Fate of *Ascaris* at various pH, temperature and moisture levels

Jenna Senecal, Annika Nordin and Björn Vinnerås

ABSTRACT

Soil-transmitted helminths (STH) are intestinal worms that infect 24% of the world's population. Stopping the spread of STH is difficult, as the eggs are resilient (can withstand high pH) and persistent (can remain viable in soils for several years). To ensure that new sanitation systems can inactivate STH, a better understanding of their resilience is required. This study assessed the inactivation of *Ascaris* eggs under various conditions, in terms of moisture content (MC) (<20 to >90%), temperature (20–50 °C) and pH (7–12.5). The results highlight that the exposure of *Ascaris* eggs to elevated pH (10.5–12.5) at temperatures ≤27.5 °C for >70 days had no effect on egg viability. Compounding effects of alkaline pH (≥10.5) or decreasing MC (<20%) was observed at 35 °C, with pH having more of an effect than decreasing MC. To accelerate the inactivation of STH, an increase in the treatment temperature is more effective than pH increase. Alkaline pH alone did not inactivate the eggs but can enhance the effect of ammonia, which is likely to be present in organic wastes.

Key words | disease transmission, helminths, nutrient recycling, sanitisation, STH

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INTRODUCTION

Soil-transmitted helminths (STH) are intestinal worms that infect 24% of the world's population (WHO 2018). The death rate from STH (*Ascaris lumbricoides*, *Trichuris trichiura*, hookworm) may be low, but they have severe health impacts such as abdominopelvic pain, symptomatic infection, wasting (low weight for height) and reduced cognitive development (Pawlowski 1982; Pullan *et al.* 2014; Strunz *et al.* 2014). People become infected by inadvertently ingesting infectious eggs attached to a piece of food and/or a particle of soil (Feachem *et al.* 1983; Asaolu & Ofoezie 2018; Jourdan *et al.* 2018). The specifics of the life cycle vary with the different STH species, but for all STH, the adult worms come to reside in either the small or large intestine, where fertilised female worms produce eggs that exit the human body with the faeces (Jourdan *et al.* 2018). The prevalence of STH is higher in tropical and subtropical regions and in areas where there is insufficient access to clean water and sanitation (Feachem *et al.* 1983; Strunz *et al.* 2014). Globally, excreta from over 4.4 billion people are not treated before entering the environment (WHO & UNICEF 2017). This lack of sanitation enables STH transmission, particularly as these worms are very prolific. For example, female *Ascaris* can produce >200,000 eggs per day and infected people can excrete eggs over years, while the eggs of STH can remain persistent in soils for years (Pawlowski 1982; Corrales *et al.* 2006; Echazú *et al.* 2015).

When assessing the effectiveness of a sanitisation treatment, *Ascaris* spp. eggs are often used as a conservative model, because they are one of the most hardy human enteric pathogens (Feachem *et al.* 1983; Jiménez 2006; Pecson *et al.* 2007; Maya *et al.* 2010). *Ascaris* eggs are difficult to inactivate chemically, as they are well protected by a four-layered shell. This comprises an outer uterine layer of acid polysaccharides and proteins, a vitelline layer of

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lipoproteins, a chitinous layer of chitin and protein and an inner lipid layer of proteins (25%) and lipids (75%) (Clarke & Perry 1988; Papajova *et al.* 2008). The lipid layer is thought to be the main permeability barrier, while the chitinous layer is the thickest and provides structural strength (Wharton 1980). This protective shell enables *Ascaris* eggs to be (i) resilient, i.e. able to withstand harsh treatment conditions (Eriksen *et al.* 1995; Gaasenbeek & Borgsteede 1998; Pecson *et al.* 2007; Papajova *et al.* 2008; Lalander *et al.* 2013; Naidoo *et al.* 2016) and (ii) persistent, i.e. remain viable in the soil for several years, even in anhydrous and anaerobic environments (Wharton 1980; Pawlowski 1982; Feachem *et al.* 1983; Clarke & Perry 1988).

Ascaris eggs are most persistent during dormancy (pre-embryo development) when the eggshell is only permeable to organic solvents, lipid-permeable vapours (such as ammonia (Weiner & Hamm 2007)) and respiratory gases (Barrett 1976; Clarke & Perry 1980). The protective eggshell needs to be permeable to respiratory gases to access oxygen (Wharton 1980). As a consequence of this, the eggshell is also permeable to water vapour (kinetic diameter 265 pm), which is a smaller molecule than oxygen gas (kinetic diameter 346 pm) (Hinton 1969). The shell restricts water loss while still allowing adequate oxygen gas diffusion (Hinton 1969; Wharton 1980). However, the ability of the shell to restrict water loss decreases exponentially with increasing temperature (Wharton 1979).

Inactivation of *Ascaris* at elevated temperatures of $\geq$50 °C (Capizzi-Banas *et al.* 2004; Naidoo & Foutch 2018) and/or exposure to ammonia (NH_3) has been relatively well studied (Ghiglietti *et al.* 1997; Mendez *et al.* 2002; Mendez *et al.* 2004; Pecson & Nelson 2005; Pecson *et al.* 2007; Nordin *et al.* 2009; Espinoza 2010; Cruz Espinoza *et al.* 2012; Fidjeland *et al.* 2013; Fidjeland *et al.* 2015; Nordin & Vinnerås 2015). However, at lower temperatures ($\leq$50 °C), the reported inactivation rates vary widely and the dominant factor affecting inactivation is often not isolated from other factors (Pecson *et al.* 2007; Maya *et al.* 2010). The effect of pH, temperature, moisture content (MC) and NH_3 are often not isolated from each other and have a wide range depending on the treatment (Eriksen *et al.* 1995; Mignotte-Cadiergues *et al.* 2001; Maya *et al.* 2010; Vu-Van *et al.* 2017). As most materials to be treated for *Ascaris* eggs will contain some nitrogen in the form of ammonium (NH_4^+) or ammonia (NH_3), an increase in pH will increase the amount of ammonia (NH_3), which will enhance the sanitisation effect from temperature and pH, especially if the treatment is performed under cover (retaining the ammonia gas) (Pecson *et al.* 2007; Nordin *et al.* 2009). The concentration of ammonia/ammonium will vary considerably depending on how the excreta has been collected (with or without the urine) and stored (open/sealed).

The objective of this study was to isolate the effect of pH, temperature and MC to better understand which of these parameters can be the driving force in the inactivation of *Ascaris* spp. eggs in the absence of NH_3. The inactivation rate of *Ascaris suum* eggs was assessed at three pH levels (7.2, 10.5 and 12.5) and three moisture level (Wet, >90% MC; Partially Wet, 60% MC and Dry, <20% MC) at five mesophilic temperatures (20, 27.5, 35, 42 and 50 °C), separately and in combination.

MATERIALS AND METHODS

Material collection

Ascaris suum eggs were retrieved by sieving swine faeces (Excelsior Sentinel, Inc., USA) and were then stored at 4 °C in a diluted formalin solution during transportation and storage prior to use. Human faeces (in total 300 g) were collected fresh from two individuals and stored in a freezer until use. At start-up, the faecal material was thawed overnight and 40 g was inoculated with *Ascaris* egg solution to a level of 100,000 eggs g^{-1} faeces. The mixture was blended for 1 min in a stomacher bag, which allowed thorough blending. The inoculated faeces were then placed in cold storage overnight. *Ascaris* was inoculated into faeces, as faecal material has been shown to have a protective effect (Eriksen *et al.* 1995; Ghiglietti *et al.* 1997; Capizzi-Banas *et al.* 2004).

Mineral soil was used as an inert medium to achieve the different moisture levels. The soil, collected from Uppsala, Sweden, was rinsed with distilled water, autoclaved wet at 120 °C for 20 min and dried at 70 °C. After this preparation, the soil was analysed. The MC and the soil organic matter (SOM) were analysed by drying ~20 g soil at 105 °C for 24 h followed by ignition at 550 °C for 4 h. The water

moisture was determined by the weight loss on drying and the SOM was determined to the weight loss on ignition. To measure the pH and EC, 50 mL of deionised water was added to 20 g soil in a small beaker with lid and places on a shaker table for 2 h (15 rpm). The pH and EC were measured in the soil/water solution (soil:water ratio 1:2.5). The analysis of total nitrogen (N_{Tot}) in the soil carried out using the Dumas combustion method. The soil had the following characteristics: pH 7.8; EC = 499 μS dm^{-1}; MC = 0.97%; SOM (% of DW) = 4.9%; N_{Tot} = 2.1 g kg^{-1}.

Preparation of treatments

There were three moisture level treatments: Wet, >90% MC; Partially Wet, 60% MC and Dry, <20% MC. For the Wet treatment, 0.1 g of inoculated faeces was added to 6 mL M/15 phosphate buffer solution (pH 7.2) in 7 mL polypropylene tubes with O-ring lined screw caps (Sarstedt AG & Co, Sweden). Faeces generally contain ammonia (Jönsson *et al.* 2005), but this would be diluted to <0.01% concentration in the tubes and would have no effect on the *Ascaris* eggs (Nordin *et al.* 2009). After sealing, the tubes were vortexed for 3 s. The Partially Wet and Dry treatments were prepared as described for the Wet treatment but prepared soil was added to the egg contaminated buffer solution to reach the targeted MC levels. The Partially Wet and Dry treatments were kept in 50 mL tubes. The pH treatments (10.5 and 12.5) were prepared as described for the Wet treatment, with the addition of 3% KOH solution to reach the targeted pHs.

The study was conducted as independent singlets using destructive sampling. The tubes were sealed and placed in incubators at one of the five target temperatures (20, 27.5, 35, 42 and 50 °C). The inactivation rates of *Ascaris* eggs were assessed in a range of temperatures, pH and moisture contents according to Table 1.

Table 1 | Inactivation was assessed at five temperatures (20, 27.5, 35, 42 and 50 °C), three pH levels (7.2, 10.5 and 12.5) and three moisture levels (wet, >90% MC; partially wet, 60% MC and dry, <20% MC)

Temp	MC	pH	Duration	*n*
20 °C	Wet	7.2	>70 d	7
		10.5	>70 d	9
		12.5	>70 d	9
	Partially wet	7.2	98 d	11
	Dry	7.2	92 d	11
27.5 °C	Wet	7.2	70 d	3
		10.5	70 d	3
		12.5	70 d	3
35 °C	Wet	7.2	>50 d	11
		10.5	>50 d	9
		12.5	>50 d	9
	Partially wet	7.2	98 d	11
	Dry	7.2	91 d	11
42 °C	Wet	7.2	16 d	5
		10.5	16 d	5
		12.5	16 d	5
50 °C	Wet	7.2	32 h	4
		10.5	32 h	4
		12.5	32 h	4

Duration of study for each treatment combination (days or hours) and the number of independent samples (*n*).

Extraction of eggs from material

The extraction procedure for the *Ascaris suum* eggs was a modification of the USEPA (2003) method. Sampling frequency varied depending on the treatment (at 20 °C, sampling was every 2 weeks, while at 50 °C, sampling was every 8 h). To extract the eggs from the soil, approximately 40 mL NaCl (0.9% w/v) peptone water with the surfactant Tween (pH 7) (hereafter called NaCl solution) were added to the tubes and shaken by hand for 30 s to help loosen the eggs from the soil. The material in the tubes was left to settle for 10 min and the liquid decanted over Tyler sieves (⌀ 38 μm, Cat. No. L3-400; ⌀ 300 μm, Cat. No. L3-50). Magnesium sulphate ($MgSO_4$, specific gravity 1.20) was added (approx. 30 mL) to the remaining soil, shaken by hand for 30 s and then centrifuged for 3 min at 4,000 rpm to float the *Ascaris suum* to the top. The supernatant was decanted over the sieves and the $MgSO_4$ washing was performed a total of three times. To wash the *Ascaris suum* eggs from the mesh of the sieves, NaCl solution was used (40–50 mL). The *Ascaris suum* eggs were then concentrated by centrifuging the washing solution for 3 min at 4,000 rpm, decanting the supernatant and adding 5 mL of 0.1 N H_2SO_4 to the remaining nugget.

Analyses

At the start of the experiment, a sample of the *Ascaris suum* eggs ($n = 100$) was observed under a microscope to confirm that the eggs were undeveloped. The initial viability was

determined by incubating three sets of the eggs at 28 °C in 0.1 N H_2SO_4 for 28 days to allow larval development (Arene 1986). All viability counts were performed under the microscope (×10 and ×20 magnification). Unfertilised eggs, which constituted a minor proportion of all eggs, were excluded from further counting. Eggs developing to the larval stage were considered viable, while pre-larval stages were not. Initial viability of the *Ascaris suum* eggs was 55% in treatment temperatures 20 and 35 °C and 77% in treatment temperatures 27.5, 42 and 50 °C.

After extraction from treatment matrixes, 100 eggs were directly examined under the microscope to assess any development or damage occurring during the treatment period. The remaining eggs (>1,000) were incubated at 28 °C in 0.1 N H_2SO_4 acid for 28 days to allow larval development (Arene 1986). Eggs developing to the larval stage were considered viable. Confidence intervals for proportions of viable *Ascaris* eggs were derived using the Wilson score interval (Minitab Inc., US).

The logarithmised egg viability data which showed a two-phase inactivation pattern were fitted against a non-linear inactivation model (Equation (1)) from Harm (1980). For some of the treatments that were repeated, the viability data were modelled as a combined data set.

$$\log_{10}N_t = \log_{10}N_0[1-(1-10^{k*t})^{10^n}] \quad (1)$$

where $\log_{10}N_t$ is the logged (base 10) proportion of viable eggs at time t (in days), and $\log_{10}N_0$ is the logged proportion of viable eggs at start. The reduction rate constant (k) described the change in viability over time during the exponential decay phase ($\log_{10}$ proportion of viable eggs day^{-1}). The k and the parameter determining lag phase duration (n; dimensionless) were determined by non-linear regression using the Gauss–Newton algorithm (Minitab 17, Minitab Inc., US). This enabled the calculation of the lag phase (Equation (2)):

$$\text{Lag phase} = l = \frac{n}{|k|} \quad (2)$$

The lag phase is the initial period where there is no significant inactivation. Many samples had no lag period according to this model. For these datasets, the model gave $n = 1$, which reduced the model to a linear regression with the y-intercept set to zero, i.e. exponential decay.

RESULTS

At 20 and 27 °C, the different pH and moisture contents studied had little or no inactivation during ≥70 days (Table 2). Even if the viability at the end of treatment was significantly lower than the initial viability for two treatments (pH 7.2 and 10.5 at 27 °C) that inactivation was still very low (−0.072 and −0.14 $\log_{10}$ respectively in 70 days). Fitting the model to such data would describe the inactivation with parameters derived by an excessive extrapolation from the measured values. This small reduction observed could better be explained by natural variation

Table 2 | Parameters for *Ascaris* egg inactivation: lag phase (days); inactivation rate constant, k ($\log_{10}d^{-1}$); time for a 3 $\log_{10}$ reduction of egg viability (days), derived from Equations (1) and (2)

Temp	MC	pH	lag (d)	k ($\log_{10}d^{-1}$)	3 $\log_{10}$ (d)
20 °C	Wet	7.2[a]	No inactivation in 94 d		
		10.5[a]	No inactivation in 126 d		
		12.5[a]	No inactivation in 126 d		
	Partially wet	7.2	No inactivation in 98 d		
	Dry	7.2	No inactivation in 94 d		
27.5 °C	Wet	7.2	−0.072 $\log_{10}$ reduction in 70 d*		
		10.5	−0.14 $\log_{10}$ reduction in 70 d*		
		12.5	No inactivation in 70 d		
35 °C	Wet	7.2[a]	23	<−0.049	<85
		10.5[a]	24	−0.077	63
		12.5	15	<−0.40	<22
	Partially wet	7.2	15	−0.029	120
	Dry	7.2	38	−0.16	57
42 °C	Wet	7.2	na	<−0.36	<8.3
		10.5	na	−0.36	8.4
		12.5	na	<−0.36	<8.4
50 °C	Wet	7.2	na	<−8.5	<0.35
		10.5	na	<−8.5	<0.35
		12.5	na	<−8.5	<0.35

Ascaris eggs were subjected to various conditions: five temperatures (20–50°C); three moisture contents (wet >90% MC; partially wet = 60% MC; dry <20% MC) and three pH levels (7.2, 10.5, and 12.5). When no inactivation was observed, the time of the viability study is given.

na = not applicable due to fast inactivation in relation to sampling frequency.

[a]Combined data from repeated study.

*Significant difference compared with initial viability but, due to low inactivation, not suitable for model fitting.

within the samples, since no decrease in egg viability was detected at pH 12.5 during the same period.

Compounding effects of temperature and pH were observed at 35 °C (Figure 1). The reduction in egg viability followed the biphasic model and inactivation parameters were derived from the data. The increase in pH from 7.2 to 12.5 decreased the time for a 3 $\log_{10}$ reduction of egg viability by four folds, from 85 days to less than 22 days (Table 2). The MC had no linear effect on the time required for a 3 $\log_{10}$ reduction of egg viability. For the Partially Wet (60% MC) treatment, the time required for a 3 $\log_{10}$ reduction in egg viability increased by 35 days to that of the Wet (>90% MC) treatment. The difference between Wet and the Dry (<20% MC) treatment decreased by 28 days.

Due to fast inactivation in relation to sampling frequency at 42 and 50 °C, the inactivation could only be described by a log-linear relationship and the derived k and the time for 3 $\log_{10}$ reduction of egg viability are conservative values (Table 2). The sampling frequency was not sufficient to detect any compounding effect of pH and temperature due to the rapid inactivation. At 42 °C, no viable eggs were observed by day 8, with the exception of the pH 10.5 treatment, which had one larva in 1,011 counted eggs (a 2.75 $\log_{10}$ reduction). At 50 °C, all eggs were inactivated below the 3 $\log_{10}$ detection limit within 8 h.

DISCUSSION

Effect of pH

During >70 days, elevated pH (10.5 and 12.5) alone did not have any effect on the inactivation rate of *Ascaris* eggs at temperatures ≤27.5 °C. The true isolated effect of pH on *Ascaris* spp. eggs has been examined in only a few previous studies and the rates of inactivation reported vary (Pecson *et al.* 2007; Maya *et al.* 2010). Our results highlight that a pH increase alone is not enough to inactivate STH during 70 days of treatment. *Ascaris* spp. eggs have also been observed to persist in low pH treatments (ranging from 2–5), with the viability of *Ascaris* eggs being affected by the uncharged carboxylic acids rather than the pH (Harroff *et al.* 2017). Elevating the pH and the temperature to 12.5 and 35 °C, respectively, had a compounding effect on the inactivation rates. Our study indicated that the

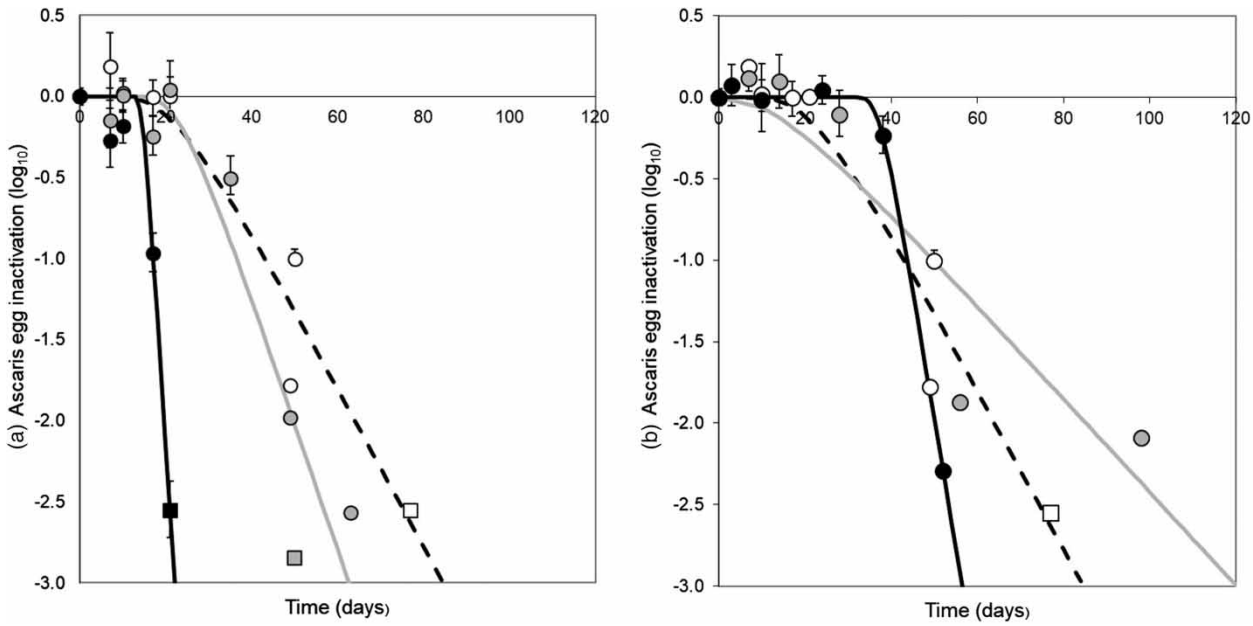

Figure 1 | Inactivation of *Ascaris suum* eggs at 35 °C in treatments with: (a) being the different pH levels under wet conditions (●/black line = pH 12.5; ●/shaded line = pH 10.5; ○/broken line = pH 7.2) and (b) being the different moisture levels at pH 7.2 (●/black line = dry; ●/shaded line = partially wet; and ○/broken line = wet). The lines show the fitted inactivation model (Equation (1). Error bars show the 95% confidence interval, however too small to be visible for some data points. Square symbols denote detection limit with no viable eggs detected.

inactivation effects reported from liming may mainly derive from elevated temperature during the mixing (Ghiglietti *et al.* 1997) and/or from ammonia being present in the material, concentration of which increased with increased pH (Gaasenbeek & Borgsteede 1998; Pecson & Nelson 2005; Pecson *et al.* 2007).

Moisture content and temperature

The effect on the inactivation rate of *Ascaris* eggs at different MC treatments (Wet (>90% MC), Partially Wet (60% MC) and Dry (20% MC)), was insignificant at 20 °C. Reported impacts of MC on *Ascaris* egg viability at lower temperature vary between studies, as does the experimental set-up. For example, Wharton (1979) observed that *Ascaris* eggs (without any other medium) placed on a slide and exposed to a constant 32.5% relative humidity at 16.5 °C collapsed due to desiccation after only 17 days, while in experimental set-ups where the eggs were covered with a medium, such as sludge, dehydration *alone* was not effective (El Hayany *et al.* 2018). Organic matter (such as faeces) may protect *Ascaris* spp. eggs from moisture loss and may explain their persistence in the environment (Barrett 1976). Anaerobic conditions may help in the survival of *Ascaris* spp. eggs, since in such conditions, the shell restricts respiration, which could help protect from the egg from water loss and the effect of pH (Gaasenbeek & Borgsteede 1998). However, longer periods of anaerobic conditions resulting in low redox potential increase the inactivation of *Ascaris* spp. eggs (Yen-Phi *et al.* 2009). Such aspects may explain the higher survival in the Partially Wet treatment at 35 °C, providing moisture as well as aeration (Table 2). In the derivation of inactivation parameters k and n (Equation (1)), the detection limit data were used when in line with the inactivation trend or showing faster inactivation than the earlier trend, so the time for inactivation could potentially be shorter.

Temperature was the driving factor for inactivation. This observed effect is likely due to the egg shell's ability to restrict water loss that decreases exponentially with increased temperature (Wharton 1980). At the elevated temperatures, pH had a compounding effect, which could be due to temperature affecting the shell's structure. The temperature at which the lipid layer starts to change in permeability and/or deteriorate depends on the amount of time that the eggs are exposed to an elevated temperature, as the effect on the lipid layer may not be permanent (Wharton 1980). Dye is reported to be able to penetrate the shell of *A. lumbricoides* eggs exposed to 44 °C, whereas eggs that were allowed to cool before being exposed to a dye retained their shell function after exposure to temperatures as high as 65 °C for 12 h (Barrett 1976; Wharton 1980). A study testing the effectiveness of chemical disinfectants on *Ascaris* spp. eggs observed that sludge gave higher protection to the eggs than wastewater (Amoah *et al.* 2017).

Ascaris persistence

Ascaris eggs are able to withstand the elevated temperatures better than the bacteria and phages (Senecal *et al.* 2018). This may be due to different stress responses and production of protective peptides and proteins. In order to be able to persist in variable conditions, organisms have adapted stress mechanisms, such as the expression of specific proteins called heat shock proteins (Maresca & Carratù 1992; Pérez-Morales & Espinoza 2015). When cells are stressed to the point of protein denaturation, heat shock proteins bind to the denaturing proteins to maintain their competent folding state (Pérez-Morales & Espinoza 2015; Bolhassani & Agi 2019). Pathogenic organisms that pass through various hosts and environments, such as *Ascaris*, can be exposed to sudden changes in their surroundings, such as temperature (Pérez-Morales & Espinoza 2015) and salinity (Abaza 2014). An increase in temperature is not the only stress to induce the expression of heat shock proteins. Other stresses, such as anoxia and ethanol (and NH_3), can also induce the expression to help protect the egg (Lindquist & Craig 1988).

Based on the minimal effect that the different moisture contents had on the *Ascaris* eggs, when treating organic material, an increase in the pH in combination with increasing temperature may be a more efficient way to accomplish faster *Ascaris* inactivation than drying the material. As excreta will contain some amount of NH_3, most treatments of excreta would benefit from being covered to retain the NH_3. The effectiveness of NH_3 treatment also increases with even small temperature increases (Nordin & Vinnerås 2015).

CONCLUSIONS

Exposure of *Ascaris suum* eggs to elevated pH (10.5–12.5) at temperatures ≤27.5 °C for >70 days was shown to have no effect on egg viability. Compounding effect of alkaline pH or moisture <20% MC was observed at 35 °C, with pH having more effect than decreasing MC. To accelerate the inactivation of STH, an increase in the treatment temperature is more effective than pH increase. However, using alkali to hygienise organic wastes can enhance the sanitising effect of ammonia if performed in closed containers.

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