Infectious helminth ova in wastewater and sludge: A review on public health issues and current quantification practices

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ABSTRACT

Raw and partially treated wastewater has been widely used to maintain the global water demand. Presence of viable helminth ova and larvae in the wastewater raised significant public health concern especially when used for agriculture and aquaculture. Depending on the prevalence of helminth infections in communities, up to 1.0×10^3 ova/larvae can be presented per litre of wastewater and 4 gm (dry weight) of sludge. Multi-barrier approaches including pathogen reduction, risk assessment, and exposure reduction have been suggested by health regulators to minimise the potential health risk. However, with a lack of a sensitive and specific method for the quantitative detection of viable helminth ova from wastewater, an accurate health risk assessment is difficult to achieve. As a result, helminth infections are difficult to control from the communities despite two decades of global effort (mass drug administration). Molecular methods can be more sensitive and specific than currently adapted culture-based and vital stain methods. The molecular methods, however, required more and thorough investigation for its ability with accurate quantification of viable helminth ova/larvae from wastewater and sludge samples. Understanding different cell stages and corresponding gene copy numbers is pivotal for accurate quantification of helminth ova/larvae in wastewater samples. Identifying specific genetic markers including protein, lipid, and metabolites using multiomics approach could be utilized for cheap, rapid, sensitive, specific and point of care detection tools for helminth ova and larva in the wastewater.

Key words | detection methods, helminth ova, public health risk, sludge, wastewater reuse

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INTRODUCTION

Freshwater resources are under pressure through climate change and increasing population with high demand of fresh water. In this situation, wastewater can be used as an alternative source of water to reduce the pressure on non-potable demand of freshwater. One of the most significant issues in relation to wastewater reuse is the potential public health risk associated with viable helminth ova/larva (Jimenez *et al.* 2007). Therefore, treatment is a must before using wastewater for non-potable purpose where there is potential for human exposure (US EPA 2003; NRMMC 2004; WHO 2006).

Due to the high level of nutrients in raw and partially treated wastewater, it has been widely used for food production such as agriculture and aquaculture, especially in the developing countries (Carr 2005; Toze 2006a; Vuong *et al.* 2007; Sidhu & Toze 2009; Pinto & Maheshwari 2010; Pritchard *et al.* 2010; Hanjra *et al.* 2012). Despite the reuse of wastewater being mainly associated with socio-economic factors such as poverty and awareness of co-existed health risks, the global trend of wastewater and sludge reuse for crop production has increased significantly in the last decade (Oleszkiewicz & Mavinic 2001; Jimenez 2006; Sidhu & Toze 2009; Pinto & Maheshwari 2010; Pritchard *et al.* 2010; Hanjra *et al.* 2012; Kelessidis & Stasinakis 2012).

Infected individuals release helminth ova/larvae into wastewater that can potentially contaminate soil, plant, and surface water depending on the wastewater reuse. In those environments, helminth ova can survive up to several years (Sanguinetti *et al.* 2005; Abaidoo *et al.* 2010). For example, eggs of *Ascaris lumbricoides* can survive up to

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15 years in an environment upon favourable conditions (Hagel & Giusti 2010). Exposure to the wastewater and sludge or soil and crops contaminated with the viable helminth ova/larvae can lead to potential public health risk. The extent of the health risk depends on the numbers of viable ova/larvae present in the environment, infective dose, exposure routes and the susceptibility of the exposed individual (Haas 1996; Haas *et al.* 1999; Navarro & Jimenez 2011).

Since a viable ovum/larva has the potential to cause infection in humans (WHO 2006; Toze 2006b), a thorough understanding of the prevalence of viable helminth ova/larvae in wastewater and sludge is essential to understand the infection potential, and the risks they pose from beneficial reuse. The main aims of this literature review are (i) to determine the prevalence of helminths ova in wastewater and sludge, (ii) overview of public health risk, and (iii) critically examine the currently available quantification methods. Information presented in this paper will encourage researchers to look for a new detection method that can accurately quantify viable helminth ova from wastewater and sludge.

Articles, reports, conference proceedings, and guidelines published from 2000 to 2016 were taken into consideration. Electronic databases including PubMed, Google Scholar, and Web of Knowledge were used to obtain the information. The literature search was performed using keywords (helminth ova in wastewater, helminth ova in sludge, wastewater treatment methods and helminth ova inactivation, sludge treatment methods and helminth ova inactivation). Since *Ascaris lumbricoides* is being used as an indicator organism, search criteria for literature were broadened using keywords (*Ascaris* in wastewater, *Ascaris* in sludge, wastewater treatment and *Ascaris* ova inactivation). Overall, 178 items of literature were reviewed and information from 134 were used to prepare this article.

PREVALENCE AND CONCENTRATION OF HELMINTH OVA AND LARVAE IN WASTEWATER AND SLUDGE

The occurrence of helminth ova/larvae in wastewater and sludge depends on the prevalence of infections in the surrounding communities (Gaspard & Schwartzbrod 2003; Hajjami *et al.* 2012; Sharafi *et al.* 2012; Bastos *et al.* 2013). Soil-transmitted helminths (STHs) (*Ascaris lumbricoides, Ancylostoma duodenale, Necator americanus,* and *Trichuris trichiura*) are detected more often than others. This is due to the fact that i) STHs are the most prevalent parasites around the world with an infection rate of 1.3×10^9; ii) a mature STH female can produce large numbers of ova each day (Bethony *et al.* 2006; Hotez *et al.* 2014; WHO 2015) (Table 1); and iii) *A. lumbricoides* has been used as a model parasite to determine the quality of treated wastewater and sludge. However, ova/larvae of other helminths such as *Enterobius vermicularis, Strongyloides stercoralis, Toxocara* spp., *Taenia* spp., *Hymenolepsis nana, Echinococcus* spp., *Trichostrongylus* spp., and *Dicrocoelium dendriticum* have also been found in wastewater and sludge samples (Gaspard & Schwartzbrod 2003; Mahvi & Kia 2006; Do *et al.* 2007; Jimenez *et al.* 2007; Wichuk & McCartney 2007; Ben Ayed *et al.* 2009; Hajjami *et al.* 2012; Sharafi *et al.* 2012; Bastos *et al.* 2013; Konate *et al.* 2013a, 2013b).

Due to the lack of a robust and uniform method, for isolation and quantification of helminth ova from wastewater and sludge, it is almost impossible to make an accurate comparative assessment of the concentration of ova in wastewater and sludge samples within and between the studies (Amoah *et al.* 2017). The concentration of ova in raw wastewater (1 L) and sludge (4 gm dry weight) can be as high as 10^3–10^4 depending upon the rate of infection in the community (Table 2). Due to the high settling velocity of helminth ova, their concentration in sludge should be higher than wastewater (Konate *et al.* 2010; Sengupta *et al.* 2011, 2012). However, Navarro & Jimenez (2011) reported high concentration of helminth ova in wastewater compared to the sludge samples. This could be due to poor recovery rate of helminth ova from sludge compared to the wastewater samples using flotation method. Gyawali *et al.* (2015a) reported poor (0.02–3.4%) recovery rate of helminth ova from sludge samples compared to the wastewater (7.1–7.4%).

Climatic conditions such as temperature and relative humidity can also influence the numbers of ova and larvae in the wastewater and sludge (Hajjami *et al.* 2012; Sharafi *et al.* 2012; Bastos *et al.* 2013). This is because helminth ova are known to develop faster at a temperature between 28 and 32 °C (Seamster 1950; Beer 1976; Smith & Schad 1989; Brooker *et al.* 2006). In addition, soil moisture and relative humidity also influence the survival of viable ova and larvae (Nwosu & Anya 1980; Udonsi *et al.* 1980; Brooker *et al.* 2006). Therefore, this potentially infects the wider population which eventually contributes more ova into the wastewater system.

POTENTIAL PUBLIC HEALTH RISKS

Wastewater and sludge contaminated with helminth ova and larvae can pose significant public health risks. People become

Table 1 | Commonly detected helminths in wastewater and sludge, infective stage and transmission mode to humans

Helminths	No of ova/larvae produced by a female	Infective stage	Transmission mode	References
Ascaris spp.	$2 \times 10^{5,a}$	Embryonated ova	OI	Knopp *et al.* (2012)
Hookworm	$3 \times 10^{4,a}$	Filariform larvae	OI/SP	Bethony *et al.* (2006)
T. trichiura	$5 \times 10^{3,a}$	Embryonated ova	OI	Knopp *et al.* (2012)
E. vermicularis	$1.6 \times 10^{4,b}$	Embryonated ova	OI	Burkhart & Burkhart (2005)
S. stercoralis	$4 \times 10^{1,a}$	Filariform larvae	SP	Ganesh & Cruz (2011)
Toxocara spp.	$2 \times 10^{5,a}$	Embryonated ova	OI	Azam *et al.* (2012)
Taenia spp.	$5 \times 10^{7,b}$	Embryonated cyst	OI	Bogitsh *et al.* (2012)
Hymenolepis spp.	NR	Embryonated cyst	OI	Bogitsh *et al.* (2012)
Echinococcus spp.	NR	Embryonated ova	OI	Bogitsh *et al.* (2012)
D. dendriticum	NR	Metacercariae larvae	OI	Bogitsh *et al.* (2012)
Fasciola spp.	$2.5 \times 10^{4,a}$	Metacercariae larvae	OI	Hanna *et al.* (2008)
Spirometra spp.	NR	Coracida larvae	OI	Bogitsh *et al.* (2012)
Schistosoma spp.	$3 \times 10^{2,a}$	Metacercariae larvae	OI	Grevelding (2004)

[a] = day.
[b] = life time.
NR = not reported, OI = oral ingestion, SP = skin penetration.

infected by exposing themselves to the wastewater and sludge contaminated with helminth ova (Figure 1). Wastewater and sludge treatment plant workers and farmers applying wastewater and sludge for agriculture and aquaculture may directly expose themselves to the helminth ova and larvae present in the wastewater and sludge during their work (Ensink *et al.* 2008; Seidu *et al.* 2008; Ackerson & Awuah 2012). Health risk among this group (wastewater workers and farmers using partially treated wastewater) is associated with their working conditions, individual behaviour, personal hygiene and use of personal protective equipment (El Kettani & Azzouzi El 2006; The World Bank 2010). The health risk in this group is significantly higher in developing countries compared to developed countries (Ensink *et al.* 2005; El Kettani & Azzouzi El 2006; Trang *et al.* 2006; The World Bank 2010), due to i) the higher prevalence rate of helminth infections in developing countries compared to the develop countries; ii) poverty; iii) lack of health education regarding the potential transmission of helminth; and iv) lack of use of personal protective equipment.

People also acquire helminth infections via indirectly exposure to helminth ova/larvae present in the wastewater and sludge. For example, handling and consuming products (vegetable and aquaculture) grown on farms that use wastewater and sludge (Ensink *et al.* 2007; Vuong *et al.* 2007; Gupta *et al.* 2009; Al-Megrm 2010; Iwamoto *et al.* 2010; Navarro & Jimenez 2011; Ackerson & Awuah 2012; Adamu *et al.* 2012).

Helminth ova contained in the wastewater (treated or raw) and sludge can contaminate both surface (e.g. spinach and parsley) and underground (e.g. carrots and radish) crops (Navarro *et al.* 2009; Navarro & Jimenez 2011; Rostami *et al.* 2016). It has been reported that up to 90% of helminths ova present in the irrigated water can get onto green and leafy vegetables such as spinach and parsley with magnitude of 1 ovum/10 gm vegetable (Amahmid *et al.* 1999; Vuong *et al.* 2007; Navarro & Jimenez 2011). The magnitude of the contamination in subsurface vegetables, however, is much (98%) lower than in leafy vegetables (Amahmid *et al.* 1999). This is because the leafy vegetables like spinach, lettuce and cabbage have large contact areas for STHs ova/larvae. In addition, the magnitude of the contamination increases in the rainy season (Vuong *et al.* 2007) due to either flooding or shifting helminth ova/larvae from the ground to the vegetables by rain. Similarly, helminth ova present in the wastewater could accumulate in fish or shellfish through a feeding mechanism where they survive until finding the human host. The potential health risks to this groups can increase with the growing trend of eating raw vegetables and seafood for the sake of nutrients. Additionally, the international transportation of fruits, vegetables and seafood has increases the human health risks in global scale.

Gardens, parks and sporting venues that use wastewater for irrigation and waterways contaminated with wastewater runoff can contain viable helminth ova. Using those land or

Table 2 | Concentration of helminth ova in raw wastewater, treated wastewater and sludge in different locations

Locations	Concentration of helminth ova in wastewater matrices			References
	Wastewater (ova/L)	Treated wastewater (ova/L)	Sludge (ova/gm)	
Australia	*NR*	41^{H}	*NR*	Water Corporation (2012)
Brazil	33^{As}, 11^{En}, 5^{St}, 4^{Ta}, 4^{Hy}, 2^{Ho}, 2^{Tr}	3^{As}, 3^{En}, 3^{Hy}	*NR*	Cutolo *et al.* (2006)
	NR	*NR*	5^{As}	Bastos *et al.* (2013)
	NR	*NR*	22^{To}, 11^{As}, 11^{Tr}	Bonatti & Franco (2016)
	700^{H}	*NR*	*NR*	Stott *et al.* (2003)
Burkina Faso	10^{As}, 4^{Ho}, 1^{Tr}	1^{As}	1327^{As}, 442^{Ho}, 20^{Tr}	Konate *et al.* (2010)
Egypt	35^{H}	9^{H}	*NR*	Stott *et al.* (2003)
France	*NR*	*NR*	5.3^{H}	Gantzer *et al.* (2001)
Ghana	*NR*	*NR*	94^{As}, 24^{Tr}	Kone *et al.* (2007)
India	72^{As}, 15^{Ho}, 4^{Tr}	60^{As}, 9^{Ho}, 2^{Tr}	*NR*	Gupta *et al.* (2009)
	72^{H}	4^{H}	*NR*	Tyagi *et al.* (2011)
Iran	NR	*NR*	225^{H}	Bina *et al.* (2004)
	12^{As}, 3^{Ta}, 2^{Ho}, 1^{En}	1^{As}	*NR*	Mahvi & Kia (2006)
Mexico	330^{H}	*NR*	177^{H}	Navarro & Jimenez (2011)
	NR	*NR*	57^{As}, 5^{Tr}, 2^{To}	Pecson *et al.* (2007)
	65^{H}	1^{H}	2^{H}	Jimenez-Cisneros *et al.* (2001)
	135^{H}	*NR*	*NR*	Cifuentes *et al.* (1999)
Morocco	23^{H}	*NR*	*NR*	Kouraa *et al.* (2002)
	NR	*NR*	0.1^{As}, 0.06^{Hy}	Moubarrad & Assobhei (2007)
Oman	517^{As}, 405^{Tr}, 33^{Ho}, 18^{To}, 12^{St}, 3^{En}	45^{As}, 18^{To}, 6^{St}	*NR*	Rivera *et al.* (2012)
Pakistan	175^{As}, 68^{Hy}, 26^{Tr}	4^{As}, 2^{Hy}	*NR*	Sharafi *et al.* (2012)
	5218^{Ho}, 672^{As}, 276^{Hy}, 62^{Tr}, 8^{Ta}	*NR*	*NR*	Ensink *et al.* (2007)
Peru	272^{H}	47^{H}	*NR*	Yaya-Beas *et al.* (2016)
Spain	10^{H}	0.9^{H}	*NR*	Reinoso *et al.* (2008)
Tunisia	960^{As}, 535^{Ev}, 208^{Ta}, 140^{Hy}	320^{Hy}, 240^{As}, 80^{Ev}, 52^{Ta}	*NR*	Ben Ayed *et al.* (2009)
	19^{H}	0^{H}	*NR*	Saddoud *et al.* (2007)
	15^{H}	2^{H}	*NR*	Riahi *et al.* (2009)
USA	8^{H}	*NR*	13^{H}	Navarro & Jimenez (2011)
	NR	*NR*	336^{As}	Engohang-Ndong *et al.* (2015)
	NR	*NR*	789^{As}	Bowman *et al.* (2003)
Vietnam	*NR*	*NR*	1200^{As}	Yen-Phi *et al.* (2010)
	5730^{As}	*NR*	*NR*	Do *et al.* (2007)

As = *Ascaris*, *Hy* = *Hymenolepsis*, *Tr* = *Trichuris*, *Ta* = *Taenia*, *En* = *Enterobius*, *To* = *Toxocara*, *Ho* = Hookworm, *St* = *Strongyloides*, *H* = Not specified, *NR* = Not reported.
The numbers present in the table is highest range reported in the study.

waterways for recreational activities is another indirect way of human exposure to helminth ova/larvae present in wastewater (Horweg *et al.* 2006; Moubarrad & Assobhei 2007; King 2010). It has been demonstrated that school-aged children playing in a wastewater-irrigated park had an 18% higher rate helminths infection than those did not used the park (Moubarrad & Assobhei 2007). Pets, especially dogs, can carry helminth ova/larvae home from outside where there is helminth contamination (Gyawali *et al.* 2013), and thereby transmit to humans. This issue however, can be reduced by adopting good hygiene practices.

PUBLIC HEALTH PREVENTION MEASURES

Various wastewater and sludge reuse guidelines have been established to minimise the public health risk associated with helminth ova/larvae present in wastewater (US EPA 2003; NRMMC 2004; WHO 2006; DEC 2012). The guidelines have identified the potential points for health risks and designed a multi-barrier approach (Figure 2).

This approach includes good safe agriculture practice, good manufacturing practice and good hygiene practices where wastewater and sludge treatment alone is not sufficient

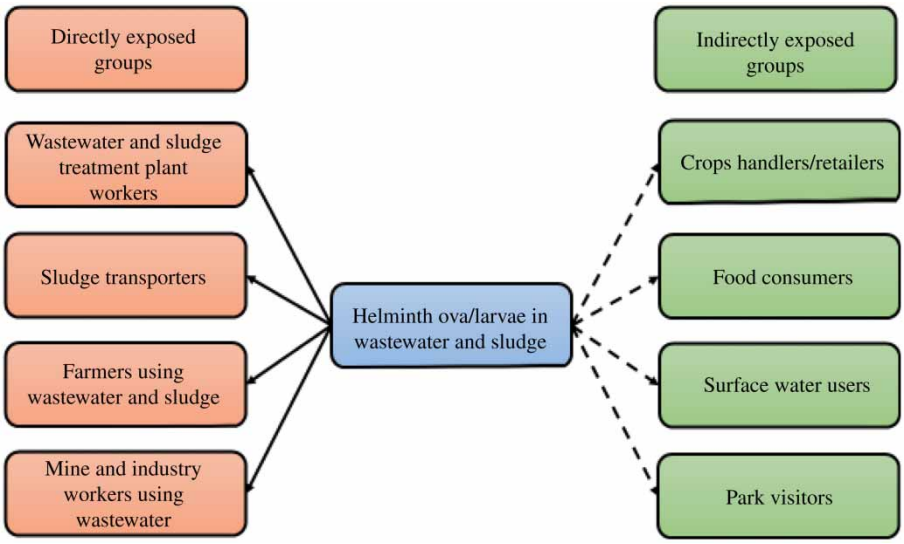

Figure 1 | Pathway of helminth infection from wastewater and sludge reuse.

to prevent the potential public health risks (WHO 2006; IWMI and IWRC 2010). An acceptable limit of helminth ova in treated wastewater and sludge depending on their final use has been recommended on the basis of cost benefit analysis of wastewater treatment methods versus potential health risks. For example, World Health Organization (WHO) has purposed value of <1 viable ovum in 1 L of treated wastewater or 4 gm of dry sludge can be used without restriction.

Wastewater and sludge treatment process is the first and most important measure towards minimising the potential health risk associated with wastewater reuse. Various wastewater and sludge treatment methods have also been proposed to inactivate helminth ova from wastewater and sludge (US EPA 2003; WHO 2006). Since the specific gravity of STHs ova is higher than wastewater, they quickly settle down into wastewater (Sengupta *et al.* 2011, 2012). Therefore, simple wastewater treatment methods such as ponding also effectively (90–99%) remove helminth ova from liquid (Toze 2006b; Reinoso *et al.* 2011; Konate *et al.* 2013a) (Table 3). However, those methods will concentrate the ova in to the sludge (solid) where they can remain viable for a long period of time (up to 20 months) (Sanguinetti *et al.* 2005).

Different methods such as aerobic digestion, anaerobic digestion, lime stabilisation and heat treatment, depending on the availability of resources and feasibility, have been used to inactivate the helminth ova from sludge (Mendez *et al.* 2002; Bina *et al.* 2004; Capizzi-Banas *et al.* 2004; Mendez-Contreras *et al.* 2009; Maya *et al.* 2010; Reinoso

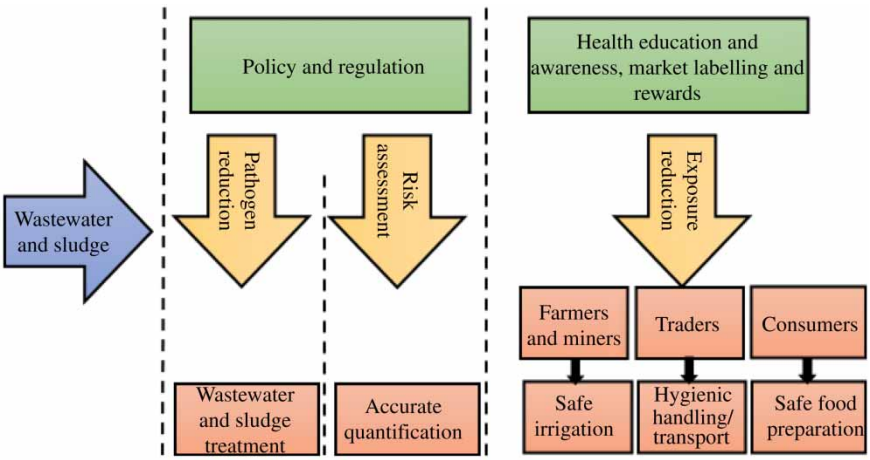

Figure 2 | Flow chart showing multi-barrier approach to minimise the public health risk associated with wastewater and sludge reuse.

Table 3 | Efficacy of different sludge treatment methods on helminth ova inactivation

Treatment methods	Treatment conditions	Helminth ova	Inactivation rate (%)	References
Lime stabilization	Ammonia 20% w/w for 2 h	[a]	83	Mendez *et al.* (2004)
	Ammonia 20% w/w with 50 °C for 2 h	[a]	100	Mendez *et al.* (2004)
	pH 10.2 for 40 days	*A.lumbricoides*	94	Endale *et al.* (2012)
	pH 11 for 120 h	[a]	56	Bina *et al.* (2004)
	pH 12 for 120 h	[a]	83	Bina *et al.* (2004)
	pH 10 with humidity 90% for 8 months of RT	*A. lumbricoides*	100	Maya *et al.* (2010)
	pH 12.5 with humidity 80% for 4 months of RT	*A. lumbricoides*	84–95	Maya *et al.* (2010)
Heat treatment	50 °C-51.5 °C for 2 h at normal pH	*A. suum*	100	Paulsrud *et al.* (2004)
	70 °C with humidity 80% for 2 h	[a]	100	Maya *et al.* (2010)
	75 °C with 5% dryness for 3 h	*A. lumbricoides*	100	Maya *et al.* (2012)
	78 °C with 5% dryness for 3 h	*A. suum*	100	Maya *et al.* (2012)
	74 °C with 5% dryness for 3 h	*T. canis*	100	Maya *et al.* (2012)
	73 °C with 5% dryness for 3 h	*T. trichiura and T. solium*	100	Maya *et al.* (2012)
	72 °C with 5% dryness for 3 h	*H. nana*	100	Maya *et al.* (2012)
Pond stabilization	Facultative pond for a 9.5 days of HRT	*A. lumbricoides* and	98	Konate *et al.* (2013a)
	Maturation pond for a 5.5 days of HRT	Hookworm	99	Konate *et al.* (2013a)
	Anaerobic pond for a 0.4 days of HRT	*A. lumbricoides* and	90	Reinoso *et al.* (2011)
	Facultative pond for a 4.1 days of HRT	Hookworm	92	Reinoso *et al.* (2011)
Anaerobic digestion	Mesophilic temperature (35 °C) for 31 days of HRT	[a]	0.3	Mendez-Contreras *et al.* (2009)
	Thermophilic temperature (55 °C) for 21 days of HRT	[a]	85–100	Mendez-Contreras *et al.* (2009)
	Thermophilic temperature (55 °C) for 120 min	*A. suum*	100	Ruiz-Espinoza *et al.* (2012)
	Thermophilic temperature (40 °C) for 120 min			Ruiz-Espinoza *et al.* (2012
	Thermophilic temperature (61 °C–62.5 °C) for 45 min			Paulsrud *et al.* (2004)
Aerobic digestion	Sludge/40 days of RT	*A. lumbricoides*	73	Endale *et al.* (2012)
	Mixed with ash/40 days of RT	*A. lumbricoides*	100	Endale *et al.* (2012)
	Mixed with soil/40 days of RT	*A. lumbricoides*	76	Endale *et al.* (2012
	Mixed with smooth soil/28 days of RT	[a]	97	Ferreira *et al.* (2002)
	Mixed with unsmooth soil/28 days of RT	[a]	89	Ferreira *et al.* (2002)

[a]Helminths not specified, RT= Retention time, HRT= Hydraulic retention time.

et al. 2011; Endale *et al.* 2012; Ruiz-Espinoza *et al.* 2012; Konate *et al.* 2013a) (Table 3). The inactivation rate of these treatment methods, however, is not consistent across the studies (Mendez *et al.* 2002; Bina *et al.* 2004; Capizzi-Banas *et al.* 2004; WHO 2006; Maya *et al.* 2010, 2012; Navarro & Jimenez 2011) (Table 3). This discrepancy between the inactivation rates between the studies could be due to the variability in recovery and detection of currently available methods.

PUBLIC HEALTH RISK MEASUREMENT METHODS

The potential public health risks associated with wastewater and sludge reuse is measured by quantifying the numbers of viable *A. lumbricoides* ova (US EPA 2003; WHO 2006). This approach may not represent the true health risk associated with wastewater and sludge reuse and needs to be changed, because types of helminths and their numbers in wastewater and sludge depend on the prevalence rate of infections in the community that generates the wastewater. Another fundamental issue related to the health risk measurement is the quantification method that is used to identify and quantitate the viable helminth ova from wastewater. Despite the advancement in quantitative detection methods, microscope dependent methods, i.e. culture-based (US EPA 2003) and vital staining (de Victorica & Galvan 2003) are being used to quantify viable helminth ova from wastewater and sludge samples.

Culture-based method

The culture-based method involves artificially hatching the ova in a laboratory. Helminth ova are incubated at 28 °C–30 °C for up to 28 days depending on the helminth, to allow the viable ova to hatch and are observed microscopically (Bowman *et al.* 2003) (Figure 3). Health regulators, including the United States Environmental Protection Agency (US EPA) and WHO, recommend this method because it has the ability to estimate the viability of helminth ova recovered from treated wastewater and sludge. The requirement of highly skilled personnel to accurately distinguish between ova/larvae of different helminths is major disadvantage of this method (Traub *et al.* 2007; Verweij *et al.* 2007). In addition, the detection limit of the method depends on the detection sensitivity of a microscope that may not be sensitive enough to detect low numbers of larvae in a sample (Weber *et al.* 1991). Gyawali *et al.* (2017b) has reported that there is a loss 33% of viable ova/larvae during visualisation. The most important limitation of the culture-based method is the lengthy wait for the result to be available which potentially increases the operational cost of the method. The main advantages and disadvantages of the culture-based method are listed in Table 4.

Vital stain method

The stain-based method is rapid, cheap and easy to use compared to the culture-based method (de Victorica & Galvan 2003). This method involves staining helminth ova with a vital stain such as Trypan blue, Congo red, Eosin Y, Hematoxylin, Methyl green, Safranin O, Methylene blue or Lugol's iodine and counting the ova under a microscope. This method takes advantage of different working mechanisms of the cell wall of viable and non-viable ova. A viable helminth ovum has three layers of intact cell walls that act as an alternative sieve and prevent the stain from entering into the cytoplasm (Matthews 1986). Once the ovum becomes non-viable, the integrity of cell wall is compromised and it becomes permeable to stain (Bae & Wuertz 2009) (Figure 3). The cell wall, however, is not permeable immediately after inactivation, and this can lead to over-estimation of viable ova in a sample (Gyawali *et al.* 2016c).

Another major disadvantage of the vital stain method is the potential loss of significant (25%) numbers of ova during visualisation (Gyawali *et al.* 2017b). This might be associated with either an inbuilt error of a microscope or an individual's error while quantifying helminth ova using the microscope (Table 4). To ease the individual error and over-estimation of the viability of the vital stain method, BacLight LIVE/DEAD staining method has been developed (Dabrowska *et al.* 2014; Karkashan *et al.* 2015). Using fluorescent microscopy and membrane permeable DNA-labelling dyes, such as Syto 9 and Propidium Iodide (PI), it differentiates the viable ova (stained green with Syto 9) with non-viable (stained red with PI). Karkashan *et al.* (2015) reported this method could improve the viability assessment of helminth ova up to 85%. There is, however, potential of generating faulty images because of various amounts of DNA, which both dyes bind (after penetration

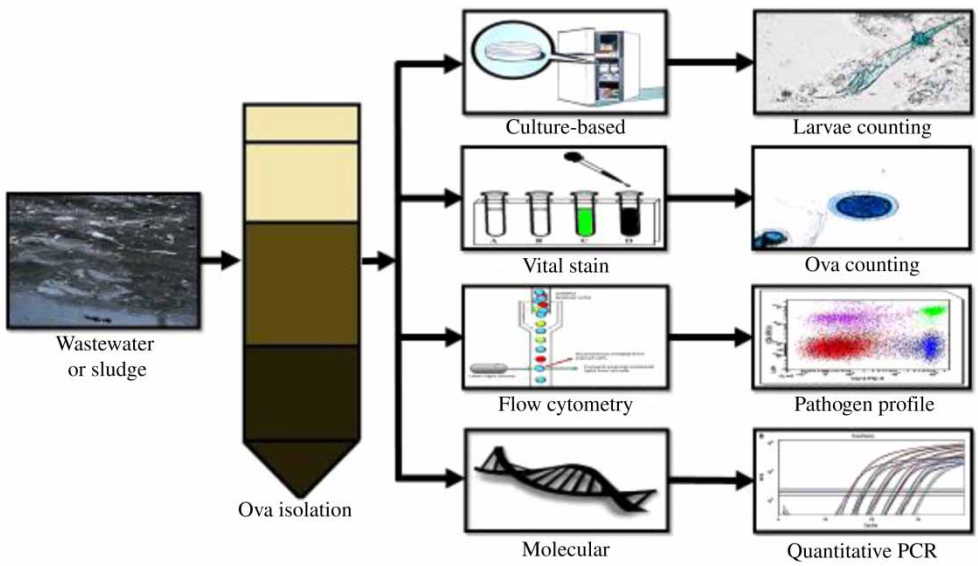

Figure 3 | Principle of different method for quantifying viable helminth ova.

Table 4 | Advantages and disadvantages of different quantitative detection methods for helminth ova

Detection methods	Advantages	Disadvantages	References
Culture-based	• Viability is possible • Cheaper chemicals and equipment • Can be done in a small scale laboratory	• Prolonged time to obtain a result • Suitable conditions and regular observation necessary • Low sensitivity and specificity	de Victorica & Galvan (2003), Bowman *et al.* (2003), US EPA (2003), Gyawali *et al.* (2017b)
Vital stain	• Cheap and easy process • Faster than culture-based method • Fewer chemicals and equipment • Can be done in small scale laboratory • Viability can be assessed	• Sensitivity depends on detection threshold of a microscope • Possibility of false positive result because stain may not penetrate the cell wall of recently inactivated ova • Differentiation between the species is impossible • Due to the toxicity of stains, ova may be inactivated during staining process	Weber *et al.*(1991), Nelson & Darby (2001), Cabaret *et al.* (2002), de Victorica & Galvan (2003), Traub *et al.* (2007), Verweij *et al.* (2007), Dabrowska *et al.* (2014), Karkashan *et al.* (2015), Gyawali *et al.* (2016c), Gyawali *et al.* (2017b)
Molecular	• Quick and easy process • High sensitivity and specificity • Automated process • [a]Multiple species can be identified from single sample • [b]Quantification is possible • [c]Viability can be assessed	• Require advanced laboratory and equipment • Genomic information is essential • Possibility of providing false positive result by extracting DNA from inactivated ova • Possibility of false negative result via inhibitors present in the samples • Sensitivity could be reduce because of multiple sets of primers • Need to run two sets of PCR for viability	Pecson *et al.* (2006), Verweij *et al.* (2007), Traub *et al.* (2008), Ngui *et al.* (2012), Gyawali *et al.* (2016a), Gyawali *et al.* (2017a), Gyawali *et al.* (2017b)

[a] = Multiplex PCR.
[b] = Quantitative PCR.
[c] = PMA-qPCR.

through intact structures of cells), emitting fluorescence, resulting in difficulty differentiating ova for visual assessment.

The method still requires skilled personnel to differentiate helminth ova with similar morphology. For example, hookworm and *Ascaris* ova of different species are morphologically identical and cannot be differentiated with microscopic observation (Traub *et al.* 2004, 2007; Do *et al.* 2007). Previous studies have noted that even skilled personnel may not be able to distinguish hookworm ova of different species as well as from other helminths (Cabaret *et al.* 2002; Traub *et al.* 2007; Verweij *et al.* 2007). Therefore, results obtained from the vital stain method may not be accurate and reliable in measuring the health risks.

Molecular methods

Developments in PCR methods have resulted in rapid, specific and sensitive detection of helminth ova from environmental samples (Gyawali *et al.* 2015b; Rudko *et al.* 2017). A high number of gene copies per ovum (Pecson *et al.* 2006; Raynal *et al.* 2012; Gyawali *et al.* 2017a) allows molecular method to detect less than an ovum from wastewater (1 L) and sludge (4 gm) samples. Gyawali *et al.* (2015b) demonstrated that the real-time PCR method has the ability to detect <1 ova from 1 L of treated wastewater. This means that the acceptable limit of viable helminth ova in treated wastewater and sludge for unrestricted use can be lower to <0.1 in treated wastewater (1 L) and sludge (4 gm) as suggested by many health regulators (IWMI and IWRC 2010). Additionally, ribosomal rRNA or rDNA of Internal Transcribed Spacer (ITS-1 and ITS-2) DNA regions contain high variability in a closely related species (Traub *et al.* 2007, 2008), thus can be used to distinguish human and zoonotic species from environmental samples.

Despite high sensitivity and specificity, the real-time PCR method is not able to quantify the concentration of helminth ova. A quantitative PCR (qPCR) method, however, can quantify helminth on the basis of amplified gene copy numbers present in the target nucleic acid (Pecson *et al.*

2006; Raynal *et al.* 2012; Gyawali *et al.* 2015a, 2016a, 2017a). Gyawali *et al.* (2017a) attempted to quantify hookworm ova from seeded wastewater samples and found this challenging due to the presence of varying numbers of gene copies (5.6×10^2–1.0×10^4) present in an ovum depending on the development stage. Similarly, Pecson *et al.* (2006) conducted a kinetic assessment of *Ascaris* ova to determine the ITS-1 rDNA and rRNA gene copy numbers in an ovum. The result of the study suggested that an ovum could have up to 600 cells before hatching a larva. Since wastewater and sludge samples may contain multiple cell staged helminth ova, a qPCR may produce variable results while quantifying them.

PCR/qPCR methods are also unable to distinguish between viable and non-viable ova. Since only viable ova are capable of hatching infectious (L_3) larvae and consequently causing infections in humans, it is important to know what fraction of the PCR-amplified ova are viable for the assessment of public health risks. Gyawali *et al.* (2016a) used propidium monoazide (PMA), a DNA intercalating dye, to eliminate the gene amplifying from non-viable ova during PCR amplification. The result of that study indicated that a PMA-qPCR could be used for the selective detection of viable helminth ova from environmental samples. The major disadvantage of this method is that it also depends on the structural integrity of viable and non-viable ova like vital stain method. Since the cell wall of inactivated ova require up to 12 h to become permeable, PMA photo activation may not be accurately achieved (Gyawali *et al.* 2016c). Other factors impact the effectiveness of PMA-qPCR depend on the concentration of stain, incubating time and light exposure (Rudi *et al.* 2005; Nocker *et al.* 2006; Wagner *et al.* 2008; Chang *et al.* 2010). However, PMA-qPCR method can be used to implement the health guidelines because the gene copy detected by PMA-qPCR can be considered as a viable ovum and, thereby, a potential health risk.

FUTURE DIRECTION

Multiomics is the analysis of genome, proteome, lipidome and metabolite information of an organism. It is the most rapidly advancing technology and has the potential to identify key markers (gene, protein, lipid and metabolites) that influence the development and infectivity of a pathogen (Preidis & Hotez 2015; Tyagi *et al.* 2015). Viability could be determined by identifying low molecular weight intracellular chemical compounds. Furthermore, studies have demonstrated that the application of the chemometric statistical analyses is able to characterise and differentiate large groups of biological data based on their metabolic profiles (Kouremenos *et al.* 2014; Tyagi *et al.* 2015). This was also demonstrated by analysing an untargeted metabolic profiling of human faecal samples infected with *Cryptosporidium* spp. (Ng *et al.* 2012). Gyawali *et al.* (2016b) also demonstrated the potential application of mass spectrometry and chemometric analysis for distinguishing viable and non-viable hookworm ova in a laboratory setting. There are, however, many challenges to the widespread application of multiomics approach within the context of the areas/countries impacted (third world endemic regions). Firstly, this approach is relatively expensive with the minimal cost for equipment being 200,000 NZ dollars. Secondly, it requires advance laboratory and well trained personnel to run the assays and analyse the data. Despite the challenges, this approach has the potential to identify key biological marker that can be used to develop a low cost devices such as strip, aptamers or bio-sensor in future that can be used for the detection of helminths from wastewater in real time.

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