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Review

A Review on the Current Knowledge and Prospects for the Development of Improved Detection Methods for Soil-Transmitted Helminth Ova for the Safe Reuse of Wastewater and Mitigation of Public Health Risks

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Abstract: Climate change, increase in population and scarcity of freshwater have led to a global demand for wastewater reuse in irrigation. However, wastewater has to be treated in order to minimize the presence of pathogens, in particular, the ova of soil-transmitted helminthes (STHs). Limiting the transmission via removal of STH ova, accurate assessment of risks and minimizing the exposure to the public have been recommended by health regulators. The World Health Organization (WHO) guideline specifies a limit of ≤1 ova/L for safe wastewater reuse. Additionally, the Australian Guidelines for Water recycling (AGWR) recommend a hydraulic retention time of over 25 days in a lagoon or stabilization pond to ensure a 4 log reduction value of helminth ova and to mitigate soil-transmitted helminths associated risks to humans. However, the lack of fast and sensitive methods for assessing the concentration of STH ova in wastewater poses a considerable challenge for an accurate risk assessment. Consequently, it has been difficult to control soil-transmitted helminthiasis despite effective mass drug administration. This limitation can be overcome with the advent of novel techniques for the detection of helminth ova. Therefore, this review presents an assessment of the current methods to detect the viable ova of soil-transmitted helminths in wastewater. Furthermore, the review focuses on the perspectives for the emerging state-of-the-art research and developments that have the potential to replace currently available conventional and polymerase chain reaction based methods and achieve the guidelines of the WHO in order to allow the safe reuse of wastewater for non-potable applications, thereby minimizing public health risks.

Keywords: wastewater reuse; soil-transmitted helminths; ova; guidelines; detection; state-of-the-art techniques

1. Introduction

Freshwater resources are under pressure through climate change and increasing population [1]. As a result, stakeholders, government regulatory bodies, water industries and researchers have formulated strategies to utilize recycled water from wastewater as an alternative to potable water in agriculture [2]. A major consideration in the reuse of wastewater is the possible risks to humans that are associated with the presence of pathogens [3,4]. Therefore, it is essential to treat wastewater in order to minimize the pathogen concentration to a safe level. The reduction of pathogens in the wastewater treatment plants in Australia is mostly achieved by lagoon detention and disinfection primarily with chlorine and ultraviolet radiation [5]. Although the pathogens such as bacteria, viruses and protozoans are susceptible to such treatment processes, helminth ova are multilayered and possess a thick shell that confers resistance to disinfection with chlorine and UV [6]. Due to a low minimal

infective dose, resistance to treatment processes and prolonged survival in the environment, the World Health Organization (WHO) has categorized helminths as being high risk compared to other microbial pathogens [7]. Furthermore, wastewater may contain up to 3000 helminth ova/L in endemic areas. Consequently, the WHO guidelines recommend ≤1 ova/L for treated wastewater and ≤1 ova/g for sludge in order to mitigate human associated helminthiasis [8].

However, in countries where sanitation system is of better standards (e.g., Australia), helminth ova are rarely detected in untreated wastewater [9]. As such, the concentration of helminth ova in Australia is ≤1 ova/L in raw wastewater even without any additional treatment, thereby adhering to the WHO guidelines [5]. However, increased migration from endemic countries and increased rates of travel to less developed nations might allow an increase in helminths associated diseases [9]. Therefore, helminth ova removal from wastewater is essential for the safe use of sludge or treated wastewater for irrigation in the developed countries. Nevertheless, the lack of a robust and uniform method poses a challenge for the comparative assessments of the concentration of helminth ova in wastewater [10]. Aimed at contributing to develop a uniform method for rapid detection of STH ova, one of the goals of this review was to assess the currently available methodologies emphasizing their advantages and disadvantages and discussing the perspectives for the development and utilization of radically innovative state-of-the-art techniques. In order to achieve the aforementioned goals, the steps required for recovering helminth ova from wastewater have also been considered.

2. Soil-Transmitted Helminths: Prevalence and Health Risks to Humans

Soil-transmitted helminths (STHs) cause infection to humans via contact with ova or larvae that survive in tropical and subtropical regions such as sub-Saharan Africa, Latin America, China and South East Asia [11,12]. Globally, over 1.5 billion people are afflicted with STHs, especially *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms (*Necator americanus* and *Ancylostoma duodenale*) [9,10,13,14]. Most of the STH infections are insidious for those who live in impoverished conditions. With a low infective dose combined with the enormous output of ova per worm, helminths are considered as pathogens that can significantly impact an individual's health and well-being (Table 1; [15,16]).

The infections caused by STH rarely causes mortality with diarrhea, abdominal pain and low hemoglobin levels as the immediate outcome. However, patients with chronic infections show reduced cognitive abilities, intellectual growth retardation and poor maternal birth outcomes [17,18]. According to the WHO, 870 million children live in the area of high prevalence [19]. Despite concerns regarding their impacts on public health, STHs are generally considered to be neglected tropical parasites [20,21].

Table 1. Daily ova output, characteristics of ova and infective stage of major STHs.

STHs	Daily Ova Output per Worm	Ova Size and Shape	Shell of Ova	Infective Stage
Ascaris	200,000	40 × 60 µm Golden brown colour for mammilated ova and clear for decorticated ova Oval shaped or slightly rounded	Smooth shell Has two layers, one thicker outer shell and one thinner inner shell Viable eggs have a defined space between inner and outer shells	Viable ova
Trichuris	3000–5000	50 × 25 µm Lemon shaped with curved sides and rounded mucoid plugs	Smooth shell No space between inner and outer shell Mucoid plugs and shell are symmetric	Viable ova
Hookworm	9000–15,000	65 × 40 µm Oval shaped	Smooth shell Outer shell appears as single, thin, black line Inner ovum is clearly segmented	Filariform larvae

The utilization of untreated wastewater for irrigation purpose is one of the main reasons for increased STH in less developed countries either via direct or indirect exposure [22,23]. In developing countries, it is estimated that raw wastewater is used to irrigate 2.0×10^7 ha of agricultural lands [24,25].

Individuals belonging to the directly exposed group to wastewater and sludge have a 40% greater risk of contracting STH infections than indirectly exposed individuals [1] (Figure 1). Infected individuals release helminth ova into wastewater that can potentially contaminate soil, plant and surface water, where helminth ova can survive up to several years [1]. Exposure to wastewater and sludge contaminated with viable STH ova can lead to potential public health risk [23,26]. This risk is based on the concentration of ova that remain viable in the environmental matrices, the infective dose, route of exposure and the susceptibility level of the exposed person [4]. The presence of ova and its concentration in wastewater relies on the incidence of infections within the surrounding communities [27].

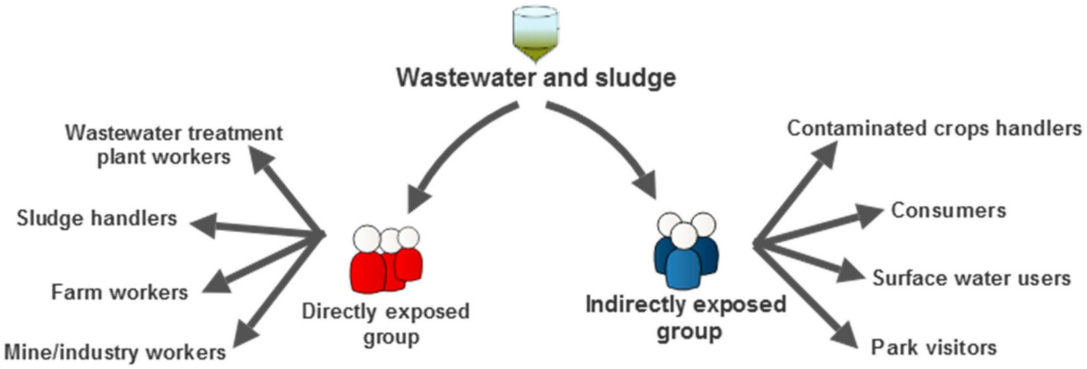

Figure 1. Risks to humans associated with usage of treated wastewater and sludge.

Although several STHs occur in wastewater, *Ascaris* species contribute 84% of the overall helminth ova genera distribution in wastewater followed by *Trichuris*, *Toxocara* and the cestode *Taenia* (Figure 2; [3]). Furthermore, *A. lumbricoides* and *A. suum* exhibit greater resistance to extreme conditions and treatment, such as heat, oxidants, detergents, pH and proteases [28]. Maya et al. assessed the rate of inactivation for the ova of *A. lumbricoides*, *A. suum*, *Toxocara canis* and *T. trichiura* following treatment at 80 °C for one hour at a pH of 12.1 and revealed that <25% of these STHs were inactivated [29]. For these reasons and their tendency to settle in sludge, *Ascaris* is considered a reference pathogen for helminths in evaluating the efficiency of the wastewater treatment processes [6,30].

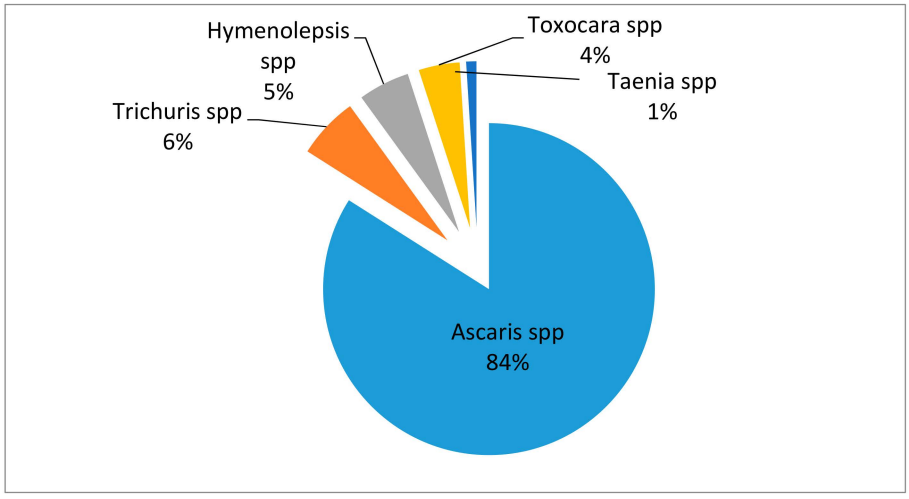

Figure 2. Distribution of helminth ova genera in wastewater from developing countries (Adapted from: [22]).

The high resistance of *Ascaris* ova to treatment processes can be due to their thick shell (Figure 3) [31,32].

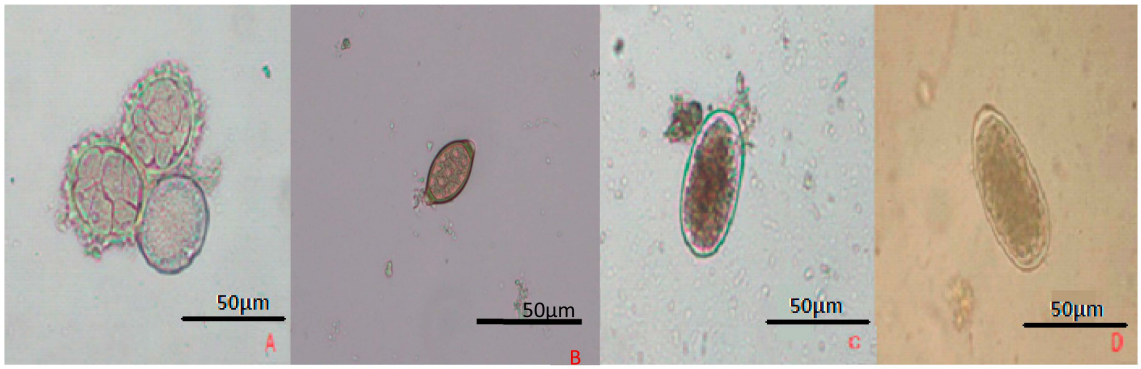

Figure 3. Photomicrographs of STH ova under 500× magnification (Scale = 50 µm): (**A**) *Ascaris lumbricoides* ova after 7 days of embryonation, (**B**) *Trichuris suis*, (**C**) *Necator americanus*, (**D**) *Ancylostoma duodenale.*

3. Mitigation of Public Health Risk

The concentration of STH ova in untreated wastewater (1 L) and sludge (4 g dry weight) can be as high as 10^3–10^4 based on the infection rates within the community (Table 2). Wastewater treatment processes minimizes helminth associated risks to humans following wastewater and sludge reuse [7].

Table 2. Helminth ova concentration in wastewater matrices in various countries.

Country	Untreated Wastewater (ova/L) *	Treated Wastewater (ova/L) *	Sludge (ova/g) *	Reference
India	72^{A}, 15^{H}, 4^{T}	60^{A},9^{H},2^{T}	NR	[33]
Iran	12^{A}, 3^{Ta}, 2^{H}	1^{A}	NR	
Burkina Faso	10^{A}, 4^{H}, 1^{T}	1^{A}	1327^{A}, 442^{H}, 11^{T}	[34]
Mexico	330^{NS}	NR	177^{NS}	[4]
Pakistan	175^{A}, 26^{T}	4^{A}	NR	[35]
Morocco	23^{NS}	NR	NR	[36]
Tunisia	960^{A}, 208^{Ta}	240^{A}, 52^{Ta}	NR	[37]
France	NR	NR	5^{NS}	[38]
Oman	517^{A}, 33^{H}, 18^{Ta}	45^{A}, 18^{Ta}	NR	[39]
Australia	NR	41^{NS}	NR	[1]
USA	8^{NS}	NR	13^{NS}	[4]

A—*Ascaris lumbricoides*, H—hookworm, T—*Trichuris trichiura*, Ta—*Taenia* species, NR—Not reported, NS—Not specified, * denotes average ova concentration.

Filtration methods such as microfiltration and ultrafiltration are effective against helminth adults and larva however they are expensive and may not completely remove helminth ova [28]. Different treatment processes such as aerobic/anaerobic digestion, lime stabilization and heat treatment have been utilized to inactivate helminth ova in sludge [40,41].

In Australia, the most common treatment process for the removal of helminth ova is lagoon detention and this process is followed primarily by disinfecting with chlorine and UV, although helminth ova are resistant to such disinfection methods. As a result, the AGWR recommend a lagoon hydraulic retention time (HRT) of over 25 days to ensure a 4 log reduction for the safe release of recycled water for non-potable purpose [42]. Despite lagoons effectively (90–99%) remove helminth ova from wastewater, the helminth ova tend to settle into the sludge and can remain viable [7]. In addition to adequate wastewater treatment processes for the health risk management, it is also essential to monitor the concentration of helminth ova in wastewater [6,30]. At present, there is no uniform method that can effectively recover helminth ova from wastewater matrices and provide accurate enumeration and quantitative detection of helminth ova.

4. Recovery Methods for STH Ova

The detection, enumeration and quantification of STH ova in wastewater and sewage sludge poses major challenges such as the need for methods that are sensitive to allow accurate estimation of STH ova whilst also being cost-effective, allowing their use in low resource setting areas where the prevalence of STH ova is higher [10]. The current methodological steps involve (1) wastewater/sludge sampling, (2) recovery and concentration of ova from the sample matrix, (3) identification and quantification of recovered STH ova and (4) evaluating STH ova viability.

4.1. Sampling of Wastewater/Sludge

Current methods available to detect helminth ova in wastewater are based on WHO guidelines which recommend a sample volume of 1 L [43,44]. However, several studies have utilized sample volume of 10 L to a maximum of 200 L for the recovery of ova [45,46]. The presence of suspended solids and turbidity in the sample may lead to lower ova recovery rate [47] as ova might be clogged or retained within the suspended solid particles during the flotation or membrane filtration method used for ova recovery [48]. The high concentration of helminth ova in wastewater can be due to the attachment and concentration of ova within the suspended solids and accumulation of ova in the sludge during wastewater treatment [49]. Due to the large size of ova compared to bacteria and viruses, the ova settle into sludge readily during primary treatment [5,50]. The amount of sludge sample is based on the dry weights, which range between 2 g and 5 g [29,51,52].

4.2. Ova Separation from Solid Matrix

Ova separation from organic compounds and polysaccharides in sewage sludge are of major concern [53]. A high concentration readily traps the ova which become refractory to subsequent purification steps such as sedimentation and floatation, resulting in lower recovery [6]. During the dewatering of sludge, coagulants may be added in order to produce more cohesive granules that enhance the total solid concentration in the sludge. The presence of high concentration of coagulants may interfere with ova recovery [54]. The removal of contaminants is essential for the proper isolation of ova.

Washing the samples with surfactants is highly desirable for the removal of ova from sludge [55]. The most common detergents used for the release of ova from the solid matrix are Tween 80, Triton X-100, 7X® and ammonium bicarbonate [47]. These surfactants dissociate the bonds that form between the outer surface of STH ova and organic compounds within the sample, thus releasing the ova [56]. However it is vital to use these surfactants at low concentrations, as it can damage the membrane integrity of the ova [57].

Ascaris ova immersed in detergents, in particular sodium lauryl sulfate and sodium dodecylbenzene sulfonate at 38 °C for 36 h has been reported to damage the membrane integrity of ova. One study denoted that the recovery of *Taenia saginata* ova that were seeded in samples from lagoons yielded a very low recovery rate with 0.8% (w/v) cetylpyridinium chloride (1.7%), SDS (6.4%) and Nonidet®P40 (4.4%) respectively [58].

The choice of the detergent in addition to the concentration is also considered a determining factor for the recovery of STH ova. Although there is no data to compare and determine the best detergent for the release of ova from wastewater matrices, a higher rate of STH ova recovery was observed when the water soluble anionic detergent 7X® was used compared to the recovery rate with Triton X and Tween 80 [49,54]. Moreover, 7X does not undergo precipitation, following reactions with flotation solutions [13].

4.3. Filtration

Following the dissociation of ova from larger particles in the sample, it is important to separate the ova from such particles. Sieves are used for retaining the larger particles whilst allowing the desired ova through the filter [59]. The choice of pore size impacts the filtration process. To allow STHs ova

having dimensions between 25 µm and 150 µm to pass via pores with the size ranging from 40 µm to 125 µm are chosen. In order to retain the ova of interest on sieves, pore sizes of 20 µm or even as low as 8 µm are used [60]. For STH ova, sieves ranging from 32 to 36 µm are chosen [38].

Filtration or sieving improves the efficiency during microscopy stage as it reduces the particulate matter that interferes with the enumeration of ova on the slides [47]. However, this treatment may also lead to reduced ova recovery via the trapping of particle clumped ova. Therefore it is important to carefully match the pore size for the desired ova since meshes that are smaller to retain *Ascaris* ova might allow the ova of *Trichuris* to pass if their orientation is on the long axis [10].

4.4. Sedimentation

Separation of ova in the filtrate is via sedimentation using the centrifuge [6]. However to date, there is no way to determine the best centrifugal speed for ova recovery [46]. Sedimentation by passive methods varies from 1 h to allowing it to settle overnight [61]. However, passive sedimentation may be influenced by sample volume and type, viscosity, the container used for sedimentation and the time required. Also, the presence of high solids in the sample may impact sedimentation eventually with adequate loss of ova [44].

Inadequate settling time can lead to more loss in ova, whereby overnight settlement of ova using methods such as the United States Environmental Protection Authority (US EPA) recommended Tulane method would be advantageous [62]. Studies conducted by Zdybel et al. to detect the viability of *Ascaris*, *Trichuris* and *Toxocara* ova in sludge from several wastewater treatment plants included centrifugation with a relative centrifugal force (RCF) of 2500 g following sedimentation and sieving [63].

4.5. Flotation

The flotation step is achieved by maintaining a gradient that allows ova to float while settling down the heavier particles that can be discarded [64].

For ova recovery, the flotation solutions must be heavier than the ova's specific gravity [65]. Most STH ova have a specific gravity between 1.05 and 1.23 [30]. The commonly used flotation solutions are listed in Table 3. The flotation solution is based on sample processing and allowing the STH ova to be recovered. Saline solutions, particularly sodium chloride, precipitate when detergents are present, whereas sucrose requires longer periods of centrifugation owing to its viscosity and specific gravity of 1.30. However sucrose is used for recovering STH ova such as *Taenia* species that possess greater specific gravity. Prolonged contact of ova with the flotation solution should be avoided as the salts may distort the embryonic development of the ova [57]. Maya et al. assessed the use of zinc sulfide (specific gravity 1.20) for recovering viable *Taenia* ova. The results indicated that the flotation solutions with specific gravity (1.20) were not effective for recovering some STH ova, and hence could lead to discrepancies in the concentration of ova within the sample. To ensure recovery of heavier ova, flotation solutions prepared with a specific gravity of 1.30 was highly recommended [29].

Table 3. STHs recovery using different flotation solutions (S.G—specific gravity).

Sample	Flotation (S.G)	References
Wastewater/Sludge	NaCl (1.18)	[66]
Wastewater/Sludge	NaCl (1.27)	[44]
Wastewater/Sludge	$ZnSO_4$ (1.18)	[67]
Wastewater/Sludge	$ZnSO_4$ (1.20)	[29]
Wastewater/Sludge	$MgSO_4$ (1.20)	[9,47]
Wastewater/Sludge	$MgSO_4$ (1.29)	[52]
Wastewater/Sludge	$NaNO_3$ (1.39)	[68]
Wastewater/Sludge	NaCl (1.19)	[57]

For the US EPA recommended Tulane method, a magnesium sulfate solution that possesses a specific gravity of 1.20 was considered to be the optimum density for the recovery of *Ascaris* ova [9,47]. Selection of the optimum flotation solution is essential for better ova recovery [64]. During the flotation step, the ova floats rapidly to the surface of the solution. Passive flotation can be utilized in resource-limited settings as the requirement of a centrifuge is not needed [13]. The duration of centrifugation during the flotation step depends on the composition of sample matrix as it may interfere with the efficiency of flotation [69]. However, for effective STH ova recovery, 800 g for 3–5 min was considered optimum for the Tulane method. In addition, few methods are based on the adherence of ova to a coverslip which in turn reduces the processing time, however the impact on the efficiency in ova recovery is not known. Hence optimization of the operating conditions is necessary for higher ova recovery [54].

4.6. Phase Extraction

This step removes lipid and ether soluble contaminants from STHs ova. The hydrophilic and lipophilic reagents used in this step separate the sample into light and heavy phases, thus concentrating the ova at the bottom [30]. Ethyl acetate and diethyl ether are widely used for the lipophilic phase, while ethanol serves as the reagent for the hydrophilic phase. Despite the removal of the major contaminants such as proteins and lipids, the reagents exert toxic effects that can result in a 95% loss of ova viability. Various studies have reported the harmful effects of such reagents on ova integrity and viability [70]. Satchwell utilized formaldehyde and diethyl ether and obtained 40% removal of contaminants from the sample containing ova of *Taenia* sp., *Ascaris* sp., and *Trichuris* sp. Despite reduction in impurities, a loss of up to 95% in the viability of the ova was observed during extraction, which was due to the toxic effects of the reagent [70]. Owing to such detrimental effects, it has been suggested to use sieving as an alternative for removing protein, lipids and other contaminant molecules [6]. Nelson and Darby recommended an exposure of 2 min with acetone and diethyl ether for the extraction step. However an exposure of more than 10 min was detrimental [51].

4.7. Factors Influencing Ova Recovery

Sequential flotation steps were stated to recover extra 10–20% of spiked ova trapped between matrix particles [64,71]. Texture and the organic content of wastewater and sludge can impact the efficiency of ova recovery [30]. The loss of ova may also be due to its adherence to the walls of the test-tubes, pipettes and beakers. Consequently, several methods specify the use of organosilane to treat the labware and glassware so that the adhesiveness is minimized [47]. However, it has also been mentioned that the utilization of organosilane in glass and plastic materials reduced the recovery yields. As such, it has been recommended that non-coated pipettes and Falcon test-tubes be utilized instead (Table 4) [72].

One of the methods to recover STH ova involves adhesion using a coverslip. Here a meniscus was created by filling the test tube with the flotation solution. A coverslip was later placed on the brim of the tube and the ova may adhere to it following centrifugal flotation, which may then be enumerated [73]. Few methods specify preprocessing of samples by sieving before performing the ova recovery steps so that the larger particles are removed from wastewater. In order to minimize the excessive loss of ova from the discarded sample, it is essential to homogenize and dissociate the ova from the sample matrix [74].

Additionally, the flotation solutions such as zinc sulfate and magnesium sulfate may be toxic and inactivate the embryonated ova during the recovery step [47]. Furthermore, ova that are incubated in formalin (1%) revealed slow development when compared with ova incubated in water or 0.1 N sulfuric acid [51]. Also the conditions for storage are crucial to maintaining ova viability. The ova of *Ascaris* and *Trichuris* species are thick shelled, which makes it easy to be stored at 4 °C for several weeks without causing any damage to viability. Hookworm ova should be recovered from fresh fecal samples as the ova do not embryonate if fecal samples are kept at 4 °C before egg recovery [74,75].

Variation in the physicochemical properties of STH ova may require optimization of recovery methods that are specific for each species.

Table 4. Helminth pva recovery method with flotation methods, tubes, pipettes and detergents (Adapted from reference [72]).

Method	Total Replicates	Average Numbers of Ova Recovered in 90 µL (Standard Deviation)	Average Ova Recovery (%)
Flotation method			
Modified McMaster	5	650 (90)	65
FLOTAC technique	5	434 (83.0)	43
Tubes and pipettes			
Non-coated Falcon tubes/glass pipettes	5	586 (168)	58
NC Falcon tubes//C glass pipettes	5	571 (171)	57
Coated Falcon tubes/glass pipettes	3	335 (120.0)	33
Coated Falcon tubes/non-coated plastic pipettes	3	374 (163)	37
Coated falcon tubes/ Non-coated glass pipettes	5	356 (188)	35
Detergent			
Deionized water	5	742 (56)	74
7X® 1%	5	897 (228)	89
Tween 80 (0.1%)	5	587 (180)	58
Benzenethonium chloride 0.1%	5	870 (129)	87
Cetylpyridinium chloride 0.1%	5	844 (178)	84

5. Detection Methods for Helminth Ova

For accurate risk assessment of STH ova as per the national and international guidelines and regulations, it is significant to determine the viable helminth ova in a sample, as only the viable ova are infective [6]. The public health risks that are associated with wastewater and sludge reuse are measured by estimating the concentration of STH ova [1]. The methods currently used to enumerate and quantify STH ova are (i) optical microscopy, (ii) PCR-based techniques and (iii) flow cytometry. This is a crucial step as the ova recovery and enumeration are considered as evaluation criteria to determine whether the treated wastewater and sludge are suitable for agriculture.

5.1. Optical Microscopy

Optical microscopy is still considered as the gold standard to enumerate and identify helminth ova during risk assessments. It involves culturing the helminth ova for up to 4 weeks followed by enumeration by optical microscopy (Figure 4) [10]. However it requires a visual sharpness owing to the presence of interferents such as pollen that could lead to false positive results. As such, removing the debris present in wastewater is crucial for accurate visualization and enumeration of the recovered helminth ova [30]. One of the studies conducted to assess the inactivation efficiency of thermal treatment in sludge indicated the risk of false positives owing to contaminants in the wastewater sample [76]. A major limitation of this method is the need for a skilled personnel for accurate identification of the STH ova [26]. Additionally, the enumeration of ova is dependent on the precision and accuracy of

the ova recovery method. Also the unembryonated ova and the ova that have developed into the larval stage have to be enumerated in the case of *Ascaris* ova [29]. Rocha et al. indicated that the different developmental stages of ova could interfere with viability determination [6]. Furthermore, treatment processes aimed at the inactivation of helminth ova can also impact the assessment of viability. Consequently, while enumeration of ova by optical microscopy, it is important that the larvae inside the ova present motility when stimulated by light [30]. The main drawback of this approach is being laborious and time-consuming [77].

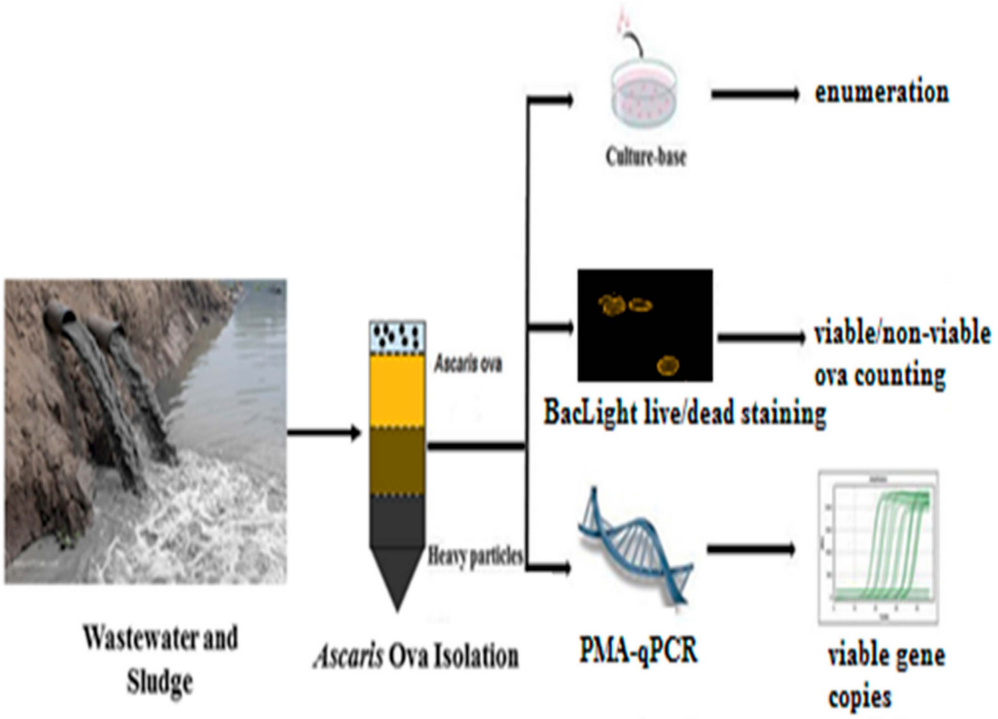

Figure 4. Various methods to enumerate, quantify and identify helminth ova (adapted from reference [1]).

Another microscopy-based method to determine viability is to differentiate live and dead helminth ova using stains or dyes. Here, vital stains such as Lugol's iodine, safranin O, trypan blue and eosin Y are used to stain and enumerate the ova under a microscope [30,78]. Despite the staining procedure being rapid and cost-effective, some vital dyes might disrupt the viable ova [26]. Another major disadvantage of the vital stain method is the significant (25%) loss of ova during visualization, which might be due to error during enumeration under a microscope [1]. To ease the individual error and over estimation of the viability of the vital stain method, the BacLight Live/Dead staining method has been utilized.

The BacLight Live/Dead bacterial viability kit was first developed to enumerate bacteria, however it is now being used to differentiate live and dead STH ova [79]. The principle depends on determining the difference in the structural integrity between live and dead ova [78]. Two DNA labeling dyes, in particular, Syto 9 and propidium iodide (PI) that possess the ability to penetrate the permeable membrane were used to differentiate *Ascaris* sp, *Trichuris* sp and *Toxocara* ova with increased efficiency, where Syto 9 stained the inner membrane of the viable ova while PI stained the inner parts of the non-viable ova (Figure 5). This study also revealed 58% viable, 38% non-viable and 3.7% partly stained STHs ova in sewage sludge samples [79]. However Karkashan et al. reported a maximum of 85% viability for *A. suum* using this staining method. This study also confirmed that staining with the BacLight Live/Dead viability kit was the only method that does not cause damage to the viability of ova [26]. This method can also determine the viability of STH ova directly from samples. Nevertheless, this method requires skilled personnel to differentiate helminth ova with

similar morphology. For instance, hookworm and *Ascaris* ova of different species are morphologically identical and cannot be differentiated with microscopic observation [80]. Therefore, results obtained from the vital stain method may be inaccurate and cannot be reliable in measuring health risks.

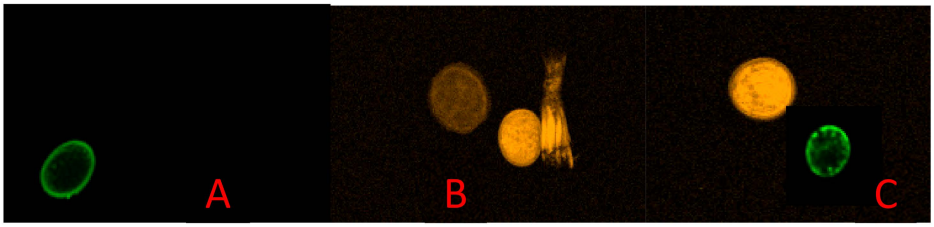

Figure 5. Confocal microscopy images of *Ascaris suum* ova stained with Syto 9 (green) and propidium Iodide (red) to determine the viability of ova: (**A**) Viable ova, (**B**) non-viable ova, (**C**) Both viable (green) and non-viable (red) ova.

5.2. PCR-Based

The inefficiency of nucleic acid (DNA/RNA) extraction methods to yield DNA with adequate quantity and purity can impact on sensitivity while detecting STH ova using molecular methods [81]. The tough ova shell of STHs such as *Ascaris* and *Trichuris* species is considered the main hindrance factor [82]. Various commercial kits have been optimized for the bacterial genomic extraction that have less tough cell walls compared to the STHs ova [83]. However for STH ova, it is essential to include proteinase enzyme which can lyse the outer protein coat of the ova shell in addition to sonication and beads [84].

Advancement in molecular technologies, particularly PCR-based techniques have been considered as potential tools for the quantification of STHs ova with precision and accuracy (Table 4) [29,41,85]. Though various PCR based methods [80,86–88] have emerged, their utilization for the identification of STHs ova in wastewater and sludge has been limited, despite its widespread use in clinical diagnostics [89].

One of the studies conducted by Raynal et al. revealed that ITS-1 signal reduced considerably in embryonated ova after 10 days following inactivation however signals persisted in viable ova [82]. The DNA that persists in the inactivated ova can be removed by treating with proteinase K and DNAase before performing RNA extraction. Further it has been demonstrated that reverse transcriptase (RT)-PCR for the mRNA of ITS1 revealed the presence of only viable ova, yet it was not sensitive enough to be considered as quantitative [6,90]. Utilization of other DNA binding dyes such as ethidium monoazide to inhibit the amplification of DNA in non-viable ova and quantitative RT-PCR in order to evaluate the expression of heat shock protein are considered as alternative approaches. However no reports on their use for assessing the viability of STH ova could be found. Also, molecular methods have a problem quantifying STH ova based on the copy number of genes, since the stage of ova development may vary [78].

As viable ova are prone to cause infection in humans, it is crucial to determine the fraction of viable ova in order to assess public health risks [79]. Propidium monoazide (PMA) is a DNA binding dye that has the potential to hinder PCR amplification of a non-viable ova resulting in the selective screening of viable ova present in wastewater matrices. However the major disadvantage is the dependency on the cell membrane integrity of viable and non-viable ova similar to vital stain method [41]. Since permeability of the inactivated ova takes 12 h, the photo-activation of PMA may not be accurately achieved [1]. However, PMA-qPCR can be used to implement health guidelines because the gene copy detected by PMA-qPCR can be considered as a viable ovum and thereby a potential health risk. Recent developments such as the utilization of PMAxx [91] and PEMAX [92] as an alternative to the original PMA are much effective in distinguishing viable and non-viable cells.

5.3. Flow Cytometry

Flow cytometry has the ability to measure and analyze various physical properties of a single cell, especially the ova/cysts when they pass via a light beam [93]. The differentiation of cells is based on the relative size, granularity and the intensity of fluorescence [94]. This approach was used to quantitatively detect the viable oocysts of *Cryptosporidium parvum* in water [95]. Recently, flow cytometry has been combined with real-time PCR and fluorescent biosensors to achieve more accurate results. Nevertheless, one of the studies comparing microscopic observation, flow cytometry and direct immunofluorescence for the screening of fecal samples for the presence of protozoan cysts revealed that flow cytometry did not show expected sensitivity than direct immunofluorescence despite being a rapid technique [96]. Furthermore, the main limitation of utilizing flow cytometry for the detection of helminth ova is the particle size limit ranging from 3 µm to 20 µm as helminth ova have a larger size range (Table 5). However, its use can be enhanced by developing the cassette that will be able to count larger particles up to 100 µm [95]. Also, the complex matrix of wastewater and sludge has to be taken into account as the interfering particulates will be challenging to remove in order for the sample to be analyzed using FACS (fluorescence activated cell sorter) without the possibility of clogging the machine. Consequently, with the development of enhanced ova recovery methods and the use of a large cassette, there might be a possibility of utilizing flow cytometry for the detection of STH ova. However, flow cytometry has not been utilized to detect STH ova to date [30].

Table 5. Advantages and drawbacks of currently available methods to enumerate and quantify helminth ova.

Methods	Advantages	Limitations	References
Optical microscopy	Viability possible Cost-effective Require less lab space Stains can differentiate viable and non-viable ova	Time-consuming Less sensitivity and specificity Possible false positive results in the determination of viability using stain-based methods Species differentiation is not possible	[47,61,96]
PCR-based	Fast, specific and sensitive Multiplex PCR is possible Quantitative detection (qPCR) of target pathogen is rapid	Not possible to distinguish viable and dead ovaneed for well-equipped laboratory Multiple primers required Requirement of skilled personnel	[79,90,96]
Flow cytometry	Accurate and reliable Differentiate cells based on complexity	Particle size detection limit ranging between 3 µm and 20 µm Expensive and require skilled personnel	[92,94]

6. Implementation of Innovative Techniques

Thus far, the focus has been on the currently available methods to recover and detect STH ova present in wastewater. However the conventional and molecular methods currently available have several limitations. According to the WHO, an ideal detection technique should follow the criteria of 'ASSURED'. Despite the term ASSURED being used for detection tests in resource-limited settings, the criteria are generic and should be adapted for all detection tests. However the tests cannot be simplified to fit the criteria [97]. Consequently, this review will now provide insights on radically innovative techniques that could potentially be implemented for detecting STH ova in wastewater.

6.1. Digital PCR

Digital droplet PCR (ddPCR) provides absolute quantification of desired genes and thus can utilized in the quantitative detection of pathogens [86]. The technique utilizes microwells that can

split the samples into several partitions in nanoliter [98]. The ddPCR relies on endpoint PCR and does not need any standard such as qPCR to determine the quantification [99]. Furthermore, it is a high throughput assay with 15,000 to 20,000 PCR reactions per well [30]. There are robots for setting up the plate and creating droplets which are more applicable for high throughput. By generating droplets, it also dilutes inhibitors much more effectively than conventional and real time PCR. One of the studies conducted to detect zoonotic pathogens in water samples from poultry found that ddPCR was effective than qPCR and culture-based methods [100]. Acosta et al. reported that dPCR can detect ≥ one *A. lumbricoides* ova present in 500 mL of reclaimed water. Droplet digital PCR has been utilized for the detection and absolute quantification of major gastrointestinal nematodes such as *Haemonchus*, *Teladorsagia* and *Trichostrongylus* in sheep [101]. Nevertheless, extensive validation for sensitivity and accuracy in the detection of STH ova in wastewater is required.

6.2. Aptamers

Aptamers are either single stranded RNA or DNA molecules which undergo changes in their conformation and fold into tertiary structures for binding the surface receptors of the target [102] (Figure 6). They exhibit high affinity and high specificity towards their targets and have the ability to differentiate proteins that are homologous and possess changes only in a few amino acids [103]. They have been utilized as a new tool in the diagnosis and drug delivery owing to their molecular properties, high stability, ease and control of their synthesis [104]. Over the past two decades, aptamers have been developed for various targets such as metal ions, whole cells, tissues, viruses and bacteria. Long et al. (2016) [105] employed an ova based egg-SELEX and identified ssDNA aptamers that specifically recognize and bind to *Schistosoma japonicum* ova. They have been successful in targeting human blood fluke (*Schistosoma japonicum*) ova trapped in liver tissues [106]. Hence the synthesis and utilization of aptamers can be effective in the detection of parasites.

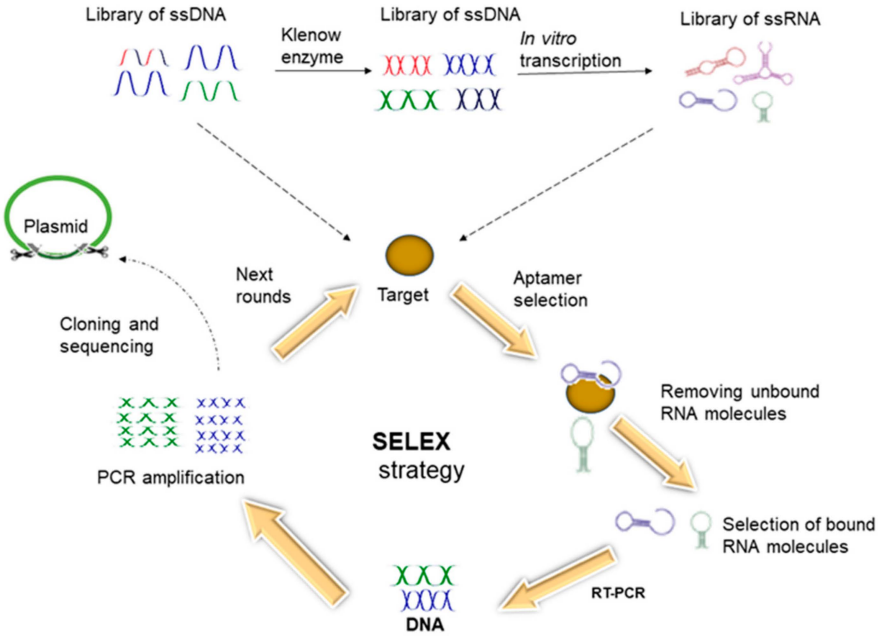

Figure 6. Schematic representation of the Systematic Evolution of Ligands by Exponential enrichment (egg-SELEX) technique to synthesize aptamers for the desired target (Adapted from reference [103]).

6.3. Gold Nanoparticle-Based Colorimetric Biosensors

The utilization of gold nanoparticles (AuNPs) for colorimetric analysis is based on the interparticle distance-dependent localized surface plasmon resonance (LSPR) property [107]. The size of the AuNP determines the controlled aggregation of the particles, thereby resulting in a change of color. This technique has been used in the analyses of specific gene sequences. It is expected that the change in

color during the aggregation of nanoparticles offer the basis for colorimetric detection using AuNPs in addition to providing differentiations in the surface enhancement abilities of Raman scattering [108].

The assays based on NP are currently being used to measure changes in the activity of enzymes with precision and accuracy. Gold nanoparticle-based colorimetric sensors have been utilized for the detection of enzymes, amino-acids and pathogenic bacteria [109,110]. This technique can be applied to detect and differentiate STHs ova based on the difference in their surface moieties [96]. Furthermore, the challenges such as turbidity encountered in wastewater and sludge samples can lead to non-specific aggregation of AuNPs, thus triggering false positive results. Therefore nanoparticles can be used in the development of biosensors and be incorporated into smart phones or portable devices for mobile sensing, however needs further innovation and validation.

6.4. Surface Enhanced Raman Scattering (SERS)

The detection of chemical transformations that occur during *in-situ* biosynthesis of metal nanoparticles are quite challenging as it occurs at the interfaces [111–113]. SERS is a surface-selective, accurate and a spectroscopic technique that is label-free and has been utilized in the detection of viable bacteria in drinking water using the synthesis of silver nanoparticles that coats the surface moiety of the bacterial cell wall [114]. The synthesized metal nanoparticles strongly exert the Surface Plasmon Resonance effect to the weak Raman signal arising from various different biological moieties on the egg surface. The utilization of metal nanoparticles enhanced the Raman signal of bacteria by 30-fold compared with that of the mixed colloid-bacterial suspension [111] (Figure 7). Additionally, this strategy was also used to differentiate three strains of *E. coli* and one strain of *S. epidermidis*. In another study, several pathogenic bacteria commonly found in systemic infections were successfully detected and discriminated in ≤5 min using SERS fingerprint at the single-cell level [112]. Moreover, SERS based detection using in situ coated metal nanoparticles has significant advantages in terms of minimal processing time, handling, minimal reactant volumes, less volume of the sample, greater sensitivity and selectivity. This strategy may be used to develop SERS-biosensors to differentiate species of STH ova, thereby providing an ultra-sensitive, rapid, and easy-to-use method of diagnosis.

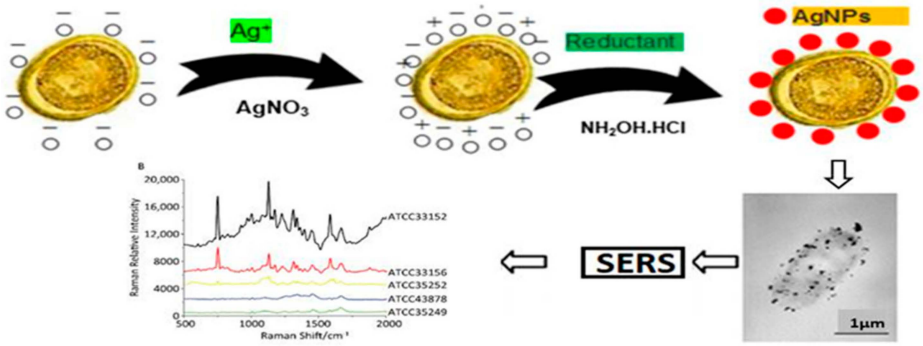

Figure 7. Surface Enhanced Raman Spectroscopy based detection using *In-situ* coating of Ag nanoparticles.

6.5. Smartphone-Based Detection

Smartphones have been utilized either alone or combined with microscopy in the detection of pathogens [115]. Furthermore, they act as a powerful tool in large epidemiological studies when used as an algorithm or app. The advantages of smartphone coupled with microscopic devices are being cost-effective and easily available. However smartphone technology based detection of parasites face challenges such as the absence of guidelines and a potential market for its application as an identification tool [116]. Nevertheless, new approaches such as the use of fluid geometrics combined with a mobile phone such as Nokia Lumia 2020 that has digital photo-microscopy would be beneficial to

detect and enumerate STH ova in endemic areas and resource limited settings [117]. In addition, further studies are required to analyze its potential to fulfill the diagnostic requirements in endemic areas.

6.6. Isothermal Amplification Assays

Recent advancements in the isothermal amplification assays for nucleic acids have resulted in incubation at a constant temperature for the amplification of nucleic acid [97]. This significantly minimized the requirement for equipment and opens a new pathway to perform nucleic acid amplification in field settings. The absence of thermocycling steps is advantageous as they reduce the time taken for amplification [118]. Rapid reaction also occurs due to multiple molecular reactions that can proceed asynchronously. The most utilized isothermal amplification methods are nucleic acid sequential amplification, signal-mediated amplification of ribonucleic acid technology, helicase dependent amplification, recombinase polymerase amplification (RPA), rolling circle amplification, multiple displacement amplification, loop-mediated isotheral amplification (LAMP) and strand displacement amplification [119].

LAMP is extremely sensitive and specific with the ability to detect genes that contain difference in a single nucleotide [120,121]. The assay utilizes a DNA polymerase that are not influenced by the presence of inhibitors and consists of a set of four primers that identify six different sequences on the DNA of interest [121,122]. However, the initiation of amplification follows binding of all the primers, thereby leading to the synthesis of an amplicon. The rapid amplification of a few DNA copies l to 10^9 within an hour significantly reduces the time required for analysis [77,123]. The possibility of assessing the reaction in real time via turbidity measurement or visualization of color change by naked eye makes it a potential method for onsite applications. LAMP has been used for detecting *Taenia* spp., hookworm and *Echinococcus granulosus* [87,124]. Despite LAMP being cost-effective and rapid, the pitfalls of LAMP are the false-positive results caused by the formation of primer-dimers and contamination. Another handicap is the complexity in designing primers and requirement of skilled personnel [77].

In addition to LAMP, one of the fastest developing isothermal amplification methods is RPA owing to its isothermal properties, simplicity, rapid turnaround and excellent sensitivity and specificity. RPA has been utilized in the detection of viruses, bacteria, protozoa and helminths from blood, cerebrospinal fluid and sputum [125,126]. RPA can be integrated with gel electrophoresis, microfluidics, lateral flow assays and performed in real time [119]. Nevertheless, its application in the laboratory-based and field-based detection of STH ova in wastewater has not been studied yet.

6.7. Paper-Based Sensors

This approach utilizes paper which creates microfluidics channels by patterning hydrophobic materials on hydrophilic paper [97]. The most widely utilized paper-based method for detection is based on colorimetric analysis where results are achieved by formation of a color that is generated due to the binding of analyte (target pathogen) and ligand. The advantages of using paper are (i) adsorption, (ii) excellent capillary action, (iii) compatibility with environmental samples, (iv) sterilization and disposal, (v) the capability for the storage and transportation of reagents in the paper matrix, (vi) lightweight and availability, (vii) low cost and (viii) simplicity [127]. Paper-based immunoassays (lateral flow and vertical flow) are being routinely performed for the detection of pathogens. Despite the advantages of paper-based sensors, limitations regarding the accuracy, sensitivity and inability to simultaneously detect more than one pathogen exist [128]. However the utilization of paper-based biosensors in the detection of STH ova remains unexplored.

7. Conclusions

Conventional detection methods have demonstrated reliability and reproducibility for several decades in the identification and enumeration of soil-transmitted helminths ova in wastewater. Despite various methods having been developed for the concentration of helminth ova, there is neither a

universally accepted nor a validated method for quality assurance and quality control. Furthermore these techniques are laborious and cumbersome, which has led to the emergence of PCR-based assays. Despite their rapidness, high sensitivity and specificity, PCR-based assays lack the ability to differentiate between viable and non-viable ova which are considered as being important for estimating the infection intensity. As such, this review has emphasized emerging alternatives that are robust. Since no single approach can satisfy the 'ASSURED' criteria for an ideal detection method, there is still a gap in research. This could nearly be fulfilled by utilizing two or more of the techniques in tandem for the detection of helminth ova. Additionally, the demand for rapid onsite (point-of-care) applications that require less technical ability is increasing enormously, especially in endemic areas and resource-limited settings. In addition to the aforementioned strategies, development of methods that require less technical assistance will also add value to the monitoring of helminth ova in wastewater, thus aiding the mitigation of helminths associated with risks in humans.

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