A Method for Determining *Ascaris* Viability Based on Early-to-Late Stage In-Vitro Ova Development

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ABSTRACT: This study suggests a new method for determining the viability of *Ascaris* spp. ova, based on in-vitro early-to-late stage development of ova. This method includes stages prior to larval development, providing an estimation of potential viability. After application of biosolids onto soil and exposure to 7°C, 22°C, or 37°C for 45 days, ova were microscopically distinguished as viable or non-viable according to progression through development categories. Results were compared to viability estimates from current methods that distinguish viable ova as motile larva. Results suggest conventional techniques underestimate viability, whereas the new method provides a more conservative approach.

INTRODUCTION

ASCARIS LUMBRICOIDES is the most common roundworm infecting humans, causing 1.3 billion illnesses worldwide [1–2]. Ascariasis is endemic in areas of Africa, Latin America, and the Far East suffering from poverty and poor sanitation [1,3–4]. Particularly, wherever people defecate around settlements and in geographical regions where night soil (human faeces) is applied as an agricultural fertilizer [1,5]. Female worms produce 240,000 ova per day, all of which are passed by the infected host via faeces [1,5–8]. Soil and fecal-oral transmissions are routine as ova are deposited in high abundances, then ingested via hand-to-mouth contact from contaminated objects, or consumed with polluted crops, meat, or water [1,3,5,9–10].

Survival of *Ascaris* spp. ova after land application of biosolids can be highly variable, depending on soil composition and climate, as well as, other abiotic and biotic factors. Williams *et al.*, indicated that exposure to different soil types and temperatures influenced *A. suum* ova inactivation [11]. *Ascaris* spp. are most prevalent in tropical and sub-tropical regions, but occur worldwide in various climates [12–13]. Yet, even though developmental time depends on geographic area and climate, there is a lack of understanding of *Ascaris* spp. survival and inactivation in arid and semi-arid climates [14–15]. One objective of this study is to determine the survivability of *A. suum* ova in arid biosolid-amended soils.

Methods to extract and concentrate *Ascaris* spp. ova from biosolids via flotation and sedimentation have proven to be adequate [16–19]. However, there is not an universally accepted assay for determining viability of ova [16]. Staining techniques are rapid, as viable ova contain multiple layers that are impermeable, whereas ova that are permeable are assumed to be non-viable [20–22]. Potential infectivity is assumed at the moment ova remain impermeable. However, some slightly permeable ova are stained with a light appearance and are assumed to be non-viable [20]. Without monitoring embryo development it is unknown whether slightly stained ova were actually viable, or if some non-viable ova remained impermeable.

Real-time PCR (qPCR) may suggest viability, as signals drastically increase throughout the progression of ova development. In principle, viable ova will grow into infective larval stages that contain approximately 600 cells, with qPCR signals increasing as more cells are produced [23]. Whereas non-viable ova will remain single-celled and provide a low signal [23]. However, this method may be subjective as it incorporates total nucleic acid, without any discrepancy for infectious and non-infectious ova.

Current microscopy methods dictate viable ova as those containing motile distinguishable larvae, but all others are non-viable [16,24–27]. The Environmental Protection Agency (EPA) method classifies *Ascaris*

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spp. ova into six stages of a life cycle, labeling unembryonated ova as non-viable [17]. Yet, these methods are subject to major constraint, as they do not monitor embryo development, and/or consider all ova displaying early-stages of embryonation, prior to motile larvae, to be non-viable.

The sequential development of *A. suum* ova outside of a host has been documented into 12 stages; 1-cell, 2-cell, 3-cell, 4-cell, early-morula, late-morula, blastula, gastrula, pre-larva 1, pre-larva 2, first-stage larva (L_1), and second stage larva (L_2) [28]. Current microscopy techniques do not include these stages in the determination of viability and are underestimating the development of ova [28–29]. Cruz *et al.* suggest that early developmental stages, prior to larvae, are capable of developing into infectious stages and must be considered when determining viability [28].

The goal of the present study was to develop a methodology for enumerating the viability of *Ascaris* spp. ova with consideration to all development stages. Therefore, a new method would not assume that ova prior to containing distinguishable larvae are non-viable. Also, the requirement for motility of cell structures and/or larvae inside the ova would be disregarded, as ova that are stationary during microscopy are often viable. Such a method would provide enumeration of potential viability, revising the viewpoint of possible infection and human health risks associated with *A. lumbricoides* ova in biosolids, wastewater, compost, and soils.

In the present study, the viability of *A. suum* ova was compared utilizing the current microscopy methods and a new development-stage enumeration technique created by the authors. *A. suum* ova were used as a model for *A. lumbricoides*, as the two are morphologically and genetically similar, yet the swine type is less infectious, easier to acquire, and more resistant to environmental stresses [10,12,30–31]. Results obtained by both methods were compared to determine any significant differences between the methods.

2. MATERIALS AND METHODS

2.1. Viable and Non-Viable Ova Controls

Concentrated *A. suum* ova were purchased from Excelsior Sentinel, Inc. (Trumansburg, NY, USA), and were initially tested by both enumeration methods to ensure viability. Briefly, a suspension containing ova was serially diluted directly from the packaging to a concentration of approximately 100 ova/ml, aliquoted into 250 µl triplicates in 24-well culture plates containing equal amounts of 0.2 N H_2SO_4 (to prevent the growth of fungi), counted for total number, and examined via light microscopy for ova development throughout a 30-day incubation at ambient temperature (22°C). Viability was determined by the criteria for each enumeration technique, as described in Section 2.6.

Briefly, 227 ova were placed into a 15 ml conical tube and submerged in a water bath at 52°C for 24 hours. Then, ova were aliquoted into designated 24-well culture plates containing equal amounts of 0.2 N H_2SO_4 and observed via microscopy. The total number of viable-intact ova were examined via microscopy prior to heat exposure. The same ova were examined via microscopy post-thermal inactivation to observe non-viable morphology. Inactive ova appeared as dark-oval shapes, often containing bubbles, and did not progress in development over the incubation period (Figure 1). The resulting heat inactivation provided a control to visualize dead (non-viable) ova.

Death (D) by heat inactivation was calculated as follows:

$$D = \frac{N_d}{N_i} \times 100 \quad (1)$$

where N_d represents the number of non-viable ova after thermal inactivation, and N_i represents the number of viable ova prior to thermal inactivation. Non-viable ova were examined over 30 days at ambient temperature (22°C) to ensure no further embryonation or development.

2.2. Microcosm Preparation

Microcosms were created to simulate the decay of *A. suum* ova in arid soil amended with biosolids incubated at 7°C, 22°C, and 37°C for up to 45 days. Class B biosolids (anaerobic digestion followed by dewatering) were obtained from a local wastewater treatment plant. The mean total solids content was determined to be 6.2 ± 0.1%, (Standard Method 2540 G) [32]. The pH of the biosolid was adjusted to 7 by the addition of 1 N NaOH.

Brazito sandy loam soil was collected from the University of Arizona Agricultural Center in Tucson, AZ using a shovel down to a 6-inch depth. Soil was mixed in a capped bucket, and then large matter was separated and removed by passage through a 2 mm sieve. The mean soil moisture content was determined to be 8.4 ± 0.1 (Standard Method D2216) [33].

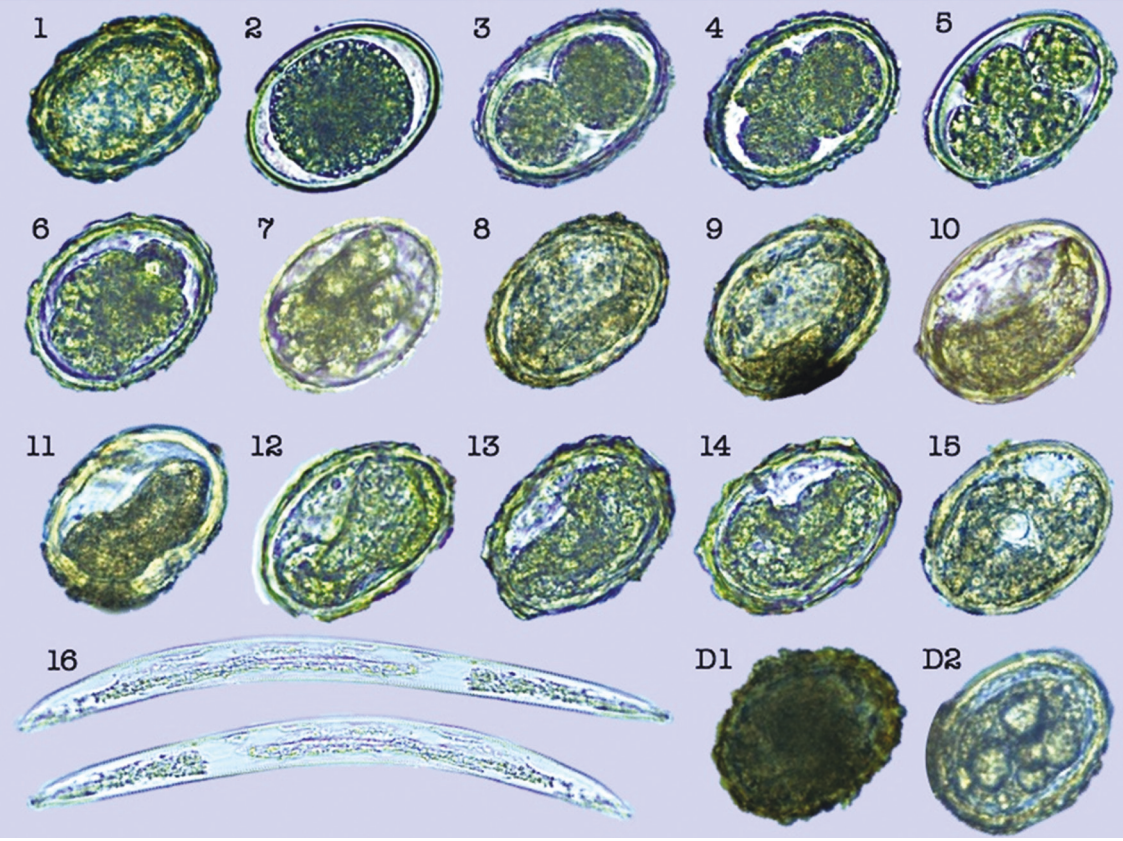

Figure 1. *A. suum ova development-stage chart for classifying ova. Unembryonated; stage 1; Embryonated, stages 2 – 7; Well-developed, stages 8 – 15; Excystation, stage 16. Dead/non-viable A. suum ova; stage D1 (disfigured dark-oval structure) and/or D2 (bubbled yolk from heat inactivation).*

Briefly, 50 µl of *A. suum* ova (3.0×10^5 ova/ml; >90% viability) was added directly to 1.5 g of Class B biosolids and mixed vigorously for several minutes in an aluminum dish to obtain a homogenous mixture of approximately 10,000 ova/g of total solids, which is the same mean concentration of night soil [5]. Using a flat spatula, the entire 1.5 g of ova-biosolid mixture was transferred to the surface of 50 g of Brazito sandy loam soil in an uncovered 50 ml beaker. The aluminum dish was rinsed with DI water to ensure the entire mixture was transferred. Then, the spatula was used to till the top few cm of the soil surface to blend in the ova-biosolid mixture. Additional deionized (DI) water was added to the biosolid-amended soil to increase the moisture content of each microcosm to approximately 22.25% (less than 25% suggested for pathogen destruction) [12,34]. A total of 48 microcosms were prepared.

2.3. Microcosm Exposure to Experimental Conditions

Triplicate microcosms were held at 7°C (refrigerator), 22°C (ambient temperature), or 37°C (incubator), and processed at time intervals of 0 (control), 5, 15, 30, and 45 days. Microcosms were not exposed to light and temperature was held constant throughout the time intervals. Ambient temperature was held constant at 22°C in a laboratory room that received pre-set automatic air conditioning without any windows or air ventilation obstructions. Temperatures were monitored via thermometers (cat no. S01639; Fisher Scientific, Waltham, MA, USA) placed alongside the microcosms. Greater than 90% moisture loss was achieved within 24 hours of incubation for samples held at 37°C, and within 48 hours for samples held at 7°C and 22°C.

2.4. Extraction of Ova from Biosolid-Amended Soil

A. suum ova were extracted from biosolid-amended soil microcosms according to a combination of modified Wisconsin and Tulane flotation methods, previously described [16,18]. The total material in each microcosm was transferred into a 250 ml polypropylene bottle, suspended in 125 ml of DI water, and agitated for 30 seconds. Then, additional DI water was added to

the bottle to create a solution volume of approximately 250 ml, and agitated for one minute. The bottle was centrifuged (Allegra® X-15R; Beckman Coulter, Inc., Brea, CA, USA) at 2383 × g for 15 minutes and the resulting supernatant was poured into a bucket containing pure bleach. The process was repeated three times, until the supernatant was clear and solids aggregated at the bottom of the bottle, to ensure sedimentation of *A. suum* ova into the pellet.

The process was repeated a fourth time with Sheather's sucrose solution (1420 ml DI water, 1.81 kg white granulated sugar, 24 ml Formalin) instead of DI water. Specific gravity of the solution was tested to be approximately 1.27 using a hydrometer. The bottle was centrifuged at 2690 × g for 30 seconds and one minute. The upper portion of the resulting supernatant, containing a Sheather's sucrose solution and *A. suum* ova mixture, was poured into stacked 63 µm (top) and 38 µm (bottom) sieves (Erie Scientific, Portsmouth, NH, USA). Large particles collected on the 63 µm sieve were rinsed with DI water into the bucket containing bleach. *A. suum* ova and other contents collected on the 38 µm sieve were rinsed with 15 ml of DI water into a 15 ml conical tube (Falcon® 352097; Becton Dickinson, Franklin Lakes, NJ, USA). The tube was centrifuged at 2690 × g for 5 minutes and the resulting supernatant was aspirated down to a 5 ml volume with a small pellet (0.1 to 0.3 ml) at the bottom. The supernatant was vortexed to dismantle the pellet and create a homogenous solution. If necessary, the solution was serially diluted with 0.2 N H_2SO_4.

To ensure the extraction process was not detrimental to ova viability, triplicate microcosms were created and inoculated with ova directly to obtain a concentration of 10,000 ova per/g. Then, the ova were extracted and viability was determined via both enumeration techniques throughout a 30-day incubation at ambient temperature in culture plates, as described below.

2.5. 30-Day Incubation and Microscopy Observations

Following extraction, 250 µl volume of concentrated ova and 250 µl of 0.2 N H_2SO_4 were added into designated 24-well culture plates (ref. no. 3526, Corning Inc., Corning, NY, USA). Each well was incubated at ambient temperature (22°C) and counted for the total number of ova after 0, 12, 15, and 30 days. Ova observations and total counts from days 0 and 30 were used to enumerate viability, as described in Section 2.6. Brief observations at days 12 and 15 were made to ensure ova remained intact, the total number of ova was consistent over time (check for human error), and there were no fungi growing in the wells. Ova were counted by scanning each well left-to-right under light microscopy (model no. 82026-630; VWR VistaVision, Radnor, PA, USA). Ova development was examined with a 100× magnification lens via light microscopy (model no. 82026-630; VWR VistaVision, Radnor, PA, USA) and confirmed with a 60× magnification image projected onto a screen via digital microscopy (EVOS XL Cell Imaging System; Advanced Microscopy Group, Bothell, WA, USA). Approximately 200 ova were collected into each well and multiplied by the appropriate serial dilution to determine the actual amount of ova contained in the original samples.

2.6. Enumeration of *A. suum* Ova Viability

Figure 2 displays how viability is determined via the conventional microscopy observations. Figure 1 displays the development stages for designating ova into categories based on the in-vitro development-stage. These techniques are described below.

2.6.1. Conventional Microscopy Technique

After the 30-day incubation in culture plates at ambient temperature (22°C), ova from each well were observed via microscopy. Ova suspected of containing a larva (Figure 2) were examined for 5–10 minutes and categorized as viable if motility was observed. Motile larvae that were currently exiting from ova (excystation) were also considered viable. All other ova, regardless of development or motility, were considered inactive and non-viable (Figure 2). Viability (V_c) was calculated as follows:

$$V_c = \frac{(N_w + N_e)}{N_t} \times 100 \qquad (2)$$

where N_w indicates the number of ova containing larvae, N_e indicates the number of motile larva with current excystation, and N_t indicates the total number of viable and non-viable ova observed.

2.6.2. In-Vitro Development-Stage Enumeration Technique

Ova observed via microscopy were distinguished based on development stages, and grouped into cat-

egories, as visually represented (Figure 1): unembryonated, cortication intact with indistinguishable cells inside (stage 1); embryonated, containing one to several individual cells (stages 2–7); well-developed, containing a distinguishable structure comprised of conglomerated cells (stages 8–15); or excystation, containing larva that was currently or had recently exited from an ovum (stage 16). These categories are based upon the 12 stages of development, previously described [28]. Ova that did not progress in development, had a dark-oval disfigured structure (Figure 1, D1), and/or contained bubbled yolk (Figure 1, D2) similar to those seen in the thermal inactivation-exposure control were considered dead/non-viable. Several visual examples of ova designated into each category are provided in the supplementary material (Figure S1 – S7).

Total counts of ova designated into each category were used to calculate the total number of ova that had progressed in development and could be considered viable. In brief, total counts of ova designated into each category via microscopy were collected during days 0 and 30 of incubation in culture plates at ambient temperature. The differences in ova assigned to each category before and after incubation were used to estimate viability. As the total number of ova increased for categories describing further development stages, the approximate number of ova capable of progressing to infectious stages could be determined. Since ova were grouped into categories, motility and development of individual ovum was not monitored. Therefore, Viability (V_d) was calculated as follows:

$$V_d = \frac{(N_{md} + N_{wd} + N_{zd}) - (N_{ui} + N_{mi} + N_{wi})}{N_t} \quad (2)$$

where N_{md} indicates the number of embryonated ova after the 30-day incubation, N_{wd} indicates the number

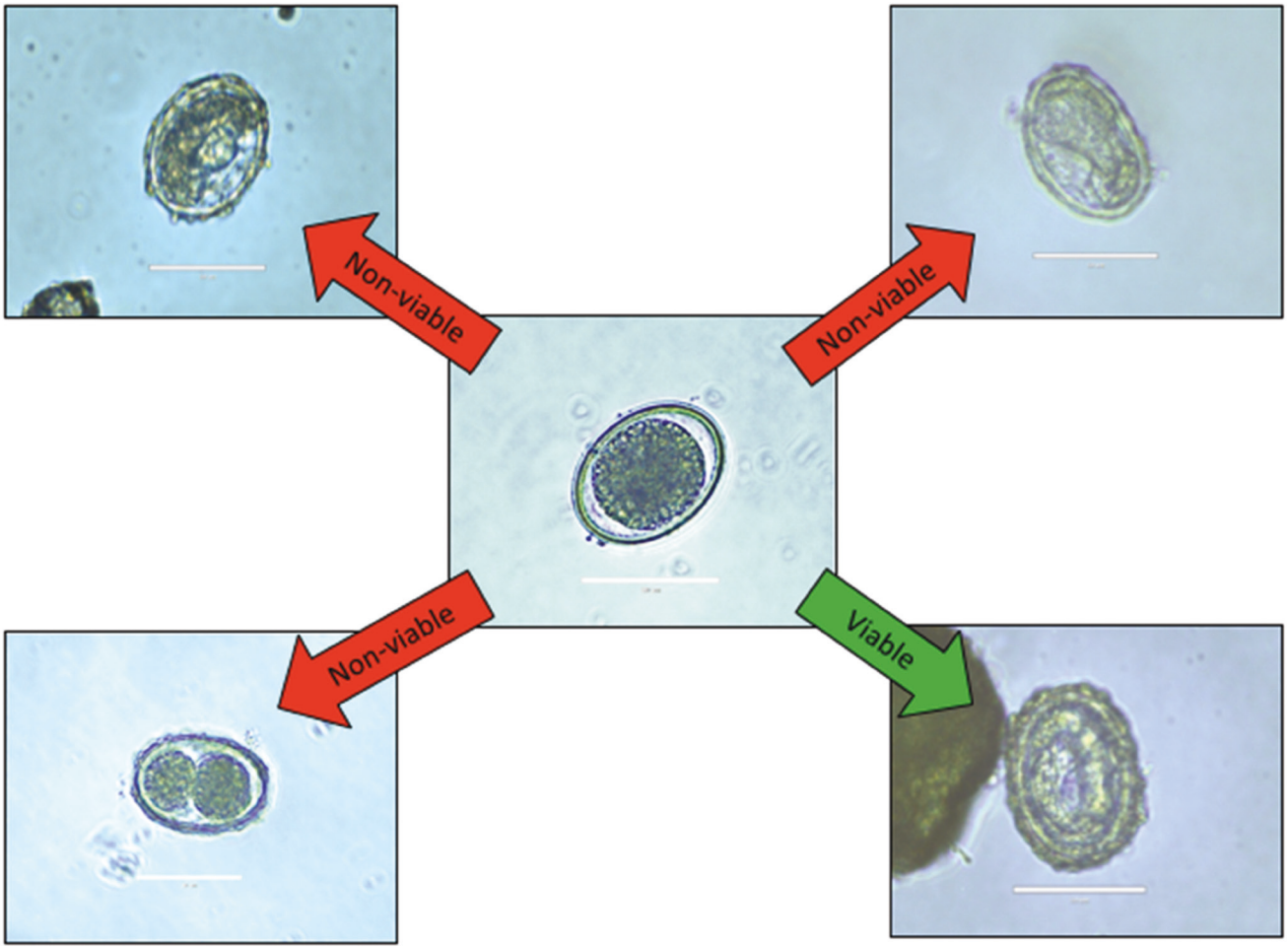

Figure 2. *Viable and non-viable ova observations determined via the conventional microscopy method. Arrows represents time elapsed between day 0 and 30 observation periods, and distinguish viable and non-viable ova based on development characteristics. Green arrows, viable; Red arrows, non-viable.*

of well-developed ova after the 30-day incubation, N_{zd} indicates the number of excyst ova after the 30-day incubation, N_{ui} indicates the number of unembryonated ova prior to incubation, N_{mi} indicates the number of embryonated ova prior to incubation, N_{wi} indicates the number of well-developed prior to incubation, and N_t indicates the total number of viable and non-viable ova.

2.7. Statistical Analysis

The number of viable ova per gram of biosolid-amended soil (V_g) was calculated as follows:

$$V_g = \frac{\left[\frac{(V_c \text{ or } V_d) \times N_i}{100}\right]}{W} \quad (4)$$

where V_c indicates the percent viability determined via the conventional enumeration method, V_d indicates the percent viability determined via the development-stage enumeration method, N_i indicates the number of ova inoculated into each microcosm, and W indicates the initial wet weight of the microcosm (soil + biosolid + *A. suum* ova + DI water).

The inactivation of viable ova per gram of biosolid-amended soil (I) was calculated as follows:

$$I = \frac{N}{N_o} \times 100 \quad (5)$$

where N is the number of viable ova after incubation, and N_o is the number of viable ova at time zero (Day 0 control).

Student's *t*-tests were performed with Microsoft Excel for Mac 2015 (Microsoft Corp., Redmond, WA, USA) to determine whether the conventional and development-stage enumeration methods resulted in significantly different numbers of viable ova per gram of biosolid-amended soil (P value was ≤ 0.05).

3. RESULTS

3.1. Viable and Non-Viable Ova Confirmations

Ova from each control were examined utilizing the conventional and development-stage enumeration techniques. Both methods verified *A. suum* purchased from Excelsior Sentinel, Inc. (Trumansburg, NY, USA), contained 98% viability (Table 1). Prior to incubation in culture plates, all ova were unembryonated. After incubation, 159 out of 162 ova developed into motile larvae and were considered viable according to the conventional method. Since these ova exhibited progression throughout the 30-day period, they were also considered viable according to the in-vitro development-stage method. Only a single ovum remained unembryonated and did not show any progression in development, so it was considered non-viable according to both methods.

Heat-exposure at 52°C for 24 hours in a water bath resulted in 226 out of 227 ova without observable viability before and after a 30-day incubation at ambient temperature. All of these ova appeared dark and often contained bubbles. Disfigured morphology and the absence of development confirmed greater than 95% inactivation (Table 1). One ovum appeared normal and was able to develop into a motile larva, so was designated as viable by both methods.

The extraction-control microcosm showed viability greater than 97% via both enumeration methods (Table 1), indicating the extraction process was not detrimental to ova that were recovered. Examinations of developmental stages and inactivated ova (Figure 1) from control samples provided visual confirmations for comparison when observing microcosm samples.

3.2. Comparison of Viability Methods

The conventional and development-stage enumera-

Table 1. Viable, Non-Viable, and Extraction Controls.

	Enumeration Technique— Mean Viability and Death	
Control	**Conventional (%)**	**Development-Stage (%)**
Prior to Exposure (Viability confirmation)	**98 ± 0.03** (159/162)	**98 ± 0.03** (159/162)
Heat Inactivation (Non-viable confirmation)	*99.5* (226/227)	*99.5* (226/227)
Extraction (Process confirmation)	**97.2 ± 0.03** (375/387)	**97.2 ± 0.03** (375/387)

Bold numbers indicate the mean viability and standard deviation of ova from triplicate control samples. Italicized numbers indicate the mean death rate of ova exposed to 52°C for 24 hours in a water bath. Numerator; total number of viable ova in the viability/process confirmation tests, or the number of non-viable ova after the heat exposure tests. Denominator; total number of ova in the control tests.

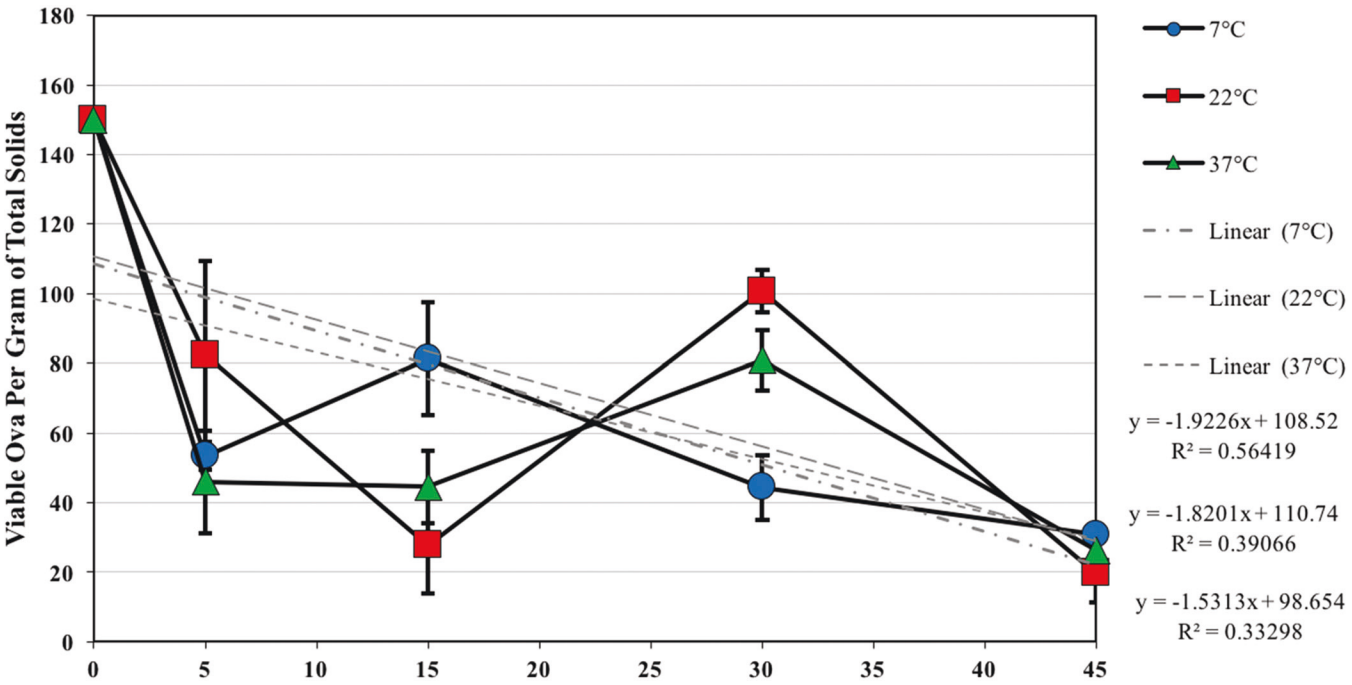

Figure 3. *Number of viable ova per gram of biosolid-amended soil enumerated via the conventional method. Viability at different temperatures (●, 7°C; ■, 22°C; ▲ 37°C). Inactivation rate determined via the slope of the best fit lines. Duration; number of days in the microcosms.*

tion methods resulted in significantly different numbers of viable ova per gram of biosolid-amended soil from the same microcosms incubated at 7°C, 22°C, or 37°C for 5 or 15 days, or 7°C for 30 days, as determined via Student's *t*-tests (*P* value ≤ 0.05) (Table 2; Figures 3 and 4). The two methods did not result in significantly different numbers of viable ova from samples that were incubated at 22°C and 37°C for 30 days, or any microcosm incubated for 45 days, regardless of temperature (Table 2; Figures 3 and 4). Enumeration via the conventional method resulted in greater inactivation and log10 reduction of viable ova in the biosolid-amended soil than the development-stage enumeration (Figures 5 and 6).

Table 2. Viability and Inactivation Based on Enumeration Methods.

		Conventional			Development-Stage			
Temperature (°C)	Time (Days)	Viable (ova/g)	Inactivation (%)	Reduction Viable (log_{10})	Viable (ova/g)	Inactivation (%)	Reduction Viable (log_{10})	*P* value
	0	150 ± 0	—	—	150 ± 0	—	—	—
7	*5*	53 ± 4	64 ± 3	1.81	111 ± 13	26 ± 8	1.40	0.002
	15	81 ± 16	46 ± 11	1.65	125 ±18	17 ± 12	1.15	0.033
	30	44 ± 9	70 ± 6	1.85	63 ± 4	58 ± 3	1.76	0.032
	45	31 ± 0	80 ± 0	1.90	39 ± 0	74 ± 0.0	1.87	—
22 (Ambient)	5	83 ± 27	45 ± 18	1.63	142 ± 20	6 ± 13	1.32	0.037
	15	28 ± 14	82 ± 9	1.91	99 ± 26	34 ± 17	1.49	0.014
	30	101 ± 6	33 ± 4	1.51	113 ± 13	25 ± 9	1.38	0.229
	45	20 ± 8	87 ± 6	1.94	22 ± 8	86 ± 5	1.93	0.771
37	5	46 ± 15	69 ± 10	1.84	130 ± 14	13 ± 9	0.99	0.002
	15	45 ± 10	70 ± 7	1.85	125 ± 23	17 ± 15	1.01	0.005
	30	81 ± 9	46 ± 6	1.66	86 ± 4	42 ± 3	1.63	0.372
	45	26 ± 7	82 ± 5	1.92	42 ± 13	72 ± 8	1.86	0.269

Mean and standard deviation viability, inactivation, and log_{10} reduction determined via the conventional and development-based enumeration methods. Temperature; indicates the degrees Celsius at which the microcosm was incubated. Time; indicates the number of days at which the microcosm was incubated. *P* value of Student's *t*-tests comparing viability (ova/g) determined via the conventional and development-stage enumeration techniques (*P* value was ≤ 0.05).

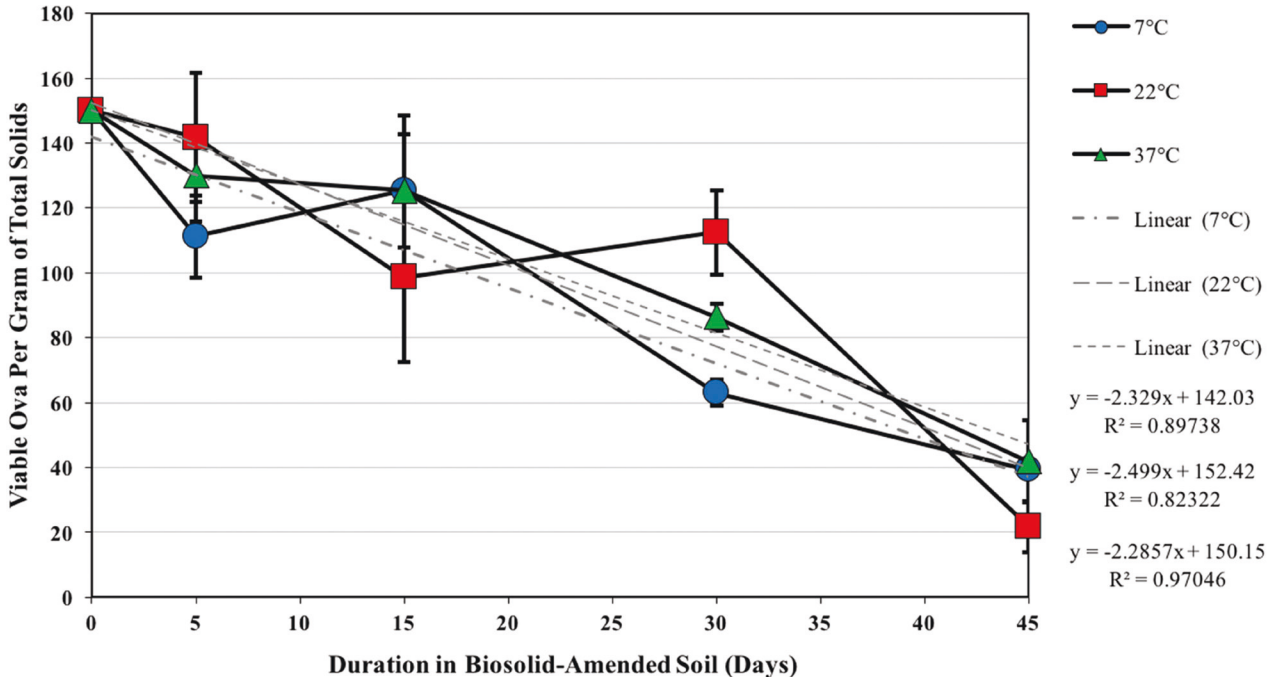

Figure 4. *Number of viable ova per gram of biosolid-amended soil enumerated via the development-stage method. Viability at different temperatures (●, 7°C; ■, 22°C; ▲ 37°C). Inactivation rate determined via the slope of the best fit lines. Duration; number of days in the microcosms.*

3.3. Temperature Effects on *A. suum* Ova Inactivation

The rate of ova inactivation was determined by the slope of the best fit line and linear regression (Figures 5 and 6). The rate of inactivation was similar at all temperatures, but was slightly greater for the development-stage method than the conventional method (Figures 5 and 6). The conventional method assumed rapid inactivation and decrease in viable ova five days after biosolids were applied onto soil (Figures 3 and 5).

4. DISCUSSION

This study created a new microscopy method that considers ova as viable, regardless of development stage, based on observations of in-vitro characteristics before and after a 30-day incubation at ambient temperature. This study also determined the survival of *A. suum*. ova in biosolids applied to arid soils. *A. suum* was used as a model organism for the development, survival, and inactivation of *A. lumbricoides*, as it is much easier to handle in the laboratory [30–31].

The new microscopy method tallies counts of ova into groups based on attributes observed via microscopy. Then, viability is enumerated based on the total number of ova that proceeded into a further development category after a 30-day incubation at ambient temperature. These ova are assumed to be capable of continuing development into infectious stages and are considered viable. This method is simple, as microscopy observations and ova counts are only needed before (day 0) and after (day 30) incubation, without the need to monitor the development of individual ova. This method is often less time consuming than the conventional microscopy method, as observing motility is not required to designate viability, which can take 5–10 minutes per ovum [28]. However, differentiating characteristics throughout ova development may be subjective and requires experience observing *Ascaris* spp. via microscopy. Results from this method were compared to the conventional microscopy technique that bases viability on motile larvae within the ova, and determined a significant difference in the assessment of viability (Student's *t*-tests P value was ≤ 0.05).

Our results suggest that the conventional method underestimates the number of potentially viable *A. suum* ova by not considering early-stages and the capability to progress into infectious stages. Microcosms containing arid soil amended with biosolids (*A. suum* inoculated) were subjected to different temperatures for 45 days. Within the first 15 days, the conventional method suggested significantly lower viability of *A. suum* ova than the development-stage method (P value was < 0.05). This was expected, as many ova in samples processed within two weeks of application onto soil

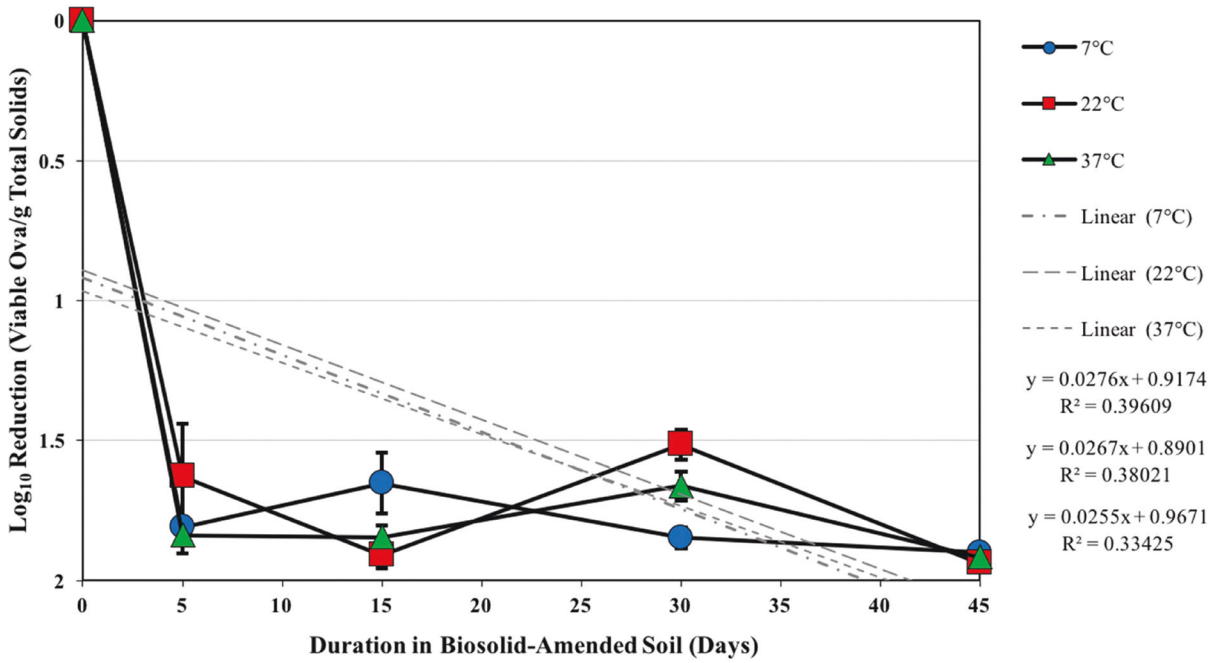

Figure 5. *Log_{10} reduction enumerated via the conventional method. Reduction at different temperatures (●, 7°C; ■, 22°C; ▲ 37°C). Inactivation rate determine via the slope of the fit lines provided in the legend. Duration; number of days in the microcosms.*

were not given enough time to form larvae, so were considered non-viable via the conventional method. Yet, the development-stage method enumerated higher viability of the same ova, by estimating the total number of ova that progressed in development, including those prior to larval stages. This enabled the assessment of viability in recently applied human fecal material, as viable and non-viable ova could be differentiated prior to the formation of larvae which usually requires more time. Consequently, the development-stage method provides a more conservative approach and is more suitable for assessing ova viability in soils recently (within 15 days) amended with biosolids and/or night soil.

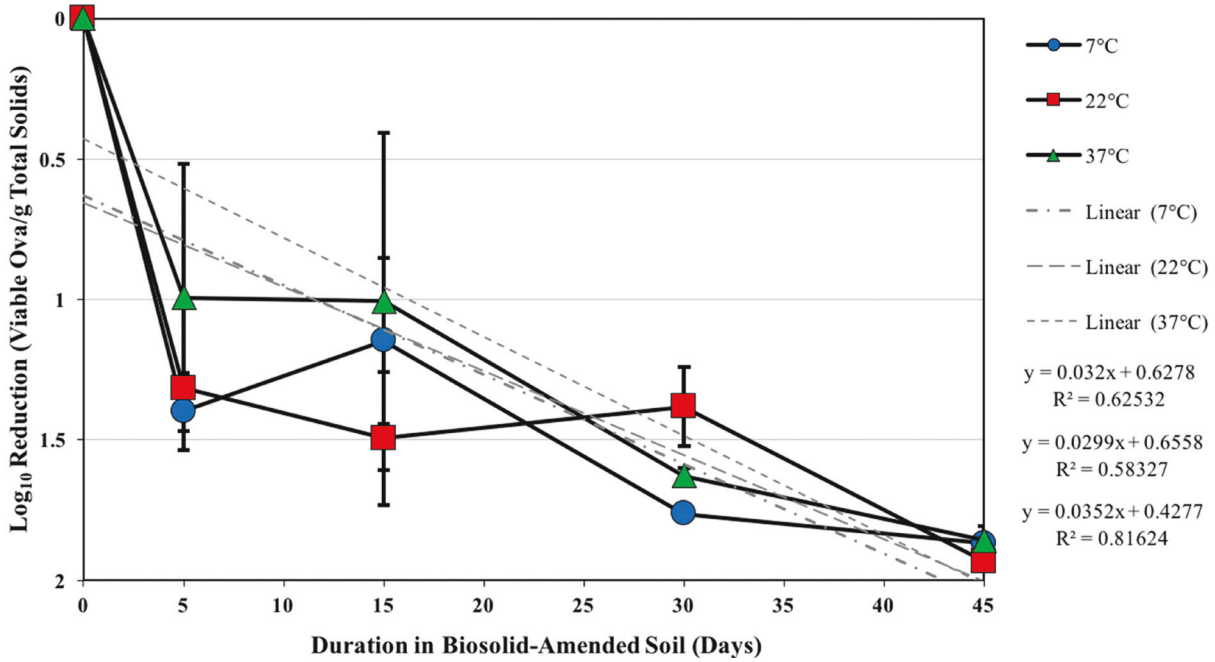

Figure 6. *Log_{10} reduction enumerated via the development-based method. Reduction at different temperatures (●, 7°C; ■, 22°C; ▲ 37°C). Inactivation rate determine via the slope of the fit lines provided in the legend. Duration; number of days in the microcosms.*

The conventional method estimated a lower number of viable ova/g of total solids in microcosms incubated longer than 15 days, except at 7°C for 30 days. However, viability assessments were not significantly different from those enumerated via the development-stage method. This was a result of environmental stresses causing increased inactivation, while slowing and/or halting development. Therefore, fewer ova were potentially viable, leading to similar assessments between the two enumeration methods. This indicates that either method may be suitable for determining ova viability in soils impacted by fecal material for more than 30 days. Yet, the development-stage technique may provide a more conservative approach, as our results indicated slightly higher numbers of viable ova/g total solids.

Practical applications for utilizing the in-vitro development-stage method are relatively unknown as this study only analyzed ova viability in arid soils amended with biosolids. We expect this method to be more applicable in tropical and sub-tropical regions where *A. lumbricoides* is prevalent and environmental conditions are favorable for ova development [12–13]. Further research is needed to determine the usefulness of this method for determining ova viability in applications other than human fecal materials applied onto agricultural soils, such as sewage sludge treatment processes.

Previous research suggests that high temperature, low moisture content, and biotic factors influence *A. suum* ova inactivation [5,15,35]. *A. lumbricoides* ova may be diminished under these conditions in the arid Southwest region of the United States. Ova have previously been reported to only survive for 2–4 weeks under dry and sunny conditions [35]. Williams *et al.*, suggested that survival within soil types is influenced by soil holding moisture ability [11]. The present study utilized the same sandy loam soil as Williams *et al.*, adjusted the moisture content to 22.25%, and created microcosms to determine *A. suum* inactivation at different temperatures. The greatest period of ova inactivation coincided with >90% moisture loss achieved within 24–48 hours, suggesting low moisture content was a major cause of ova death.

Whereas, the rate of ova inactivation seemed to be independent of temperature conditions. *Ascaris* spp. ova are typically inactivated when held at temperatures greater than 45°C (lethal temperature) for long periods of time [7]. This study exposed *A. suum* ova to conditions lower than the lethal temperature for 45 days. Accordingly, the rate of inactivation was similar for all microcosms held at 7°C, 22°C, and 37°C (Figures 5 and 6). However, our results suggest that inactivation fluctuated over time, as the number of viable ova increased in samples held at constant 22°C between 15 and 30 days, and 7°C between 5 and 15 days (Figures 5 and 6). This was most likely due to separate microcosms being processed for each time point, causing ova to be exposed to unknown inconsistencies between samples and/or extraction procedures. Nonetheless, all microcosms had similar numbers of viable ova after 45 days in land applied biosolids, suggesting that inactivation was not influenced by temperature over long periods of time.

Biotic factors that occur naturally in soil may have influenced *A. suum* ova in the microcosms. In particular, fungi may have interfered with ova development [35]. Ova extracted from microcosms were incubated in 0.2 N H_2SO_4 to prevent the growth of fungi in culture plates. However, soil was not autoclaved prior to creating the microcosms. Thus, fungi may have affected the survival and inactivation of *A. suum* ova during the simulation of contaminated land applied fecal material, especially over time. Since we did not incorporate a sterilize-soil control, we are not able to disclose the influence that biotic factors had on ova development. Therefore, we suggest that in arid soils with temperatures below 40°C, ova inactivation primarily results from low moisture content and/or biotic factors.

In conclusion, this study details a new method for assessing the viability of *Ascaris* spp. in biosolid-amended soils. This method incorporates the potential for early-stage ova to develop into infectious stages. Whereas, conventional microscopy methods underestimate viability by disregarding ova that do not contain motile larvae. When comparing the two enumeration techniques, the in-vitro development-stage method suggested significantly higher viability of ova in recently amended soils. Therefore, the in-vitro development-stage method provides a more conservative estimation of potential viability that agencies can consider when creating regulations and guidelines intended to minimize human health risks associated with *A. lumbricoides* ova in soils amended with human fecal materials. Also, this study demonstrates that ova inactivation in arid soils is primarily due to biotic factors and/or low moisture conditions.

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