Water Research X 5 (2019) 100036

Contents lists available at ScienceDirect

Water Research X

journal homepage: https://www.journals.elsevier.com/water-research-x

Current time-temperature relationships for thermal inactivation of *Ascaris* eggs at mesophilic temperatures are too conservative and may hamper development of simple, but effective sanitation

Lauren A. Harroff [a], Janice L. Liotta [b], Dwight D. Bowman [b], Largus T. Angenent [c, *]

[a] Department of Biological and Environmental Engineering, Cornell University, Ithaca, NY, 14853, USA
[b] Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA
[c] Center for Applied Geosciences, University of Tübingen, 72074, Tübingen, Germany

ARTICLE INFO

Article history:
Received 1 March 2019
Received in revised form
19 August 2019
Accepted 30 August 2019
Available online 3 September 2019

Keywords:
Ascaris
Pathogens
Mesophilic
Thermal inactivation
Sanitation
Fecal sludge

ABSTRACT

Ascaris eggs are commonly used as indicators for pathogen inactivation during the treatment of fecal sludge and wastewater due to their highly resistant lipid membrane and ability to survive in the environment for long periods of time. Current guidelines suggest that thermal treatment alone cannot inactivate *Ascaris* eggs at temperatures below 45 °C, although some evidence in the literature suggests this to be incorrect. Here, we performed a controlled experiment to test the effect of mesophilic temperatures on *Ascaris* inactivation. We exposed *Ascaris suum* eggs to a temperature gradient between 34°C and 45 °C under anaerobic and aerobic conditions to observe the required exposure times for a 3-log reduction. Indeed, we found that temperatures lower than 45 °C did inactivate these eggs, and the required exposure times were up to two orders of magnitude shorter than suggested by current guidelines. Results from the anaerobic exposures were used to develop a time-temperature relationship that is appropriate for *Ascaris* inactivation at mesophilic temperatures. Data from the literature demonstrated that our relationship is conservative, with faster inactivation occurring under environmental conditions when *Ascaris* eggs were suspended in fecal sludge or manure. A specific aerobic relationship was not developed, but we demonstrated that aerobic conditions cause faster inactivation than anaerobic conditions. Therefore, the anaerobic relationship provides a conservative guideline for both conditions. We demonstrate that relatively low temperatures can considerably impact *Ascaris* viability and suggest that mesophilic temperatures can be used in waste treatment processes to inactivate pathogens. The development of safe, low-input, mesophilic treatment processes is particularly valuable for ensuring universal access to safe sanitation and excreta management.

1. Introduction

The intestinal roundworm *Ascaris lumbricoides* is widely considered to be the most resistant pathogen to disinfection processes in human fecal sludge and wastewater (Feachem et al., 1983; US EPA, 2003). *A. lumbricoides* infects approximately 1.3 billion people worldwide and is spread by fecal-oral transmission, particularly in areas with poor access to sanitation (De Silva et al., 1997). Eggs are passed into the environment through feces and have been shown to survive and retain infectivity for many years (Brudastov et al., 1970). Due to their extreme resistance, *Ascaris* eggs are often used as indicators of pathogen inactivation when testing treatment methods for wastewater and fecal sludge.

The resistance of *Ascaris* eggs to thermal inactivation is of particular interest here. Time-temperature recommendations for using heat to inactivate fecal pathogens are provided by Feachem et al. (1983) and the US Environmental Protection Agency (US EPA) 40 CFR Part 503 regulations (US EPA, 2003). Both guidelines consider *Ascaris* eggs to be among the most resistant pathogens to thermal inactivation (Feachem et al., 1983; US EPA, 2003). According to the US EPA regulations, *Ascaris* viability is not substantially reduced at temperatures between 32 °C and 38 °C, and complete reduction at 38°C–50 °C is still not guaranteed (US EPA, 2003). Therefore, Feachem et al. and the US EPA suggest a minimum temperature of 45 °C and 50 °C, respectively, for any thermal

* Corresponding author. Hölderlingstr. 12, 72074, Tübingen, Germany.
E-mail address: l.angenent@uni-tuebingen.de (L.T. Angenent).

https://doi.org/10.1016/j.wroa.2019.100036

treatment, disqualifying many treatment options such as mesophilic (<45 °C) anaerobic or aerobic digestion (Feachem et al., 1983; US EPA, 2003).

Previous work already demonstrated that the Feachem and US EPA guidelines were extremely conservative at thermophilic temperatures (Aitken et al., 2005; Popat et al., 2010). Manser et al. (2015) demonstrated that mesophilic inactivation was feasible with *Ascaris* inactivation observed at 35 °C after exposure times of 16–24 days in anaerobic digesters and in phosphate buffer solutions. Several additional studies have investigated the effect of temperature on inactivation by other measures such as ammonia, pH, and peracetic acid (Fidjeland et al., 2015; Ghiglietti et al., 1995; Maya et al., 2012; Nordin et al., 2009; Pecson et al., 2007). All of these studies show that small changes in mesophilic temperatures have a large effect on inactivation rates, and the control treatments from some of these studies can also be used to examine the effect of temperature alone. The combined evidence from these studies clearly indicates that the recommended minimum temperature threshold for *Ascaris* inactivation is too high, but little work has been performed to decisively prove this or to establish a time-temperature relationship that is appropriate for mesophilic temperatures.

The Feachem and US EPA guidelines also make no distinction between time-temperature requirements for anaerobic and aerobic conditions, but inactivation has been found to be faster under aerobic conditions than under anaerobic conditions (Manser et al., 2015). Embryonic development of larvae occurs only in the presence of oxygen, and the eggshell is expected to weaken during this process, leaving the larvae more vulnerable to inactivation. This process is further supported by studies that have found faster inactivation of embryonated eggs compared to unembryonated eggs (Maya et al., 2012). The effect of anaerobic versus aerobic conditions may be particularly important at mesophilic temperatures compared to thermophilic temperatures because longer exposure times will be required during which embryonic development can occur.

Current beliefs about the temperature limits for thermal inactivation may be preventing development of useful, low-input treatment processes that could help provide safe excreta management for the 4.5 billion people that currently live without it (WHO and UNICEF, 2017). To better understand these temperature limits, we exposed *Ascaris suum* eggs to temperatures between 34 °C and 45 °C for a range of exposure times under anaerobic and aerobic conditions. We used this data to: 1) determine exposure times required to achieve a 3-log reduction of *Ascaris* viability at each temperature; 2) develop a new time-temperature relationship that can be used to predict *Ascaris* inactivation in this range; and 3) compare with evidence from the literature to confirm that our relationship is conservative but reasonable.

2. Methods

A. suum eggs were collected from fecal material in the intestines of naturally infected pigs and used as surrogates for *A. lumbricoides* in this study. Collected fecal material was passed through a series of six sieves ranging from US 10-US 100 to remove large debris, and eggs were collected on a US 500 sieve. The eggs were later purified from additional small debris by centrifugal flotation in a magnesium sulfate solution (specific gravity = 1.2) at 800×*g* for 5 min. Eggs were stored at 4 °C in 0.1 N H_2SO_4 until use.

A series of four exposure trials was conducted, with results from earlier trials used to select appropriate treatment conditions in subsequent trials (Table 1). Temperatures ranged between 34 °C and 45 °C. Temperatures below 34 °C were not tested because *Ascaris* eggs have been shown to form mobile larvae when incubated aerobically at temperatures up to 34 °C (Arene, 1986). For each treatment, 1000 *A. suum* eggs were suspended in 1.5 mL 0.1 N H_2SO_4 in a 2.0-mL microcentrifuge tube. Anaerobic and aerobic conditions were tested. The tube caps for all treatments were left open and sealed with Parafilm (Bemis NA; Neenah, WI) to allow for gas exchange. Tubes for anaerobic treatments were kept in gas-tight chambers that were made anaerobic using GasPak EZ Anaerobe Sachets (BD, Sparks, MD), and indicator strips (BD, Sparks, MD) were used to confirm anaerobic conditions. Tubes were stored in the dark in incubators, and temperatures were monitored using data loggers (HOBO UX100-003, Bourne, MA and Lascar Electronics EL-USB-1, Eerie, PA). All treatments were performed in duplicate.

After the designated exposure times, eggs were removed from incubators and transferred to 24-well plates, where they were suspended in 0.1 N H_2SO_4 and incubated aerobically at 28 °C for 3 weeks in the dark to promote larval development. The 24-well plates were monitored throughout the incubation period, and additional 0.1 N H_2SO_4 was added as needed to replace evaporated solution. Eggs were examined microscopically before and after the 3-week incubation. For eggs treated under anaerobic conditions, pre-incubation examination confirmed that they had not begun developing during treatment, which would have indicated oxygen exposure. For all treatments, viability was quantified after the 3-week incubation period. Approximately 500 eggs were examined in each well. Eggs that had formed larvae were considered viable, and all others were considered nonviable. If no viable eggs were found in the initial 500, the remaining eggs in the well were examined, to a maximum of 1000 eggs. Reported percent viabilities are normalized to the baseline viability of the *A. suum* eggs used for a given trial (Table 1). Baseline viability was determined by incubating *A. suum* eggs aerobically at 28 °C for 3 weeks with no prior treatment.

When chemically or thermally treating *Ascaris* eggs, a lag period is expected with minimal loss of viability followed by a region of exponential inactivation (Nordin et al., 2009; Pecson et al., 2007). For most treatments in the present study, the initial sampling time occurred after the lag period. Therefore, only exponential decline was observed, and inactivation was modeled using linear regression,

$$ln\left(\frac{N}{N_0}\right) = -kt + b \quad (1)$$

where N_0 is the baseline percent viability of the *A. suum* eggs used for the trial (Table 1), N is the percent viability of the *A. suum* eggs exposed to a given temperature for time t in days, k is the first-order inactivation constant, and b is the y-intercept, which gives some indication of the expected but unobserved lag period. If viability was below the detection limit for multiple exposure times, then only the shortest time was used in the regression analysis, and viability at that time was assumed to equal the detection limit. The linear regression equations for each temperature were then used to calculate the exposure time required to achieve a 3-log reduction in viability (t_3).

Next, linear regression of the base-ten logarithms of the anaerobic t_3 values versus temperature were used to develop a new time-temperature relationship for *Ascaris* inactivation at mesophilic temperatures. The upper limit of the 95% confidence interval for each t_3 value was used to develop a conservative relationship, and a literature review was performed to compile additional evidence of *Ascaris* inactivation at temperatures below 45 °C. All statistical analysis was performed using R (R Core Team, 2018), and confidence intervals were calculated using the chemCal package (Ranke, 2018).

Table 1
Summary of treatment conditions.

Trial	Baseline Percent Viability (SE[a])	Temperature (°C)	Anaerobic/Aerobic	Exposure Times (d)
1	73.6 (2.1)	36	Anaerobic	10, 15, 20, 24, 30, 42
1	73.6 (2.1)	36	Aerobic	10, 15, 30
2	67.5 (1.6)	40	Anaerobic	4, 8, 12, 16
2	67.5 (1.6)	40	Aerobic	2, 4, 8, 12
2	67.5 (1.6)	45	Anaerobic	0.75, 2, 4, 5
2	67.5 (1.6)	45	Aerobic	0.75, 1, 2, 4
3	67.5 (1.6)	36	Aerobic	2, 5, 8, 12
4	69.1 (1.2)	34	Aerobic	3, 5, 10, 15, 20
4	69.1 (1.2)	37	Anaerobic	6, 11, 16, 21, 40
4	69.1 (1.2)	39	Anaerobic	5, 11, 16, 21, 32

[a] Standard error of replicate samples tested for baseline viability. Three samples of 500 eggs were counted for Trial 1, and two samples of 500 eggs were counted for Trials 2–4.

Finally, we compared our time-temperature relationship to relationships provided by the Feachem guidelines and the US EPA Part 503 guidelines for producing Class A biosolids (Feachem et al., 1983; US EPA, 2003). Feachem et al. (1983) provides a figure with a "zone of safety" of time-temperature combinations that are expected to cause inactivation of *Ascaris* eggs based on literature studies, but the detection limits or degrees of inactivation in these studies are not known. The equation for the boundary of the "zone of safety" is also not given but was derived elsewhere (Equation (2)), where $t_{Feachem}$ is the required exposure time in hours, and T is the temperature in °C (Vinnerås et al., 2003).

$$t_{Feachem} = \left(1.77 \times 10^{2}\right) \times 10^{-0.1944(T-45)} \quad (2)$$

The US EPA recommended exposure time was calculated based on the time-temperature equation for sewage sludge with less than 7% total solids and a contact time of at least 30 min (Equation (3)), where t_{EPA} is the required exposure time in days, and T is the temperature in °C (US EPA, 2003).

$$t_{EPA} = \left(5.007 \times 10^{7}\right) \times 10^{-0.14T} \quad (3)$$

Equation (3) is expected to create Class A biosolids that contain less than 1 viable *Ascaris* egg per 4 g biosolids (dry weight basis). Equation (3) represents the least stringent of the four time-temperature relationships given by the EPA and was chosen here because our experimental data was collected with *Ascaris* eggs suspended in aqueous solution. The corresponding log reduction would depend on the initial concentration of viable eggs in the biosolids. It should be noted that the Feachem equation and the US EPA equation were only intended for use at temperatures ≥45 °C and ≥50 °C, respectively, but we have extended them here below the recommended minimums for comparison purposes.

3. Results and discussion

3.1. Ascaris inactivation under anaerobic conditions

Under anaerobic conditions, we observed *Ascaris* inactivation at temperatures between 37 °C and 45 °C when no additional inactivating agents were present (Fig. 1A), and we used linear regression of graphs of ln (N_0/N) versus time to determine inactivation parameters (k and b) and the time required for a 3-log reduction in *Ascaris* viability (t_3) (Table 2). At 45 °C, we observed rapid inactivation under anaerobic conditions, with viability reduced below the detection limit within 2 days (Fig. 1A, Table S1). Correspondingly, the t_3 calculated by linear regression was 2.12 days (Table 2). At 40 °C under anaerobic conditions, we observed a 2.19-log reduction after a maximum exposure time of 16 days (Fig. 1A, Table S1), and the calculated t_3 from linear regression was 20.5 days (Table 2). We did not test longer exposure times to confirm the t_3 value, but we have high confidence in the regression because all four time points occurred along the exponential portion of the inactivation curve (Fig. 1A), and the regression provided a good fit to the data with all inactivation parameters significant at $p < 0.05$ and $R^2 = 0.954$ (Table 2). Under anaerobic conditions at 39 °C, the exponential portion of the inactivation curve was fully captured, and we found no viable eggs after a 32-day exposure time (Fig. 1A, Table S1). Our linear regression fit the data well and yielded a t_3 of 31.8 days (Table 2).

The longest exposure time tested under anaerobic conditions at 37 °C was 40 days, at which point the results from the duplicate samples varied. Viability was reduced below detection (2.84-log reduction) for one sample, but only a 1.37-log reduction was observed for the second sample (Fig. 1A, Table S1). Correspondingly, the t_3 calculated by linear regression (57.5 days) was longer than 40 days (Table 2). The regression line fit the data well ($R^2 = 0.759$) but would be improved with an additional time point beyond 40 days to demonstrate viability below detection in both replicates and an additional point between 21 and 40 days to refine the rate of decline. Unfortunately, the three-week incubation time required between the end of the exposure period and examining the eggs for viability prevented us from recognizing the need for these treatments until after the experiment was completed. Longer exposure times were also needed to improve inactivation predictions under anaerobic conditions at 36 °C. The longest exposure time we tested was 42 days, and we observed minimal inactivation (0.321-log reduction), indicating that the exponential portion of the inactivation curve was not observed (Fig. 1A, Table S1). As expected, the resulting linear regression provided a poor fit to the data (Table 2).

3.2. Ascaris inactivation under aerobic conditions

Under aerobic conditions, we observed *Ascaris* inactivation at temperatures between 34 °C and 45 °C, with *Ascaris* viability reduced below detection during the tested exposure times at all temperatures. Inactivation at 45 °C under aerobic conditions was similar to the inactivation observed under anaerobic conditions with no viable eggs found after a 2-day exposure time (Fig. 1B, Table S1). For temperatures between 34 °C and 40 °C, mesophilic inactivation of *Ascaris* eggs was even more effective under aerobic conditions than under anaerobic conditions (Fig. 1B). Under aerobic conditions at 40 °C, no viable eggs were found in samples with an 8-day exposure time (detection limit = 2.69-log reduction). However, low viability (2.33-log reduction) was still observed in samples with a 12-day exposure time, and exposure times longer than 12 days were not tested (Fig. 1B, Table S1). The corresponding t_3 value calculated by linear regression was 12.6 days, which was

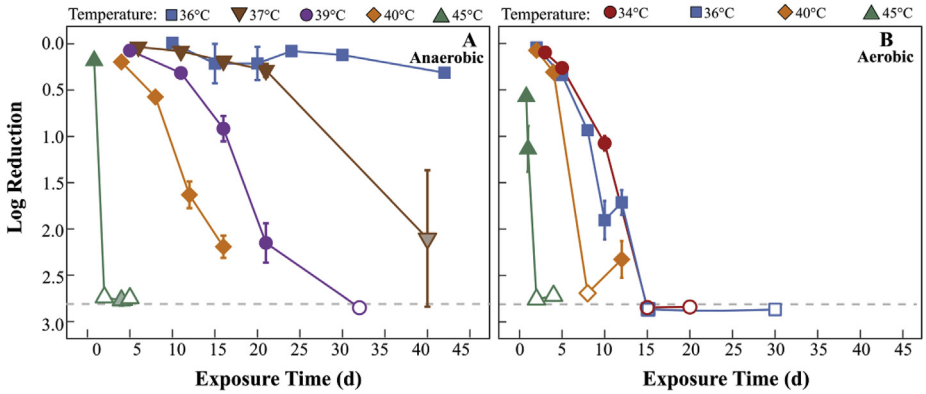

Fig. 1. Inactivation profiles of *Ascaris* eggs when exposed to mesophilic temperatures between 34°C and 45°C under anaerobic (A) and aerobic (B) conditions. Error bars show the range between duplicate samples. Open symbols indicate that no viable eggs were found in either sample, and log reduction is plotted as equal to the detection limit of the individual treatment. Symbols with a shaded fill (anaerobic, 37 °C, 40 d and anaerobic, 45 °C, 4 d) indicate that no viable eggs were found in one of the two duplicate samples. The dashed gray line marks the cumulative average detection limit of 2.81-log reduction. Raw data for each treatment is included as supplemental information (Table S1).

Table 2
Inactivation parameters determined by linear regression of experimental data and inactivation times recommended by three time-temperature relationships ($t_{Feachem}$, t_{EPA}, and $t_{Harroff}$). Standard errors for inactivation parameters are given in parentheses. Exposure time required for a 3-log reduction (t_3) was calculated using the inactivation parameters, and the lower and upper bounds of the 95% confidence interval are given in parentheses. Inactivation times required by the Feachem ($t_{Feachem}$) and US EPA (t_{EPA}) guidelines are given as comparison, as well as inactivation times given by the time-temperature relationship developed in the current study ($t_{Harroff}$, Equation (4)).

Anaerobic/Aerobic	Temperature (°C)	Linear regression of experimental data				Inactivation times predicted by time-temperature relationships		
		k (SE)	*b* (SE)	R^2	t_3 (d) (95% CI)	$t_{Feachem}$ [c] (d)	t_{EPA} [d] (d)	$t_{Harroff}$ [e] (d)
Anaerobic	36	0.0148 [a] (0.00976)	−0.0285 [a] (0.251)	0.187	465 [b] (−187; 1120)	414	457	151
	37	0.147 (0.0292)	1.53 (0.648)	0.759	57.5 [b] (32.4; 82.7)	265	331	95.4
	39	0.270 (0.0326)	1.67 (0.567)	0.907	31.8 (23.2; 40.4)	108	174	38.1
	40	0.406 (0.0363)	1.41 (0.397)	0.954	20.5 [b] (16.8; 24.2)	69.2	126	24.1
	45	4.71 (0.0703)	3.12 (0.106)	0.999	2.12 (2.03; 2.23)	7.38	25.1	2.44
Aerobic	34	0.521 (0.0583)	1.72 (0.541)	0.930	16.6 (12.1; 21.1)	1014	870	377
	36	0.501 (0.0435)	1.35 (0.422)	0.930	16.5 (13.1; 19.8)	414	457	151
	40	0.618 (0.140)	0.900 [a] (1.06)	0.763	12.6 (5.40; 19.9)	69.2	126	24.1
	45	3.94 (0.331)	1.50 (0.450)	0.973	2.13 (1.74; 2.52)	7.38	25.1	2.44

[a] Parameter is not significant at $p < 0.05$.
[b] Viability was not reduced below the detection limit during the exposure times tested. Inactivation times are therefore extrapolated.
[c] Feachem et al. (1983).
[d] US EPA (2003).
[e] Current study.

substantially shorter than the 20.5 days calculated for anaerobic conditions at the same temperature (Table 2). Inactivation rates under aerobic conditions at 34 °C and 36 °C were similar, with no viable eggs found after exposure times of 15 days for both temperatures (Fig. 1B, Table S1). The corresponding t_3 values calculated by linear regression were 16.6 and 16.5 days, respectively, which are less than half of the longest exposure time tested for 36 °C under anaerobic conditions that resulted in minimal inactivation (Fig. 1, Table 2).

3.3. We developed a time-temperature relationship for mesophilic temperatures and anaerobic conditions that is reasonable and conservative

Our results demonstrate that thermal inactivation of *Ascaris* eggs consistently occurs at temperatures between 37 °C and 45 °C under anaerobic conditions and between 34 °C and 45 °C under aerobic conditions, which directly contradicts current guidance from Feachem et al. (1983) and the US EPA (2003) that require minimum temperatures of 45 °C and 50 °C, respectively. Even if lower temperatures were considered sufficient, the time-temperature relationships provided by these guidelines predict substantially longer inactivation times than we found from our t_3 values (Table 2). Under anaerobic conditions, the Feachem guidelines suggest exposure times that are 3–5 times larger than our t_3 values, and the US EPA guidelines suggest times that are up to 12 times larger than ours (Table 2). Under aerobic conditions, the differences are even larger, particularly at lower temperatures of 34 °C and 36 °C. The recommended exposure time by Feachem et al. at 34 °C is two orders of magnitude larger than our t_3 value (Table 2). Therefore, a new time-temperature relationship specific for mesophilic temperatures is clearly necessary. We developed this relationship using linear regression of the base-ten logarithm of our anaerobic t_3 values as a function of temperature, rearranged to produce Equation (4),

$$t_{Harroff} = \left(2.20 \times 10^{9}\right) \times 10^{-0.199T} \quad (4)$$

where $t_{Harroff}$ is the exposure time in days required for a 3-log reduction, and T is temperature in °C. The upper limit of the 95% confidence interval was used for each t_3 value to develop a more conservative relationship. The t_3 calculated for anaerobic inactivation at 36 °C was excluded due to the poor fit of the linear regression model. Therefore, the model should only be applied with

confidence to temperatures between 37 °C and 45 °C under anaerobic conditions.

To evaluate the appropriateness of our time-temperature relationship outside of the current study, we compared it to mesophilic inactivation that has been observed previously in literature under anaerobic conditions (Fig. 2A, Table S2) (Cruz Espinoza et al., 2012; Johansen et al., 2013; Manser et al., 2015; Nordin et al., 2009; Pecson et al., 2007; Scheinemann et al., 2015). We selected literature data points that measured *Ascaris* viability without addition of external inactivating agents (*e.g.,* ammonia or volatile fatty acids) so inactivation is assumed to occur primarily due to temperature, although some variation is expected due to differences in moisture content, sludge properties, and intrinsic concentrations of inactivating compounds (Cruz Espinoza et al., 2012; Senecal et al., 2018). Many of the relevant literature data points were control treatments from larger studies. Individually, these data points prove little about mesophilic thermal inactivation, but they provide convincing evidence when combined. Details about each literature data point in Fig. 2, as well as other studies that are discussed here, are included as supplementary data (Table S2). Details in Table S2 include exposure conditions, how inactivation times were determined, and the degree of inactivation that was measured in each study.

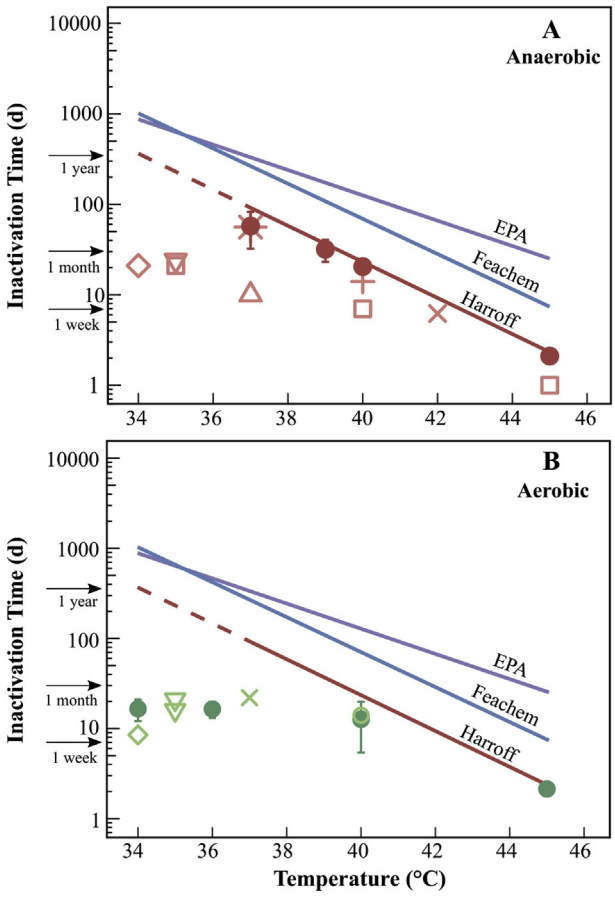

Fig. 2. Proposed time-temperature relationship for a 3-log reduction of *Ascaris* eggs at 34°C-45°C. Our relationship (Harroff) is compared to the Feachem and US EPA guidelines. Data points show t_3 values from this study (●) with error bars representing the 95% confidence interval. Literature data points are also shown from Cruz Espinoza et al., 2012) (□), Ghiglietti et al. (1995) (○), Johansen et al. (2013) (△), Manser et al. (2015) (▽), Nordin et al. (2009) (◇), Pecson et al. (2007) (+), Scheinemann et al. (2015) (∗), and Tharaldsen and Helle (1989) (×). All data points in panel A (red) were observed under anaerobic conditions, and all data points in panel B (green) were observed under aerobic conditions. Our relationship (Harroff) is shown as a dashed lined between 34 °C and 37 °C because the experimental data used to develop it did not extend to these temperatures. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

With all literature data points falling at or below our time-temperature relationship and the t_3 values generated from regression of experimental data, we conclude that the time-temperature relationship developed here is more reasonable than the Feachem and US EPA relationships for temperatures between 37 °C and 45 °C, while still being conservative. The conservative nature of our time-temperature relationship can be attributed to the use of aqueous solutions for exposing eggs in our experimental results versus the use of manure or fecal sludge matrices for the literature points. Past work looking at *Ascaris* inactivation in autoclaved versus non-autoclaved anaerobic fecal material at 30 °C showed that compounds may be biologically produced during anaerobic fermentation that contribute to *Ascaris* inactivation (Harroff et al., 2017). Other studies examining *Ascaris* inactivation by ammonia have also found that results from exposures in aqueous solutions are conservative compared to those in manures and fecal sludge (Nordin et al., 2009; Pecson et al., 2007; Schuh et al., 1985). Here, the relationship that we developed using aqueous exposures appears more conservative at lower temperatures than at higher temperatures, indicating that intrinsic inactivating compounds in sludge became less important at warmer temperatures within the mesophilic spectrum (Fidjeland et al., 2015).

Even though overly conservative guidelines may hamper the use of simple treatment, the use of a conservative time-temperature relationship is still important for protecting public health when developing waste treatment strategies. For this reason, our time-temperature relationship should still be confirmed at temperatures less than 37 °C using exposures in aqueous solutions. Although literature values demonstrate inactivation at 34 °C and 35 °C at exposure times less than those predicted by our relationship (Cruz Espinoza et al., 2012; Manser et al., 2015; Nordin et al., 2009), intrinsic factors in those treatments, such as ammonia and solids concentration, may have affected inactivation times. For example, Manser et al. (2015) observed 99% inactivation of *Ascaris* eggs after 24 days in an anaerobic digester at 35 °C, but another study observed only 50% inactivation after 35 days in a 35 °C digester (Johnson et al., 1998). Both times are far below the 238 days required for a 3-log (99.9%) inactivation by our time-temperature relationship, but they suggest a need for more information.

3.4. The time-temperature relationship we developed also provides conservative recommendations under aerobic conditions

Recent work by Manser et al. (2015) compared inactivation times required for *Ascaris* inactivation under anaerobic and aerobic conditions at 34 °C (24 days and 16 days, respectively) and concluded that aerobic conditions cause faster inactivation. An earlier study compared anaerobic and aerobic digestion at 37 °C and 47 °C and reached the opposite conclusion that anaerobic conditions caused faster inactivation. However, the authors acknowledged that the actual temperatures in the aerobic digesters were uncertain due to the use of circulating air to keep them oxygenated. Therefore, the results of that study are inconclusive (Kato et al., 2003). Our experimental data supports the conclusion of Manser et al. (2015) at a range of mesophilic temperatures between 34 °C and 40 °C (Fig. 1), which has not been shown before. In addition, our data shows that the effect of oxygen exposure decreases as temperature increases. Three temperatures (36 °C, 40 °C, and 45 °C) were tested under both anaerobic and aerobic conditions. We cannot directly compare the 36 °C treatments because

exponential decline was not observed under anaerobic conditions, but the t_3 calculated for 37 °C under anaerobic conditions is 3.5 times larger than the t_3 predicted for 36 °C under aerobic conditions (Table 2). Meanwhile, at 40 °C the t_3 under anaerobic conditions is only 1.6 times larger than the t_3 under aerobic conditions (Table 2). The t_3 values at 45 °C for anaerobic and aerobic conditions are equal, which indicates that the effect of oxygen becomes negligible at this temperature (Table 2). Aerobic conditions likely cause faster inactivation because the larvae undergo embryonic development when they have access to oxygen, and they consume lipids and carbohydrates within the eggshell to cause structural and chemical changes that may leave the larvae more susceptible to environmental conditions (Arene, 1986; Fairbairn, 1957; Manser et al., 2015). However, inactivation at 45 °C is likely too rapid (2.1 days) to allow for significant embryonic development, which causes the effect of oxygen presence to be negated.

Previous studies have shown aerobic inactivation at times and temperatures consistent with ours, but they did not directly compare inactivation rates under anaerobic and aerobic conditions (Fig. 2B, Table S2). In control treatments of a pH 7 saline solution, Ghiglietti et al. (1995) observed 99% inactivation of *Ascaris* eggs after exposure to 40 °C for 14 days (Fig. 2B). Aerobic conditions were not explicitly indicated in the Ghiglietti study, but embryonic development was observed during the exposure time for treatments at lower temperatures so we can assume that oxygen was present in the treatments (Ghiglietti et al., 1995). In comparison, our t_3 for 40 °C under aerobic conditions was slightly lower at 12.6 days. Tharaldsen and Helle (1989) found that *Ascaris* eggs in mechanically aerated pig manure slurry at 37 °C were 99% inactivated between 14 and 22 days, which is shown in Fig. 2B as 22 days, and we observed similar inactivation at 36 °C with a predicted t_3 of 16.5 days.

Nordin et al. (2009) examined *Ascaris* inactivation from different ammonia concentrations at 34 °C and reported results that also indicate an effect of aerobic conditions. In one treatment of fecal material containing 43 mM NH_3 ammonia (maximum pH = 8.3, total ammonia [NH_3+NH_4] = 247 mM), 99% inactivation was predicted after 21 days using linear regression. For a treatment using diluted urine containing 40 mM NH_3 ammonia (maximum pH = 8.7, total ammonia [NH_3+NH_4] = 131 mM), 99% inactivation was predicted after only 8.5 days. With similar NH_3 ammonia concentrations, the study concluded that unmonitored factors contributed to the different inactivation rates. We propose here that oxygen was the primary unmonitored factor. For the fecal material treatment, eggs were contained in mesh bags and inserted in 200 g of fecal material in sealed containers, leading to anaerobic conditions. For the urine treatment, the mesh bags were suspended in urine contained in a sealed 50-mL tube. However, the tube was opened on days 1, 2, 7, and 10 for sample collection, allowing the urine to be oxygenated each time and decreasing the required exposure time for inactivation. A control treatment of 0.9% NaCl solution at the same temperature also provided an interesting result because eggs were only 7% inactivated after 31 days. Based on our experimental results, we expect eggs in aerobic conditions at 34 °C to be 99.9% inactivated after 16.6 days. However, results for the control are only reported after 31 days. Without the frequent sampling interval used for the urine treatment, the control would have been anaerobic, and we would not expect to see significant inactivation after 31 days. Results reported for the urine treatment are expectedly faster than ours due to the added inactivating effects of ammonia in the urine.

Other studies have examined the effect of temperature on rates of embryonic development in *Ascaris* and found that eggs could not fully develop into motile larvae at temperatures between 34 °C and 45 °C (Arene, 1986; Gaspard et al., 1996; Seamster, 1950). We cannot predict the required exposure times for these studies because the eggs were never removed from the warmer temperatures, but embryonic development is expected to occur within 14–21 days, and therefore we can assume that required exposure times would be less than 21 days for temperatures between 34 °C and 45 °C. Similarly, all of our predicted inactivation times for the same temperature range under aerobic conditions were less than 21 days.

Based on the faster inactivation observed under aerobic conditions than under anaerobic conditions (Fig. 1, Table 2), a separate time-temperature relationship should be developed for aerobic conditions. However, more experimental data is needed to accurately capture the effect of oxygen and how it increases at lower temperatures (Table 2). Therefore, we recommend using the time-temperature relationship developed under anaerobic conditions (Equation (4)) as a conservative predictor for aerobic conditions. By comparing our data points under aerobic conditions, results from literature under aerobic conditions, and our time-temperature relationship given by Equation (4), we confirm that anaerobic conditions present a conservative case (Fig. 2B).

These results also demonstrate the importance of strictly maintaining and monitoring anaerobic and aerobic conditions when testing *Ascaris* inactivation by temperature and other mechanisms. Inactivation that is observed under aerobic conditions may not occur under anaerobic conditions. Monitoring of additional pathogens may also be required for mesophilic treatment systems, particularly under aerobic conditions. Previous work has shown that *Escherichia coli* and *Salmonella* are also inactivated faster under aerobic conditions than under anaerobic conditions (Pandey et al., 2016), but the extent to which different pathogens are affected is not known, and it is possible that other pathogens will prove more resilient than *Ascaris* eggs under certain conditions.

3.5. Limitations

Caution must be used when applying the results and time-temperature relationship shown here to large-scale systems, where required exposure times for inactivation may be affected by uneven heating, fluctuating temperatures, and shielding effects of solids (Popat et al., 2010; Senecal et al., 2018). For example, composting systems often do not reach expected temperatures, leading to poor pathogen inactivation (Mehl et al., 2011). Those that do reach proper temperatures have large spatial and temporal variations that make thermal inactivation difficult to predict. In one study, measured temperatures ranged between <30 °C and nearly 50 °C in different portions of a compost heap over a 120 day period, and over 100 days of exposure was required to observe >99% *Ascaris* inactivation (Jensen et al., 2009). Another study observed temperatures >60 °C. *Ascaris* inactivation would typically be expected within minutes at such high temperatures, but 6 days were required to reduce viability below detection, indicating pockets with a lower temperature (Szabová et al., 2010). For this reason, the US EPA guidelines require longer exposure times for sewage sludges with higher solids content (US EPA, 2003). In addition to variation within treatment systems, Pecson and Nelson (2005) also have observed variability in temperature resiliency between batches of *Ascaris* eggs. Temperature must be closely controlled and monitored for any treatment system relying on thermal inactivation of pathogens. For mesophilic systems in particular, pathogen inactivation should be verified for each unique system.

4. Conclusions

- Given adequate exposure times, *Ascaris* eggs are consistently inactivated under anaerobic conditions at mesophilic temperatures between 37 °C and 45 °C and under aerobic conditions at

mesophilic temperatures between 34 °C and 45 °C without the addition of external inactivating agents.

- The relationship $t_{Harroff} = (2.20 \times 10^9) \times 10^{-0.199T}$ can be used to conservatively estimate exposure times (t, days) required to achieve a 3-log reduction of *Ascaris* viability at temperatures (T) between 37 °C and 45 °C under both anaerobic and aerobic batch conditions, when uniform temperature occurs.
- This relationship is still conservative, but much less conservative than current relationships that are used as standards. Therefore, reduction of minimum temperature thresholds in regulations for thermal waste treatment processes should be considered.
- Mesophilic waste treatment processes should be given greater consideration when considering how to treat the excreta of the 4.5 billion people globally who currently lack access to safely managed sanitation services (WHO and UNICEF, 2017). Mesophilic systems can be less expensive and easier to operate than thermophilic systems, and they are shown here to potentially provide adequate pathogen treatment.
- Thermal inactivation of *Ascaris* eggs is faster under aerobic conditions than under anaerobic conditions. Therefore, presence of oxygen should be monitored more closely in research. New waste treatment systems should be developed that use this advantage of oxygen to inactivate pathogens.
- *Ascaris* is less resistant to thermal inactivation than is often stated, and more work is needed to evaluate the circumstances under which it can be used as a reliable indicator organism.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

LAH thanks Lucinda Li for valuable laboratory assistance and acknowledges support from the Cross-Scale Biogeochemistry and Climate NSF IGERT program (Award #1069193) and the NSF Graduate Research Fellowship Program (Award #1650441). LTA acknowledges support from the Alexander von Humboldt Foundation in the framework of the Alexander von Humboldt Professorship endowed by the Federal Ministry of Education and Research in Germany. We acknowledge support by Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of University of Tübingen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wroa.2019.100036.

References

Aitken, M.D., Sobsey, M.D., Blauth, K.E., Shehee, M., Crunk, P.L., Walters, G.W., 2005. Inactivation of *Ascaris suum* and poliovirus in biosolids under thermophilic anaerobic digestion conditions. Environ. Sci. Technol. 39 (15), 5804–5809.

Arene, F., 1986. *Ascaris suum*: influence of embryonation temperature on the viability of the infective larva. J. Therm. Biol. 11 (1), 9–15.

Brudastov, A., Krasnonos, L., Lemelev, V., Kholmukhamedov, S., 1970. Infectivity of *Ascaris lumbricoides* eggs to man and Guinea-pigs after 10 years in the soil. Med. Parazitol. Parazit. Bolezni 39 (4).

Cruz Espinoza, L.M., Yeh, D., Vinnerås, B., Rajaram, L., Whiteford, L., Corvin, J., Izurieta, R., 2012. Inactivation of *Ascaris suum* by ammonia in feces simulating the parameters of the solar toilet. J. Appl. Sci. Environ. Sanit. 7 (3).

De Silva, N.R., Chan, M.S., Bundy, D.A.P., 1997. Morbidity and mortality due to ascariasis: re-estimation and sensitivity analysis of global numbers at risk. Trop. Med. Int. Health 2 (6), 513–518.

Fairbairn, D., 1957. The biochemistry of *Ascaris*. Exp. Parasitol. 6 (5), 491–554.

Feachem, R.G., Bradley, D.J., Hemda, G., Mara, D.D., 1983. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management (Washington, DC).

Fidjeland, J., Nordin, A., Pecson, B.M., Nelson, K.L., Vinnerås, B., 2015. Modeling the inactivation of *Ascaris* eggs as a function of ammonia concentration and temperature. Water Res. 83, 153–160.

Gaspard, P., Wiart, J., Schwartzbrod, J., 1996. A method for assessing the viability of nematode eggs in sludge. Environ. Technol. 17 (4), 415–420.

Ghiglietti, R., Rossi, P., Ramsan, M., Colombi, A., 1995. Viability of *Ascaris suum, Ascaris lumbricoides* and *Trichuris muris* eggs to alkaline pH and different temperatures. Parassitologia 37 (2–3), 229.

Harroff, L.A., Liotta, J.L., Bowman, D.D., Angenent, L.T., 2017. Inactivation of *Ascaris* eggs in human fecal material through in situ production of carboxylic acids. Environ. Sci. Technol. 51 (17), 9729–9738.

Jensen, P.K., Phuc, P.D., Konradsen, F., Klank, L.T., Dalsgaard, A., 2009. Survival of *Ascaris* eggs and hygienic quality of human excreta in Vietnamese composting latrines. Environ. Health 8 (1), 57.

Johansen, A., Nielsen, H.B., Hansen, C.M., Andreasen, C., Carlsgart, J., Hauggard-Nielsen, H., Roepstorff, A., 2013. Survival of weed seeds and animal parasites as affected by anaerobic digestion at meso- and thermophilic conditions. Waste Manag. 33 (4), 807–812.

Johnson, P., Dixon, R., Ross, A., 1998. An in-vitro test for assessing the viability of *Ascaris suum* eggs exposed to various sewage treatment processes. Int. J. Parasitol. 28 (4), 627–633.

Kato, S., Fogarty, E., Bowman, D.D., 2003. Effect of aerobic and anaerobic digestion on the viability of *Cryptosporidium parvum* oocysts and *Ascaris suum* eggs. Int. J. Environ. Health Res. 13 (2), 169.

Manser, N.D., Wald, I., Ergas, S.J., Izurieta, R., Mihelcic, J.R., 2015. Assessing the fate of *Ascaris suum* ova during mesophilic anaerobic digestion. Environ. Sci. Technol. 49 (5), 3128–3135.

Maya, C., Torner-Morales, F., Lucario, E., Hernández, E., Jiménez, B., 2012. Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH and dryness. Water Res. 46 (15), 4770–4782.

Mehl, J., Kaiser, J., Hurtado, D., Gibson, D.A., Izurieta, R., Mihelcic, J.R., 2011. Pathogen destruction and solids decomposition in composting latrines: study of fundamental mechanisms and user operation in rural Panama. J. Water Health 9 (1), 187–199.

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 (3), 662–667.

Pandey, P.K., Cao, W., Wang, Y., Vaddella, V., Castillo, A.R., Souza, A., del Rio, N.S., 2016. Simulating the effects of mesophilic anaerobic and aerobic digestions, lagoon system, and composting on pathogen inactivation. Ecol. Eng. 97, 633–641.

Pecson, B.M., Barrios, J.A., Jiménez, B.E., Nelson, K.L., 2007. The effects of temperature, pH, and ammonia concentration on the inactivation of *Ascaris* eggs in sewage sludge. Water Res. 41 (13), 2893–2902.

Pecson, B.M., Nelson, K.L., 2005. Inactivation of *Ascaris suum* eggs by ammonia. Environ. Sci. Technol. 39 (20), 7909–7914.

Popat, S.C., Yates, M.V., Deshusses, M.A., 2010. Kinetics of inactivation of indicator pathogens during thermophilic anaerobic digestion. Water Res. 44 (20), 5965–5972.

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Satistical Computing, Vienna, Austria.

Ranke, J., 2018. chemCal: Calibration Functions for Analytical Chemistry. R package version 0.2.1.

Scheinemann, H.A., Dittmar, K., Stöckel, F.S., Müller, H., Krüger, M.E., 2015. Hygienisation and nutrient conservation of sewage sludge or cattle manure by lactic acid fermentation. PLoS One 10 (3).

Schuh, R., Philipp, W., Strauch, D., 1985. Influence of Sewage Sludge with and without Lime Treatment on the Development of Ascaris suum Eggs. Elsevier Applied Science Publishers, London.

Seamster, A.P., 1950. Developmental studies concerning the eggs of *Ascaris lumbricoides* var. *suum*. Am. Midl. Nat. 43 (2), 450–470.

Senecal, J., Nordin, A., Simha, P., Vinnerås, B., 2018. Hygiene aspect of treating human urine by alkaline dehydration. Water Res. 144, 474–481.

Szabová, E., Juriš, P., Papajová, I., 2010. Sanitation composting process in different seasons. *Ascaris suum* as model. Waste Manag. 30 (3), 426–432.

Tharaldsen, J., Helle, O., 1989. Survival of parasite eggs in livestock slurry utilized for compost heat. Acta Agric. Scand. 39 (4), 381–387.

US EPA, 2003. Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge (Including Domestic Septage) under 40 CFR Part 503 (Cincinnati, OH).

Vinnerås, B., Björklund, A., Jönsson, H., 2003. Thermal composting of faecal matter as treatment and possible disinfection method––laboratory-scale and pilot-scale studies. Bioresour. Technol. 88 (1), 47–54.

WHO, UNICEF, 2017. Progress on Drinking Water, Sanitation and Hygiene: 2017 Update and SDG Baselines (Geneva, Switzerland).