

Research Paper

Inactivation of *Ascaris* for thermal treatment and drying applications in faecal sludge

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ABSTRACT

Ascaris lumbricoides is the most common helminth of human health importance, and the most resilient helminth found in faecal sludge. There are numerous types of sludge treatments; however, heating and drying are most commonly used for pathogen inactivation. *Ascaris suum* eggs were heated in a water bath at 40–55 °C for 10 seconds to 60 minutes in water, as well as heated in both urine diversion dry toilet and ventilated improved pit latrine sludge at 40 °C, 60 °C and 80 °C for times ranging from 5 seconds to 120 minutes. Eggs were also spiked into sludges of different moisture contents and incubated over 12 weeks at 25 °C, with samples analysed weekly. Overall, we concluded that eggs were inactivated at temperatures >50 °C, that the temperature–time relationship directly impacted the efficacy of heat treatment, that suspension medium had no effect, and that eggs survived better in wet rather than dry sludges.

Key words | *Ascaris*, exposure time, heating, inactivation, moisture content, temperature

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INTRODUCTION

Approximately 2.3 billion people globally lack access to basic sanitation facilities and one-third of the world's population is infected with soil-transmitted helminths (STHs) (JMP 2017; Cooper & Hollingsworth 2018). *Ascaris lumbricoides*, also known as the human roundworm, is the most common STH of human health importance (Brownell & Nelson 2006). Infection with *A. lumbricoides* is most prominent in areas that lack a potable source of water, improved sanitation and proper hygiene practices, with an estimated 804 million people infected worldwide (Jourdan *et al.* 2018). Mild infections may be asymptomatic; however, heavy worm infections can lead to symptoms such as diarrhoea, bloating, abdominal blockages and discomfort, malnutrition,

and impaired growth and cognitive development (Cooper & Hollingsworth 2018). Diarrhoeal diseases are the cause of 1.3 million deaths per year, of which one in eight are children under the age of five years (Kotloff 2017).

Ascaris eggs can withstand harsh environmental conditions and are considered the most resilient organisms found in faecal sludge, as they are able to survive for up to seven years in the soil (Pecson & Nelson 2005). *Ascaris* spp. eggs are therefore commonly used as indicator organisms of faecal contamination and for inactivation experiments (Maya *et al.* 2012).

Heat treatment technologies for sludge have become common practice due to increased pathogen inactivation success (Belcher *et al.* 2015). There have been a number of studies globally that have focused on heating or drying for pathogen inactivation and sludge sanitisation (Brownell & Nelson 2006; Maya *et al.* 2010; Szabová *et al.* 2010; Buttar

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et al. 2013; Andes & Paller 2018). Composting systems and drying beds are feasible treatment options that operate at lower temperatures over prolonged periods of time, and are common practice in Africa. Koné *et al.* (2007) reported that heat generation due to the composting process in a drying bed resulted in 90–100% *Ascaris* inactivation after 80 days at ≥ 55 °C. Septien *et al.* (2018) reported that pelletisation and infrared drying of faecal sludge resulted in the inactivation of *Ascaris* eggs.

Naidoo & Foutch (2017) collated results from various heat treatment studies in order to establish a temperature–time relationship relative to *Ascaris* inactivation. Few studies have investigated the isolated effects of heat on *Ascaris* eggs, as most include the effects of other treatment factors, such as pH and relative humidity (Pecson & Nelson 2003; Capizzi-Banas *et al.* 2004), or involve some type of treatment technology, such as a viscous heater (Belcher *et al.* 2015). There are also gaps in the literature in terms of isolated heat treatment of helminth eggs at lower temperatures (40–50 °C), where such data may be useful to sanitation practitioners employing drying beds, composting systems and low heat technologies for sludge treatment. This study therefore aimed at extending the temperature–time relationship profile towards the lower end of the temperature scale, based on work previously investigated by Naidoo & Foutch (2017) and Naidoo *et al.* (2019) that focused on heat treatment at 60–80 °C in water. Eggs were heated in water at 40, 45, 50 and 55 °C for times ranging from 10 seconds to 60 minutes.

According to Jebri *et al.* (2013), the suspension medium of the eggs plays a role in the efficacy of heat treatment. Buttar *et al.* (2013) went on to report that sludge, or any similar suspension medium, may act as insulation against heat, thus higher temperatures and longer exposure times might be needed for successful inactivation. The role of the suspension medium on the inactivation of *Ascaris* eggs was therefore investigated in this study, testing water, urine diversion dry toilet (UDDT, to be referred to as UD from henceforth) sludge and ventilated improved pit (VIP) latrine sludge where eggs were heated at 40, 60 and 80 °C, for various exposure times that were selected based on previous data (Naidoo *et al.* 2019).

Drying leads to a decrease in moisture content of treated sludge. While previous studies have focused on the effects of reduced sludge moisture on bacterial and viral inactivation

(Romdhana *et al.* 2009) and the effects of dryness in combination with heating and pH alteration (Maya *et al.* 2012), none have explored the effects of dryness alone on the survival of helminth eggs. These effects were thus explored in the present study for both UD and VIP sludge.

Ascaris eggs are used as indicator organisms for overall pathogenic contamination of sludge, as it is assumed that if all *Ascaris* eggs have been killed off, then all other pathogens would have died as well (Sidhu & Toze 2009). Due to ethical and logistical issues it is often difficult to source *Ascaris lumbricoides* eggs, thus eggs of the pig roundworm, *Ascaris suum*, are often used as a surrogate. Both species are morphologically identical in all developmental stages (Daughschies *et al.* 2013), thus *A. suum* eggs were used for inactivation testing in this study.

MATERIALS AND METHODS

Sample collection and sludge characterisation

The effects of heating were tested in three separate experiments. UD sludge was collected from a stockpile that was collected from UD toilets within the eThekweni Municipality (Durban, South Africa) and transported to the Isipingo Wastewater Treatment Plant in KwaZulu-Natal for treatment. VIP sludge was collected directly from household latrines in Bester (within the eThekweni Municipality), and wet VIP sludge was collected directly from the vacuum trucks that were emptying pits in the Bester area at the time of sampling. Water was added to the UD sludge to match the moisture content of the wet VIP sludge, for the purposes of consistency during treatment. The sludge characteristics are summarised in Table 1. *Ascaris suum* eggs were purchased from Excelsior Sentinel Inc. (USA) and were stored at 4 °C until needed.

Experiment 1: Heat treatment of eggs in water

The first experiment was a continuation of Naidoo *et al.* (2019) that focused on heating at 60, 65, 70, 75 and 80 °C. Eggs were heated at 40, 45, 50 and 55 °C for 10 and 30 seconds, and at 1, 2, 5, 10 and 60 minutes. Plastic 15 mL test tubes containing tap water were preheated in a water

Table 1 | Characteristics of the collected UD, VIP (from latrines) sludge, wet VIP (from vacuum tanker, used for Experiment 2) and wet UD (diluted UD sludge used for Experiment 2)

Sludge	Moisture content (g/g wet sample)	Total solids (g/g wet sample)	Volatile solids (g/g dry sample)	Ash content (g/g dry sample)
UD	0.789 ± 0.005	0.211 ± 0.005	0.576 ± 0.045	0.424 ± 0.045
VIP	0.877 ± 0.033	0.123 ± 0.033	–	–
Wet VIP	0.901 ± 0.045	0.099 ± 0.045	0.644 ± 0.016	0.356 ± 0.016
Wet UD	0.902 ± 0.030	0.098 ± 0.030	–	–

UD, urine diversion; VIP, ventilated improved pit latrine.

bath to the respective test temperature. Approximately 500 *Ascaris* eggs (suspended in 1 mL of water) were spiked into each heated test tube and exposed for the test time. The tubes were then removed from the water bath and the contents brought back to room temperature immediately by emptying into iced tap water, in order to prevent prolonged heat exposure. Samples were treated, processed and analysed using light microscopy, both before and after incubation according to the methodology used by Naidoo et al. (2019).

Experiment 2: The effects of the suspension medium on the efficacy of heat treatment

The second experiment involved the heating of *Ascaris* eggs in both VIP and UD sludge, and water as a control. Test parameters included 30, 60 and 120 minutes at 40 °C, 30 seconds, 2 minutes and 5 minutes at 60 °C, and 5 and 10 seconds and 1 minute at 80 °C. The UD sludge was diluted to match the moisture content of the wet VIP sludge. Sludge samples of approximately 10 g each were weighed out into aluminium cups (70 mm × 40 mm) and covered with sheets of aluminium foil. The water bath was preheated to the respective test temperature, after which the aluminium cups containing the sludge were inserted into holes cut into a polystyrene sheet (350 mm × 250 mm × 15 mm), being fully immersed in the water. Once the entire system reached the test temperature, eggs were spiked into the sludge and mixed, and the cups were immediately covered again to prevent moisture loss. The samples were exposed for the respective test times, after which the cups were removed and placed into plastic bowls containing iced water, and approximately 30 mL iced water was poured into the sludge sample and mixed to allow the sample to return to room temperature and prevent prolonged heating.

The samples were then processed according to the PRG Helminth Method (2018). Ammonium bicarbonate was poured into the aluminium cups until the sludge was just covered and mixed well. The contents of the cup were then poured over a set of drum sieves (100 µm over a 20 µm sieve). The sample was washed thoroughly in the sieve, using pressure from a hose on the tap, and by breaking any clumps using the back of a gloved hand. The 100 µm sieve was then removed and the retentate discarded. The retentate (containing the *Ascaris* eggs) on the 20 µm sieve was then washed thoroughly and collected into four plastic 15 mL test tubes. These were centrifuged at 1,512 × g (3,000 rpm) for 10 minutes and the supernatant discarded. Zinc sulphate (ZnSO₄) was added to each tube in 3 mL aliquots to a total of 14 mL while vortexing to break up the pellet and homogenise the suspension. The test tubes were then centrifuged at 672 × g (2,000 rpm) for 10 minutes, to allow eggs to float up into the liquid column above the sediment. The supernatant was then poured onto a smaller 20 µm sieve, washed with water, and collected into a single 15 mL tube. The final samples were centrifuged at 1,512 × g (3,000 rpm) for 3 minutes, after which the supernatant was discarded and the final pellet microscopically analysed. The samples were then washed back into the test tubes, incubated for 28 days at 25 °C and re-analysed microscopically. Eggs were scored and categorised according to morphology and viability, as per Naidoo et al. (2019).

Experiment 3: The effects of moisture content on *Ascaris* survivability

The third experiment involved the incubation of eggs in sludge samples of different moistures. Both VIP and UD sludges were dried down to different approximate moistures – 60, 50, 40, 30 and 20%, and undried sludge was used as a

control. Sludge was dried in an oven at 105 °C until the respective moisture content was reached. These dried samples were further weighed out into 5 g samples, spiked with approximately 400 *Ascaris* eggs per sample and incubated at 25 °C for a period of 12 weeks. Each week, samples were processed using the PRG Helminth Method described in Experiment 2, then analysed microscopically.

For this study, the criterion set for successful inactivation was <10% viable eggs recovered after treatment (Ayçiçek et al. 2001; Naidoo et al. 2019). Statistical analyses included the Kolmogorov–Smirnov test for normality of data, followed by a nested analysis of variance (ANOVA), the Shapiro–Wilk test for normality of residuals and the Levene’s test for homogeneity of variance of residuals, both from the nested ANOVA. The analyses were run on IBM SPSS Statistics (version 25) and R (version 3.5.2). Percentage viability was calculated as follows:

$$\begin{aligned} & \text{Percentage viable eggs recovered} \\ & = (\text{Total viable eggs recovered} / \\ & \quad \text{Total number of eggs recovered}) * 100 \end{aligned}$$

RESULTS AND DISCUSSION

Table 2 presents the statistical analyses of the results of the nested ANOVA and compares the percentage of viable eggs recovered between each variable and respective combinations. The in-depth description of the statistical analyses (including the exact nested designs and *p* values for specific variable combinations) were not included in this article, but can be made available upon request.

Experiment 1

Results from the nested ANOVA (Table 2) for Experiment 1 indicate that alone, temperature had a significant effect on *Ascaris* egg inactivation ($p < 0.001$). In combination with the second and third levels of nestedness (exposure time and point of analysis, respectively), significant effects were also observed ($p < 0.001$). Experiment 1 indicated that 40 and 45 °C was not sufficient for successful *Ascaris* inactivation within the tested exposure time frame ($p = 0.844$ and $p = 0.866$). After 60 minutes of heat exposure, egg viability was 81.4% before incubation and 76.6% after incubation at 40 °C, and 83.2% before incubation and 83.5% after incubation at 45 °C, thus not meeting our inactivation criterion (<10% viable eggs recovered) (Figure 1(a) and 1(b)). Treatment at 50 and 55 °C led to a statistically significant egg inactivation after 60 minutes ($p < 0.001$) and 5 minutes ($p < 0.001$) of exposure, respectively. At 50 °C for 10 minutes of heat exposure, egg viability before incubation was 85.5%, and a decline in viability was seen after incubation (65.5%; $p = 0.762$), but this did not meet the inactivation criterion for this study. After 60 minutes of heat exposure, egg viability was 86.5% before incubation and 1.9% after incubation ($p < 0.001$). Treatment at 55 °C rendered an egg viability of 89, 92 and 84% before incubation, and 3.8, 0.2 and 0% after incubation, for 5 minutes ($p < 0.001$), 10 minutes ($p < 0.001$) and 60 minutes ($p < 0.001$) of heat exposure, respectively, meeting the inactivation criterion for this study (Figure 1(d)). The 5 °C difference between heat treatment at 50 and 55 °C appears to be crucial in terms of inactivation efficacy, as the most effective exposure time for each temperature ranges from 60 down to 5 minutes. It should also be noted that complete inactivation was not observed after 1 hour of treatment at temperatures below 50 °C.

Table 2 | Statistical results of the general ANOVA from the nested ANOVA design for Experiments 1, 2 and 3

Experiment	Primary variable	F _(df)	p-Value	^a Variable combinations	p-Value
1	Temperature	F _(6, 174) = 10.58	<0.001	Temperature/Exposure time Temperature/Exposure time/point of analysis	<0.001 <0.001
2	Temperature	F _(5, 90) = 10.58	<0.001	Temperature/Exposure time Temperature/Exposure time/suspension medium	0.001 0.860
3	Time (week)	F _(1, 216) = 10.58	<0.001	Time/Moisture content Time/Moisture content/suspension medium	<0.001 0.223

^aVariable combinations represent the nestedness of the statistical design. Point of analysis refers to whether eggs were examined before or after incubation.

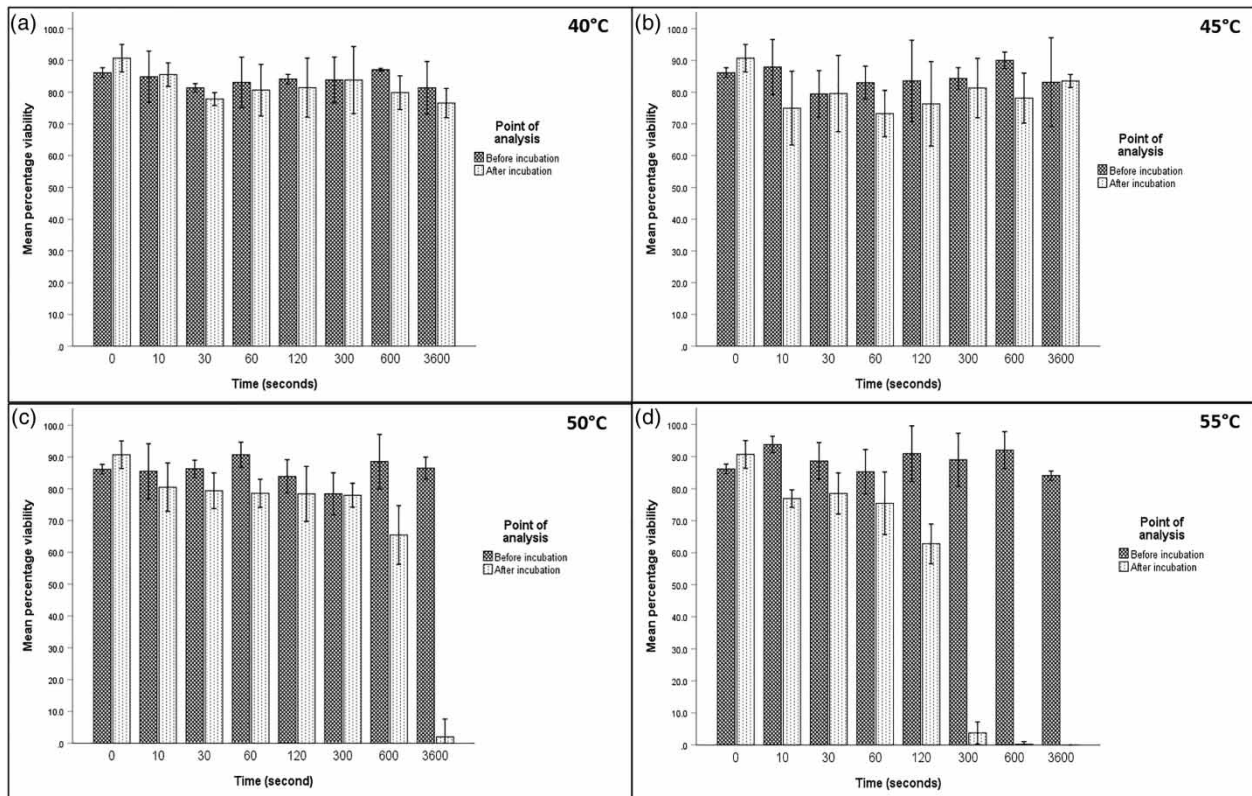


Figure 1 | The effects of heat treatment on *Ascaris* eggs at: (a) 40 °C, (b) 45 °C, (c) 50 °C and (d) 55 °C, both before and after incubation at 25 °C for 28 days, for exposure times of 10, 30 and 60 seconds; 2, 5, 10 and 60 minutes; 0 seconds represents the control tests in water ($n = 3$).

Brannen *et al.* (1975) and Brandon (1978) both investigated the effects of direct heating of *Ascaris* eggs in a water bath. The former reported almost 100% inhibition of egg development when exposed to 55 °C for 4 minutes and the latter reported that egg viability was reduced to almost 0% when heated at 55 °C for 60 minutes. Pecson & Nelson (2003) reported that complete *Ascaris* egg inactivation was achieved after treatment for 24 hours at 48 °C, and 1 hour at 54 °C, in various buffered solutions. These studies support the findings of the current study in terms of the temperature–time relationship for *Ascaris* egg inactivation.

The major difference in egg viability between analyses conducted before and after incubation, particularly for 50 and 55 °C, should be noted. Naidoo *et al.* (2019) reported that at higher temperatures (≥ 60 °C), morphological damage to eggs was easily identifiable and egg death very apparent with a globular embryo or disintegrated larva. Damage was, however, not as visible at lower temperatures. Drawing on our experience, we know that eggs sometimes

appear healthy at the one-celled undeveloped stage after treatment at low temperatures; however, upon incubation, no further development occurs, indicating successful inactivation. This scenario explains the difference in viability seen in Figure 1(c) at 50 °C and Figure 1(d) at 55 °C, where eggs appeared healthy before incubation, but did not develop further when incubated, and were thereafter scored as dead. The incubation step (at 25 °C for 28 days, which is the duration of the environmental development steps of the life cycle of *Ascaris* spp.) is therefore imperative, especially for *Ascaris* inactivation studies conducted at low temperatures. Note that the opposite could also occur, where an egg sometimes appears somewhat damaged, but upon incubation, it would develop further, indicating that inactivation was not successful.

Experiment 2

Results from the nested ANOVA (Table 2) for Experiment 2 indicated that alone, temperature had a significant effect on

Ascaris egg inactivation ($p < 0.001$). In combination with time (second level of nestedness), significant differences in egg viability were observed ($p < 0.001$). However, when taking into consideration the suspension medium (third level of nestedness), no significant differences were noted in egg viability after treatment ($p = 0.860$). Based on data from Naidoo *et al.* (2019) and from Experiment 1, it is known that at lower temperatures, damage to the eggs after heat treatment may not always be visible, thus the importance of incubation has already been established. Statistical and graphical analyses therefore only included egg viability after incubation (Table 2; Figure 2).

At 40 °C, significant inactivation was not achieved after the maximum exposure time of 2 hours in water (66.5%; $p = 0.981$), UD sludge (56.9%; $p = 0.261$) and VIP sludge (65.5%; $p = 1.000$), thus none of the above met the inactivation criterion for this study (Figure 2(a)). Figure 2(b) shows that significant inactivation was achieved at 60 °C after 30 seconds of exposure in water (5.6%; $p < 0.001$), UD sludge (12.7%; $p < 0.001$) and VIP sludge (3.6%; $p < 0.001$). A near complete inactivation of *Ascaris* eggs was obtained at 2 minutes' exposure ($p < 0.001$) and complete inactivation was achieved at 5 minutes' exposure ($p < 0.001$), at 60 °C in all three suspension media. At 80 °C, significant inactivation was achieved after 5 seconds' exposure in water (3.3%; $p < 0.001$), UD sludge (0.0%; $p < 0.001$) and VIP sludge (3.6%, $p < 0.001$). After 10 seconds of treatment, egg viability was almost negligible and at 60 seconds, complete die-off was observed (Figure 2(c)). It should be noted that the suspension medium did not impact treatment and resultant inactivation efficacy. This implies that laboratory testing of *Ascaris* inactivation for technology design could be done in water rather than sludge or faecal simulants, saving time and avoiding the handling of hazardous material.

Popat *et al.* (2010) investigated the effects of thermophilic anaerobic digestion on *Ascaris* eggs in a sludge of 98% moisture content. A 2-log reduction in egg viability was reported after 2 hours of exposure at 55.5 °C. Thomas *et al.* (2015) investigated the effects of shear and temperature, both together and independently, on the inactivation of *Ascaris* eggs, using methods similar to the current study. It was found that after 60 seconds at 47, 51 and 55 °C, there was 94.7%, 91.1% and 89.7% egg development, respectively,

after treatment. A similar inactivation pattern was seen in the current study, where successful inactivation was only observed after treatment at 55 °C for ≥ 5 minutes, and at 60 °C after ≥ 30 seconds, in sludges of similar moisture content.

Neither Brannen *et al.* (1975) nor Brandon (1978) reported any significant difference in *Ascaris* inactivation between treatment in water or sludge, suggesting that inactivation follows a similar pattern, irrespective of the suspension medium of the eggs. Similar data were observed in the current study (Experiment 2, Figure 2), where inactivation patterns were similar across all three suspension media – water, UD sludge and VIP sludge.

Experiment 3

Results from the nested ANOVA (Table 2) for Experiment 3 indicated that alone, time (week), significantly affected egg survivability and development ($p < 0.001$). In combination with moisture content (second level of nestedness), there was a significant effect on survivability as well ($p < 0.001$). Egg development (at the expected developmental rate as per the *Ascaris* spp. life cycle) was the most consistent in sludges of 50% moisture (Figure 3) and much slower in drier sludges (20% and 30% moisture), indicating that *Ascaris* eggs survived longer in wetter sludges (Figure 3). There was however, no significant difference between incubation of eggs in UD sludge and VIP sludge (suspension medium; third level of nestedness; $p = 0.223$), meaning that eggs developed or died in a similar manner in both sludges. The findings from this study indicate that drying sludge to a low moisture content creates unfavourable conditions for egg survival and development. Long-term storage of sludge, which may result in a reduction of moisture, can promote the inactivation of *Ascaris* eggs.

Maya *et al.* (2012) investigated the effects of temperature, pH and dryness on the inactivation of *A. suum* and *A. lumbricoides* eggs (together with four other parasite species), where eggs were spiked into sterilised sludge. They stated that at 80 °C, 3 hours' exposure was sufficient to inactivate undeveloped eggs in dry sludges (5–10% moisture), and at 60 °C, 1 hour was sufficient in sludges of 5–15% moisture. It was also reported that drier conditions resulted in increased inactivation, and that larval eggs were more

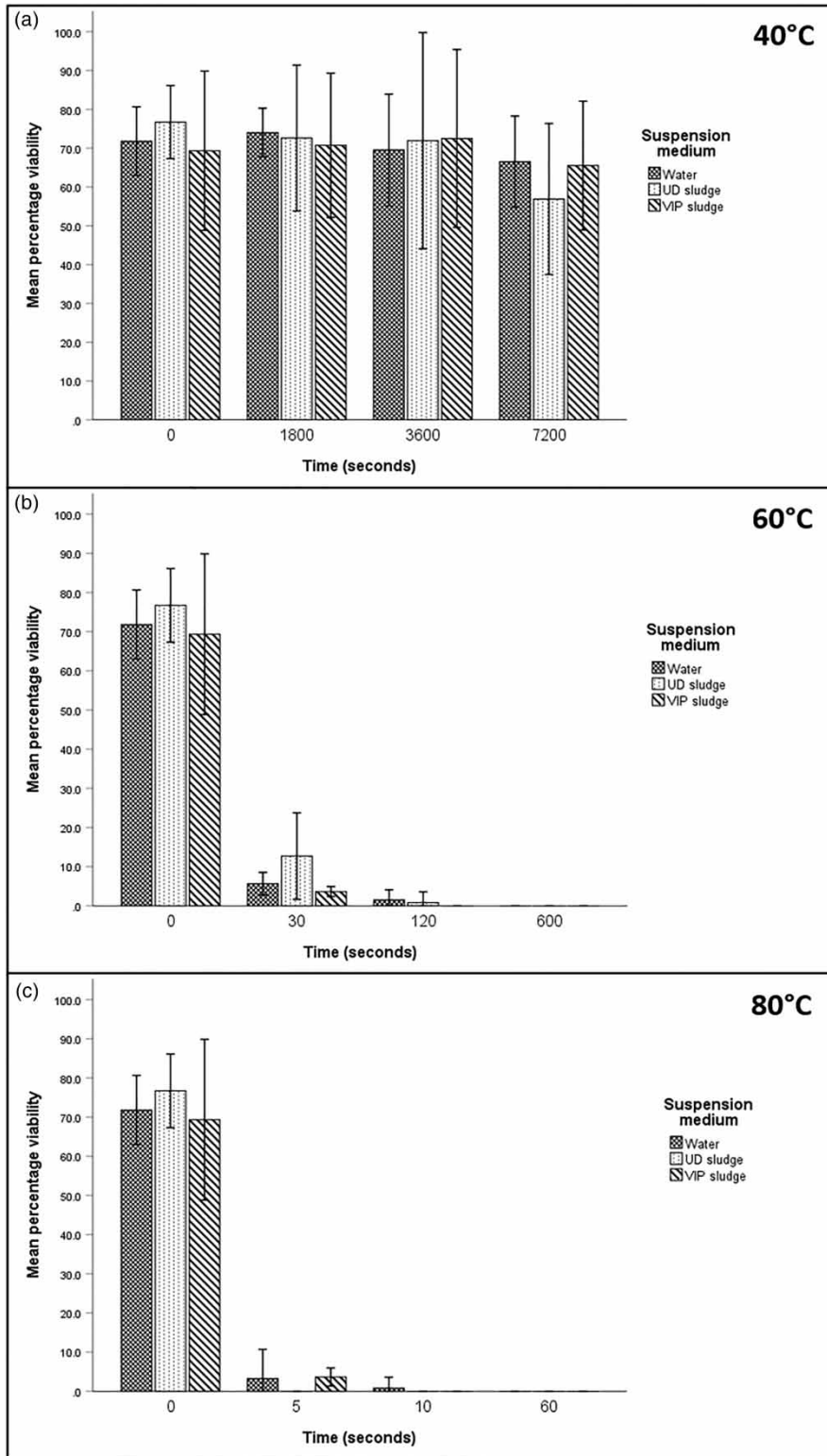


Figure 2 | Testing the role of the suspension medium on *Ascaris* egg inactivation by heat treatment at: (a) 40 °C for 30, 60 and 120 minutes, (b) 60 °C for 30, 120 and 600 seconds and (c) 80 °C for 5, 10 and 60 seconds, in water, UD sludge and VIP sludge after incubation at 25 °C for 28 days; 0 seconds represents the controls ($n = 3$).

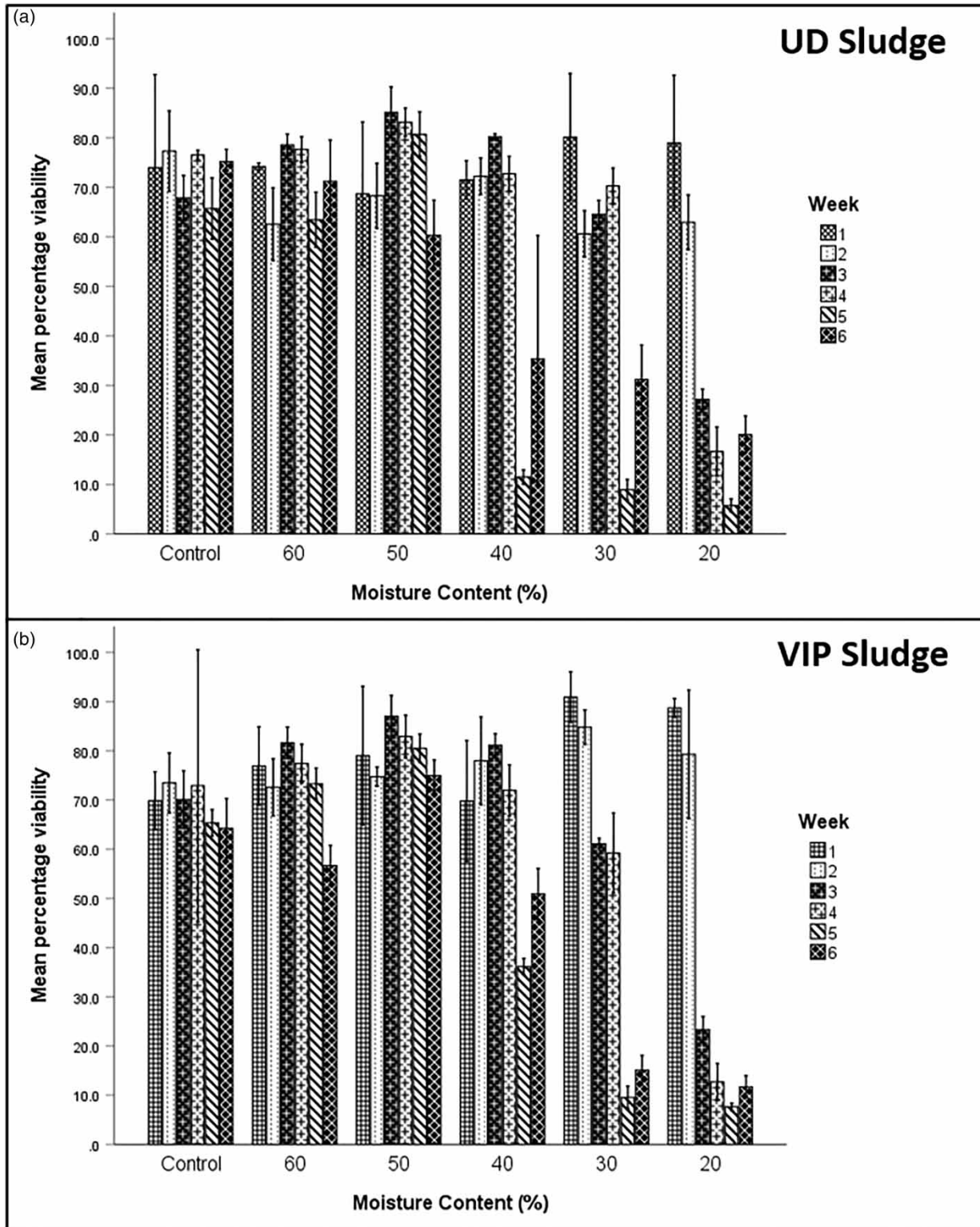


Figure 3 | The effects of different levels of moisture of the suspension medium on the development and survival of *Ascaris* eggs when incubated at 25 °C for 6 weeks, in (a) UD sludge and (b) VIP sludge ($n = 3$).

susceptible to environmental stressors as opposed to undeveloped eggs, supporting the findings of this study.

During the 12-week incubation period, there was development of fungal contamination within the samples. Data from weeks 7 to 12 were therefore excluded from analyses, as studies have found that certain strains of fungi may have ovicidal properties (Ferreira et al. 2011; Blaszkowska et al. 2014). Filamentous growth was observed around eggs isolated from sludge samples that had visible contamination and, in most cases, these eggs were very damaged and dead.

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

The conclusion and recommendations for future work are as follows:

- This study was aimed at determining the effects of low temperatures, suspension medium and storage conditions on *Ascaris* eggs.
- Heat treatment at 40 and 45 °C after 60 minutes, and treatment at 40 °C after 120 minutes were insufficient for *Ascaris* inactivation, providing critical data for the planning related to slow heating technologies such as drying beds.
- At 50 and 55 °C, 60 minutes and 5 minutes, respectively, are sufficient for successful *Ascaris* inactivation.
- Furthermore, morphological damage was not necessarily visible before incubation. The incubation step is therefore imperative when testing any kind of heat treatment in order to verify inactivation.
- The suspension medium did not play a role in the efficacy of heat treatment of the eggs – inactivation patterns were the same for water and UD and VIP sludge, meaning that heat treatment of *Ascaris* eggs can be done in water rather than having to source sludge
- Time had an effect on *Ascaris* egg survival and development in sludge, indicating the potential for egg inactivation during long-term storage.
- In combination with moisture content, a significant effect was also observed. Eggs preferred wetter sludges for development, with 50% moisture being the optimum

condition, and development was much slower in drier sludges (20–30% moisture).

- Fungal growth is inevitable over time, supporting egg inactivation during long-term storage of sludge.

The exact exposure time for successful inactivation at low temperatures (40–49 °C) is still unknown (it fell outside the time frame of this project), in terms of isolated heat treatment. Further work is therefore necessary in order to complete the full temperature–time relationship for *Ascaris* egg inactivation. Fungal development in stored sludge could be advantageous in terms of developing biological control protocols for *Ascaris*. Studies have indicated the total ovicidal potential of certain strains of fungus, which have been used for the control of plant nematodes in agriculture. Further research is needed for the application to faecal sludge management.

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