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Time-Temperature Model for Bacterial and Parasitic Annihilation from Cow Dung and Human Faecal Sludge: A Forthcoming Bio-Fertilizer

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Abstract

The present study investigated the optimum time and temperature for inactivation of bacteria and parasites in cow dung and pit faecal sludge, a forthcoming fertilizer.

Samples were collected from different areas of Bangladesh and were examined through modified centrifugal flotation and conventional culture techniques to isolate parasites and bacteria respectively. A cow dung sample from Gopalganj and a pit sample from Dohar that were found to be the most contaminated among the samples tested were heated to annihilate the pathogens present there. After 30 min of exposure at 60°C, all bacteria lost their ability to grow on culture media except *enterococci*.

Among the parasites found in the pit sample, *Entamoeba histolytica* was the least heat resistant, which was killed at 60°C within 30 min followed by *Ancylostoma duodenale* larva, *Strongyloides stercoralis* larva, *Trichuris trichiura*, *Ancylostoma duodenale* eggs and *Strongyloides stercoralis* egg. *Ascaris lumbricoides* and *Hymenolepis nana* were the most resistant to heat, inactivated at 75°C within 15 min. In cow dung, *Paramphistomum* was the most resistant, became inactivated at 65°C within 60 min whereas *Haemonchus* at 65°C within 30 min.

The study findings showed the best time dependent temperature to deactivate the pathogens present in faecal sludge in Bangladesh context.

Keywords: Pathogen; Inactivation; Cow dung; Faecal sludge

Introduction

Studies have been conducted on agronomic effectiveness of humanure as an important basis of nutrients for crop fertilization in countries like Mexico, Germany, USA, Sweden, Denmark and Zimbabwe, China, Korea, and Japan [1,2]. Nearly 200 million farmers in China, India, Vietnam, sub-Saharan Africa and Latin America harvest grains and vegetables from fields using untreated human waste as fertilizer [3]. Contamination of non-ruminant food sources and vegetables associated with outbreaks were found to have been grown in soil layered with faecal manure [4,5]. Faecal sludge may contain disease causing microorganisms even after keeping it undisturbed in the pit for months. Farmers of developing countries using human faeces as fertilizer due to availability and lower costs are at greatest risk [6].

The occurrence, survival and infectivity of pathogens in human sewage and animal wastes disposed on land had been extensively reviewed [7]. After bacteria, protozoa are the most important microorganisms to consider in faecal sludge and cow dung treatment. Along with coliform bacteria and helminths other indicators of faecal contamination that have been used in pathogen die-off studies include *Clostridium perfringens, Bacteroides*, enterococci, and *Bifidobacterium* spp. [8].

The most probable number of coliforms per gram of dried sludge must be less than 1000, and the parasitic contamination should be less than one viable egg of helminths in four grams of dried sludge and less than one egg per liter of effluent [9]. The application of lime in composting is recommended in some countries like USA, France and Brazil. However, the efficiency of the stabilization processes depends of the operational quality and of the pathogen characteristics present in the sewage sludge [10]. Helminth eggs are the most difficult biological parasites to be inactivated in wastewater and sludge. The eggs remain viable for 1-2 months in crops and for many months in soil, fresh water, and sewage. They may remain viable for several years in faeces, night soil and sludge [11]. Because of their viability after using chlorine, UV-light or ozone they are considered highly resistant biological structures. To inactivate helminth eggs it is recommended either to raise the temperature (above 40°C), to reduce moisture (below 5%) or to maintain both of these conditions for an extended period of time [12]. Not all treatment processes are efficient enough to inactivate the eggs contained in sludge in developing countries, reliably or affordably [13]. Therefore the present study was conducted to find out the optimum time-temperature for inactivation of bacteria and parasites in cow dung and human faecal sludge.

Materials and Methods

Sample collection and processing

The current investigation was a laboratory based inactivation of parasites and bacteria aiming to produce pathogen-free bio-fertilizer from night soil/ faecal sludge and cow dung. A series of time-temperature framework was developed to assess the optimum temperature and time required to kill a particular bacterium or parasite. A total of 40 cow-dung samples were collected from Dhaka (Dohar and Keranigonj), Mymensingh and Gopalganj and 40 pit faecal sludge samples were collected from Dhaka (Dohar, Hazaribagh and Keranigonj) and Gopalganj, 10 from each site. The collected samples were examined for bacteria (total coliforms, faecal coliforms, *Escherichia coli* and enterococci) and parasites using drop plate and modified centrifugal floatation techniques respectively following the standard procedures [14,15]. Two highly contaminated samples from cow dung and pit soil were heated in a water bath at 50°C to 80°C at different times to inactivate pathogens.

The number of ova, larvae and cysts retrieved was expressed in parasite per gram of dry solids. The percentage of total solids was determined by drying 10 gm of two selected samples in an oven until no change in weight is observed. The weight of the sample after drying was recorded.

Isolation and identification of parasites

To concentrate the parasitic stages from faecal sludge and cow dung, one gram of the collected sample was suspended into 5 ml sterile normal saline and homogenized using a vortex machine. The large particulates were removed by filtering through a strainer (160 µm pore size). The filtrate was then centrifuged for 3.5 min at 1800 rpm. The supernatant was discarded leaving a small amount of fluid just above the sediment. Five ml salt ($ZnSO_4$) solution (S.G-1.20) was added and the sediment was re-suspended using a vortex machine. Homogenized sample was centrifuged for 1.5 min at 1500 rpm. The flotation procedure yielded a surface layer that contains parasite eggs, larvae and cysts. One ml of surface layer was transferred into a microcentrifuge tube and a drop of eosin methylene blue was added and homogenized to check the viability. The tube was then kept undisturbed for two min. After two min the sample was homogenized again and examined under microscope. Each sample was placed longitudinally on a smooth microscope slide and the identification of parasites (eggs, larvae and cysts) was performed with the aid of a 40X objective from an optical microscope using identification keys based on morphology following the standard procedures described by Cheesbrough [14].

Isolation and identification of bacteria

To isolate *E. coli*, 100 µl of serially diluted samples (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were inoculated on mTEC agar (Difco, MD, USA) plates using drop plate technique [15]. Plates were incubated at 37°C for 2 hours, and then at 44.5°C for 18–24 hours. After overnight incubation, purple colored colonies were counted as *E. coli* [16]. Similarly for enterococci, samples were dropped on Slanetz and Bartley Agar (SBA) plates [17]. Plates were incubated at 37°C for 48 hours. Dark brown colored colonies were selected and suspected as enterococci and for further confirmation suspected colonies were sub cultured on enterococci agar plate and incubated at 44°C for 2 hours. Colonies with black hue were confirmed and enumerated as enterococci [16]. For isolation of faecal coliforms and total coliforms, m-FC agar plates were used and incubated at 44°C for 22-24 hours and at 37°C for 22-24 hours respectively [18].

Inactivation of bacterium and parasites

From the two highly contaminated samples, 10 gm from each sample was taken into two separate 4 OZ bottles. Temperature was increased by 5°C starting from 50°C and rising up to 80°C. Three bottles, each containing 10g of particular sample were kept in water bath at each temperature point. All three bottles were removed subsequently after exposing to a fixed temperature for 15 min, 30 min and 60 min and observed under microscope after processing. The concept of time-temperature based inactivation of bacteria and parasites in this study was adopted from the previous studies [19,20].

Data analysis

All the data were systematically recorded which were analyzed using Statistical Package for the Social Sciences (SPSS) software for Windows (Version 20.0). The relationship between time and temperature for inactivation of bacteria and parasites was studied using Pearson Correlation Coefficient. The uncertainties of estimates were assessed using p values.

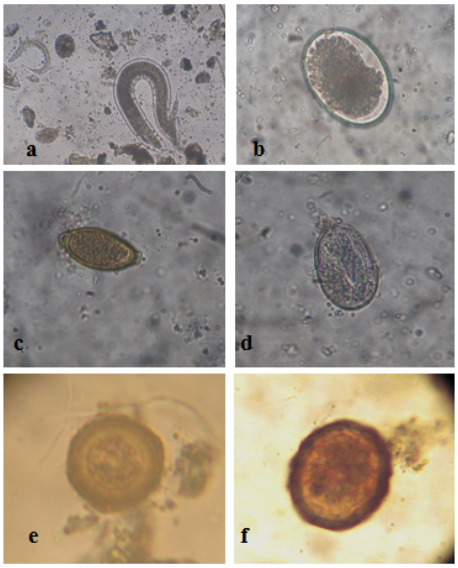

Figure 1: Microscopic images of different parasites observed at 40X magnification. (a) *A. duodenale* larva, (b) *A. duodenale* egg, (c) *T. trichiura* egg, (d) *S. stercoralis* egg, (e) *H. nana* egg, (f) *A .lumbricoides* egg.

Results and Discussions

It is the first laboratory based experiment which was carried out to establish a model approach of killing or inactivating pathogens (bacteria and parasites) through an optimum time and temperature from cow dung and human pit, usual objects used in agriculture as fertilizer in Bangladesh. A total of seven different parasite species were found from pit soil sample including one species of protozoa, one species of cestode and five species of nematode. From cow dung

samples, a total of five different parasite species were found including three nematodes, one trematode and one protozoan. Microscopic images of some of the identified parasites are shown in Figure 1. A cow dung sample from Gopalganj and a pit sample from Dohar were found to be the most contaminated that were heated to annihilate the studied bacteria and parasites found.

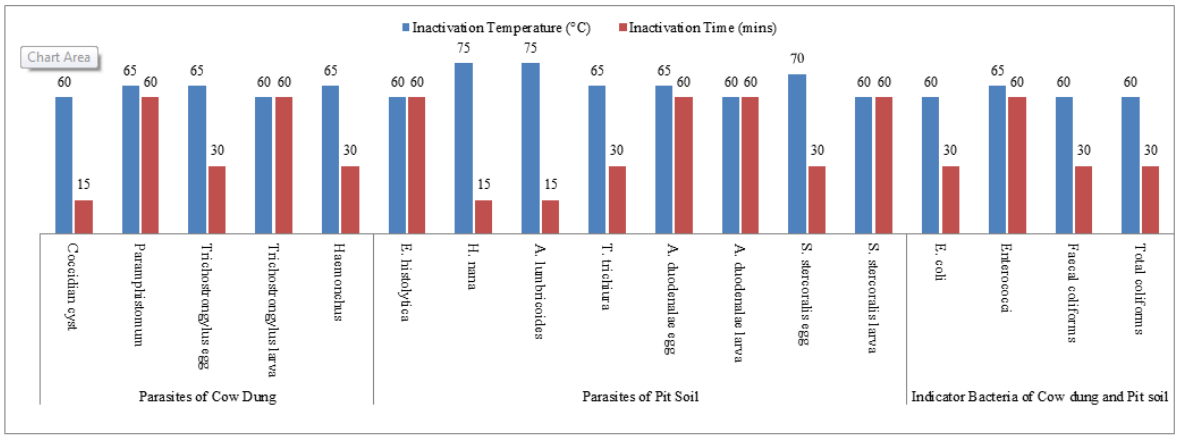

Figure 2: Time-temperature relationship for inactivation of bacteria and parasites. X axis represents the different parasites and bacteria in cow dung and pit soil and Y axis represents temperature and time for inactivation of different parasites and bacteria.

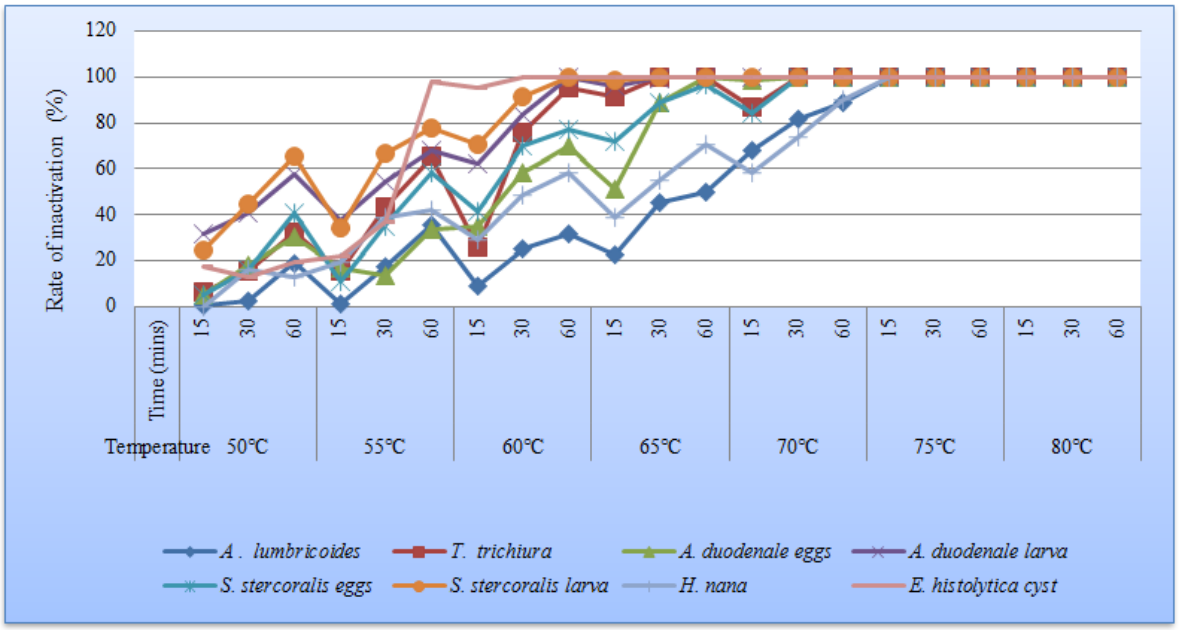

Figure 3: The rates of inactivation of different parasites, at different time and temperature, in human pit soil.

Both in cow dung and pit soil, enterococci were killed at 65°C after 60 min of heat treatment, whereas other bacteria were killed at 60°C after 30 min of exposure. In case of cow dung, the parasite *Paramphistomum* survived longer (65°C, 60 min) and coccidian cysts survived lesser (60°C, 15 min). On the other hand, *A. lumbricoides* and *H. nana* from pit soil became nonviable/inactivated at 75°C after 15 min of heat treatment, whereas *E. histolytica*, *A. duodenalae* larva and *S. stercoralis* larva were inactivated at 60°C after treating for 60 min (Figure 2). The rates of annihilation of parasites in human pit soil and cow dung varied species to species. In human pit soil, *A. lumbricoides* and *H. nana* were 100% killed at 75°C for 15 mins, followed by *S. stercoralis* eggs at 70°C for 30 mins, *A. duodenalae* eggs at 65°C for 60 mins, *E. histolytica* cysts, *A. duodenalae* larva and *S. stercoralis* larva at 60°C for 60 mins (Figure 3). In case of cow dung, *Paramphistomum* were 100% killed at 65°C for 60 mins followed by *Trichostrongylus* egg at 65°C for 30 mins, *Haemonchus* at 65°C for 30 mins, *Trichostrongylus* larva at 60°C for 60 mins and coccidian cysts at 60°C for 15 minutes (Figure 4).

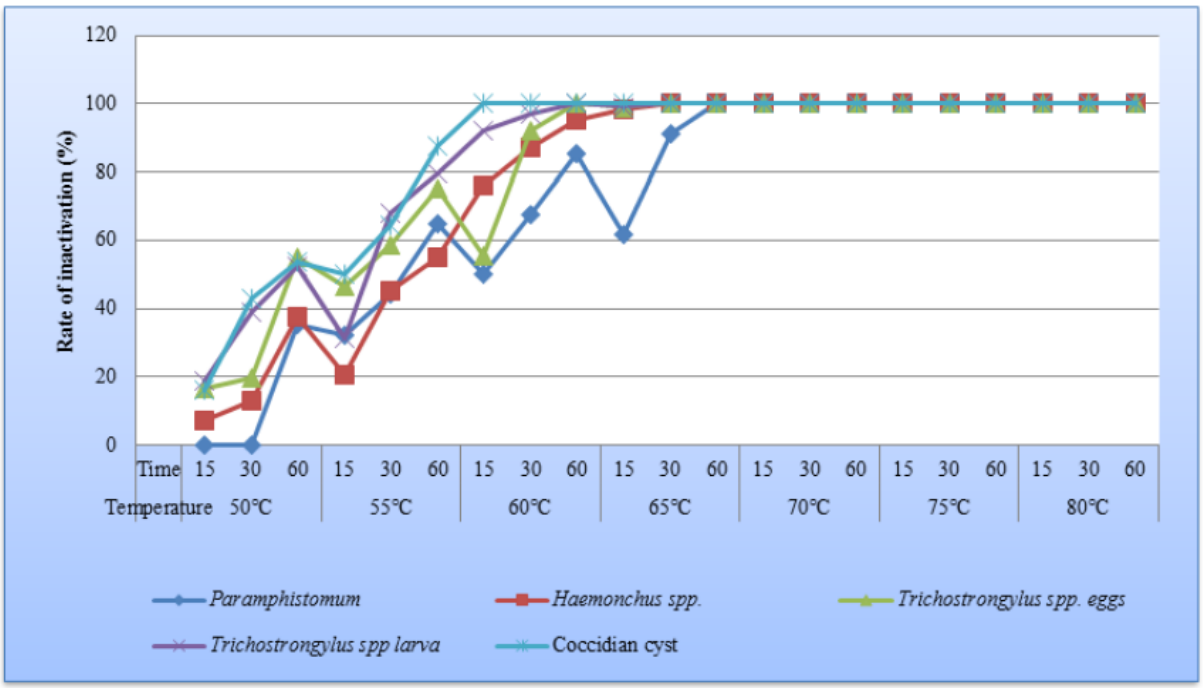

Figure 4: The rates of inactivation of different parasites, at different time and temperature, in cow dung.

Name of organisms	Temperature		Time	
	Correlation coefficient (r)	P value	Correlation coefficient (r)	P value
Enterococci	-.560**	.008	-.331	.143
E. coli	-.517*	.016	-.199	.387
Faecal coliforms	-.451*	.040	-.352	.118
Total colidorms	-.420	.058	-.325	.150
Paramphistomum	-.834**	.000	-.265	.246
Haemonchus	-.830**	.000	-.151	.514
Trichostrongylus	-.799**	.000	-.238	.299
Coccidian cyst	-.740**	.000	-.174	.450
A. lumbricoides	-.937**	.000	-.183	.428
T. trichiura	-.827**	.000	-.273	.231
A. duodenale	-.832**	.000	-.233	.309
H. nana	-.942**	.000	-.220	.339
S. stercoralis	-.781**	.000	-.270	.236
E. histolytica	-.742**	.000	-.152	.512

**Correlation is significant at the 0.01 level (2-tailed).

*Correlation is significant at the 0.05 level (2-tailed).

Table 1: Time temperature correlation in inactivation of bacteria and parasites.

The time-temperature correlations for bacteria and parasites were found negative (Table 1). With the increased time and temperature the number of bacteria and parasites decreased gradually but at a critical level (100% inactivation temperature) it was decreased dramatically. The critical temperatures were different for different agents. *Paramphistomum* from cow dung, and *A. lumbricoides* and *H. nana* were the most resistant ones.

There are several processes to make the sludge become pathogen free, but almost all of them use different chemicals like lime (CaO), soda lime (NaOH), ammonia (NH_3) and so on [21,22]. Those chemicals are either acidic or basic, so after treatment the pH changes and become useless whereas the present study was conducted based on heat treatment, a safer treatment than others. Heat treated fertilizer adds natural nutrients to soil, increases soil organic matter, improves soil structure and water holding capacity, reduces soil crusting problems and erosion from wind and water as well as slow and consistent release of nutrients. Claire Turner (2002) recommended that keeping temperatures in excess of 55°C for 2 hours are required for inactivation of *Escherichia coli* 11943 [23]. *Enterococcus faecalis* were the most heat resistant of the bacteria studied by Spinks et al. [20] followed by the pathogenic *Escherichia coli* O157:H7 and the non-pathogenic *E. coli* O3:H6. Previous study suggested that the temperature range from 55°C to 65°C is critical for effective elimination of enteric/pathogenic bacteria components. Thermal inactivation of working suspension of *Enterococcus faecium* was performed between temperature ranges 60°C to 65°C that supports current study [24].

The thermal death time for spiked *A. lumbricoides* ova was reported to be 60 min when a suspension of 1.7×10^5 viable ova per ml was held at 60°C or 30 min at 65°C [25]. To make the compost pathogen free it was required to treat at greater than 55°C for at least 3 days [19]. It was reported that *Ascaris lumbricoides* eggs, *Taenia saginata*, *Necator americanus* (hookworm) and *Entamoeba histolytica* cysts were killed within 1 hour at temperature over 50°C, within a few min at 55°C, within 50 min at 45°C and within a few min at 45°C respectively [26] which is quite different from the present findings because, in the present study faecal sludge were heated directly, whereas the above studies used spiked parasites. It was estimated that *Eimeria oocysts* were morphologically unaffected after 50 days at 35°C, 20°C, and 4°C and after 24 h at 53°C [27]. A previous study observed the relationship between temperature and treatment time on the treatment effect [28]. A longer treatment time of three hours was required for pasteurization of sewage sludge at 55°C to reduce infectivity of *Taenia saginata* eggs by 98.6%. The present study provided the data about inactivation of nonspore forming bacteria and parasites in faecal sludge and cow dung aiming to use these as bio-fertilizer or disposal in a secured way.

Finally, 75°C was found lethal for all the pathogens studied. Within 15 min at 75°C all the parasites and bacteria were found to be inactivated. Cow dung and humanure is a valuable resource suitable for agricultural purposes and has been recycled for such purposes by large segments of the world's human population for thousands of years. However, they contain the potential for harboring human pathogens, thereby can contribute to the spread of disease when improperly managed. Thermal processing produces no waste, no pollutants and no toxic by-products and don't require any chemicals, so that the pH remains same along with no stress on our ecosystems. After achieving an optimum temperature to inactive all the pathogens, the manure is thereby converted into a hygienically safe and suitable for soil applications for the purpose of human food production. During heat treatment *Ascaris* and *Hymenolepis* were found to be the most resistant and for the future studies they can be used as indicator organisms.

Conclusion

To date in Bangladesh, no standard method exists for measuring helminth ova inactivation temperature of waste made bio-fertilizer, wastewater sludge or faecal excreta. To obtain safe pathogen concentrations, the Environmental Protection Agency (EPA) has established a set of effective, pre-approved treatment options. Studies have shown that thermophilic conditions can reduce pathogens effectively while mesophilic conditions alone cannot. This study has carried out research to find the best time dependent temperature to deactivate the pathogens present in faecal sludge in Bangladesh context. This has revealed an option to design pre-treatment or sanitization unit in large biogas or scale organic fertilizer production factories. Besides, the present study provided the information about prevalence of parasite in different areas of Bangladesh which should be carried out in other areas to find out level of risk and socio-economic status.

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References

1. Mnkeni P, Austin L, Haneklaus S, Hera C, Rietz R, et al. (2008) Effectiveness of human manure from ecological sanitation toilets as a source of nutrients for cabbage. Proc 15th Int Symp Int Sci Centre for Fertilisers: Fertiliser and Fertilization for Sustainability in Agriculture: The First World meets the Third World-Challenges for the Future.
2. Steineck S, Stintzing R, Rodhe L, Elmquist H, Jakobsson C (1999) Plant nutrients in human urine and food refuse. DIAS Report Plant Production (Denmark).
3. Eichenseher T (2010) Human waste used by 200 million farmers, study says.
4. Khanum H, Ahmed S, Uddin M, Rahman A, Dey R, et al. (2008) Prevalence of intestinal parasites and anaemia among the slum male children in Dhaka city. J Biol Sc.
5. Waters JR, Sharp JC, Dev VJ (1994) Infection caused by Escherichia coli O157: H7 in Alberta, Canada, and in Scotland: a five-year review, 1987-1991. Clin Infect Dis 19: 834-843.
6. Pell AN (1997) Manure and microbes: public and animal health problem? J Dairy Sci 80: 2673-2681.
7. Strauch D (1996) Occurrence of microorganisms pathogenic for man and animals in source separated biowaste and compost importance, control, limits and epidemiology. The science of composting 1: 224-232.
8. Feachem R, Bradley D, Garelick H, Mara D (1983) Sanitation and Disease: Health Aspects of Wastewater and Excreta Management. World Bank studies in water supply and sanitation 3.
9. WHO (1989) Health guidelines for the use of wastewater in agriculture and aquaculture: report of a WHO scientific group [meeting held in Geneva from 18 to 23 November 1987].
10. Paulino RC, Castro EA, Thomaz-Soccol V (2001) Helminth eggs and protozoan cysts in sludge obtained by anaerobic digestion process. Rev Soc Bras Med Trop 34: 421-428.

11. Koné D, Cofie O, Zurbrügg C, Gallizzi K, Moser D, et al. (2007) Helminth eggs inactivation efficiency by faecal sludge dewatering and co-composting in tropical climates. Water Res 41: 4397-4402.
12. Jiménez Cisneros BE (2009) Helminth ova control in wastewater and sludge for agricultural reuse. Encyclopaedia of Biological, Physiological and Health Sciences, Water and Health 2: 429-449.
13. Jiménez B, Wang L (2006) Sludge Treatment and Management. Chapter 10 in Municipal Wastewater Management in Developing Countries: Principles and Engineering. Ujang Z. and Henze M. Editors, 237-292 pp. IWA Publishing, London, UK.
14. Cheesbrough M (2006) District laboratory practice in tropical countries. Cambridge university press.
15. Hoben H, Somasegaran P (1982) Comparison of the pour, spread, and drop plate methods for enumeration of Rhizobium spp. in inoculants made from presterilized peat. Appl Environ Microbiol 44: 1246-1247.
16. Myers D, Stoeckel D, Bushon R, Francy D, Brady A (2007) Fecal indicator bacteria (ver. 2.0): US Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7, section 7.
17. Slanetz LW, Bartley CH (1957) Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. J Bacteriol 74: 591-595.
18. Britton L, Greeson P (1989) Methods for collection and analysis of aquatic biological and microbiological samples: US Geological Survey Techniques of Water-Resources Investigations, book 5, chapter A4.
19. Wichuk KM, McCartney D (2007) A review of the effectiveness of current time-temperature regulations on pathogen inactivation during composting. Journal of Environmental Engineering and Science 6: 573-586.
20. Spinks AT, Dunstan R, Harrison T, Coombes P, Kuczera G (2006) Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Res 40: 1326-1332.
21. Nordin A, Nyberg K, Vinnerås B (2009) Inactivation of Ascaris eggs in source-separated urine and feces by ammonia at ambient temperatures. Appl Environ Microbiol 75: 662-667.
22. Yilmaz V, Icemer GT (2014) Sludge Characteristic and Pathogen Inactivation of Two Different Wastewater Treatment Plants in Antalya. The International Journal of Engineering and Science 3: 63-67.
23. Turner C (2002) The thermal inactivation of E. coli in straw and pig manure. Bioresour Technol 84: 57-61.
24. Spelina V, Schlemmerova L, Landfeld A, Kyhos K, Mericka P, et al. (2007) Thermal inactivation of Enterococcus faecium. Czech J Food Sci 25: 283-290.
25. Wiley BB, Westerberg SC (1969) Survival of human pathogens in composted sewage. Appl Microbiol 18: 994-1001.
26. Gotaas HB (1956) Composting; sanitary disposal and reclamation of organic wastes. Monogr Ser World Health Organ 31: 1-205.
27. Olsen JE, Nansen P (1987) Inactivation of some parasites by anaerobic digestion of cattle slurry. Biological wastes 22: 107-114.
28. L'Hermite P (2013) Processing and Use of Organic Sludge and Liquid Agricultural Wastes: Proceedings of the Fourth International Symposium held in Rome, Italy, 8-11 October 1985: Springer Science & Business Media.