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# Experimental evaluation of *Ascaris* egg survival during vermicomposting of faeces

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Tiger worm toilets (TWTs) are a form of non-sewered sanitation that uses composting worms, such as *Eisenia fetida* (tiger worms), to convert human waste into vermicompost. Previous studies have shown low concentrations of faecal coliforms in vermicompost. However, this is the first study to assess whether TWT-produced vermicompost contains surviving parasitic helminth eggs. To evaluate this, six laboratory-scale biodigesters, replicating the tiger worm loadings and bedding layer types in a TWT, and three control units (the same setup without tiger worms) were fed identically with pig faeces spiked with a known concentration of *Ascaris* eggs for four weeks. After this time, the pig faeces in the biodigesters had been transformed into vermicompost, whereas the pig faeces in the control units remained unchanged. A standard multiple-straining method was then used to analyse and count *Ascaris* eggs in the coir, gravel, and end products of both biodigesters and control units. Findings revealed that the tiger worms did not degrade the *Ascaris* eggs, and viability analysis confirmed that the eggs that were present in the vermicompost remained viable. In the biodigesters, most *Ascaris* eggs were found in the coir layer (48–72% of the total eggs), whereas in the control units, they were primarily concentrated in the pig faeces layer at the top (71–74% of the total eggs). Eggs were also detected in the gravel and effluent of biodigesters, but not in that of the control units, likely due to the tiger worms' movement between layers of the biodigester. There was a significant difference in egg distribution across biodigester layers ($p$ = 0.03), indicating that some layers consistently retain higher *Ascaris* egg concentrations. This study, therefore, indicates that TWT end products, including vermicompost and effluent, are not considered safe for agricultural use, as per World Health Organization guidelines. Therefore, these products require safe disposal or further treatment before use, particularly in settings with a high prevalence of ascariasis.

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## Water impact

This study demonstrated that *Eisenia fetida* (tiger worms), commonly used in tiger worm toilets (an emerging on-site sanitation technology), do not remove or inactivate *Ascaris* eggs in faeces under conditions replicating the toilets. This has important implications for how vermicompost produced within such toilets is used (*e.g.* in agriculture) and safely disposed of in ascariasis-endemic regions of the world.

## 1. Introduction

Tiger worm toilets (TWTs) are a relatively new form of non-sewered sanitation technology, with the oldest TWTs now having been in continuous operation worldwide for only approximately 10 years.[1] TWTs employ composting worms, such as *Eisenia fetida*, also known as tiger worms, to degrade human waste into vermicompost and carbon dioxide, thereby reducing latrine fill rates, fly presence, and odour.[1] Over the long term, TWTs are cost-effective due to reduced emptying frequency as compared to traditional pit latrines.[2,3] Moreover, studies on human waste treated through vermifiltration have reported reductions of up to 98.6% in faecal coliforms, along with significant decreases in chemical oxygen demand (COD) and total solids.[1,4] The resulting vermicompost is safer to handle, transport, and dispose of compared with untreated faecal sludge, thereby improving the safety of latrine emptying. A laboratory-scale biodigester treating blackwater produced high-quality effluent, achieving a 7.45 log reduction of *E. coli*, 99% removal of biochemical oxygen demand (BOD), 98% removal of COD, and 97–99% nutrient removal.[5] Similarly, a recent *meta*-analysis reports BOD removal ranging from 70–95% and COD removal from

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65–90%, with faecal coliform reductions exceeding 99%.[6] A full-scale vermifilter in Switzerland, treating domestic blackwater and sedimented greywater, achieved >75% removal of BOD, COD, and total suspended solids, and 91 ± 15% removal of organic micropollutants.[7] In addition, low concentrations of microplastic fibres were detected in the effluent, and *E. coli* was reduced by 3.67 log.[8] Furthermore, a vermifiltration study on sewered wastewater reported approximately 90% reductions in total dissolved solids, suspended solids, and turbidity.[9] Though these findings are encouraging, further treatment would be required to comply with World Health Organization (WHO) guidelines for the safe use of latrine-derived end products in agriculture.

Indeed, TWT end products (vermicompost and effluent) contain appreciable nutrient levels, indicating their potential value for agricultural applications.[8,10,11] However, reuse of this material must adhere to WHO guidelines, which recommend that treated end products (*i.e.* the vermicompost derived from TWTs) intended for safe disposal and reuse contain no more than one viable helminth egg per gram of total solids.[12] This threshold is established to mitigate the risk of soil-transmitted helminth (STH) infections, which remain among the most prevalent infections worldwide, affecting over 1.5 billion individuals, or approximately 24% of the global population.[13,14] STHs comprise four main nematodes: *Ascaris lumbricoides* (*A. lumbricoides*), *Trichuris trichiura*, and the two hookworms (*Necator americanus* and *Ancylostoma duodenale*). Among these, *Ascaris* is the most prevalent, affecting approximately 1.2 billion people, and is considered a conservative indicator for assessing sanitation safety due to its high persistence and resistance to treatment relative to other pathogens.[15,16] The WHO recommends large-scale deworming to prevent STH infections, but long-term control also requires sustained improvements in water, sanitation, and hygiene.[17–19]

Evidence on the fate of helminth eggs during vermicomposting remains limited and somewhat contradictory. Therefore, the potential ability of tiger worms to inactivate or degrade parasitic helminth eggs remains unclear. This is an important question to answer to ensure the protection of public health during vermicompost handling, land application, and food production.

In one study, vermicomposting of septic tank sludge, which underwent 30 days of pre-composting (short aerobic treatment to release ammonia and partially break down the sludge) followed by 60 days of composting by tiger worms, reduced viable helminth eggs to fewer than 1 egg per gram of dry weight.[10] In another study using a physically mixed feedstock of 70% municipal sewage sludge and 30% chopped/shredded water hyacinth (stems/leaves), the final vermicompost contained no detectable helminth eggs.[20] However, the initial feed had a low egg concentration (0.63 eggs per g of dry weight), and egg counts in the control system (without tiger worms) also decreased to 0.5 eggs per g of dry weight, suggesting that the reduction cannot be attributed solely to vermicomposting.[20] Field vermicomposting of biosolids at 15–20% total solids (~80–85% moisture) led to rapid reductions in *Ascaris* egg counts, up to 1.9 log (~98.9%) within 144 hours in a tiger worm-treated unit.[21] However, control systems also showed a 0.6 log (~74.2%) reduction, making it unclear whether the decreases were due to tiger worm-mediated inactivation or simply physical loss of *Ascaris* eggs.[21] Yet another study, using a mixture of 50% sewage sludge and 50% cow dung, resulted in substantial but incomplete reductions (~80–90%) of *Ascaris* eggs.[22] However, once again, approximately 74% reduction also occurred in the control systems.

The present study aims to evaluate the presence and viability of parasitic helminth eggs in vermicompost produced under TWT conditions. Unlike previous studies, this research did not use pre-treated feedstocks or co-substrates[10,20–22] but instead replicated TWT conditions to isolate and assess the specific role of tiger worms in the degradation of parasitic helminth eggs in vermicompost. To achieve this, laboratory-scale biodigesters were set up to replicate the tiger worm mass and bedding layer types in TWTs, along with control units without tiger worms. These systems were fed pig faeces spiked with known concentrations of *Ascaris suum* (*A. suum*) eggs obtained from infected pigs. The setup was used to assess the presence, absence, and viability of *A. suum* eggs under controlled conditions.

## 2. Methods

### 2.1 Setting up the laboratory-scale experiment

The laboratory-scale setup, tiger worm density, and feeding regime of this study were adapted from the TWT prototype developed by Furlong *et al.*[4] A 30 L polypropylene container with a tap at the bottom and a cover on top was used. The container had a height of 41 cm and a diameter of 38.5 cm. The container was smaller than the vermifiltration system in the reference study, in order to allow multiple replicates, facilitate practical handling, and avoid eggs being lost in a large container. This enables the main research question to be addressed: whether the tiger worms inactivate or degrade *Ascaris* eggs. The depths of the drainage and bedding layers were calculated based on the container's surface area and scaled accordingly. To create the biodigesters, first, a drainage layer composed of gravel of varying sizes between 20 and 30 mm was placed at the bottom of the container. The drainage layer maintains aerobic conditions and ensures the tiger worms can breathe.[23] Secondly, an organic bedding layer was prepared by soaking 500 g of dry, compressed coconut coir in 4 L of water for 1 hour, allowing it to expand into a loose, fibrous structure. The expanded coconut coir was then fully drained by hand pressing, ensuring it was neither waterlogged nor compressed. The moist coconut coir was then added on top of the drainage layer.

Six laboratory-scale biodigesters and three control units (without tiger worms) were thus installed in a controlled-environment room at Ghent University

 

(Belgium) in March 2025 and maintained at 23 ± 1 °C. Each container had a 15 cm deep gravel layer at the bottom (step 1 in Fig. 1), topped with a 15 cm layer of coconut coir (step 2 in Fig. 1), with the remaining 11 cm left as headspace. Each of the six biodigesters (B1–6) contained around 500 g of tiger worms (supplier: Wormenkwekerij Wasse, the Netherlands) (step 3 in Fig. 1), while the control units (C1–3) had the same setup without tiger worms. Every day, 100 g of fresh pig faeces from helminth-free farm pigs was used to feed all of the containers (step 4 in Fig. 1).

Moisture content within the coir layer was measured during each feeding using a moisture meter. The coir layer functioned as both the bedding material and the primary habitat for tiger worms, maintaining moisture within the optimal range of 65–80% for their activity.[4] Consumption of pig faeces by tiger worms was monitored using photographs captured twice daily: before and after feeding in the morning, and again approximately eight hours later in the evening. Visual observations of the biodigesters indicated that the tiger worms facilitated extensive decomposition of pig faeces over time, as evidenced by the images (panel A of Fig. 2). In contrast, the pig faeces in the control units remained largely unchanged, as expected (panel B of Fig. 2).

Pig faeces and *A. suum* eggs have been used in a sanitation-related helminth study,[24] supporting the relevance of pig-derived materials in the present study. More broadly, *A. suum* has been widely used as a surrogate for *A. lumbricoides* in studies on sanitation systems and pathogen inactivation in matrices such as human faeces and urine, septic tank sludge, raw wastewater, and faecal sludge.[25–29] In addition, the genomes of the pig roundworm and the human roundworm share over 99% sequence identity, and the two parasites form an interbreeding species complex.[30] Similarly, *A. lumbricoides* and *A. suum* eggs are morphologically identical and cannot be differentiated using optical microscopy.[31–33] Moreover, both species are capable of infecting both humans and pigs, as demonstrated by experimental human infections with *A. suum* and by field evidence.[30,34,35] Furthermore, pig manure closely resembles human faeces in key physicochemical properties, including nitrogen, phosphorus, organic matter, and carbon-to-nitrogen ratio.[36–39] In addition, pigs are widely recognised as translational models for humans in biomedical research due to similarities in gastrointestinal anatomy, physiology, and intestinal microbiota, supporting their use as a surrogate for human faecal material in experimental studies.[40,41] Hereafter, *A. suum* will be referred to as '*Ascaris*' in this paper.

The experiments on the laboratory-scale biodigesters were carried out in two stages. Two sequential experiments were performed using the same experimental setup, but with different objectives. The first stage focused on evaluating the performance of the multiple-straining method for the recovery of *Ascaris* eggs from the vermicompost (March to May 2025). Following this evaluation, the second stage was conducted in which pig faeces spiked with a known concentration of viable *Ascaris* eggs were fed into the systems. The resulting end products were analysed using the multiple-straining method on samples collected from different layers of the biodigesters and the control units at 6, 10, and 14 weeks post-feeding (May to October 2025).

### 2.2 The first-stage experiment: evaluating the multiple-straining method to detect the presence of *Ascaris* eggs in vermicompost

The method for analysing the presence of *Ascaris* eggs in vermicompost developed in this study builds on the standard multiple-straining method, originally developed to detect STH eggs in human faecal sludge from pit latrines or septic tanks.[42] Since vermicompost is also derived from sanitation waste, it was hypothesised that the multiple-straining method would also be suitable to detect eggs in vermicompost. Fig. 3 shows the standard operating procedure (SOP) for the multiple-straining method, with detailed steps provided in the SI1.

First, one needs to extract the vermicompost from the TWT. Vermicompost primarily accumulates within the coconut coir layer as a result of tiger worm activity and,


**Fig. 1** Sequential stages in the setup of the laboratory-scale biodigesters. Shown from left to right: (step 1) placement of a gravel layer at the base of the container, (step 2) addition of a coir layer above the gravel, (step 3) topping up tiger worms above the coir, and (step 4) daily feeding of pig faeces.

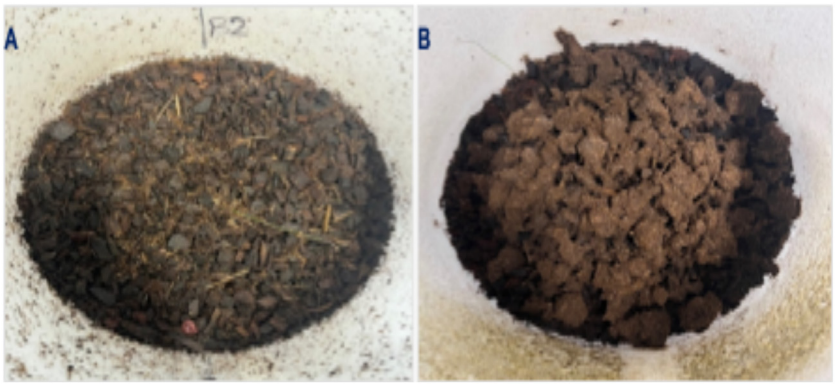


Fig. 2 Appearance of pig faeces one week post-feeding in the biodigester (panel A) and control system (panel B).

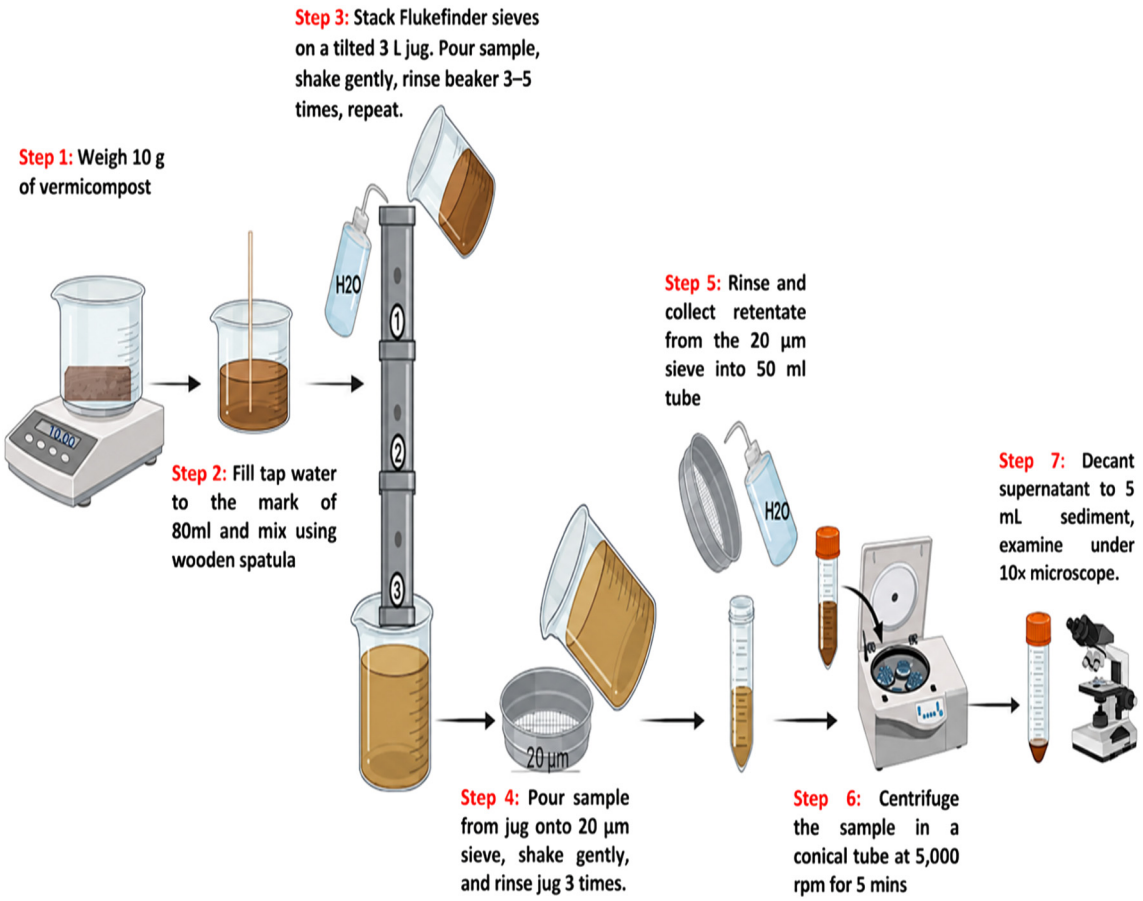


Fig. 3 Schematic flow diagram of the SOP for the multiple-straining method used in this study.

because of its fine texture, gradually passes from the surface through the cracks and gaps in the coir. Therefore, this study developed a protocol for vermicompost separation from the coir layer, thanks to systematic daily observations of biodigesters and through practical experimentation. The step-by-step separation procedure is provided in SI2. To separate the vermicompost from the coir layer, the coir material was air-dried at 25 ± 1 °C to facilitate differentiation between the fibrous coir and the finer vermicompost (see Fig. S2.1–S2.4 in SI2). Similarly, the gravel layer was air-dried, and adhering vermicompost was removed by manual brushing using a soft-bristled brush. Subsamples of both the coir and gravel layers were processed in fresh and dried states to ensure complete recovery of *Ascaris* eggs. Air drying was performed solely to facilitate vermicompost separation and was not intended to assess changes in egg counts. Fresh and dried samples represent different processing stages of the same layer, rather than independent experimental units.

 

Therefore, egg counts were summed across both conditions to represent total egg abundance per layer. Second, once vermicompost was extracted from the biodigesters by air-drying the layers, the performance of the multiple-straining method was evaluated. In parallel, it was also used on pig faeces, which is primarily present in the control units, to determine the recovery rate of *Ascaris*. The hypothesis was that differences in physical properties and texture between vermicompost and pig faeces may influence the retention of *Ascaris* eggs within these materials, potentially leading to differences in recovery efficiency. To assess the performance of the multiple-straining method in its ability to recover *Ascaris* eggs, one needs to spike a known number of *Ascaris* eggs in vermicompost and pig faeces separately and perform the method on each. Therefore, spiking concentrations of 50, 100, 200, and 400 *Ascaris* eggs were selected based on values reported in a study assessing the performance of the multiple-straining method on human-derived sludge.[42] These concentrations were then separately spiked into 10 g of vermicompost and 10 g of pig faeces. Negative control samples, without the addition of *Ascaris* eggs, were also analysed to confirm the absence of *Ascaris* eggs in the vermicompost (collected during the first stage experiment) and fresh pig faeces. Eight replicates were prepared for each egg concentration, and four microscope slides were examined per replicate, giving a total of 32 slide readings for every spiking level.

The *Ascaris* eggs used in this study were intact and contained fully developed third-stage larvae, as confirmed by replicate readings of microscopic slides. A stock solution was prepared by mixing *Ascaris* eggs with potassium dichromate at a 1:6 dilution ratio. The potassium dichromate was used to prevent microbial growth. A 50 mL stock solution of *Ascaris* eggs was prepared every Monday and used throughout the week. The number of viable eggs in the stock solution was determined by examining a total of twelve 20 μL aliquots under a microscope, using a 10× objective for initial observation and a 40× objective for confirmation. This comprised eight primary replicates and two additional replicates performed independently by each of two laboratory technicians to assess reproducibility. The mean egg count across these aliquots was taken as the number

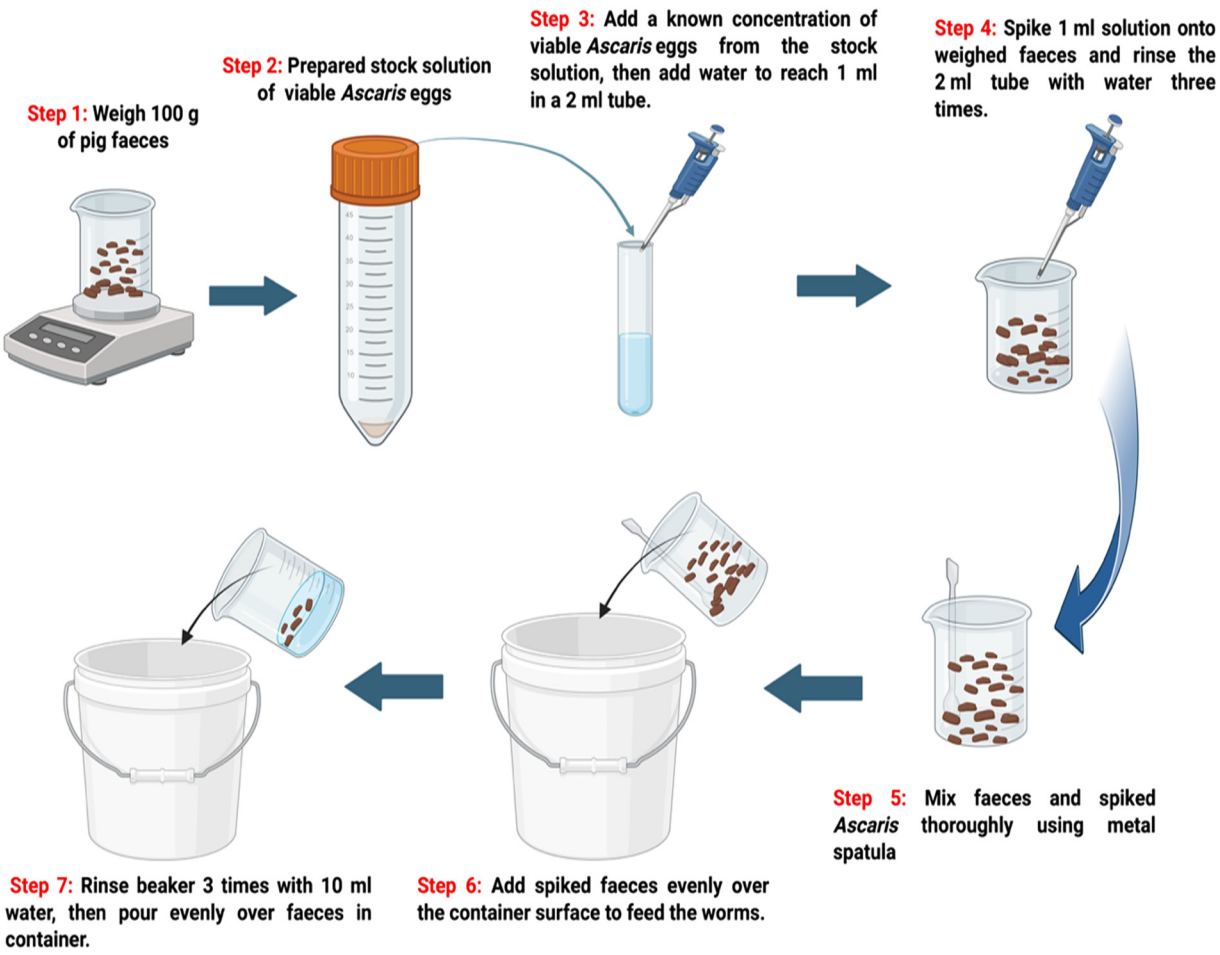


**Fig. 4** Overview of pig faeces spiking with *Ascaris* eggs and tiger worms feeding procedures.

of eggs per 20 μL and subsequently divided by 20 to estimate the egg concentration (eggs per μL).

### 2.3 The second-stage experiment: assessing the survival of *Ascaris* eggs in vermicomposting

**2.3.1 Method to spike pig faeces with *Ascaris* eggs.** After vermicompost was removed from the biodigester for the first stage experiment, this second stage experiment was initiated. In this phase, a known quantity of *Ascaris* eggs added to the pig faeces is fed into the biodigesters and the control units.[43] Fig. 4 summarises the protocol for pig faeces spiking and tiger worm feeding, with detailed procedures provided in the SI3.

A spiking concentration of 80 eggs per gram (EPG) was applied to the pig faeces used for feeding the tiger worms. This concentration was selected based on field data from endemic areas in Ethiopia, where approximately 80 EPG in human faeces was associated with an estimated ascariasis prevalence of 25% in the population.[44] The selection of a 25% prevalence condition was based on WHO guidelines,[45] indicating that a prevalence above 20% represents a transmission setting requiring preventive chemotherapy and reflects a realistic endemic contamination level for sanitation studies. In addition, the selected concentration (80 EPG) ensured sufficient *Ascaris* eggs remained available for recovery and quantification after potential losses during handling, processing, and recovery procedures. In this experiment, 100 g of pig faeces was used to feed all biodigesters ($n$ = 6) and control systems ($n$ = 3) for four weeks. Each daily feed, therefore, contained 8000 eggs.

**2.3.2 Assessment of the presence of *Ascaris* eggs post-faeces feeding.** Post-feeding, the biodigesters and control units were left undisturbed. This allowed the tiger worms time to process any remaining pig faeces in biodigesters. Samples from the layers of both the biodigesters and control units were then analysed using the multiple-straining method to quantify *Ascaris* eggs in each layer. Analyses were conducted at different time points: B1, B2, and C1 at 6 weeks; B3, B4, and C2 at 10 weeks; and B5, B6, and C3 at 14 weeks, to evaluate whether the number of *Ascaris* eggs changed when tiger worms were allowed additional time.

In biodigesters, the coir and gravel layers were air-dried to facilitate the separation of vermicompost. Subsamples from each layer were weighed and analysed to quantify the number of *Ascaris* eggs they contained. Fresh samples were analysed immediately before drying. Dried samples were analysed after vermicompost extraction. The extracted vermicompost was also examined for *Ascaris* egg counts. In the control units, layers of pig faeces, coir, and gravel were collected separately. These layers were weighed and analysed without drying, as no vermicompost was present in the control units. For quantifying the *Ascaris* eggs using the multiple-straining method, four sample replicates were prepared for each layer, and four slides were examined per replicate, resulting in 16 egg counts per layer.

The water added to maintain optimal moisture in the containers percolated over time through the bedding layer (coir) and accumulated in the drainage layer (gravel). This water, referred to as effluent, was common in both the biodigesters and the control units. During the feeding phase, effluent was collected from the bottom tap when the effluent level in the drainage layer reached 10 cm. This prevented water from backing up into the bedding layer, as the total depth of the drainage layer was 15 cm. The volume of collected effluent was measured, and the entire sample was processed using the multiple-straining method to count *Ascaris* eggs.

Following *Ascaris* quantification, the moisture content and total solids of each layer, including coir, gravel, vermicompost, pig faeces, and effluent, were measured using the oven-drying method.[46] These measurements were subsequently used to determine the dry weight of each layer for the calculation of EPG on a dry weight basis.

**2.3.3 Conducting viability analyses on retrieved *Ascaris* eggs.** The viability of *Ascaris* eggs spiked into both biodigesters and control systems was assessed using vital dye staining. This protocol was originally used to evaluate the viability of *Giardia* cysts in dog faeces.[47] Fig. 5 shows the SOP for viability analysis using the vital dye, with the detailed procedure in the SI4. In summary, propidium iodide in 1× phosphate-buffered saline (PBS) was used to distinguish viable from non-viable *Ascaris* eggs based on membrane integrity. Non-viable eggs take up the dye and fluoresce red under a fluorescence microscope, whereas viable eggs remain unstained.

The viability of eggs in each layer was assessed by preparing samples according to the established viability protocol and examining slides under a fluorescence microscope, with 20 *Ascaris* eggs evaluated per layer. This number was selected to balance statistical representation with practical feasibility. Indeed, counting 20 eggs typically required the preparation and examination of approximately 50 slides per layer, representing a considerable analytical workload.

Before applying the viability protocol, the propidium iodide staining method was validated using a control sample of *Ascaris* eggs with known viability status. In this control sample, non-viable *Ascaris* eggs were generated through heat treatment (100 °C for 15 minutes), aiming to disrupt the integrity of the eggshell. The sample was then stained with propidium iodide and examined under a fluorescence microscope to assess whether non-viable eggs could be consistently identified. All eggs in the heat-inactivated control sample were stained with propidium iodide, indicating the reliability of the method for distinguishing viable from non-viable eggs.

## 3. Results

### 3.1 The performance of the multiple-straining method during the first stage experiment

The numerical summary of the performance of the multiple-straining method on vermicompost collected from

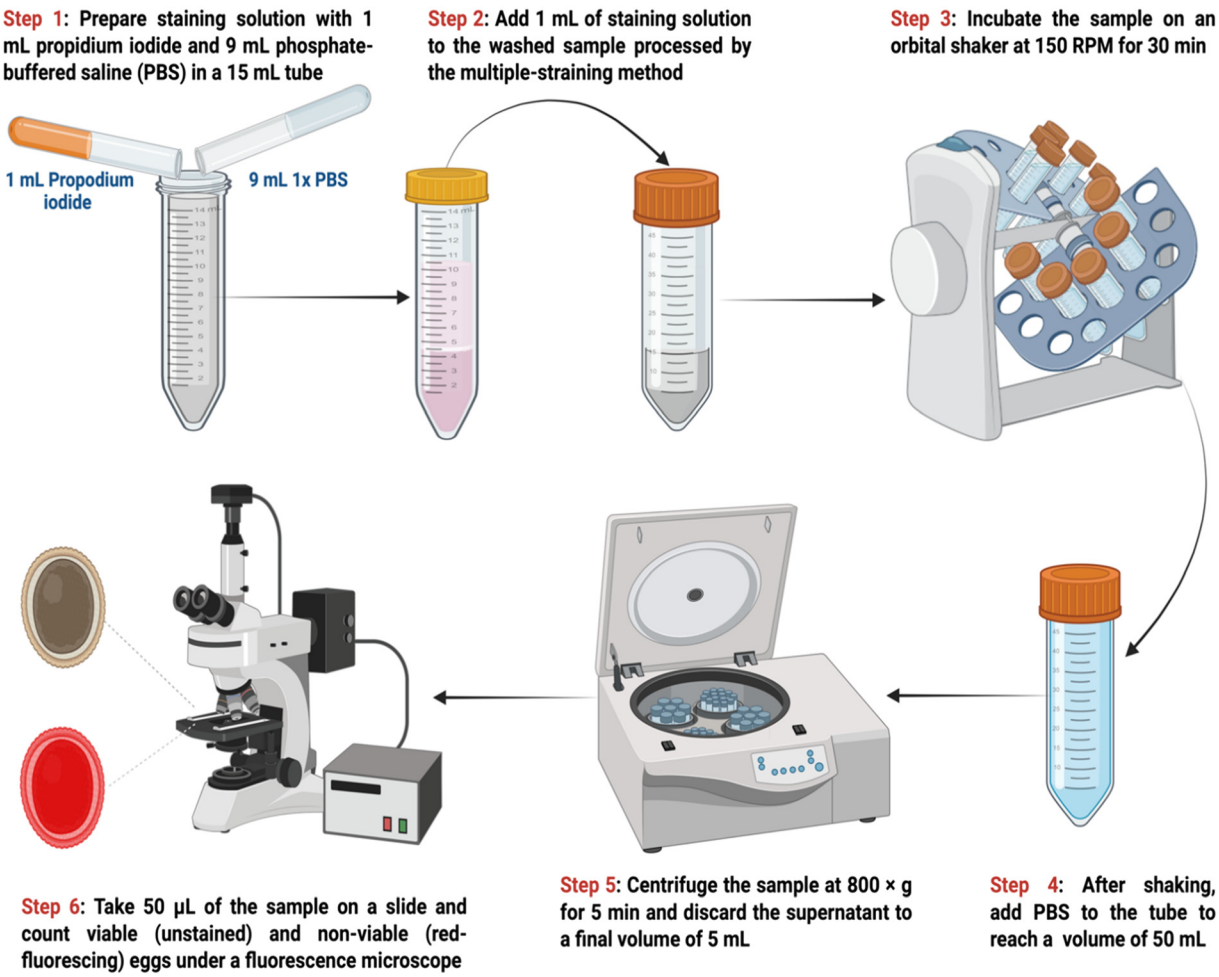


**Fig. 5** Viability analysis of *Ascaris* eggs using vital dye staining.

the biodigesters is presented in Table S5. The corresponding results for pig faeces collected from the control units are shown in Table S6. Fig. 6 shows the relationship between the number of eggs spiked (*X*-axis) and the percentage of positive replicates detected (*Y*-axis). The 95% Wilson confidence intervals (CIs) are displayed in red, providing accurate intervals even at extreme proportions (0% and 100%).

Fig. 6A illustrates vermicompost, where detection was variable at the lowest spike level (50 eggs), with five of eight replicates positive. Fig. 6B shows pig faeces, which displayed a similar pattern, with six of eight replicates positive at 50 eggs and a correspondingly wide CI. The upper confidence limit for the zero-spike (negative control) samples in both vermicompost and pig faeces reflects statistical uncertainty rather than the presence of eggs. From 100 spiked eggs onward, all replicates in both sample types (vermicompost and pig faeces) were positive. Overall, the multiple-straining method demonstrated reliable detection in both matrices, with all replicates positive at ≥100 eggs. The median recovery varied across spiking egg concentrations. In vermicompost samples, median recovery ranged from 50 to 84%, while in pig faeces, it ranged from 75 to 84%. Overall, across all spike levels, the median recovery was approximately 72% for vermicompost and 78% for pig faeces.

### 3.2 Recovery of *Ascaris* eggs from biodigesters and control units during the second stage experiment

A total of 200 000 *Ascaris* eggs were added equally in all biodigesters and the control units. Samples from the biodigesters and the control units were analysed identically at set time intervals to evaluate changes in *Ascaris* egg counts over time. See Table S7 for the total *Ascaris* egg counts recovered after the experiments and 95% CIs. *Ascaris* eggs were found to be unevenly distributed in different layers, especially in those of the biodigesters.

**3.2.1 Relative distribution of *Ascaris* eggs across the biodigester and control layers.** Fig. 7 illustrates the distribution of *Ascaris* eggs across the different layers of both the biodigesters and the control units over the study period.

During sample processing and analysis, some *Ascaris* eggs were inevitably lost, primarily during the washing and sieving steps of the multiple-straining method. Overall,

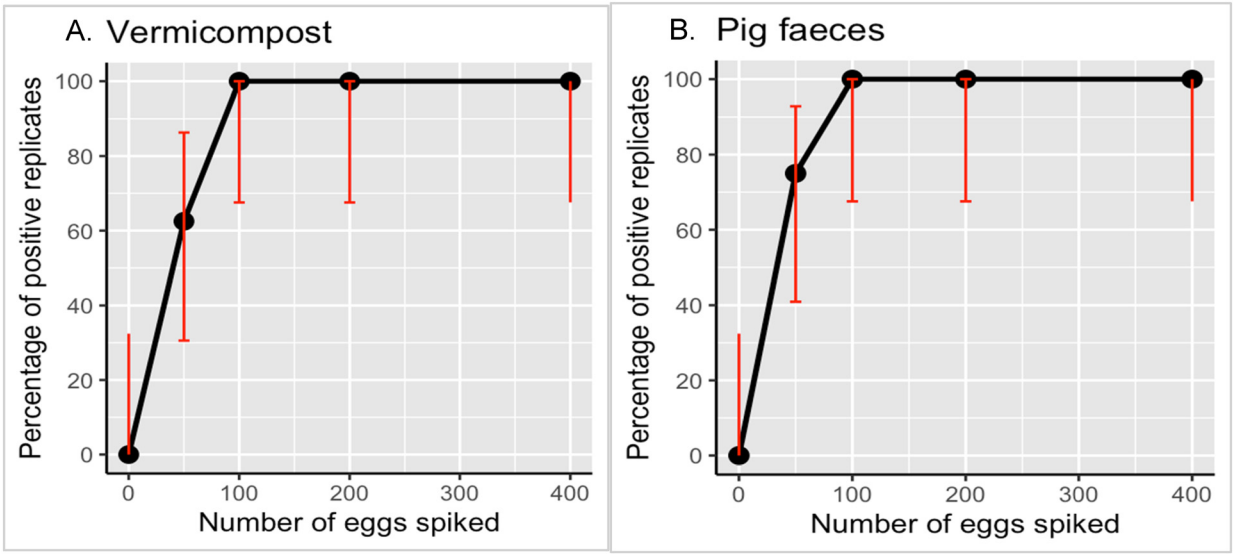


**Fig. 6** Percentage of positive replicates detected across spiked egg levels in vermicompost (A) and pig faeces (B), with 95% Wilson confidence intervals shown in red.

approximately 21–28% of *Ascaris* eggs were not recovered from the biodigesters, and 22–27% were not recovered from the control units, which is consistent with the observed median recovery efficiency of the method.

The majority of *Ascaris* eggs were found in the coir layer of the biodigester, accounting for approximately 48–72% of the total number of eggs, whereas the pig faeces layer of the control systems contained the highest *Ascaris* egg counts (71–74%). However, over time, more *Ascaris* eggs were detected in the gravel (5–21%) and effluent (0.15–0.59%) of the biodigester, while no eggs were recovered from these layers in the control units. This migration of eggs in the biodigesters over time is likely due to tiger worms moving independently across layers, which dislodged *Ascaris* eggs, causing them to move downward under gravity. The vermicompost layer contained 1.5–2.5% of the total number of eggs, which was generally lower than in all other layers, except for the effluent.

Differences in the relative distribution of *Ascaris* eggs between layers were evaluated using a pairwise Wilcoxon signed-rank test, as the data were not normally distributed. To perform this analysis, paired observations from the six biodigesters and the three control units were evaluated separately. All possible pairwise combinations of layers

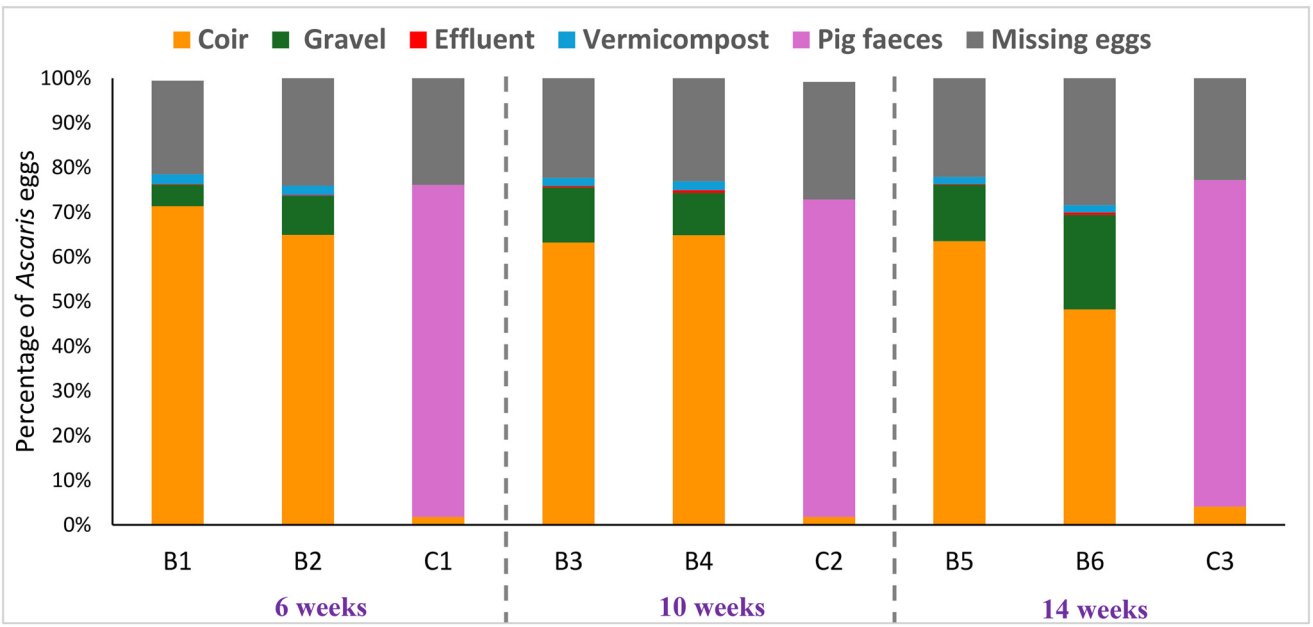


**Fig. 7** Percentage distribution of *Ascaris* eggs across all layers of the biodigester and the control units over time, expressed relative to the initial spiked load of 200 000 eggs introduced during feeding.

within each system were considered, resulting in six layer comparisons for each system. See Table S8, which summarises these pair-wise comparisons, reports the median egg difference for each pair, along with the corresponding *p*-values for each comparison.

For the biodigesters, the Wilcoxon signed-rank test revealed statistically significant differences for all layer comparisons at the 5% significance level. The coir layer differed significantly from all other layers, including gravel, effluent, and vermicompost ($p$ = 0.03). Gravel also showed significant differences when compared with effluent and vermicompost ($p$ = 0.03). Furthermore, effluent significantly differed from vermicompost ($p$ = 0.03).

For the control units, the pig faeces layer contained the majority of *Ascaris* eggs, representing 71–74% of the total (Fig. 7), with a mean EPG of 183 per dry weight (Fig. 8). In contrast, other layers, such as coir, gravel, and effluent, had substantially lower proportions and mean EPG values. None of the pairwise comparisons among control layers showed statistically significant differences at the 5% significance level ($p$ = 0.18 for all comparisons), although eggs were concentrated in the pig faeces layer. This is likely attributable to the small sample size (three control units) and the presence of zero *Ascaris* egg counts in the gravel and effluent layers. These factors reduced the power of the rank-based test, limiting its ability to detect consistent directional differences, even though the pig faeces contained higher egg counts than the other layers.

**3.2.2 Egg per gram (EPG) in the biodigester and the control units.** The *Ascaris* egg concentrations (EPG) were measured across all layers and subsequently evaluated against WHO guidelines for the safe reuse and disposal of latrine end products (<1 helminth egg per gram of total solids). Fig. 8 shows the EPG distribution per dry weight of layers in the biodigesters and control systems. To obtain EPG on a dry weight basis, wet EPG values for all layers were corrected using moisture content and total solids. Eggs per gram dry weight were calculated by dividing the total number of eggs recovered per wet sample by the corresponding dry mass (total solids) of each layer.

In the biodigesters (Fig. 8A), coir and vermicompost consistently exhibited the highest egg concentrations. Coir had a mean *Ascaris* concentration of 134 eggs per dry weight (95% CI: 59–193), while vermicompost had a mean of 40 eggs per dry weight (95% CI: 22–59). Although the proportion of *Ascaris* eggs in the vermicompost relative to the total eggs was lower than in other layers (Fig. 7), this likely reflects the comparatively lower mass of vermicompost extracted from the biodigesters compared with the other layers. Effluent showed much lower egg concentrations, with a mean of 7 eggs per dry weight (95% CI: 4–11). Gravel contained the lowest concentration (mean of 1 per dry weight; 95% CI: 1–2).

In the control units (Fig. 8B), a distinct pattern was observed, with *Ascaris* eggs largely confined to the pig faeces layer (mean of 183 eggs per dry weight; 95% CI: 103–263), while coir contained low egg concentrations (mean of 12 eggs per dry weight; 95% CI: 6–29), and no eggs were detected in the gravel or effluent.

### 3.3 Viability of *Ascaris* eggs

Across all layers in both the biodigester and the control, all eggs that were present were considered viable. *Ascaris* eggs remained morphologically intact at all sampling points in time and space throughout the experimental period. This indicates that the presence and activity of tiger worms within biodigesters did not result in the inactivation of the *Ascaris* eggs.

## 4. Discussion

The multiple-straining method recovered approximately 72–79% of *Ascaris* eggs from the biodigesters sample and 74–78% from the control units sample, consistent with

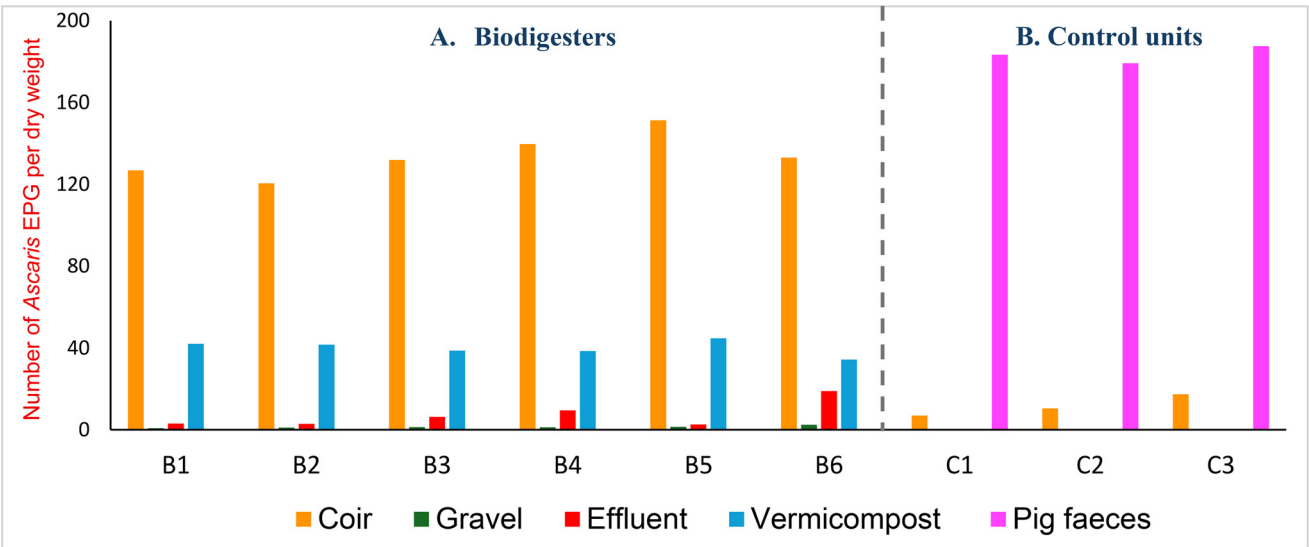


**Fig. 8** *Ascaris* egg concentration (EPG) for each layer in the biodigesters (A) and control units (B). EPG are expressed on a dry weight basis.

the recovery rates of vermicompost (72%) and pig faeces (78%). This indicates that approximately 28–21% of *Ascaris* eggs in the biodigesters and 27–22% in the control units were not recovered during sample processing and analysis. These losses may have occurred during washing and sieving in the straining method or due to limitations in the visual identification of *Ascaris* eggs under microscopy. No correction factor was applied to account for these losses. Considering the recovery rates and the similar total number of eggs in both biodigesters and control units at the end of the experiment (Table S7), no degradation of *Ascaris* eggs attributable to tiger worms was observed in this study. This contrasts with a previous vermicomposting study on sludge-derived biosolids, which reported a 1.9 log reduction in *Ascaris* eggs, although a 0.6 log reduction was also observed in the control system.[21] This suggests that part of the observed removal in the previous study may have been driven by factors unrelated to tiger worm activity. In the present study, viability analysis further confirmed that *Ascaris* eggs remained fully viable and morphologically intact after 14 weeks of prolonged post-feeding exposure to tiger worms. This is consistent with findings from a study on vermicomposting of human faeces diluted with coconut coir (30 : 70, wet weight), which reported no significant inactivation or change in *Ascaris* egg viability in tiger worm-treated systems over 13 weeks.[48]

The distribution of *Ascaris* eggs was uneven in the layers of the biodigesters compared to within the control units, as explained earlier. This heterogeneity appears to be strongly influenced by the activity of tiger worms within the biodigester system. Their own continuous movement facilitated the transport of *Ascaris* eggs into the gravel layer, which functions as a drainage medium. This is supported by the observation that there was no movement of eggs in the control units, as egg counts were zero in the gravel and effluent from these units.

Tiger worm biomass increased consistently over the experimental period, indicating favourable reproduction conditions within the biodigester system. After 6 weeks, tiger worm biomass increased by 6.12% in B1 and 8.09% in B2. At week 10, this increase became more pronounced, reaching 17.94% in B3 and 15.21% in B4. By week 14, biomass gains were highest, with compounded increases of 20.33% in B5 and 26.32% in B6. These trends are consistent with findings from the tiger worm-based toilet study, which reported a steady increase in tiger worm numbers over time, suggesting sustained reproduction and survival under biodigester conditions.[5]

Post-experimental observations further indicated that the own movement of tiger worms in the drainage layer (gravel) increased over time. The proportion of total tiger worm biomass present in the gravel layer rose progressively across biodigesters, from 25.05% in B1 and 31.99% in B2 to 51.40% in B3 and 43.43% in B4 and reached 60.79% in B5 and 74.55% in B6. The downward movement of tiger worms was likely driven by foraging behaviour, with the gravel layer offering higher moisture content than the bedding layer (coir), making it a more favourable environment. This increasing concentration of tiger worms within the gravel layer likely enhanced the physical transfer of *Ascaris* eggs into this zone. Consistent with this pattern, the proportion of recovered *Ascaris* eggs in the gravel layer, as shown in Fig. 7, increased over time. At 6 weeks, gravel samples B2 and B1 accounted for 8.72% and 4.76%, respectively, of the total recovered eggs. At 10 weeks, this proportion increased, with 12.30% recovered from B3 and 9.46% from B4. By 14 weeks, gravel from B6 contained 21.21% of the total recovered *Ascaris* eggs, followed by B5 with 12.58%. Not only fresh but also air-dried materials processed to separate vermicompost from the biodigesters contained *Ascaris* eggs, including dry coir (2–4%), and dry gravel (1–2%) of the total recovered eggs. This indicates that air-drying at 25 °C did not inactivate or degrade them. Effective inactivation requires thermophilic conditions, with guidelines recommending sustained temperatures of approximately 50–55 °C or higher.[12,15,27]

In addition, *Ascaris* eggs were detected in the effluent collected through the tap at the bottom of the containers, indicating downward transport within the system. The depths of the drainage layer (gravel) and bedding layer (coir) in the experimental system were smaller than those of a typical full-scale TWT.[4] This reduced scale may have altered the movement and spatial distribution of tiger worms, facilitating vertical movement and the redistribution of *Ascaris* eggs within the system and into the effluent. This represents a limitation of the study, as laboratory-scale hydraulic conditions and dimensions do not fully replicate those of full-scale TWTs. In full-scale systems, deeper bedding layers, larger reactor volumes, and more complex flow pathways may restrict vertical movement of tiger worms into the drainage layer. Consequently, *Ascaris* egg concentrations in the drainage layer and effluent are expected to be lower in the field than in those observed in this study. Additionally, previous studies showed that *Ascaris* eggs tend to adhere to dense, solid-rich matrices, leading to lower recovery efficiencies compared to slurry or liquid samples.[24,49] Nevertheless, the recovery rate observed in this study (72%) is higher than values reported for semi-solid sludge from pit latrines (57%) in the literature.[42] Therefore, the multiple-straining method is considered acceptable for TWT vermicompost. A further limitation of this study is that it was conducted using pig-derived substitutes, namely pig faeces and *A. suum* eggs. These materials were selected because pig-derived matrices remain a widely accepted and practical proxy in controlled sanitation research.[24–29] This choice is supported by the similarities in environmental tolerance between *A. suum* and *A. lumbricoides*, as well as the comparable characteristics of pig and human faeces.[30–41] However, variability in pig diet and gut microbiome may influence faecal properties and egg recovery dynamics, and matrix-specific effects may limit direct extrapolation of the findings to human sanitation contexts. Nevertheless, despite evidence supporting their suitability as experimental substitutes, their use remains a methodological limitation of

 

the study. Further research is therefore needed to assess the ability of tiger worms to degrade or inactivate *A. lumbricoides* in human-derived sludge.

The present study was conducted under conditions intended to represent the egg counts in faeces in regions with 25% prevalence of ascariasis in the human population.[44,45] These findings emphasise the need for further treatment of TWT-derived vermicompost in endemic regions to ensure safe reuse or disposal within a circular economy framework. Post-treatment methods such as anaerobic digestion[26] or thermal processing[27] can improve helminth egg inactivation and support safe resource recovery for agricultural applications. These approaches may enhance sustainability through nutrient recovery and organic waste valorisation while contributing to United Nations Sustainable Development Goal (SDG) 6 by supporting safely managed sanitation and reducing environmental contamination.

Policy frameworks should require an additional validated treatment barrier after vermicomposting to ensure adequate helminth egg inactivation before agricultural application, particularly in high ascariasis prevalence settings. Where testing capacity is limited, vermicompost should be restricted to lower risk applications such as non-food crops, forestry, or land restoration. Policies should also adopt a multi-barrier approach that includes treatment, safe handling practices, crop selection controls, and withholding periods to reduce exposure to residual helminth eggs.

Practitioners should avoid describing TWT-derived material as "safe compost" without validated evidence of pathogen reduction. Adequate inactivation of *Ascaris* eggs before land application is essential to minimise infection risks, enable safe nutrient recycling, and protect public health. Failure to achieve sufficient pathogen reduction may undermine progress towards SDG 2 through reduced food safety, SDG 3 through increased helminth exposure and disease burden, and SDG 15 through the introduction of persistent pathogens into terrestrial ecosystems.

## 5. Conclusions

The present study indicates that TWT systems do not achieve sufficient inactivation of *Ascaris* eggs to meet WHO standards for safe agricultural use[12] of the vermicompost under the conditions tested. Guidance for TWT operation and management should mandate helminth egg testing of end products (vermicompost and effluent), particularly in high ascariasis prevalence regions, to assess potential transmission risks associated with emptying, handling, and agricultural reuse. This should be incorporated as a minimum safety requirement within sanitation standards to support safely managed sanitation and protect public health.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data generated during this experimental study were recorded in Excel file and are available from the corresponding author upon reasonable request. The dataset will be provided in Excel format, along with the associated R scripts used for data analysis.

Supplementary information (SI): the SI provides detailed standard operating protocols used for experimental analysis and additional results supporting the main findings. See DOI: https://doi.org/10.1039/d6ew00117c.

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