Food and Waterborne Parasitology 28 (2022) e00174

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Potentials and challenges in the isolation and detection of ascarid eggs in complex environmental matrices

Patrick Waindok [1], Marie-Kristin Raulf [1], Christina Strube [*]

Institute for Parasitology, Centre for Infection Medicine, University of Veterinary Medicine Hannover, Hanover, Germany

ARTICLE INFO

Keywords:
Ascaris
Toxocara
Egg recovery
Soil
Environmental contamination

ABSTRACT

Ascarid infections constitute a major concern for both human and animal health risk assessment. Although being effectively transmitted by soil, water and contaminated food, reliable detection of ascarid eggs in environmental media often remains challenging. However, contamination of the environment with ascarid ova has gained more attention as a decisive part of proper risk assessment in recent years. Due to various factors, such as sample matrices, dissociation detergents and flotation solutions, defined and standardised protocols for the isolation of eggs from complex environmental matrices are difficult to establish and therefore limited. Thus, this study reviews common techniques used for the recovery of ascarid eggs from environmental media with special emphasis on sampling strategies, purification procedures and microscopic as well as molecular detection of egg contamination. Despite various advancements, mainly in the field of molecular methods leading to more reliable and sensitive detection, it can be concluded that there is still a need for unified guidelines for sampling and recovery of ascarid eggs derived from complex environmental matrices.

1. Introduction

Parasitic ascarid roundworms infect animals since at least 240 million years when mammals evolutionarily began to diverge from their ancestors in the Triassic period (Silva et al., 2014). Nowadays, the infraorder Ascaridomorpha comprises >50 genera of monoxenous and heteroxenous species, characterised as medium to large worms, often with three lips on the anterior end of the adult worms. Hosts acquire infections by ingestion of eggs containing infective third-stage larvae (L3) or of L3 present in intermediate (e.g. Crustacea for *Anisakis simplex*) or paratenic hosts. In the vertebrate definitive host, helminths generally parasitise the stomach or the intestinal tract (Nadler and Hudspeth, 2000), whereas in intermediate as well as paratenic host the L3 tend to remain in an arrested stage in different organs without developing into adults (Bowman, 2020).

The human roundworm *Ascaris lumbricoides*, one of the most important representatives of the ascarids, affects at least 447 million people worldwide (Crompton, 2001), with over 1 billion children requiring preventive chemotherapy for soil-transmitted helminthoses in 2020 (WHO, 2022). After ingestion of infective L3, larval migration through the pulmonary tissue may result in acute lung inflammation with clinical pulmonary signs. Manifestation of adult *A. lumbricoides* in the intestine may lead to abdominal distension, pain, nausea and diarrhoea (Crompton, 2001). The majority of infections tend to be asymptomatic, while an estimated

* Corresponding author at: Institute for Parasitology, Centre for Infection Medicine, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hanover, Germany.
E-mail address: christina.strube@tiho-hannover.de (C. Strube).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fawpar.2022.e00174
Received 27 October 2021; Received in revised form 5 July 2022; Accepted 1 August 2022
Available online 9 August 2022
2405-6766/© 2022 The Author(s). Published by Elsevier Inc. on behalf of International Association of Food and Waterborne Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1
Human infecting ascarid soil-transmitted helminths, their hosts and main sources of infection/environmental contamination.

Species	Definitive hosts (excretion of eggs)	Paratenic hosts	Sources of infection/environmental contamination	References
Ascaris lumbricoides	Humans, occasionally pigs	–	Toilets/latrines and their surroundings, housings, backyards, vegetables, fruits (via fertilisation and irrigation)	Bowman, 2021; Dold and Holland, 2011
Ascaris suum	Pigs, occasionally humans, rarely sheep and cattle	–	Areas of husbandry (stables, pastures), vegetables, fruits (via fertilisation and irrigation)	Bowman, 2021; Dold and Holland, 2011
Baylisascaris procyonis	Racoons, dogs, skunks, badgers	Rodents, lagomorphs, humans, primates, carnivores, birds	Raccoon latrines, play areas/sandboxes, fireplaces, wood chips/piles, food from garbage cans	Graeff-Teixeira et al., 2016
Toxascaris leonina	Canids and felids	Mice, rabbits, chickens, occasionally humans	Public parks, play areas/sandboxes, backyards	Rostami et al., 2020
Toxocara cati	Felids	Humans, rodents, lagomorphs, chickens and other birds	Public parks, play areas/sandboxes, backyards, raw and undercooked meat	Nijsse et al., 2020
Toxocara canis	Canids	Humans, primates, rodents, lagomorphs, chickens and other birds	Public parks, play areas/sandboxes, backyards, raw and undercooked meat	Nijsse et al., 2020

8–15% (120–220 million cases) of infected humans suffer from high worm burdens associated with increased morbidity and mortality (Chan, 1997; Dold and Holland, 2011). Children are particularly affected as infections may cause stunted development due to malnutrition (Chan, 1997). A model facilitated calculation of the disability-adjusted life years (DALYs), which translates disabilities experienced into years of healthy life lost, revealed a loss of 10.5 million DALYs in 1990 (Chan, 1997) due to ascariosis. In 2019, a reduction to 0.75 million DALYs was calculated, possibly as a result of deworming programmes and socio-economic developments (Else et al., 2020; IHME, 2021). Moreover, the infection of humans with the pig roundworm *Ascaris suum* has been debated for years. Recently, an experimental infection of volunteers with infective L3 of *A. suum* reinforced its zoonotic capacity and indicated that this parasite can cause *A. lumbricoides*-like symptoms in humans (da Silva et al., 2021). Some researchers even propose that both parasites are a single species (Alves et al., 2016). Thus, it can be presumed that human ascarosis is not only caused by *A. lumbricoides* and that *A. suum* accounts for at least a part of human cases.

Other ascarids with high zoonotic potential are the dog and cat roundworm *Toxocara canis* and *T. cati* In contrast, *Toxascaris leonina* also infects canid and felid hosts, but its zoonotic relevance is limited (Rostami et al., 2020). *Toxocara* spp. affect humans as paratenic hosts after accidental infection causing toxocarosis with symptoms ranging from abdominal pain to irreversible blindness or meningitis and cognitive disorders (Fan et al., 2015). Wildlife animals like foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) can also be infected with *T. canis* and these animals play an important role in the transmission of *Toxocara* to domestic and synanthropic cycles as the contact between wildlife, domestic animals and humans constantly increased in the past (Duscher et al., 2015). This further entails the risk of spillovers from sylvatic to domestic or synanthropic cycles of *Baylisascaris* species, roundworms of bears and lower carnivores like raccoons (*Procyon lotor*), badgers (*Meles meles*) and skunks (family Mephitidae). The raccoon roundworm *Baylisascaris procyonis* is considered as an important pathogen causing clinical *larva migrans* in humans, in which the aggressive larval invasion of the central nervous system may result in fatal or severe neurological deficits (Graeff-Teixeira et al., 2016; Sorvillo et al., 2002). An overview on the ascarid species addressed in this review, their definitive- and paratenic hosts and the sources of infection/environmental contamination are listed in Table 1.

Although ascarids infect various hosts, adults as well as eggs of the different ascarids are morphologically homologous with only minor variations among genera. In general, eggs are oval to spherical shaped with a brownish colour (Fig. 1). They are protected by a thick outer surface shell composed of multi-layered lipids, ascarosides as well as chitin and vitelline, which facilitate the resistance against desiccation and penetration of polar substances (Quiles et al., 2006). Eggs of various ascarid species are covered by a web-like albuminous coat, giving them characteristic surface structures including narrow pitted surfaces (e.g. *Baylisascaris* spp.), intermediate pitted surfaces (e.g. *Toxocara* spp., often referred to as golf ball structure) or wider pitted surfaces (e.g. *Ascaris* spp.) (Ubelaker and Allison, 1975; Uga et al., 2000). Thus, ova are highly resistant to environmental stressors and may survive in soils for years (Uga et al., 2000). However, as the faecal material commonly disperses, due to for instance leaching, over a short period in soil (Wong and Bundy, 1990), mostly resulting in low egg concentrations in soil surface layers (Storey and Phillips, 1985) that are often difficult to detect, evidence for environmental contamination with ascarid eggs might be impeded.

Numerous studies analysed environmental contamination with different ascarid ova to evaluate the infection risk for humans and animals. Nevertheless, there is no standardised method for the detection and quantification of these eggs in environmental samples available until to date. Furthermore, accurate detection and quantification of ova is largely influenced by numerous factors such as the composition and characteristics of sampled soil (content of minerals, organic matter, nutrients, humidity and pH) or the amount of sample and the technique used for quantification (Amoah et al., 2017; Collender et al., 2015). Hence, comparative evaluation of egg concentrations in different sample matrices and between different locations remains challenging (Collender et al., 2015). Nevertheless,

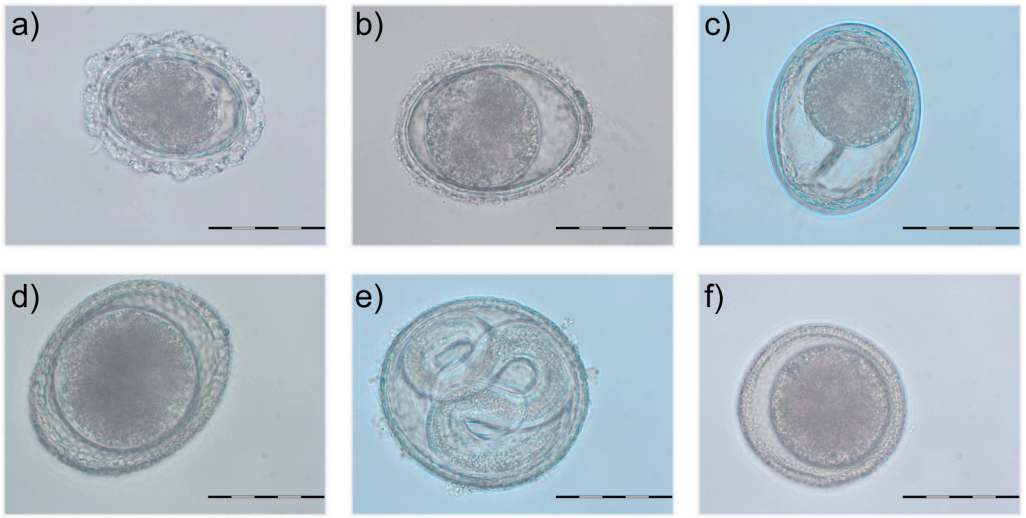

Fig. 1. Eggs of different ascarid species with importance for human and animal health. a) *Ascaris suum*, b) *Baylisascaris procyonis*, c) *Toxascaris leonina*, d) *Toxocara canis*, e) *Toxocara canis* containing infective larva, f) *Toxocara cati*. Scale bars represent 50 µm.

appropriate detection of ascarid eggs is crucial for reliable infection risk assessments for humans and animals. As *Ascaris* and *Toxocara* spp. exhibit a high zoonotic risk, most studies regarding ascarid soil contamination are related to ova of these genera. Therefore, this review aims to give a comprehensive overview about different techniques and their combination to detect and quantify eggs of *Ascaris* and *Toxocara* spp. as representatives of the order Ascaridida.

2. Sampling and isolation of ascarid ova from environmental media

2.1. Sampling strategy for soil

Numerous studies from different regions of the world evaluated the local soil contamination with helminth eggs to define a potential risk of human and animal infection with ascarids (cf. Table 2), which is essential for the development and implementation of effective prevention strategies (Carabin et al., 1998). The comparison of results obtained from different studies remains difficult as soil-sampling techniques differ and many studies usually do not define an adequate spatial sampling strategy (Jarosz et al., 2010). Although the majority of organic material spreads over wide surface areas in a relatively short time due to weather conditions, trampling and coprophagic organisms, ascarid eggs are not evenly dispersed in the environment (Kraglund et al., 1998; Mizgajska, 1997; Wong and Bundy, 1990). The contamination of study areas with helminth eggs mainly occurs by faecal contamination and animals have preferred defecation sites e.g. shaded areas, near walls or in sandy substrates like sand pits (Collender et al., 2015). Therefore, a spatial sampling strategy adapted to the study hypotheses is a necessity to strengthen the significance of the respective results.

Purposive sampling is characterised by defined sampling sites chosen by the investigator and is often used for the assessment of contamination in areas posing a high risk for infection or areas that are assumed to have high contamination rates (Collender et al., 2015). For instance, preferred human and animal defecation sites like sandboxes for cats (Fajutag and Paller, 2013) or areas providing optimal conditions for egg survival like shaded or moist areas are often sampled (Fig. 2a) (Horiuchi et al., 2013). Appropriate sampling sites for the assessment of infection risks for humans and animals with *Ascaris* spp. are e.g. surroundings of latrines (Baker and Ensink, 2012), feeding/dunging area of pig farms (Roepstorff et al., 2001) or back yards (Horiuchi et al., 2013). In order to evaluate the infection risk for children with for instance *Toxocara* spp. at playgrounds, places where children preferably like to play such as sandboxes, swings or seesaws should be selected as primary sampling sites (Fig. 2b) (Eisen et al., 2019).

In contrast, soil contamination can be evaluated by spatial sampling e.g. by dividing sampling areas into homogeneous subdivisions. These homogenous subdivisions can be sampled either randomly (Fig. 2c) or systematically based on predetermined patterns (Fig. 2d and e) (Collender et al., 2015; Wang et al., 2012). Accordingly, subdivisions and sampling patterns vary between studies. While some investigators use grid-based subdivisions with sampling every single (Bojanich et al., 2015) to fourth or fifth square meter (Raissi et al., 2020), other researchers' subdivisions are numbered with sampling spots being randomly selected by numeration or lot (Fig. 2f) (Carabin et al., 1998). Moreover, predetermined patterns such as the classification of areas into compass directions or free- or self-designed patterns can be used for the determination of sampling spots (Fig. 2g) (Mizgajska-Wiktor, 2005; Rocha et al., 2011). Otherwise, equidistant sampling or sampling at equal intervals along a determined route like the diagonal of the area, a meandering pattern or a W-shaped route, are commonly chosen for soil sampling (Fig. 2h) (Jarosz et al., 2010; Kleine et al., 2017; Mejer and Roepstorff, 2006). Overall, systematic spatial sampling often covers the entire studied location and gives therefore a holistic approximation on soil contamination of the sampled area (Collender et al., 2015). Thus, this method gives more reliable estimates than biased purposive sampling often over- or underestimating environmental egg contamination (Carabin et al., 1998; Collender et al., 2015).

Along with an adequate spatial sampling strategy adopted to the study hypothesis, considerations have to be made on how many samples need to be drawn in order to have an appropriate estimate of environmental egg contamination. On the one hand, sample size is dependent on the dimensions of the study area: the bigger the area, the more samples should be taken. Many of the above-mentioned strategies avoid this problem by sampling in defined intervals such as square meters (Bojanich et al., 2015; Raissi et al., 2020). On the other hand, contamination heterogeneity critically influences the sample size. The higher the heterogeneity, the narrower should be the sampling grid to cover as many areas of differential contamination intensity as possible to reduce the uncertainty of spatial sample estimation (Wang et al., 2012). Moreover, sample size volume or weight as well as depth of sampling are important parameters influencing the detection of ascarid eggs in soil. Although rain water and the activity of invertebrates like beetles or earthworms result in deposition eggs to a depth of about 40 cm (Kraglund et al., 1998; Mizgajska, 1997), the majority of ascarid eggs stay in the upper 5 cm of the ground (Mizgajska, 1993, 1997). Soil is defined by the primary constituent particle size and by the fractions of each soil separate (sand, silt, and clay) present in the sample and classified by the United States Department of Agriculture in 12 major classes (García-Gaines and Frankenstein, 2015). These soil types differ in various properties, such as texture, which may affect the concentration of ascarid eggs in the environment. For instance, sand has high egg infiltration rates while clay is less permeable for ova due to its high mechanical compaction (Nunes et al., 1994). Therefore, sampling amount and depth should be, whenever possible, adjusted to the occurring soil type and the estimated local prevalence of the ascarid eggs (Nunes et al., 1994; Oge and Oge, 2000). Due to its loose compaction, sandy soil should be drawn more wide-ranging as eggs tend to disperse more easily than in clay. Moreover, weather conditions should be considered as for instance long-lasting heavy rain can lead to the leaching of eggs into deeper soil layers (Storey and Phillips, 1985; Wong and Bundy, 1990).

2.2. Isolation of ascarid eggs from soil

The quantification of ascarid eggs in environmental matrices is of major concern to evaluate soil contamination. For reliable

detection, ova has to be concentrated and isolated from the environmental matrix. Although being easily said, the reliability of the isolation is greatly affected by a manifold of experimental factors such as methodological approaches, the sample texture and available reagents/materials etc. Commonly, extracting ascarid eggs from soil involves key processes like the homogenisation of the sample, chemical dissociation of eggs from matrix particles, filtration, sedimentation and flotation.

2.2.1. Homogenisation and dissociation

As eggs are often unevenly distributed within environmental samples, thorough homogenisation of sampled soil is necessary for reliable estimation of contamination with minor variabilities between replicates and different samples.

The external coat of ascarid eggs consists of proteins and mucopolysaccharides, leading to adhesive properties of the eggs (Kleine et al., 2016; Meng et al., 1981; Uga et al., 2000). The degree of adhesion of the eggs differs according to the material they get in touch with. For instance, *Toxocara* eggs adhere heavily to plastic and to a lesser extent to glass, what should be considered when drawing and storing soil samples (Kleine et al., 2016). Furthermore, the recovery efficacy is influenced by adhesion of eggs to laboratory consumables used for the processing of samples (Gaspard et al., 1994; Kleine et al., 2016). However, coating of lab ware with organosilane, a water-repellent substance which is normally used to coat car windshields, did not enhance egg recovery (Jeandron et al., 2014). There are other agents available for coating such as LiquiGlide, which is a lubricant that is mainly used in food technology to reduce adhesive properties of surface structures (Smith et al., 2013). However, this or other substances like polytetrafluorethylen (Teflon®) have not been tested for coating of laboratory consumables so far.

Ascarid eggs also tend to adhere to organic and inorganic particles derived from soil (Landa-Cansigno et al., 2013). As ionic forces are implicated in adhesion of the eggs, different detergents are widely supplemented during different experimental procedures to dissociate ascarid eggs from particles present in soil and faeces. Commonly used detergents either have cationic (benzethonium chloride 0.1% and cetylpyridinium chloride CPC 0.1%) or anionic (detergent 7X®) properties. Furthermore, non-ionic tensids like Triton® X-100 (Forslund et al., 2010; Molleda et al., 2008) and Tween®20/40/80 as well as chemical compounds like ammonium bicarbonate (Moodley et al., 2008; Trönnberg et al., 2010), sodium hydroxide or acetoacetic acid (Ruiz De Ybanez et al., 2000) have frequently been used for dissociation of helminth eggs from environmental particles (Table 2). Although a broad variety of detergents and chemical compounds are utilised, studies comparing the impact of detergents on egg recovery rates under defined conditions are not always available. Steinbaum et al. (2017) and Gnani Charitha et al. (2013) tested different detergents indicating that 7X®, glycine and Tween®80, either supplemented to homogenisation- or flotation solutions, lead to high egg recovery rates from both soil and vegetables. Moreover, a comparative analysis of the studies listed in Table 2 confirms these assumptions by showing that Tween®80, glycine and 7X® seem to be superior to other detergents as indicated by higher recovery rates when these agents were used (Fig. 3a). Moreover, Tween®20/40/80 or 7X® have been used by numerous investigators as both are soluble in water at any given concentration and 7X® does not form precipitates with highly concentrated salts used for flotation (Bowman et al., 2003).

2.2.2. Filtration

After dissociation of eggs from larger organic and inorganic particles, separation of eggs from particles is commonly conducted via filtration through sieves (Bowman et al., 2003; Engohang-Ndong et al., 2015; Katakam et al., 2014), in which the choice of pore size is crucial to optimise egg recovery from different sample matrices. More precisely, ascarid eggs have varying dimensions ranging from approximately 45–75 µm for *Ascaris* spp., 45–90 µm for *Ascaridia galli*, 45–75 µm for *Heterakis gallinarum,* 90–100 µm for *Parascaris* spp., 60–95 µm for *Toxocara* spp. and 65–85 µm for *Baylisascaris* spp. (Zajac et al., 2021) (Fig. 1). Thus, sieve sizes of at least 100 µm are used to withhold larger particles, while sieves with pore sizes of maximally 36 µm enable the collection of ascarid eggs with smaller particles being discarded in the flow-through (Katakam et al., 2014). Comprehensive studies regarding the influence of pore sizes of sieves on the recovery of eggs from different sample matrices have not been reported so far. Although filtration may result in lower recovery rates due to trapping of particle-associated or clotted eggs (Collender et al., 2015), sieving with varying mesh sizes may reduce unwanted matrix material in the sample, thus enhancing the accuracy of egg identification and quantification during microscopic examination (Smith, 1998).

2.2.3. Sedimentation

An unwanted side effect of chemical dissociation and filtration is the unavoidable increase in sample volume, further on leading to difficulties in processing or microscopic examination. In order to reduce the volume of the sample, sedimentation is conducted to separate solid particles, including the eggs, from the liquid phase. Efficacy and velocity of sedimentation is influenced by various factors like the sample matrix, viscosity of the matrix, the matrix-liquid ratio, size and density of eggs, their properties to coagulate with other particles as well as the size and shape of the container used for sedimentation (da Rocha and Braga, 2016). The duration of sedimentation depends on these factors and can therefore vary greatly. For instance, *A. suum* eggs were shown to have a settling velocity of 0.0612 mm s^{-1} in tap water, whereas velocities were comparably higher in wastewater and sediment suspensions after bed shear stress with 0.1582 mm s^{-1} and 0.9 mm s^{-1}, respectively (Sengupta et al., 2012; Sengupta et al., 2011). Hence, the sedimentation time is critically dependent on the experimental setup und therefore often ranges between 1 h to overnight (Amoah et al., 2017). The sedimentation process can be actively accelerated by centrifugation. However, centrifugation speed and duration is influenced by the above-mentioned factors, thus speed and duration has to be adjusted to the experimental settings (Amoah et al., 2017; Smith, 1998).

2.2.4. Flotation

Filtration and sedimentation alone are often insufficient to remove the majority of matrix particles. Thus, further egg separation is commonly conducted by flotation, but methods and protocols are broad ranging. In principal, separation via flotation is achieved by a

certain density of the flotation solution in which particles with a lower specific gravity (like eggs) float while particles with a higher specific gravity (like matrix particles) sediment. Specific gravities of eggs of the most widespread ascarid species range between 1.05 and 1.13, but as illustrated in Table 2, recovery rates are influenced by various factors and differ between procedures applied. A variety of flotation solutions like zinc sulphate, magnesium sulphate, sodium nitrate and sucrose solutions with a specific gravity of >1.2 are commonly used for ova purification, but the use of e.g. sodium chloride solutions with equal or lower specific gravity is also frequently described (Amoah et al., 2017; Ruiz De Ybanez et al., 2000). The here compiled studies rather indicate that flotation solutions with higher specific gravities result in superior egg recovery rates (Table 2, Fig. 3b). Comparative evaluation of several different flotation solutions by Quinn et al. (1980), Ruiz De Ybanez et al. (2000), Oge and Oge (2000) or Horn et al. (1990) show that high-density flotation solutions frequently exceed recovery rates of 10%. In contrast, recovery rates of flotation solutions with a specific gravity of <1.2, especially low-gravity sodium chloride, sucrose and sodium nitrate solutions often only range between 0% and 10% (Fig. 3b). Thus, solutions with a specific gravity >1.2 should be preferably used for the isolation of ascarid eggs from environmental matrices. Nevertheless, adverse effects of high-density flotation solutions like viscosity or chemical interactions with the eggs' outer surfaces proteins seem to play an eminent role in recovery. For instance, the osmotic pressure might result in distortion of eggs (Steinbaum et al., 2017) or the viscosity of a sucrose solution may interfere with the flotation speed of eggs (Bowman et al., 2003).

The flotation process can be accelerated by centrifugation, but the centrifugation speed and time should be adapted to the used solution as for instance sucrose requires longer periods of centrifugation because of its viscosity. To increase egg recovery rates, flotation steps are often repeated as ova may still be entrapped between matrix particles (Quinn et al., 1980). This entrapment is dependent on the soil type. For instance, sandy soils are homogenous and are composed of large particles, thus eggs are only loosely withheld. In contrast, clay soils contain smaller particles resulting in stronger attachment of eggs and therefore inconsistent and lower recovery after flotation compared to sandy soil (Nunes et al., 1994). To circumvent egg-particle adherence, detergents are often supplemented to flotation solutions. However, it should be considered that salts tend to precipitate when certain detergents, such as Tween®20/40/80, are present (da Rocha and Braga, 2016). Another important point in selecting a flotation solution constitutes the reliability, reproducibility and the hazardousness of the substance. Flotations involving chemicals like zinc sulphate, zinc chloride, sodium nitrate, sodium dichromate or mercury(II) iodide result in good egg recovery rates, but these substances exhibit toxic and environmentally harmful properties and must be disposed as hazardous waste in accordance with local/regional/national/international regulations. In contrast, the hazard potential of sucrose, sodium chloride and magnesium sulphate is rather low, wherefore the use of one of these flotation solutions, especially those with a specific gravity >1.2, should be preferred.

2.3. Isolation of ascarid eggs from wastewater and sludge

Wastewater and sludge are commonly used for irrigation and as fertilisers, thus being a source of contamination for agriculture products. To reduce the infection risk for humans and animals, sludge must be treated by appropriate chemical, physical or thermal methods. In certain countries of the EU, sludge, no matter whether treated or untreated, may not be used on agricultural land on which fruit and vegetable crops are grown. Furthermore, grassland or forage land that will be grazed by animals or will be harvested in the following three weeks shall not be fertilised with sludge. However, the use of sludge as a fertiliser is not generally prohibited, provided that country-specific guidelines are fulfilled (EU, 2018). For this case, health regulators like the WHO or the U.S. EPA published guidelines to regulate the pathogen load of wastewater and sludge. Accordingly, treated wastewater and sludge should contain ≤1 ova/L for wastewater or ≤ 1 ova/g for sludge of human-pathogenic helminths (U.S. EPA, 2003; WHO, 2006). Furthermore, health regulators recommend appropriate methods for an accurate risk and exposure assessment of these matrices. In principle, isolating ascarid eggs from wastewater or sludge comprise identical steps as isolating ova from soil. The WHO recommends a sample volume of 1 L (Mes, 2003; Sengupta et al., 2011), but several studies also used higher sample volumes ranging from 10 L to a maximum of 200 L (Levantesi et al., 2010; Molleda et al., 2008). Concerning sludge, a dry weight of approximately 2 to 5 g is frequently used (Maya et al., 2012; Shamma and Al-Adawi, 2002). Equal to soil, organic compounds in wastewater and sewage sludge also tend to coagulate with ascarid ova, why a dissociation step using detergents is recommended. Here, the detergent 7X® has been reported to result in higher recovery efficiency compared to other dissociation agents like Triton® X-100, Tween®80 and benzethonium chloride (Amoah et al., 2018; Amoah et al., 2017). To reduce the sample volume and to concentrate ova, filtration and sedimentation as well as flotation are often described for purifying ova from wastewater or sludge.

2.4. Isolation of ascarid eggs from food

Food-borne transmission represents an important route for the spread of ascarid infections. Vegetables, fruits or herbs can be contaminated with infective ascarid eggs by human and animal faeces or are introduced by polluted water during the production, harvesting, transportation, preparation, and/or processing of the vegetables (Mohamed et al., 2016). Consequently, the consumption of vegetables and fruits without thermal processing or proper washing or peeling before ingestion is a frequent source of infection for humans and animals (Lynch et al., 2009). Various studies evaluated the contamination of leafy and soil-grown vegetables like lettuce, carrots, potatoes, onions, zucchini, spinach, cucumbers or cress mostly with eggs of *Toxocara* or *Ascaris* spp. (Fallah et al., 2012; Klapec and Borecka, 2012; Kozan et al., 2005; Maikai et al., 2012). Examination of vegetables regarding ascarid egg contamination has been neglected from food regulation committees worldwide, why officially recommended protocols in the assessment of vegetable contamination are not available so far. The United States Food and Drug Administration (USFDA) published a protocol (USFDA, 2021), initially designed to isolate protozoans from contaminated water (Bier, 1991), that was adapted for isolating helminth eggs from food. Nevertheless, recovery of *Ascaris* eggs was rather low (Matosinhos et al., 2016).

Table 2
Summary of selected studies evaluating the recovery of ascarid eggs in artificially spiked soil samples. This overview illustrates how recovery rates are influenced by various factors like the soil substrate, flotation solution and its specific gravity (SG) as well as dissociation solution and its concentration (conc.).

Ascarid species	Soil		Spike level [total eggs]	Method/ according to	Flotation solution	SG	Dissociation		Recovery [%]	Reference
	type	weight					Detergent	conc.		
Ascaris spp.										
Ascaris spp.	n.d.[a]	10 g	100	Santarem et al., 2009	$ZnSO_4$ saturated	1.2	none		9.5	Gnani Charitha et al., 2013
			200						5.42	
		10 g	100	O'Lorcain, 1994	$NaNO_3$ saturated	1.35	Tween® 80	n.d.	66.5	
			200						79.59	
		10 g	100	Kazacos, 1983	$NaNO_3$ saturated	1.35	Tween® 40	n.d.	47	
			200						53.83	
A. suum	clay	1 g	20	CFT[b]	$ZnSO_4$	1.2	water		10.6	David, 1977
		1 g	20	CFT	$ZnSO_4$	1.2	NaOH	0.1 N	27.9	
Ascaris spp.	loam	15 g	1000	U.S. EPA, 2003	$ZnSO_4$		7X®	1%	37.2	Steinbaum et al., 2016
A. suum	loam	15 g	931	U.S. EPA, 2003 initial	$ZnSO_4$	1.2	Tween® 80	0.10%	37.2	Steinbaum et al., 2017
				U.S. EPA, 2003 improved	$ZnSO_4$	1.25	7X®	1%	72.7	
A. suum	sewage sludge	5 g	1156	Tulane	$MgSO_4$	1.2	7X®	1%	96.7	Bowman et al., 2003
A. suum	sewage sludge	100 ml	10,353	CFT	sucrose	1.26	lactalbumin hydrolysate	3%	46.5	O'Donnell et al., 1984
A. suum	sewage sludge									Karkashan et al., 2015
	15% dry solid content	50 ml	7440	Tulane	$MgSO_4$	1.2	7X®	1%	33.3	
			1860						69.7	
			465						73.3	
	3% dry solid content	200 g	7440	Tulane	$MgSO_4$	1.2	7X®	1%	41.8	
			1860						59.7	
			465						63.6	
Ascaris spp.	sewage sludge	10 g	5	CFT	$NaNO_3$	1.36	Tween® 20	0.0025%	10	Zdybel et al., 2016
			10						12.5	
			50						13.4	
			100						23.6	
			200						27.3	
			400						28.4	
			800						30.6	
A. suum	sewage sludge	1 l	1000	Bowman et al., 2003 modified	$MgSO_4$	1.25	7X®	1%	42.0	Shahsavari et al., 2017
				WHO					11.0	
A. suum	sewage sludge	50 g	100	U.S. EPA, 2003	$MgSO_4$	1.2	7X®	1%	74.0	da Rocha and Braga, 2016
A. suum	waste water and sludge	n.d.	1000	Tulane modified	$MgSO_4$	1.25	7X®	1%	69.0	Ravindran et al., 2019
				Tulane modified	$MgSO_4$	1.25	none		19.0	
A. suum	lettuce	30 g	100	CST[c]			distilled water		81.7	Matosinhos et al., 2016
							Tween® 20	0.1%	61.7	
							glycine	1 M	99.3	
							$NaC_{12}H_{25}SO_4$	1%	43.3	
	arugula	30 g	100	CST			glycine	1 M	58.1	
			50						61.8	
			20						65.0	
			11						58.1	
			5						70.0	
A. suum	lettuce	30 g	blinded	CST			glycine	1 M	57.1	Pineda et al., 2021
	arugula	30 g	blinded	CST			glycine	1 M	50.7	
Toxocara spp.										
T. canis	n.d.	25 g	400	CFT	$ZnSO_4$ (33%)	1.09	Tween® 80	0.0025%	1.8	Quinn et al., 1980
					$ZnSO_4$ saturated	1.27			27.5	
					$MgSO_4$ (33%)	1.07			26.8	
					$MgSO_4$ (50%)	1.14			72.0	

(*continued on next page*)

Table 2 (*continued*)

Ascarid species	Soil		Spike level [total eggs]	Method/ according to	Flotation solution	SG	Dissociation		Recovery [%]	Reference
	type	weight					Detergent	conc.		
					$MgSO_4$ saturated	1.28			82.5	
					NaCl saturated	1.21			51.3	
T. canis	n.d.	1 g	3	CFT	$NaNO_3$	1.2	none		6.0	Rosa Xavier et al., 2010
			5						8.8	
			10						3.8	
			25						4.9	
			50						8.5	
			100						7.1	
			200						4.9	
		1 g	3	CFT	$ZnSO_4$	1.2	none		6.0	
			5						6.0	
			10						8.5	
			25						5.7	
			50						7.9	
			100						7.1	
			200						6.7	
T. canis	n.d.	50 g	50	CFT	$MgSO_4$	1.28	none		47.2	
			100						34.0	
			200						39.2	
Toxocara spp.	n.d.	10 g	100	Santarem et al., 2009	$ZnSO_4$ saturated	1.2	none		7.7	Gnani Charitha et al., 2013
			200						7.4	
		10 g	100	O'Lorcain, 1994	$NaNO_3$ saturated	1.35	Tween® 80	n.d.	71.0	
			200						74.7	
		10 g	100	Kazacos, 1983	$NaNO_3$ saturated	1.35	Tween® 40	n.d.	46.8	
			200						54.0	
T. canis	sandy	100 g	280,400	CFT	sucrose	1.2	distilled water		3.2	Ruiz De Ybanez et al., 2000
					NaCl saturated	1.2			2.7	
					$ZnSO_4$ saturated	1.2			8.2	
					sucrose	1.27			99.9	
					$MgSO_4$ saturated	1.28			23.2	
					$MgSO_4$ saturated + KI (5%)	1.35			18.9	
					$NaNO_3$	1.35			51.5	
	sandy	100 g	280,400	CFT	sucrose	1.2	NaOH	0.1 N	2.1	
					NaCl saturated	1.2			6.7	
					$ZnSO_4$ saturated	1.2			6.3	
					sucrose	1.27			35.3	
					$MgSO_4$ saturated	1.28			22.6	
					$MgSO_4$ saturated + KI (5%)	1.35			13.6	
					$NaNO_3$	1.35			39.7	
	sandy	100 g	280,400	CFT	sucrose	1.2	Tween® 20	1%	2.1	
					NaCl saturated	1.2			3.1	
					$ZnSO_4$ saturated	1.2			5.8	
					sucrose	1.27			29.1	
					$MgSO_4$ saturated	1.28			11.4	
					$MgSO_4$ saturated + KI (5%)	1.35			7.0	
					$NaNO_3$	1.35			8.9	
	sandy	100 g	280,400	CFT	sucrose	1.2	$C_4H_6O_3$	0.2 M	3.9	
					NaCl saturated	1.2			10.7	
						1.2			13.7	

(*continued on next page*)

Table 2 (*continued*)

Ascarid species	Soil		Spike level [total eggs]	Method/ according to	Flotation solution	SG	Dissociation		Recovery [%]	Reference
	type	weight					Detergent	conc.		
					$ZnSO_4$ saturated sucrose	1.27			39.1	
					$MgSO_4$ saturated	1.28			50.2	
					$MgSO_4$ saturated + KI (5%)	1.35			25.1	
					$NaNO_3$	1.35			82.9	
T. canis	sand	50 g	10	Deumer, 1984	$ZnSO_4$-NaCl	1.3	none		0.0	Oge and Oge, 2000
			100						12.0	
			500						4.8	
		50 g	10	Düwel, 1984	NaCl	1.19	none		0.0	
			100						7.0	
			500						5.0	
		50 g	10	Quinn et al., 1980	$MgSO_4$	1.27	Tween® 80	n.d.	20.0	
			100						13.0	
			500						5.6	
		50 g	10	Dunsmore et al., 1984	$NaNO_3$	1.22	Tween® 80	n.d.	10.0	
			100						15.0	
			500						14.6	
		50 g	10	Dada and Lindquist, 1979	$ZnSO_4$	1.2	NaOH	n.d.	0.0	
			100						6.0	
			500						5.8	
		50 g	10	Kazacos, 1983	$ZnSO_4$	1.2	Tween® 40	n.d.	0.0	
			100						15.0	
			500						9.0	
T. canis	sand	250 g	1	CFT	NaCl saturated	n.d.	Tween® 80	n.d.	30.0	Kleine et al., 2016
			5						38.0	
			10						45.0	
			25						47.2	
			50						36.4	
			75						46.8	
			100						51.6	
			150						39.0	
			200						51.4	
T. cati	sand	250 g	1	CFT	NaCl saturated	n.d.	Tween® 80	n.d.	30.0	
			5						22.0	
			10						28.0	
			25						20.0	
			50						48.8	
			75						31.9	
			100						39.1	
			150						23.5	
			200						34.8	
T. canis	sand	100 g	10	Köhler et al., 1980	$ZnSO_4$ (45%)	n.d.	none		70.0	Horn et al., 1990
				Deumer, 1984	$ZnSO_4$-NaCl	1.3	none		20.0	
				Kasieczka, 1982	$ZnSO_4$	1.21	NaClO	12–13%	2.1	
				Boreham and Capon, 1982	NaCl saturated	1.2	Tween® 80	0.0025%	2.1	
				Tharaldsen, 1982	$ZnSO_4$-NaCl	1.3	dishwasher detergent	n.d.	8.1	
				Quinn et al., 1980	$MgSO_4$ saturated	1.27	Tween® 80	0.0025%	14.0	
				Kazacos, 1983	$ZnSO_4$	1.2	Tween® 80	0.83%	0.0	
				Kazacos, 1983	$NaNO_3$ saturated	1.35	Tween® 80	0.83%	4.1	
				Dada and Lindquist, 1979	$ZnSO_4$	1.2	NaOH	0.1 N	0.0	
				Stoye and Horn, 1986	sucrose	1.25	Tween® 80	0.83%	44.0	
				Stoye and Horn, 1986	sucrose	1.25	Pepsin-HCl	n.d.	14.0	
		100 g	100		$ZnSO_4$ (45%)	n.d.	none		17.9	

(*continued on next page*)

Table 2 (*continued*)

Ascarid species	Soil		Spike level [total eggs]	Method/ according to	Flotation solution	SG	Dissociation		Recovery [%]	Reference
	type	weight					Detergent	conc.		
				Köhler et al., 1980						
				Deumer, 1984	$ZnSO_4$-NaCl	1.3	none		12.3	
				Kasieczka, 1982	$ZnSO_4$	1.21	NaClO	12–13%	5.7	
				Boreham and Capon, 1982	NaCl saturated	1.2	Tween® 80	0.0025%	0.9	
				Tharaldsen, 1982	$ZnSO_4$-NaCl	1.3	dishwasher detergent		4.0	
				Quinn et al., 1980	$MgSO_4$ saturated	1.27	Tween® 80	0.0025%	20.0	
				Kazacos, 1983	$ZnSO_4$	1.2	Tween® 80	0.83%	15.0	
				Kazacos, 1983	$NaNO_3$ saturated	1.35	Tween® 80	0.83%	12.1	
				Dada and Lindquist, 1979	$ZnSO_4$	1.2	NaOH	0.1 N	17.3	
				Stoye and Horn, 1986	sucrose	1.25	Tween® 80	0.83%	48.5	
				Stoye and Horn, 1986	sucrose	1.25	pepsin-HCl	n.d.	15.2	
		100 g	1000	Köhler et al., 1980	$ZnSO_4$ (45%)	n.d.	none		36.3	
				Deumer, 1984	$ZnSO_4$-NaCl	1.3	none		12.0	
				Kasieczka, 1982	$ZnSO_4$	1.21	NaClO	12–13%	5.4	
				Boreham and Capon, 1982	NaCl saturated	1.2	Tween® 80	0.0025%	0.0	
				Tharaldsen, 1982	$ZnSO_4$-NaCl	1.3	dishwasher detergent		6.7	
				Quinn et al., 1980	$MgSO_4$ saturated	1.27	Tween® 80	0.0025%	28.7	
				Kazacos, 1983	$ZnSO_4$	1.2	Tween® 80	0.83%	0.9	
				Kazacos, 1983	$NaNO_3$ saturated	1.35	Tween® 80	0.83%	8.1	
				Dada and Lindquist, 1979	$ZnSO_4$	1.2	NaOH	0.1 N	16.7	
				Horn, 1986	sucrose	1.25	Tween® 80	0.83%	36.9	
				Horn, 1986	sucrose	1.25	pepsin-HCl	n.d.	10.9	
T. canis	clay	1 g	210	CFT	$ZnSO_4$	1.18	water		66.2	Dada and Lindquist, 1979
					$Na_2Cr_2O_7$	1.2			56.0	
					$ZnSO_4$	1.2			62.9	
					HgI2	1.63			55.2	
		1 g	210	CFT	$ZnSO_4$	1.18	NaOH	0.1 N	61.4	
					$Na_2Cr_2O_7$	1.2			52.2	
					$ZnSO_4$	1.2			58.7	
					HgI_2	1.63			49.1	
T. canis	clay	1 g	20	CFT	$ZnSO_4$	1.2	water		55.8	David, 1977
		1 g	20	CFT	$ZnSO_4$	1.2	NaOH	0.1 N	55.0	
T. canis	clay slit	50 g	10,000	CFT	$ZnSO_4$	1.2	none		8.2	Nunes et al., 1994
	sandy								24.4	
	silty clay								1.9	
	sand								43.5	
	clay slit	50 g	10,000		$Na_2Cr_2O_7$	1.35	none		13.9	
	sandy								38.0	
	silty clay								7.5	
	sand								62.5	
T. canis	sewage sludge	100 g	2073	CFT	sucrose	1.26	lactalbumin hydrolysate	3%	16.2	O'Donnell et al., 1984
Toxocara spp.	sewage sludge	10 g	1	CFT	$NaNO_3$	1.36	Tween® 20	0.0025%	20.0	Zdybel et al., 2016
			3						30.0	
			5						22.0	
			10						28.0	
			50						24.6	
			100						28.0	
			200						32.7	

(*continued on next page*)

Table 2 (*continued*)

Ascarid species	Soil		Spike level [total eggs]	Method/ according to	Flotation solution	SG	Dissociation		Recovery [%]	Reference
	type	weight					Detergent	conc.		
			400						36.2	
			800						28.2	
Toxocara spp.	lettuce	300 g	20	sieving	$ZnCl_2$	n.d.	none		5/5[d]	Guggisberg et al., 2020
			4		$ZnCl_2$	n.d.	none		2/5[e]	

[a] n.d.: not determined.
[b] CFT: Centrifugation-flotation-technique.
[c] CST: Centrifugation-sedimentation-technique.
[d] *Toxocara* eggs could be detected in 5 of 5 spiked specimens by microscopy (PCR 5/5).
[e] *Toxocara* eggs could be detected in 2 of 5 spiked specimens by microscopy (PCR 5/5).

Generally, different techniques have been used for the isolation of ascarid ova from contaminated vegetables, whereas most of these methods are modifications of techniques established for the analysis of faecal material or water. However, only few studies analysed the effectivity of methods utilised for the recovery of ascarid eggs from vegetables, fruits or herbs (Matosinhos et al., 2016; Pineda et al., 2021). Matosinhos et al. (2016) established a technique to recover helminth eggs from leafy vegetables like lettuce and arugula. Within the scope of this study, the method was standardised in an inter-laboratory approach, which was later on pursued and extended by Pineda et al. (2021). Briefly, a total of 30 g of vegetables was sealed in a plastic bag with 1 M glycine solution. After manual shaking for 3 min, the solution was filtered through a 1 mm sieve and left 2 h for sedimentation. The resulting pellet was centrifuged and screened for helminth eggs under a light microscope. The inter-laboratory confirmation of the procedure resulted in an average recovery of 52.1% (±37.9) with the detection of at least one egg in 96.3% of samples spiked with *A. suum* eggs (Matosinhos et al., 2016). The utilisation of the protocol by Pineda et al. (2021) yielded in a mean recovery efficiency of 57.1% (±37.6) for the lettuce samples and 50.7% (±29.0) for the arugula samples with a proposed detection limit of eleven eggs per gram of vegetable leaves. A further approach is described by Guggisberg et al. (2020), who implemented a sequential sieving system to isolate a variety of parasite infective stages based on their size by concentration in nylon filters of different mesh sizes (105 µm, 40 µm for the detection of ascarid eggs and 21 µm for the detection of taeniid eggs) in a flow-through system. Here, 300 g of lettuce were washed with 0.2% Tween® 20. The washing solution was passed through the sieving system and the filters were thoroughly washed with water. Afterwards, 40 µm-filtered material was centrifuged and the pellets were used for microscopic and/or molecular detection of ascarid eggs, with further

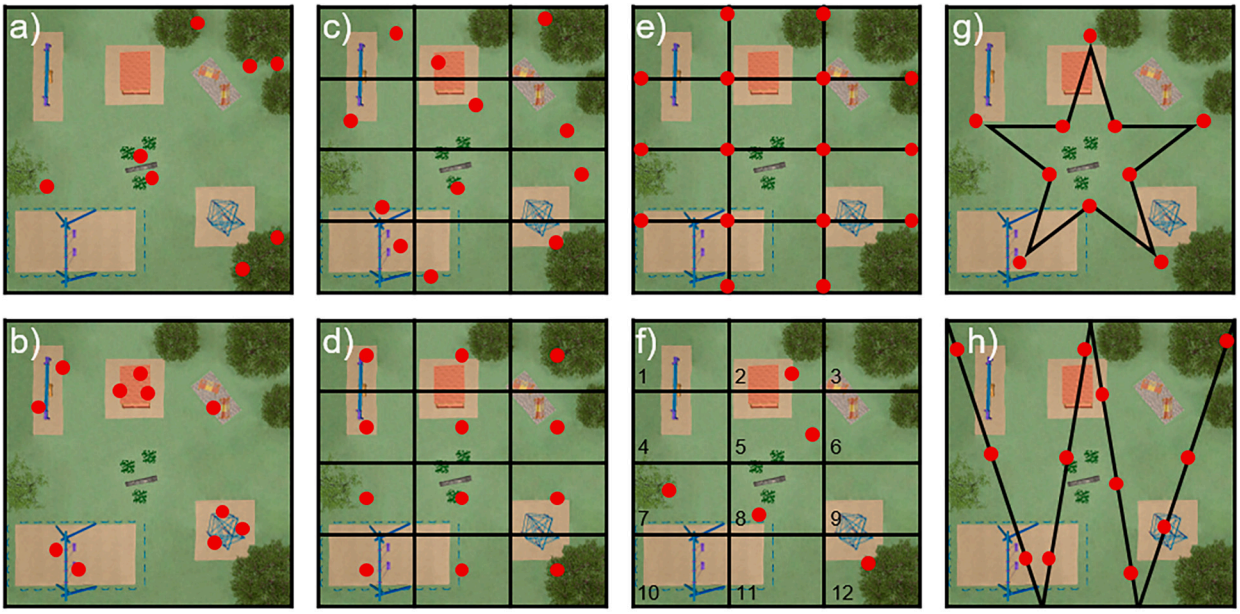

Fig. 2. Examples of spatial sampling patterns to determine the environmental contamination at sampling sites. a) Sampling at preferred defection sites or areas providing optimal conditions for egg survival, b) sampling at spots of particular interest e.g. where children preferably play. Spatial stratification by dividing sampling areas into homogeneous subdivisions, which can be sampled either c) randomly in each square, or d, e) systematically based on predetermined patterns d) in the centre of each square or e) at the intersections of the grid. f) Subdivisions can be numbered and sampling spots are selected based on the numeration or by lot. g) Sampling spots with predetermined, equidistant pattern, h) sampling at intervals along a determined route e.g. W-shaped route. Playground site was designed with the 3D playground designer (https://playgroundideas.org/, retrieved 10/07/2021).

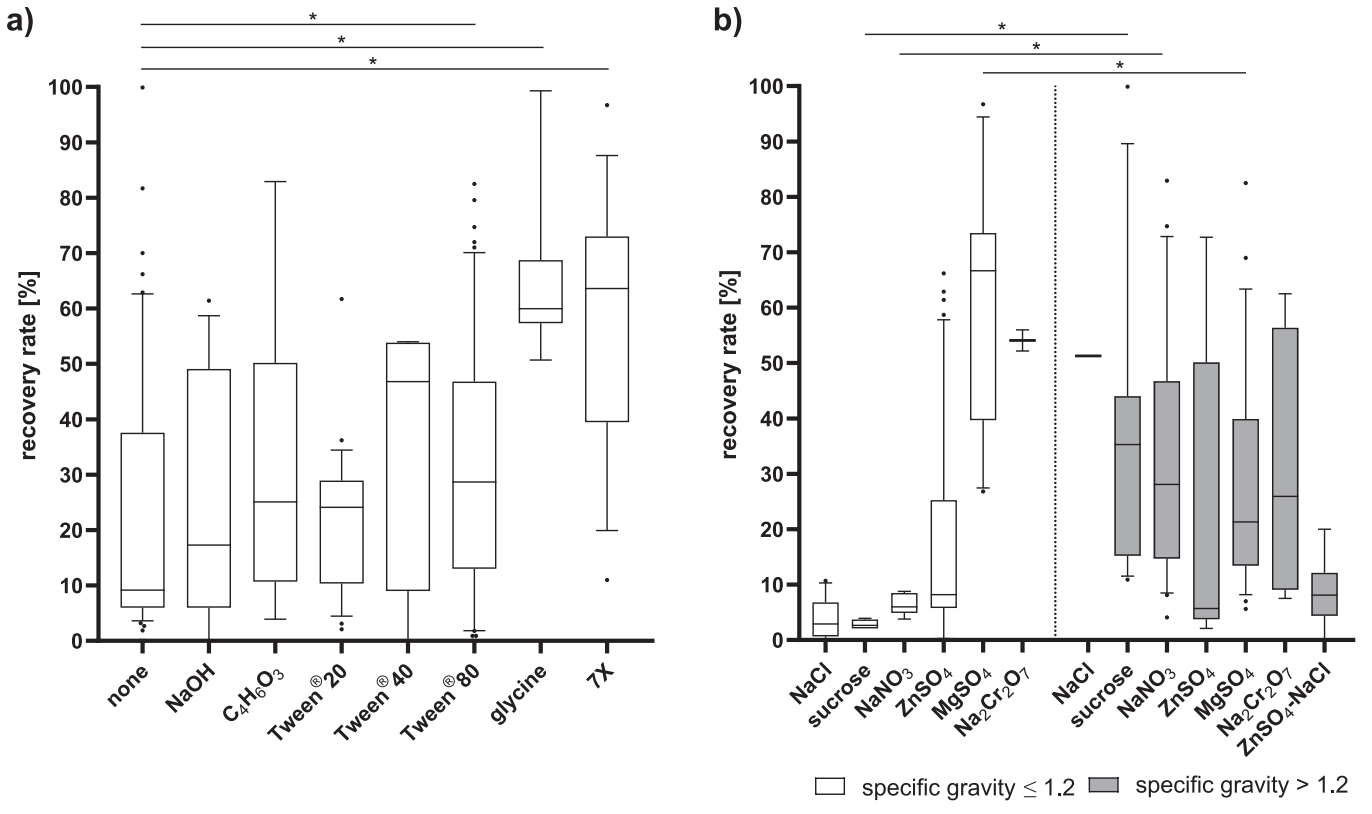

Fig. 3. Comparative analysis of egg recovery rates from studies listed in Table 2 with categorisation into a) detergents and b) flotation solutions. Error bars define the 10th and 90th percentiles with dots representing individual data points beyond mentioned percentiles. The line indicates the median. Statistical significance was evaluated using Mann-Whitney test. A p-value ≤ 0.5 was considered statistically significant and asterisks indicate a significant difference to a) no detergent (none) or b) the respective flotation solution with a specific gravity ≤ 1.2.

Table 3
Comparison of selected studies encompassing DNA extraction methods and molecular assays for determination of ascarid egg contamination. SDS: sodium dodecyl sulfate, EL: enzymatic lysis, MD: mechanical disruption, TD: thermal disruption, Ct: Cycle threshold of applied qPCR, EPG: eggs per gram faeces/soil/sludge, LOD: limit of detection, cLOD: calculated limit of detection, GE: genome equivalents, n.a.: not available, n.d.: not determined.

Ascarid species	Matrix	Concentration [eggs/volume]	Flo-tation	DNA extraction/purification	Dissociation/ detergent	Egg shell disruption	Detection method/ gene	Quantification	Reference
Ascaris spp.									
Ascaris spp.	faeces	1 to 50,000/1 g	–	Maxwell RSC PureFood GMO and Authentication kit (Promega)	SDS, proteinase K	EL, MD	qPCR, Hex-IABkFQ probe/ITS1	cLoD 5 EPG [Ct 34.75]	Zendejas-Heredia et al., 2021
A. suum	H_2O	83/n.a.	–	PowerWater DNA isolation kit (MO BIO)	SDS	MD	qPCR, SYBR Green/ ITS1	Ct 27.54	Amoah et al., 2020
				PowerLyzer PowerSoil DNA isolation kit (MO BIO)	SDS	MD		Ct 26.33	
				PowerSoil DNA isolation kit (MO BIO)	SDS	MD		Ct 25.25	
				PowerLyzer Ultraclean Microbial DNA isolation kit (MO BIO)	SDS	MD		Ct 25.84	
				PowerFecal DNA isolation kit (MO BIO)	SDS	MD		Ct 28.66	
				QIAamp Fast DNA Stool Mini kit (Qiagen)	InhibitEX, proteinase K	EL		Ct 30.11	
A. lumbricoides	sludge	94/20 g	$ZnSO_4$	PowerSoil DNA isolation kit (MO BIO)	SDS	MD		Ct 20.53	
				PowerLyzer Ultraclean Microbial DNA isolation kit (MO BIO)	SDS	MD		Ct 23.37	
				QIAamp Fast DNA Stool Mini kit (Qiagen)	InhibitEX, proteinase K	EL		Ct 28.45	
	wastewater	343/1 L	$ZnSO_4$	PowerSoil DNA isolation kit (MO BIO)	SDS	MD		Ct 22.69	
				PowerLyzer Ultraclean Microbial DNA isolation kit (MO BIO)	SDS	MD		Ct 23.71	
				QIAamp Fast DNA Stool Mini kit (Qiagen)	InhibitEX, proteinase K	EL		Ct 24.90	
A. lumbricoides	faeces	n.a./500 mg	–	n.a., Qiasymphony (Qiagen)	n.a., proteinase K	EL, TD, MD	qPCR, Texas Red-BHQ-2 probe/ITS1	n.a.	Ayana et al., 2019
A. lumbricoides	faeces	n.a./500 mg	–	QIAsymphony DSP Virus/ Pathogen Midi kit (Qiagen)	n.a., proteinase K	EL, TD, MD	qPCR, Texas Red-BHQ-2 probe/ITS1	1.12 GE/ml	Cools et al., 2019
A. lumbricoides	H_2O	1 to 50/50 µl	–	–	–	TD, MD	qPCR, FAM-TMR probe/ITS1	LoD 1 egg [Ct n.a.]	Acosta Soto et al., 2017
	reclaimed water	1 to 10/500 mL	–	phenol/chloroform/isoamyl alcohol	SDS, proteinase K	EL, TD, MD	qPCR, FAM-TMR probe/ITS1	LoD 1 egg [Ct n.a.]	
							dPCR/ITS1	LoD 5 eggs [Ct n.a.]	
		1 to 50/10 L					qPCR, FAM-TMR probe/ ITS1	LoD 20 eggs [Ct n.a.]	
							dPCR/ITS1	n.d.	
A. lumbricoides	faeces	n.a./n.a.	–	PowerSoil DNA isolation kit (MO BIO)	SDS	MD	PMA-qPCR, FAM-TAMRA probe/ITS1	cLoD 1 egg	Gyawali et al., 2016
A. lumbricoides	coprolite	n.a./10 g	Glucose, NaCl	PowerLyzer PowerSoil DNA isolation kit (MO BIO)	SDS	MD	PCR/Cox1, 18S rRNA	n.d.	Søe et al., 2015
A. suum	faeces	0 to 20/100 mg	–	NucliSens easyMAG (bioMérieux)	Triton X-100	– MD	qPCR, n.a./n.a.	LoD 30 EPG [Ct 40.69] LoD 10 EPG [Ct 40.40]	Andersen et al., 2013

(*continued on next page*)

Table 3 (*continued*)

Ascarid species	Matrix	Concentration [eggs/volume]	Flo-tation	DNA extraction/purification	Dissociation/ detergent	Egg shell disruption	Detection method/ gene	Quantification	Reference
A. lumbricoides	faeces	n.a./100 mg	–	QIAamp Mini kit (Qiagen)	n.a., proteinase K	EL	qPCR, ROX-BHQ-2 probe/ITS1	1 copy per gram [Ct n.a.]	Basuni et al., 2012; Basuni et al., 2011
A. suum	sand	5 to 1020/5 g	–	PowerMax Soil DNA isolation kit (MO BIO)	SDS	MD	qPCR, Red610-BHQ-2 probe/18S rRNA	LoD 2 EPG [Ct 33.70]	Durant et al., 2012
A. suum	0.1 M H_2SO_4	10 to 1000/1 mL	–	UltraClean Faecal DNA isolation kit (MO BIO)	n.a.	MD	qPCR, FAM-TMR probe/ITS1	LoD 10 to 50 eggs [Ct n.a.]	Raynal et al., 2012
A. suum	0.5% formalin	1400/n.a.	–	UltraClean Microbial DNA and RNA isolation kit (MO BIO)	SDS	MD	qPCR, FAM-TMR probe/ITS1	cLoD 90 single-celled eggs, 1 larvated egg [Ct n.a.]	Pecson et al., 2006
Ascaris spp.	coprolite	n.a.	–	phenol/chloroform/isoamyl alcohol	N-lauryl sarcosyl, proteinase K	ultrasonication	PCR/18S rRNA	n.d.	Loreille et al., 2001
Toxocara spp.									
T. canis	H_2O	1 to 1000/n.a.	–	NucliSens MiniMAG (bioMérieux)	–	EL TD MD	qPCR, FAM-BHQ-1 probe/ITS2	LoD 100 eggs [Ct n.a.] cLoD 7 eggs [Ct n.a.] cLoD 7 eggs [Ct n.a.]	Jarosz et al., 2021
T. canis	sand	1 to 10,000/10 g	–	DNeasy® PowerMax® Soil kit/ AMPure beads (Qiagen)	SDS	MD		cLoD 0.4 EPG [Ct 34.25 1 EPG]	
				FastDNA™ SPIN kit for Soil/ AMPure beads (MP Biomedicals)	SDS	MD		LoD 1000 EPG [Ct 37.14]	
	soil	1 to 10,000/10 g	–	DNeasy® PowerMax® Soil kit/ AMPure beads (Qiagen)	SDS	MD		cLoD 4.6 EPG [Ct 35.34 10 EPG]	
				FastDNA™ SPIN kit for Soil/ AMPure beads (MP Biomedicals)	SDS	MD		LoD 10 EPG [Ct 37.17]	
T. canis *T. cati*	faecal extracts faecal extracts	75 and 150/1 µL	NaCl or $ZnSO_4$	–	–	TD	qPCR, EvaGreen/ 28S rDNA	n.a. 0.003 eggs	Demeler et al., 2013
T. cati	sand	5 to 100/5 g	–	PowerMax Soil DNA isolation kit (MO BIO)	SDS	MD	qPCR, Cy5-BHQ-3 probe/ITS2	LoD 2 EPG [Ct n.a.]	Durant et al., 2012
T. canis	sand	1 to 7/10 g	NaClO	NaOH, 95 °C	–	NaClO	LAMP/ITS2	LoD 0.3 EPG	Macuhova et al., 2010
T. canis	sand	1 to 20/2.5 g	–	NucleoSpin Tissue kit (Macherey-Nagel), GeneReleaser (Bioventures)	proteinase K	EL	PCR/ITS2	LoD n.d.	Krämer et al., 2002
				NucleoSpin Tissue kit (Macherey-Nagel), Maximator (Connex)	proteinase K	EL		LoD 1.2 EPG	
Baylisascaris procyonis									
B. procyonis	faeces	20 to 20,000/1 g	–	QIAamp DNA Micro kit (Qiagen)	n.a., proteinase K	EL, MD	PCR/Cox2 qPCR, SYBR Green/ Cox2	LoD 20 EPG LoD 20 EPG [Ct 36.01]	Dangoudoubiyam et al., 2009
B. procyonis	sand lake water concen-trates	5 to 250/500 mg 5 and 25/0.5 mL	–	UltraClean Soil DNA Isolation kit (MO BIO)	n.a.	MD	qPCR, JVBPP beacon probe/Cox2	LoD 10 EPG [Ct 34.00] LoD 10 EPG [Ct 38.00]	Gatcombe et al., 2010

flotation steps being necessary for microscopy (Guggisberg et al., 2020). All *Toxocara*-spiked replicates captured in the 40 μm filter fraction were positive in PCR, indicating that the method has a high sensitivity with a detection limit of at least four eggs. Microscopic evaluation was less sensitive than DNA analysis with *Toxocara* eggs being detected in all five replicates from lettuce spiked with 20 eggs, but only in 40.0% of replicates spiked with four eggs.

3. Detection methods for egg contamination

Ascarid egg contamination of soil and faeces can be determined by various methods, which are often divided into conventional or more recent molecular methods. Conventional methods mainly include microscopic examination, with eggs often being isolated and purified from the sample matrix beforehand (see sections above). Considerable drawbacks of microscopic egg examination are the labour- and time-intensive process, the need for experienced personal or specific training, and the probability of misdiagnoses. For instance, eggs of several parasite species are difficult to differentiate via morphologic traits, such as *T. canis* and *T. cati*. *Ascaris lumbricoides* and *A. suum* eggs are indistinguishable with some researchers even proposing that both parasites are a single species (Alves et al., 2016). Moreover, purification methods can lead to the distortion of eggs, thus impeding diagnosis (Collender et al., 2015) and pseudoparasites including pollen as well as parts of plants, fungal spores and psocid as well as grain mites may be mistaken for parasite eggs. Unfortunately, standardised methods that allow an easy and cost-effective microscopy-based egg quantification, such as Kato-Katz (filtration), McMaster (filtration and flotation), (Mini-)FLOTAC (filtration and flotation) and FECPAK^{G2} (filtration, sedimentation and flotation) used for faecal examination (Bosch et al., 2021; Cools et al., 2019) are not available for assessment of environmental egg contamination. However, artificial intelligence-based methods, utilising algorithms for the identification of captured helminth eggs, recently emerged and have the potential to eliminate examiner-dependent inconsistency. Jiménez et al. (2020) established a Helminth Egg Automatic Detector (HEAD) that is capable of differentiating seven helminth species derived from wastewater, sludge, biosolids, faeces and soils. Furthermore, Lee et al. (2021) developed a Helminth Egg Analysis Platform (HEAP) with the ability to discriminate between helminth eggs of 17 species and simultaneous quantification of the faecal egg count.

3.1. DNA isolation, purification and preparation from eggs present in complex environmental matrices

Over the last years, molecular methods, mostly PCR-based techniques, are more frequently used for the detection of egg contamination in environmental samples. Not only do these methods void the drawbacks of microscopy, but they also have the potential for more specific, sensitive and therefore reliable detection of eggs. A critical step for successful PCR detection is the isolation of sufficient and enough intact DNA from the ova, which is especially challenging when eggs are present in complex environmental matrices. For instance, environmental egg contamination may be very low with only few eggs present in sampled media, thus frequently resulting in insufficient amounts of isolated DNA, particularly when low-yielding DNA extraction methods are used (Amoah et al., 2020; Salonen et al., 2010). Furthermore, quality of DNA is affected by environmental factors such as UV radiation, desiccation of eggs, high temperatures or environmental chemicals as well as enzymes, leading to rapid degradation of DNA (Buxton et al., 2017). Another factor that impedes the isolation of DNA is the thick outer surface shell of ascarid eggs that has to be disrupted for proper DNA extraction. Recently, Jarosz et al. (2021) compared the most frequently utilised egg shell disruption methods, namely enzymatic lysis via proteinase K, thermal disruption with repeated freeze-thaw/boiling cycles and bead-based mechanical disruption for the isolation of DNA from *T. canis* ova. While both mechanical and thermal disruption yielded high amounts of qPCR-detectable DNA, enzymatic lysis was less successful (cf. Table 3). Indeed, most of the currently available protocols utilise bead-beating or a combination of this method with thermal disruption and/or enzymatic lysis for efficient destruction of ascarid egg shells (Table 3), indicating that mechanical disruption seems to be superior to other methods.

Besides DNA, various organic and inorganic matters of complex environmental matrices are carried along during isolation which tend to inhibit subsequent PCR assays. Thus, interfering substances like humic acids, polysaccharides, salts, lipids, proteins and other organic molecules should be removed prior to molecular detection as another critical step for successful PCR detection (Amoah et al., 2020; Collender et al., 2015; Smith, 1998). While isolation of ova from matrices by sedimentation and/or flotation can be helpful and should be applied to remove at least some of the inhibitory contents, anti-inhibitory additives are often supplemented to improve the performance of DNA-based detection assays (Collender et al., 2015; Krämer et al., 2002). These substances are, for instance, ion exchangers, resins or blotting papers that scavenge and precipitate interfering factors such as salts and proteins (Scheibner, 2000). Otherwise, DNA can also be separated from inhibitors via clean-up steps utilising DNA-binding beads (Jarosz et al., 2021). Furthermore, obtained DNA is often diluted to minimise detrimental effects of inhibitory matrix components, however, with concomitant reduction of detection sensitivity (Amoah et al., 2020; Scheibner, 2000).

Both, mechanical disruption by bead beating and anti-inhibitory additives are frequently implemented in commercial kits designated for the extraction of DNA from soil or other complex matrices. Presumably, most of these kits use similar anti-inhibitory additives as mentioned above. However, the exact composition of these supplier-derived patented agents is unknown as most companies normally withhold any specifications. Although often being similarly structured, the DNA recovery rate of kits can vary greatly as recently shown by Amoah et al. (2020). In particular, a kit lacking a bead-beating step showed poorest recovery of DNA (Amoah et al., 2020), highlighting the need for comparative evaluation of differential DNA extraction methods and the implementation of mechanical disruption and anti-inhibitory additives when isolating DNA from complex environmental matrices.

3.2. Molecular detection methods of egg contamination

In the last decade, various efforts have been made to detect ascarid eggs via molecular tools, with quantitative real-time PCR (qPCR) being the by far mostly utilised technique (Table 3). Although also conventional PCR is often superior to microscopically-based methods, it is rarely used for the detection of ascarid eggs as it requires a subsequent visualisation step and is hardly quantifiable (Manuel et al., 2021). However, it has to be considered that PCR can only be successful if the preceding DNA isolation is adjusted to sampled media and worked out properly. Presumably, microscopic examination might be more sensitive than PCR in case of low intensity contamination due to previous purification and concentration of eggs, which is often not implemented prior to DNA isolation procedures (cf. Table 3).

One of the protruding characteristics of qPCR is its outstanding sensitivity. Some of the developed assays were able to detect DNA derived from a single egg (Acosta Soto et al., 2017; Gyawali et al., 2016; Pecson et al., 2006) or even less as determined by dilution series (Demeler et al., 2013). Another characteristic is the possibility for quantification of detected DNA, feasible due to the utilisation of DNA-intercalating dyes such as SYBR Green, YO-PRO-1 as well as BEBO (Gudnason et al., 2007) or fluorophore-tagged probes like TaqMan, locked nucleic acid (LNA) as well as molecular beacon (Gasparic et al., 2010). DNA-intercalating dyes bind unspecifically to double-stranded DNA, but are easy to use and less cost-intensive than fluorophore-tagged probes. In contrast, probes need to hybridise to the designated target sequence to generate a positive signal, and are therefore more reliable and specific. Furthermore, they offer the opportunity for multiplexing to assess, for instance, multiple pathogenic agents in a single qPCR run, which can be useful to save costs and DNA while achieving very good comparability.

Absolute quantification can be achieved via the establishment of a standard row, consisting e.g. of a serial dilution of defined amounts of isolated DNA or the desired DNA fragment. It has to be considered that, depending on the genetic target and the developmental stage of the egg, gene copy numbers are varying (Manuel et al., 2021). Standard rows enable a normalised quantification, if for instance referenced to DNA isolated from a single-celled egg, for the expression of results in genome equivalents/mL (GE/mL) (Cools et al., 2019). Out of convenience and for reproducibility, the target region amplified in qPCR is often cloned into a plasmid, allowing the isolation of large quantities of highly pure and specific DNA (Acosta Soto et al., 2017; Basuni et al., 2012; Basuni et al., 2011; Pecson et al., 2006). However, especially when DNA of eggs present in complex matrices is extracted, plasmid-derived standards might distort obtained results as they are devoid of PCR inhibitors and other agents inevitably carried along during DNA isolation from various organic and inorganic matters of these matrices. Therefore, reference samples of soil, wastewater, sludge or food spiked with a defined amount of eggs that are treated equally to samples to be diagnosed are often included in qPCR-based assays (Acosta Soto et al., 2017; Durant et al., 2012; Gatcombe et al., 2010; Jarosz et al., 2021). However, it has to be mentioned that normalisation is especially challenging in case of environmental samples as the obtained eggs tend to have varying developmental stages, ranging from unembryonated to fully embryonated eggs, thus having differential gene copy numbers. Therefore, quantification via standard rows should be considered as an approximation rather than an exact determination of egg contamination in environmental samples.

A downside of DNA-based methods, including qPCR, is the missing discrimination between viable and non-viable ova or contained larvae since DNA is also present in dead organisms or may be released during the dying process. To overcome this problem, propidium monoazide (PMA) qPCR can be applied. PMA is a DNA-intercalating molecule that is able to penetrate the membrane of damaged or dead cells. Once forming covalent high-affinity bonds with the DNA, it has inhibitory properties in PCR, thereby selectively hindering the amplification of DNA derived from dead organisms (Gyawali et al., 2016) (for details on other methods for the determination of egg viability see Collender et al., 2015 and Amoah et al., 2017).

Other emerging DNA-based tools to detect ova contamination are loop-mediated isothermal assay (LAMP), digital PCR (dPCR) and a variety of dPCR called digital droplet PCR (ddPCR). Certainly, these methods have their advantages and disadvantages: Briefly, LAMP is cost-effective but does not offer the possibility for multiplexing, whereas dPCR and ddPCR do not require a standard for quantification but are cost-intensive (for a detailed review on these methods see Manuel et al., 2021 and Amoah et al., 2017). Nevertheless, qPCR is still the method of choice when it comes to molecular detection of egg contamination.

4. Concluding remarks

Assessing the environmental contamination with ascarid eggs is key for proper and reliable human and animal health risk assessment. However, adequate risk assessment is critically dependent on standardised methods to guarantee comparability of the acquired data. Although many researchers proposed more uniform protocols for the recovery of STH eggs from environmental matrices in the past (reviewed by Collender et al., 2015 and Amoah et al., 2017), comparably little has changed in recent years. Standardisation is a tremendous challenge considering the mass of factors affecting the sampling and isolation of eggs from complex matrices. Sampling regimes have to be adjusted to the study hypothesis (e.g. infection risk for humans vs. general prevalence estimation) and the variations in matrices influence the application of techniques, i.e. dissociation, sedimentation and flotation, utilised for ascarid egg isolation. With its different textural classes, soil is a highly diverse matrix whereas wastewater and sludge show less variation. Isolation of ova from food has been mainly performed with leafy vegetables, but established protocols can presumably be applied to other foods as well. In general, some agents are preferably used by many investigators, including Tween®20/40/80 or 7X® for the dissociation of eggs from matrix particles or non-toxic saturated sodium chloride or magnesium sulphate solution for flotation. Moreover, there are also certain parallels in applied procedures such as the implementation of centrifugation for accelerated flotation and sedimentation. Nevertheless, a protocol that can be applied to the multitude of different matrices is still not available and will also be difficult to establish in the future. In contrast, the detection of ascarid eggs has progressed substantially with molecular methods, most of all qPCR, being more frequently established and applied. Molecular methods are fast, highly sensitive and often species-specific, thus paving the

way for more accurate and reliable detection and quantification of ascarid egg contamination of complex environmental matrices. However, isolation of egg DNA from complex environmental matrices is challenging with the possibility of misdiagnosis due to the recovery of degraded or insufficient amounts of DNA and PCR inhibitors impeding molecular detection, especially if the DNA isolation procedure is not adapted to complex environmental matrices. In contrast, microscopy-based detection methods do not harbour these difficulties and are cost-effective, being an important economic factor for e.g. diagnostic laboratories, and are therefore still frequently applied.

Funding

A postdoc grant of the Karl-Enigk-Foundation (grant no. S0229/10020/20) to MKR is gratefully acknowledged. This Open Access publication was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) within the programme LE 824/10-1 "Open Access Publication Costs" and University of Veterinary Medicine Hannover, Foundation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

References

Acosta Soto, L., Santisima-Trinidad, A.B., Bornay-Llinares, F.J., Martin Gonzalez, M., Pascual Valero, J.A., Ros Munoz, M., 2017. Quantitative PCR and digital PCR for detection of *Ascaris lumbricoides* eggs in reclaimed water. Biomed. Res. Int. 2017, 7515409.

Alves, E.B.D.S., Conceição, M.J., Leles, D., 2016. *Ascaris lumbricoides*, *Ascaris suum*, or "*Ascaris lumbrisuum*"? J. Infect. Dis. 213, 1355.

Amoah, I.D., Singh, G., Stenstrom, T.A., Reddy, P., 2017. Detection and quantification of soil-transmitted helminths in environmental samples: a review of current state-of-the-art and future perspectives. Acta Trop. 169, 187–201.

Amoah, I.D., Reddy, P., Seidu, R., Stenstrom, T.A., 2018. Concentration of soil-transmitted helminth eggs in sludge from South Africa and Senegal: a probabilistic estimation of infection risks associated with agricultural application. J. Environ. Manag. 206, 1020–1027.

Amoah, I.D., Singh, G., Troell, K., Reddy, P., Stenstrom, T.A., Bux, F., 2020. Comparative assessment of DNA extraction procedures for *Ascaris* spp. eggs. J. Helminthol. 94, e78.

Andersen, L.O.B., Röser, D., Nejsum, P., Nielsen, H.V., Stensvold, C.R., 2013. Is supplementary bead beating for DNA extraction from nematode eggs by use of the NucliSENS easyMag protocol necessary? J. Clin. Microbiol. 51, 1345–1347.

Ayana, M., Cools, P., Mekonnen, Z., Biruksew, A., Dana, D., Rashwan, N., Prichard, R., Vlaminck, J., Verweij, J.J., Levecke, B., 2019. Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. PLoS Negl. Trop. Dis. 13, e0007778.

Baker, S.M., Ensink, J.H., 2012. Helminth transmission in simple pit latrines. Trans. R. Soc. Trop. Med. Hyg. 106, 709–710.

Basuni, M., Muhi, J., Othman, N., Verweij, J.J., Ahmad, M., Miswan, N., Rahumatullah, A., Aziz, F.A., Zainudin, N.S., Noordin, R., 2011. A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. Am. J. Trop. Med. Hyg. 84, 338–343.

Basuni, M., Mohamed, Z., Ahmad, M., Zakaria, N.Z., Noordin, R., 2012. Detection of selected intestinal helminths and protozoa at Hospital Universiti Sains Malaysia using multiplex real-time PCR. Trop. Biomed. 29, 434–442.

Bier, J.W., 1991. Isolation of parasites on fruits and vegetables. Southeast Asian J. Trop. Med. Public Health 22 (Suppl), 144–145.

Bojanich, M.V., Alonso, J.M., Caraballo, N.A., Itati Scholler, M., Lopez Mde, L., Garcia, L.M., Basualdo, J.A., 2015. Assessment of the presence of *Toxocara* eggs in soils of an arid area in Central-Western Argentina. Rev. Inst. Med. Trop. Sao Paulo 57, 73–76.

Boreham, P.F.L., Capon, A.G., 1982. Environmental contamination with canine zoonotic helminths in Brisbane. Aust. Vet. Pract. 12, 14–16.

Bosch, F., Palmeirim, M.S., Ali, S.M., Ame, S.M., Hattendorf, J., Keiser, J., 2021. Diagnosis of soil-transmitted helminths using the Kato-Katz technique: what is the influence of stirring, storage time and storage temperature on stool sample egg counts? PLoS Negl. Trop. Dis. 15, e0009032.

Bowman, D.D., 2020. History of *Toxocara* and the associated larva migrans. Adv. Parasitol. 109, 17–38.

Bowman, D.D., 2021. *Ascaris* and *Toxocara* as foodborne and waterborne pathogens. Res. Vet. Sci. 135, 1–7.

Bowman, D.D., Little, M.D., Reimers, R.S., 2003. Precision and accuracy of an assay for detecting *Ascaris* eggs in various biosolid matrices. Water Res. 37, 2063–2072.

Buxton, A.S., Groombridge, J.J., Zakaria, N.B., Griffiths, R.A., 2017. Seasonal variation in environmental DNA in relation to population size and environmental factors. Sci. Rep. 7, 46294.

Carabin, H., Gyorkos, T.W., Kokoskin, E., Payment, P., Joseph, L., Soto, J., 1998. Comparison of methods of sampling for *Toxocara* species and fecal coliforms in an outdoor day care environment. Can. J. Infect. Dis. 9, 149–156.

Chan, M.S., 1997. The global burden of intestinal nematode infections-fifty years on. Parasitol. Today 13, 438–443.

Collender, P.A., Kirby, A.E., Addiss, D.G., Freeman, M.C., Remais, J.V., 2015. Methods for quantification of soil-transmitted helminths in environmental media: current techniques and recent advances. Trends Parasitol. 31, 625–639.

Cools, P., Vlaminck, J., Albonico, M., Ame, S., Ayana, M., Jose Antonio, B.P., Cringoli, G., Dana, D., Keiser, J., Maurelli, M.P., Maya, C., Matoso, L.F., Montresor, A., Mekonnen, Z., Mirams, G., Correa-Oliveira, R., Pinto, S.A., Rinaldi, L., Sayasone, S., Thomas, E., Verweij, J.J., Vercruysse, J., Levecke, B., 2019. Diagnostic performance of a single and duplicate Kato-Katz, Mini-FLOTAC, FECPAK^{G2} and qPCR for the detection and quantification of soil-transmitted helminths in three endemic countries. PLoS Negl. Trop. Dis. 13, e0007446.

Crompton, D.W.T., 2001. Ascaris and ascariasis. Adv. Parasitol. 48, 285–375.

Dada, B.J.O., Lindquist, W.D., 1979. Studies on flotation techniques for the recovery of helminth eggs from soil and the prevalence of eggs of *Toxocara* spp. in some Kansas public places. J. Am. Vet. Med. Assoc. 174, 1208–1210.

Dangoudoubiyam, S., Vemulapalli, R., Kazacos, K.R., 2009. PCR assays for detection of *Baylisascaris procyonis* eggs and larvae. J. Parasitol. 95, 571–577.

David, E.D., 1977. Evaluation of a Modification of the Dada Technique for the Recovery of Helminth Eggs from Soil, and Determination of the Specific Gravity of Certain Helminth Eggs Using Sucrose Density Gradient Centrifugation. Thesis. Kansas State University, Manhattan, Kansas.

Demeler, J., Ramunke, S., Wolken, S., Ianiello, D., Rinaldi, L., Gahutu, J.B., Cringoli, G., von Samson-Himmelstjerna, G., Krucken, J., 2013. Discrimination of gastrointestinal nematode eggs from crude fecal egg preparations by inhibitor-resistant conventional and real-time PCR. PLoS One 8, e61285.

Deumer, J.W., 1984. Untersuchungen über den Endoparasitenbefall von Hunden in München. Die Kontamination von öffentlichen Sandspielplätzen mit parasitären Entwicklungsstadien und ihr Verhalten gegenüber Umwelteinflüssen. Thesis. Ludwig-Maximilians-Universität München, Munich.

Dold, C., Holland, C.V., 2011. *Ascaris* and ascariasis. Microbes Infect. 13, 632–637.

Dunsmore, J.D., Thompson, R.C., Bates, I.A., 1984. Prevalence and survival of *Toxocara canis* eggs in the urban environment of Perth, Australia. Vet. Parasitol. 16, 303–311.

Durant, J.F., Irenge, L.M., Fogt-Wyrwas, R., Dumont, C., Doucet, J.P., Mignon, B., Losson, B., Gala, J.L., 2012. Duplex quantitative real-time PCR assay for the detection and discrimination of the eggs of *Toxocara canis* and *Toxocara cati* (Nematoda, Ascaridoidea) in soil and fecal samples. Parasit. Vectors 5, 288.

Duscher, G.G., Leschnik, M., Fuehrer, H.-P., Joachim, A., 2015. Wildlife reservoirs for vector-borne canine, feline and zoonotic infections in Austria. Int. J. Parasitol. Parasites Wildl. 4, 88–96.
Düwel, D., 1984. The prevalence of *Toxocara* eggs in the sand in children's playgrounds in Frankfurt/M. Ann. Trop. Med. Parasitol. 78, 633–636.
Eisen, A.K.A., Demoliner, M., de Oliveira, K.G., Troian, E.A., Mallmann, L., Filippi, M., de Almeida, P.R., Spilki, F.R., 2019. Soil contamination of a public park by human and canine mastadenovirus, as well as hookworms and *Toxocara* spp eggs. Rev. Inst. Med. Trop. Sp. 61.
Else, K.J., Keiser, J., Holland, C.V., Grencis, R.K., Sattelle, D.B., Fujiwara, R.T., Bueno, L.L., Asaolu, S.O., Sowemimo, O.A., Cooper, P.J., 2020. Whipworm and roundworm infections. Nat. Rev. Dis. Primers 6, 1–23.
Engohang-Ndong, J., Uribe, R.M., Gregory, R., Gangoda, M., Nickelsen, M.G., Loar, P., 2015. Effect of electron beam irradiation on bacterial and *Ascaris* ova loads and volatile organic compounds in municipal sewage sludge. Radiat. Phys. Chem. 112, 6–12.
EU, 2018. Sewage Sludge Directive - Council Directive 86/278/EEC European Commission.
Fajutag, A.J.M., Paller, V.G.V., 2013. *Toxocara* egg soil contamination and its seroprevalence among public school children in Los Banos, Laguna, Philippines. Se Asian J. Trop. Med. 44, 551–560.
Fallah, A.A., Pirali-Kheirabadi, K., Shirvani, F., Saei-Dehkordi, S.S., 2012. Prevalence of parasitic contamination in vegetables used for raw consumption in Shahrekord, Iran: influence of season and washing procedure. Food Control 25, 617–620.
Fan, C.-K., Holland, C.V., Loxton, K., Barghouth, U., 2015. Cerebral toxocariasis: silent progression to neurodegenerative disorders? Clin. Microbiol. Rev. 28, 663–686.
Forslund, A., Ensink, J.H.J., Battilani, A., Kljujev, I., Gola, S., Raicevic, V., Jovanovic, Z., Stikic, R., Sandei, L., Fletcher, T., Dalsgaard, A., 2010. Faecal contamination and hygiene aspect associated with the use of treated wastewater and canal water for irrigation of potatoes (*Solanum tuberosum*). Agr. Water Manage. 98, 440–450.
García-Gaines, R.A., Frankenstein, S., 2015. USCS and the USDA Soil Classification System: Development of a Mapping Scheme. Cold Regions Research and Engineering Laboratory (U.S.) Engineer Research and Development Center (U.S.).
Gaspard, P., Wiartz, J., Schwartzbrod, J., 1994. Étude expérimentale de l'adhésion des oeufs d'helminthes (*Ascaris suum*): conséquences pour l'environnement. Rev. Sci. Eau. 7, 367–376.
Gasparic, M.B., Tengs, T., La Paz, J.L., Holst-Jensen, A., Pla, M., Esteve, T., Zel, J., Gruden, K., 2010. Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. Anal. Bioanal. Chem. 396, 2023–2029.
Gatcombe, R.R., Jothikumar, N., Dangoudoubiyam, S., Kazacos, K.R., Hill, V.R., 2010. Evaluation of a molecular beacon real-time PCR assay for detection of *Baylisascaris procyonis* in different soil types and water samples. Parasitol. Res. 106, 499–504.
Gnani Charitha, V., Rayulu, V.C., PM, K., Srilatha, C., 2013. Comparative evaluation of flotation techniques for the detection of soil borne parasites. J. Parasit. Dis. 37, 260–263.
Graeff-Teixeira, C., Morassutti, A.L., Kazacos, K.R., 2016. Update on baylisascariasis, a highly pathogenic zoonotic infection. Clin. Microbiol. Rev. 29, 375–399.
Gudnason, H., Dufva, M., Bang, D.D., Wolff, A., 2007. Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic Acids Res. 35, e127.
Guggisberg, A.R., Rojas, C.A.A., Kronenberg, P.A., Miranda, N., Deplazes, P., 2020. A sensitive, one-way sequential sieving method to isolate helminths' eggs and protozoal oocysts from lettuce for genetic identification. Pathogens. 9, 624.
Gyawali, P., Ahmed, W., Sidhu, J.P.S., Nery, S.V., Clements, A.C., Traub, R., McCarthy, J.S., Llewellyn, S., Jagals, P., Toze, S., 2016. Quantitative detection of viable helminth ova from raw wastewater, human feces, and environmental soil samples using novel PMA-qPCR methods. Environ. Sci. Pollut. Res. 23, 18639–18648.
Horiuchi, S., Paller, V.G.V., Uga, S., 2013. Soil contamination by parasite eggs in rural village in the Philippines. Trop. Biomed. 30, 495–503.
Horn, K., 1986. Untersuchungen über das Vorkommen exogener Stadien humanpathogener Darmhelminthen der Fleischfresser im Sand öffentlicher Kinderspielplatze in Hannover. Thesis. University of Veterinary Medicine Hannover, Hanover.
Horn, K., Schnieder, T., Stoye, M., 1990. Quantitative comparison of various methods for detecting eggs of *Toxocara canis* in samples of sand. J. Veterinary Med. Ser. B 37, 241–250.
IHME, 2021. Ascariasis - Level 4 Cause. http://www.healthdata.org/results/gbd_summaries/2019/ascariasis-level-4-cause. Access date 21 October 2021.
Jarosz, W., Mizgajska-Wiktor, H., Kirwan, P., Konarski, J., Rychlicki, W., Wawrzyniak, G., 2010. Developmental age, physical fitness and *Toxocara* seroprevalence amongst lower-secondary students living in rural areas contaminated with *Toxocara* eggs. Parasitology. 137, 53–63.
Jarosz, W., Durant, J.F., Irenge, L., Fogt-Wyrwas, R., Mizgajska-Wiktor, H., Gala, J.L., 2021. Optimized DNA-based identification of *Toxocara* spp. eggs in soil and sand samples. Parasit. Vectors 14, 426.
Jeandron, A., Ensink, J.H., Thamsborg, S.M., Dalsgaard, A., Sengupta, M.E., 2014. A quantitative assessment method for *Ascaris* eggs on hands. PLoS One 9, e96731.
Jiménez, B., Maya, C., Velásquez, G., Barrios, J.A., Pérez, M., Román, A., 2020. Helminth egg automatic detector (HEAD): improvements in development for digital identification and quantification of helminth eggs and its application online. MethodsX. 7, 101158.
Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S.R., Deighton, M., 2015. Comparison of methodologies for enumerating and detecting the viability of *Ascaris* eggs in sewage sludge by standard incubation-microscopy, the BacLight live/dead viability assay and other vital dyes. Water Res. 68, 533–544.
Kasieczka, J., 1982. Zur Kontamination öffentlicher Grünflachen und Kinderspielplätze in Wien mit Dauerstadien humanpathogener Endoparasiten von Hund und Katze. Thesis. University of Vienna, Vienna.
Katakam, K.K., Mejer, H., Dalsgaard, A., Kyvsgaard, N.C., Thamsborg, S.M., 2014. Survival of *Ascaris suum* and *Ascaridia galli* eggs in liquid manure at different ammonia concentrations and temperatures. Vet. Parasitol. 204, 249–257.
Kazacos, K.R., 1983. Improved method for recovering ascarid and other helminth eggs from soil associated with epizootics and during survey studies. Am. J. Vet. Res. 44, 896–900.
Klapec, T., Borecka, A., 2012. Contamination of vegetables, fruits and soil with geohelmints eggs on organic farms in Poland. Ann. Agr. Environ. Med. 19, 421–425.
Kleine, A., Janecek, E., Waindok, P., Strube, C., 2016. Flotation and adherence characteristics of *Toxocara canis* and *T. cati* and a reliable method for recovering *Toxocara* eggs from soil. Vet. Parasitol. 227, 35–41.
Kleine, A., Springer, A., Strube, C., 2017. Seasonal variation in the prevalence of *Toxocara* eggs on children's playgrounds in the city of Hanover, Germany. Parasit. Vectors 10, 248.
Köhler, G., Jörren, R., van Knapen, F., 1980. Untersuchungen zur Kontamination von Spielkastensanden mit Eiern von Fleischfresseraskariden. Bundesgesundheitsblatt 23.
Kozan, E., Gonenc, B., Sarimehmetoglu, O., Aycicek, H., 2005. Prevalence of helminth eggs on raw vegetables used for salads. Food Control 16, 239–242.
Kraglund, H.O., Gronvold, J., Roepstorff, A., Rawat, H., 1998. Interactions between the nematode parasite of pigs, *Ascaris suum*, and the earthworm *Aporrectodea longa*. Acta Vet. Scand. 39, 453–460.
Krämer, F., Vollrath, T., Schnieder, T., Epe, C., 2002. Improved detection of endoparasite DNA in soil sample PCR by the use of anti-inhibitory substances. Vet. Parasitol. 108, 217–226.
Landa-Cansigno, O., Duran-Alvarez, J.C., Jimenez-Cisneros, B., 2013. Retention of *Escherichia coli*, *Giardia lamblia* cysts and *Ascaris lumbricoides* eggs in agricultural soils irrigated by untreated wastewater. J. Environ. Manag. 128, 22–29.
Lee, C.C., Huang, P.J., Yeh, Y.M., Li, P.H., Chiu, C.H., Cheng, W.H., Tang, P., 2021. Helminth egg analysis platform (HEAP): an opened platform for microscopic helminth egg identification and quantification based on the integration of deep learning architectures. J. Microbiol. Immunol. Infect. 2, S1684–S1782.
Levantesi, C., La Mantia, R., Masciopinto, C., Bockelmann, U., Ayuso-Gabella, M.N., Salgot, M., Tandoi, V., Van Houtte, E., Wintgens, T., Grohmann, E., 2010. Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe. Sci. Total Environ. 408, 4923–4930.
Loreille, O., Roumat, E., Verneau, O., Bouchet, F., Hanni, C., 2001. Ancient DNA from *Ascaris*: extraction amplification and sequences from eggs collected in coprolites. Int. J. Parasitol. 31, 1101–1106.
Lynch, M.F., Tauxe, R.V., Hedberg, C.W., 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiol. Infect. 137, 307–315.

Macuhova, K., Kumagai, T., Akao, N., Ohta, N., 2010. Loop-mediated isothermal amplification assay for detection and discrimination of *Toxocara canis* and *Toxocara cati* eggs directly from sand samples. J. Parasitol. 96, 1224–1227.
Maikai, B.V., Elisha, I.A., Baba-Onoja, E.B.T., 2012. Contamination of vegetables sold in markets with helminth eggs in Zaria metropolis, Kaduna state, Nigeria. Food Control. 28, 345–348.
Manuel, M., Ramanujam, K., Ajjampur, S.S., 2021. Molecular tools for diagnosis and surveillance of soil-transmitted helminths in endemic areas. Parasitologia. 1, 105–118.
Matosinhos, F.C., Valenzuela, V.C., Silveira, J.A., Rabelo, E.M., 2016. Standardization of a method for the detection of helminth eggs and larvae in lettuce. Parasitol. Res. 115, 1827–1834.
Maya, C., Torner-Morales, F.J., Lucario, E.S., Hernandez, E., Jimenez, B., 2012. Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH and dryness. Water Res. 46, 4770–4782.
Mejer, H., Roepstorff, A., 2006. *Ascaris suum* infections in pigs born and raised on contaminated paddocks. Parasitology. 133, 305–312.
Meng, X.Q., Wang, S.S., Wang, B.X., Ying, G.H., Li, X.Y., Zhao, Y.Z., 1981. The membranous structure of eggs of *Ascaris lumbricoides* as revealed by scanning electron-microscopy. Scan. Electron Microsc. 187–190.
Mes, T.H., 2003. Technical variability and required sample size of helminth egg isolation procedures. Vet. Parasitol. 115, 311–320.
Mizgajska, H., 1993. The distribution and survival of eggs of *Ascaris suum* in six different natural soil profiles. Acta Parasitol. 38, 170–174.
Mizgajska, H., 1997. The role of some environmental factors in the contamination of soil with *Toxocara* spp. and other geohelminth eggs. Parasitol. Int. 46, 67–72.
Mizgajska-Wiktor, H., 2005. Recommended method for recovery of *Toxocara* and other geohelminth eggs from soil. Wiad. Parazytol. 51, 21–22.
Mohamed, M.A., Siddig, E.E., Elaagip, A.H., Edris, A.M.M., Nasr, A.A., 2016. Parasitic contamination of fresh vegetables sold at central markets in Khartoum state, Sudan. Ann. Clin. Microbiol. Antimicrob. 15, 1–7.
Molleda, P., Blanco, I., Ansola, G., de Luis, E., 2008. Removal of wastewater pathogen indicators in a constructed wetland in Leon, Spain. Ecol Eng. 33, 252–257.
Moodley, P., Archer, A., Hawksworth, D., 2008. Standard Methods for the Recovery and Enumeration of Helminth Ova in Wastewater, Sludge, Compost and Urine-Diversion Waste in South Africa. WRC Report No. TT322/08, South Africa.
Nadler, S.A., Hudspeth, D.S., 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J. Parasitol. 86, 380–393.
Nijsse, R., Overgaauw, P., Ploeger, H., Mughini-Gras, L., 2020. Sources of environmental contamination with *Toxocara* spp.: an omnipresent parasite. Adv. Parasitol. 109, 585–614.
Nunes, C.M., Sinhorini, I.L., Ogassawara, S., 1994. Influence of soil texture in the recovery of *Toxocara canis* eggs by a flotation method. Vet. Parasitol. 53, 269–274.
O'Donnell, C.J., Meyer, K.B., Jones, J.V., Benton, T., Kaneshiro, E.S., Nichols, J.S., Schaefer, F.W., 1984. Survival of parasite eggs upon storage in sludge. Appl. Environ. Microbiol. 48, 618–625.
Oge, H., Oge, S., 2000. Quantitative comparison of various methods for detecting eggs of *Toxocara canis* in samples of sand. Vet. Parasitol. 92, 75–79.
O'Lorcain, P., 1994. Prevalence of *Toxocara canis* ova in public playgrounds in the Dublin area of Ireland. J. Helminthol. 68, 237–241.
Pecson, B.M., Barrios, J.A., Johnson, D.R., Nelson, K.L., 2006. A real-time PCR method for quantifying viable *Ascaris* eggs using the first internally transcribed spacer region of ribosomal DNA. Appl. Environ. Microbiol. 72, 7864–7872.
Pineda, C.O., Leal, D.A.G., de Lima, R., Ribeiro, P.P., Rodrigues, A., Martini, M.H., Stancari, R.C.A., Matosinhos, F.C.L., Mine, J.C., Greinert-Goulart, J.A., Grott, S.C., Diefenbach, L.M.G., Araujo, B., Franco, R.M.B., 2021. Parasites in fresh produce: a Brazilian inter-laboratory evaluation of a standardized methodology for the detection of *Ascaris* sp. in leafy vegetables. Food Anal. Methods 14, 989–996.
Quiles, F., Balandier, J.Y., Capizzi-Banas, S., 2006. In situ characterisation of a microorganism surface by Raman microspectroscopy: the shell of *Ascaris* eggs. Anal. Bioanal. Chem. 386, 249–255.
Quinn, R., Smith, H.V., Bruce, R.G., Girdwood, R.W., 1980. Studies on the incidence of *Toxocara* and *Toxascaris* spp. ova in the environment. 1. A comparison of flotation procedures for recovering *Toxocara* spp. ova from soil. J. Hyg. (Lond.) 84, 83–89.
Raissi, V., Raiesi, O., Etemadi, S., Firoozeh, F., Getso, M., Hadi, A.M., Zibaei, M., 2020. Environmental soil contamination by *Toxocara* species eggs in public places of Ilam, Iran. Ann. Agr. Environ. Med. 27, 15–18.
Ravindran, V.B., Surapaneni, A., Crosbie, N.D., Schmidt, J., Shahsavari, E., Haleyur, N., Soni, S.K., Ball, A.S., 2019. A modified approach to recover and enumerate *Ascaris* ova in wastewater and sludge. PLoS Negl. Trop. Dis. 13, e0007020.
Raynal, M., Villegas, E.N., Nelson, K.L., 2012. Enumeration of viable and non-viable larvated *Ascaris* eggs with quantitative PCR. J. Water Health 10, 594–604.
da Rocha, M.C.V., Braga, M.C.B., 2016. Assessment of the accuracy of a method used for quantification of *Ascaris* eggs in sewage sludge. Mod. Environ. Sci. Eng. 2, 31–36.
Rocha, S., Pinto, R.M.F., Floriano, A.P., Teixeira, L.H., Bassili, B., Martinez, A., da Costa, S.O.P., Caseiro, M.M., 2011. Environmental analyses of the parasitic profile found in the sandy soil from the Santos municipality beaches, Sp, Brazil. Rev. Inst. Med. Trop. Sp. 53, 277–281.
Roepstorff, A., Murrell, K.D., Boes, J., Petkevicius, S., 2001. Ecological influences on transmission rates of *Ascaris suum* to pigs on pastures. Vet. Parasitol. 101, 143–153.
Rosa Xavier, I.G., Ramos, B.C., Santarem, V.A., 2010. Recovery threshold of *Toxocara canis* eggs from soil. Vet. Parasitol. 167, 77–80.
Rostami, A., Riahi, S.M., Fallah Omrani, V., Wang, T., Hofmann, A., Mirzapour, A., Foroutan, M., Fakhri, Y., Macpherson, C., Gasser, R.B., 2020. Global prevalence estimates of *Toxascaris leonina* infection in dogs and cats. Pathogens. 9, 503.
Ruiz De Ybanez, M.R., Garijo, M., Goyena, M., Alonso, F.D., 2000. Improved methods for recovering eggs of *Toxocara canis* from soil. J. Helminthol. 74, 349–353.
Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović, M., Kekkonen, R.A., Palva, A., de Vos, W.M., 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 81, 127–134.
Santarem, V.A., Magoti, L.P., Sichieri, T.D., 2009. Influence of variables on centrifuge-flotation technique for recovery of *Toxocara canis* eggs from soil. Rev. Inst. Med. Trop. Sao Paulo 51, 163–167.
Scheibner, H.M., 2000. Methoden zur Isolierung von Ribonukleinsäure (RNA) zum Virusgenomnachweis mittels Polymerasekettenreaktion nach Reverser Transkription (RT-PCR). Thesis. University of Veterinary Medicine Hannover, Hanover.
Sengupta, M.E., Thamsborg, S.M., Andersen, T.J., Olsen, A., Dalsgaard, A., 2011. Sedimentation of helminth eggs in water. Water Res. 45, 4651–4660.
Sengupta, M.E., Andersen, T.J., Dalsgaard, A., Olsen, A., Thamsborg, S.M., 2012. Resuspension and settling of helminth eggs in water: interactions with cohesive sediments. Water Res. 46, 3903–3912.
Shahsavari, E., Schmidt, J., Aburto-Medina, A., Khallaf, B., Balakrishnan, V., Crosbie, N.D., Surapaneni, A., Ball, A.S., 2017. A modified assay for the enumeration of *Ascaris* eggs in fresh raw sewage. MethodsX. 4, 186–190.
Shamma, M., Al-Adawi, M.A., 2002. The morphological changes of *Ascaris lumbricoides* ova in sewage sludge water treated by gamma irradiation. Radiat. Phys. Chem. 65, 277–279.
Silva, P.A., Borba, V.H., Dutra, J.M., Leles, D., da Rosa, A.A., Ferreira, L.F., Araujo, A., 2014. A new ascarid species in cynodont coprolite dated of 240 million years. An. Acad. Bras. Cienc. 86, 265–269.
da Silva, T.E., Barbosa, F.S., Magalhães, L.M.D., Gazzinelli-Guimarães, P.H., Dos Santos, A.C., Nogueira, D.S., Resende, N.M., Amorim, C.C., Gazzinelli-Guimarães, A. C., Viana, A.G., Geiger, S.M., Bartholomeu, D.C., Fujiwara, R.T., Bueno, L.L., 2021. Unraveling *Ascaris suum* experimental infection in humans. Microbes Infect. 23, 104836.
Smith, H.V., 1998. Detection of parasites in the environment. Parasitology. 117, 113–141.
Smith, J.D., Dhiman, R., Anand, S., Reza-Garduno, E., Cohen, R.E., McKinley, G.H., Varanasi, K.K., 2013. Droplet mobility on lubricant-impregnated surfaces. Soft Matter 9, 1772–1780.
Søe, M.J., Nejsum, P., Fredensborg, B.L., Kapel, C.M.O., 2015. DNA typing of ancient parasite eggs from environmental samples identifies human and animal worm infections in Viking-age settlement. J. Parasitol. 101, 57–63.

Sorvillo, F., Ash, L.R., Berlin, O., Yatabe, J., Degiorgio, C., Morse, S.A., 2002. *Baylisascaris procyonis*: an emerging helminthic zoonosis. Emerg. Infect. Dis. 8, 355.
Steinbaum, L., Njenga, S.M., Kihara, J., Boehm, A.B., Davis, J., Null, C., Pickering, A.J., 2016. Soil-transmitted helminth eggs are present in soil at multiple locations within households in rural Kenya. PLoS One 11.
Steinbaum, L., Kwong, L.H., Ercumen, A., Negash, M.S., Lovely, A.J., Njenga, S.M., Boehm, A.B., Pickering, A.J., Nelson, K.L., 2017. Detecting and enumerating soil-transmitted helminth eggs in soil: new method development and results from field testing in Kenya and Bangladesh. PLoS Negl. Trop. Dis. 11, e0005522.
Storey, G.W., Phillips, R.A., 1985. The survival of parasite eggs throughout the soil profile. Parasitology. 91, 585–590.
Tharaldsen, J., 1982. Parasitter fra hund OR katt i prover fra sandkasser i daginstitusjoner i Oslo. Nord Vet. 94, 251–254.
Trönnberg, L., Hawksworth, D., Hansen, A., Archer, C., Stenstrom, T.A., 2010. Household-based prevalence of helminths and parasitic protozoa in rural KwaZulu-Natal, South Africa, assessed from faecal vault sampling. Trans. R. Soc. Trop. Med. Hyg. 104, 646–652.
U.S. EPA, 2003. Appendix I: Test Method for Detecting, Enumerating, and Determining the Viability of *Ascaris* Ova in Sludge. U.S. EPA Environmental regulations and technology: control of pathogens and vector attractions in sewage sludge.
Ubelaker, J.E., Allison, V., 1975. Scanning electron microscopy of the eggs of *Ascaris lumbricoides*, *A. suum*, *Toxocara canis*, and *T. mystax*. J. Parasitol. 802–807.
Uga, S., Matsuo, J., Kimura, D., Rai, S.K., Koshino, Y., Igarashi, K., 2000. Differentiation of *Toxocara canis* and *T. cati* eggs by light and scanning electron microscopy. Vet. Parasitol. 92, 287–294.
USFDA, 2021. BAM Chapter 19: Parasitic Animals in Foods. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19-parasitic-animals-foods. Access date 5 October 2021.
Wang, J.F., Stein, A., Gao, B.B., Ge, Y., 2012. A review of spatial sampling. Spat Stat-Neth. 2, 1–14.
WHO, 2006. Guidelines for the safe use of wastewater, excreta and greywater. In: WHO (Ed.), Wastewater Use in Agriculture. Switzerland, WHO, Geneva.
WHO, 2022. Soil-Transmitted Helminthiases. Number of Children (Pre-SAC and SAC) Requiring Preventive Chemotherapy for Soil-Transmitted Helminthiasis: 2020. https://apps.who.int/neglected_diseases/ntddata/sth/sth.html. Access date 23 February 2022.
Wong, M.S., Bundy, D.A., 1990. Quantitative assessment of contamination of soil by the eggs of *Ascaris lumbricoides* and *Trichuris trichiura*. Trans. R. Soc. Trop. Med. Hyg. 84, 567–570.
Zajac, A.M., Conboy, G.A., Little, S.E., Reichard, M.V., 2021. Veterinary Clinical Parasitology, nineth ed. Wiley, New Jersey.
Zdybel, J., Karamon, J., Rozycki, M., Bilska-Zajac, E., Klapec, T., Cencek, T., 2016. Characterisation of a new, highly effective method for detecting nematode eggs (*Ascaris* spp., *Toxocara* spp., *Trichuris* spp.) in sewage sludge containing flocculants. Exp. Parasitol. 170, 198–206.
Zendejas-Heredia, P.A., Colella, V., Hii, S.F., Traub, R.J., 2021. Comparison of the egg recovery rates and limit of detection for soil-transmitted helminths using the Kato-Katz thick smear, faecal flotation and quantitative real-time PCR in human stool. PLOS Neg. Trop. Dis. 15, e0009395.