010). KAW thanks the Agriculture and Environment Foundation and Berliner Hochschule für Technik (BHT) for the Ph.D. fellowships.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Doktor Elias, Tsegaye Tabi, Kajela Daniel, and Edao Osu for their support in setting up and maintaining the composting trials. Thanks to Khaliel El-Said for providing photos of *Ascaris* eggs and the Mini-FLOTAC chamber.

Conflicts of Interest: The authors declare no conflict of interest.

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