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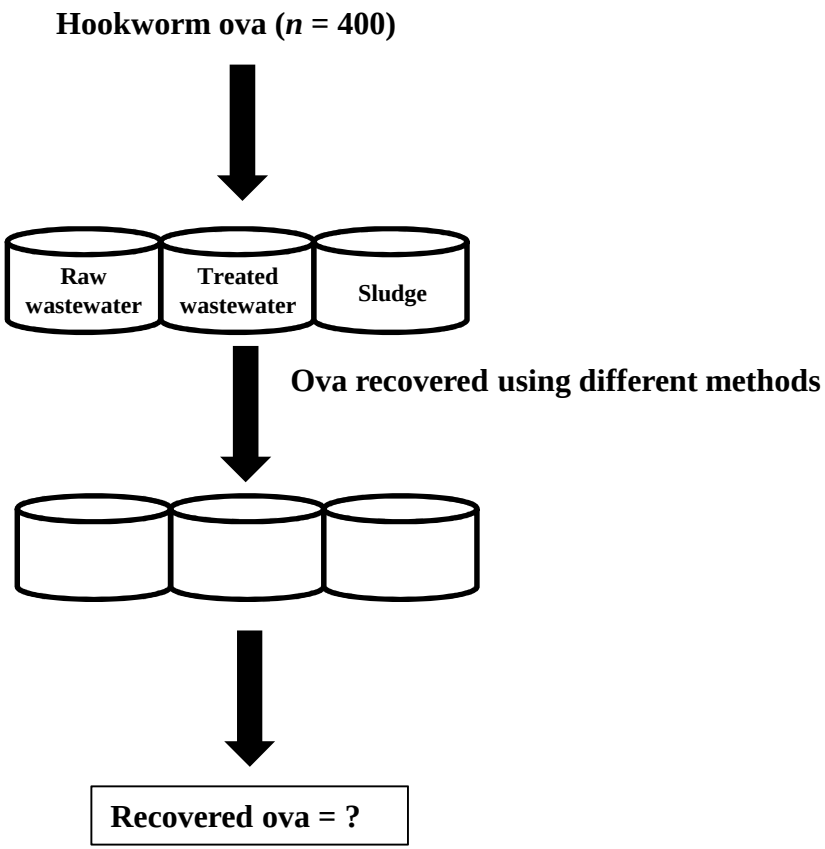

Comparison of Concentration Methods for Rapid Detection of Hookworm Ova in Wastewater Matrices Using Quantitative PCR

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Abstract

Hookworm infection contributes around 700 million infections worldwide especially in developing nations due to poor sanitation. The effective recovery of hookworm ova from wastewater matrices is difficult due to their low concentrations and heterogeneous distribution. In this study, we compared the recovery rates of (i) four rapid hookworm ova concentration methods from municipal wastewater, and (ii) two concentration methods from sludge samples. *Ancylostoma caninum* ova were used as surrogate for human hookworm (*Ancylostoma duodenale* and *Necator americanus*). Known concentration of *A. caninum* hookworm ova were seeded into wastewater (treated and raw) and sludge samples collected from two wastewater treatment plants (WWTPs) in Brisbane and Perth, Australia. The *A. caninum* ova were concentrated from treated and raw wastewater samples using centrifugation (Method A), hollow fiber ultrafiltration (HFUF) (Method B), filtration (Method C) and flotation (Method D) methods. For sludge samples, flotation (Method E) and direct DNA extraction (Method F) methods were used. Among the four methods tested, filtration (Method C) method was able to recover higher concentrations of *A. caninum* ova consistently from treated wastewater (39-50%) and raw wastewater (7.1-12%) samples collected from both WWTPs. The remaining methods (Methods A, B and D) yielded variable recovery rates ranging from 0.2 to 40% for treated and raw wastewater samples. The recovery rates for sludge samples were poor (0.02-4.7), although, Method F (direct DNA extraction) provided 1-2 orders of magnitude higher recovery rate than Method E (flotation). Based on our results it can be concluded that the recovery rates of hookworm ova from wastewater matrices, especially sludge samples, can be poor and highly variable. Therefore, choice of concentration method is vital for the sensitive detection of hookworm ova in wastewater matrices.

Keywords: Hookworm ova, Wastewater and sludge, Concentration methods, Recovery rate, Public health

1. Introduction

It is estimated that almost a quarter of the world's population is infected with soil transmitted helminths (STHs) (WHO, 2015). Among the STHs, hookworm infection contributes around 700 million infections worldwide, especially in tropical and subtropical regions of developing nations due to poor sanitation and hygiene practices (Bethony et al., 2006; Brooker, 2010; Knopp et al., 2012). Depending on the prevalence of infections in the community, high concentrations of viable hookworm ova can be present in human wastewater. The presence of ova in wastewater does not pose a direct health risks to humans. However, viable hookworm ova can be hatched into infective larvae (L3) under favorable conditions, and may survive up to 90 days in wastewater matrices (Ben Ayed et al., 2009). When wastewater is used as irrigation water for crop production, agricultural workers may get infected with hookworm larvae through skin penetration (Gupta et al., 2009; Sidhu and Toze, 2009; Navarro and Jimenez, 2011). The infectious dose of hookworm is quite low (1 viable ovum) (WHO, 2006), and therefore, it is vital to detect and quantify these ova in wastewater matrices using traditional or molecular methods. This is crucial for assessing the health risks of exposure to ova/larvae contaminated wastewater matrices.

The distribution of hookworm ova in wastewater matrices could be patchy. Therefore, detection and quantification of hookworm ova by traditional or molecular methods in wastewater matrices require concentration of the hookworm ova. Ideally, any concentration method should be rapid and have the ability to consistently recover high concentrations of ova from wastewater matrices. The concentration method developed by the US EPA has been the most commonly used to recover hookworm ova from wastewater and sludge samples (US EPA, 1999). The recovery rate of this method can be ranged from 65-74% from wastewater samples (Maya et al., 2006). This method, however, is laborious and time-consuming due to the requirement of multiple steps of washing and concentrating the samples (Ferguson et al., 2004).

Several methods such as centrifugation (Whitmore and Carrington, 1993; Higgins et al., 2003), hollow-fiber ultra filtration (HFUF) (Simmons et al., 2001; Ferguson et al., 2004; Hill et al., 2005; Hill et al., 2007), filtration (Nieminski et al., 1995; Maya et al., 2006; Alli et al., 2011), and flotation

(Bowman et al., 2003; de Victorica and Galván, 2003; Bastos et al., 2013) have also been used to recover various microorganisms including ova from water and soil samples. Some of these methods are rapid and can potentially be used to concentrate hookworm ova from wastewater matrices.

The aim of this study was to evaluate the performance of various concentration methods to recover hookworm ova from wastewater and sludge samples. For wastewater samples, (A) centrifugation, (B) HFUF, (C) filtration, and (D) flotation, and for sludge samples, (E) flotation, and (F) direct DNA extraction were chosen and compared. A newly developed rapid quantitative PCR (qPCR) assay was developed in this study and used to measure the concentrations of seeded known concentrations of *Ancylostoma caninum* ova in wastewater and sludge samples in order to identify the best performing method(s).

2. Materials and methods

2.1. Isolation and enumeration of *Ancylostoma caninum* ova from dog fecal samples

We used dog hookworm (*A. caninum* ova) as a surrogate for human hookworm due to the low prevalence of the latter in the Australian population. For the isolation of *A. caninum* ova, dog fecal samples were collected from the School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia. Ova were isolated from ~20 gm of dog fecal samples using the flotation method described elsewhere (Bowman et al., 2003). After isolation, ova were preserved in 0.5% formalin and stored at 4ºC. The concentrations of ova were estimated by microscopic observation using a Sedgewick-Rafter Counting Chamber (Pyser-SGI, UK), and enumerated in each grid at 40 × magnification in triplicate.

2.2. Determination of ITS-1 rDNA gene copy concentrations in *A. caninum* ova

DNA was extracted from 400 ± 40 (mean ± standard deviation) ova in replicates (n = 6) using a MO BIO Power Soil DNA Extraction Kit (Mo Bio, Carlsbad, CA) with minor modifications. All samples were mixed with lysis buffer C1, and freeze-thawed for 10 min (repeated 5 times). In addition, the protocol was amended to allow all the supernatant to be removed at each step, and therefore, increased volumes of solutions C3 and C4 were added to compensate. Extracted DNA was eluted through the spin filter membranes by adding 100 µL of Solution C6, and stored at -80ºC until

processed. The concentrations of ITS-1 rDNA gene copies in *A. caninum* DNA samples were determined using a qPCR assay (see below for methodological details).

2.3. Sample preparation

Ten liters of raw and treated wastewater samples were collected from two metropolitan wastewater treatment plants (WWTPs) in Brisbane, Queensland (WWTP-1) and Perth, Western Australia (WWTP-2), Australia. The WWTP-1 is a large biological treatment facility, whereas the WWTP-2 is a ponding facility. Treated and raw wastewater samples were transported to the laboratory, and stored at 4°C in the dark until processing. The pH of the wastewater samples were determined to be 7.2 ± 0.1 (treated wastewater; WWTP-1), 8.9 ± 0.2 (raw wastewater; WWTP-1) and 7.2 ± 0.1 (treated wastewater; WWTP-2), 6.7 ± 0.3 (raw wastewater; WWTP-2). The turbidity values of the wastewater samples were determined to be 86 ± 8 (treated wastewater; WWTP-1), 197 ± 17 NTU (treated wastewater; WWTP-1), and 286 ± 6 (raw wastewater; WWTP-2), 246 ± 4 NTU (raw wastewater; WWTP-2). The pH and turbidity were measured using 90 FL-T field lab analyser (McVan Instruments, Pty Ltd, Melbourne, Australia).

Sludge samples were collected from the dewatering belt from WWTP-1 and from the facultative pond from WWTP-2 in 500 mL sterile polyethylene zip-locked bags. Samples were then placed on ice for transportation to the laboratory and kept at 4^{o}C in dark until processing. The background levels of *A. caninum* ITS-1 rDNA gene copies ova in all samples (treated wastewater, raw wastewater and sludge) were determined using a qPCR assay (see below). All samples were determined to be free of *A. caninum* ITS-1 rDNA. Approximately, 400 ± 40 *A. caninum* ova were seeded into 1 L of treated wastewater, raw wastewater and sludge (~ 4 gm dry weight) samples. Three repeat trials were undertaken, and all samples were tested in triplicates in each trial.

2.4. Ova recovery from wastewater matrices

Ova concentration methods flow chart is shown in Fig 1. These methods are referred to as Method A [centrifugation (Whitmore and Carrington, 1993)], Method B [HFUF (Hill et al., 2005)], Method C [filtration (Hawksworth et al., 2012)], Method D [flotation (Bowman et al., 2003)] for wastewater matrices, and Method E [flotation (Bowman et al., 2003)], and Method F [Direct DNA extraction (Ahmed et al., 2015)] for sludge samples.

Method A began with the centrifugation of each sample (1 L) in a bucket at 5,200 *g* for 30 min (Allegra ×-15R, Beckman Coulter, USA) in two consecutive steps. The pellet was then transferred into a 50 mL polycarbonate tube, further centrifuged at 5,200 *g* for 10 min, and stored at -20ºC until DNA was extracted.

Method B involved amending the sample with sodium hexametaphosphate ($NaPO_3$) (Sigma Aldrich, Australia) to achieve a final concentration in the water samples of 0.01%. Each water sample was pumped with a peristaltic pump in a closed loop with sterile high-performance, platinum-cured L/S 36 silicone tubing (Masterflex, Cole-Parmer Instrument Co.). Tubing was sterilized by soaking in 10% bleach for 30 min, washed with sterile distilled water, and autoclaved at 121ºC for 15 min prior to use. A Fresenius Hemoflow F80A polysulfone dialysis filter with a surface area of 1.8 m^2 and a fiber inner diameter of 200 µm (Fresenius Medical Care, Lexington, MA) was used to process the treated and raw wastewater samples. A new filter cartridge was used for each sample. The sample (1 L) was concentrated to approximately 150-200 mL, depending on the turbidity. A 500-mL elution solution consisting of 0.01% Tween 80, 0.01% NaPP, and 0.001% Antifoam A was recirculated through the filter for 5 min, and then allowed to concentrate to 150 mL (Hill et al., 2007). This elution solution was added to the concentrated sample to achieve a final volume of approximately 300-350 mL. Secondary concentration of *A. caninum* ova from the HFUF concentrated samples was performed by centrifugation at 5,200 *g* for 15 min. After the centrifugation, the supernatant was discarded and the pellet was stored at -20ºC for DNA extraction.

Method C began with filtering a sample through series of sieves (800-38 µm pore size) (Rowe scientific Pty Ltd, Australia) with the help of a stream of tap water. Particles including ova retained in the smallest pore sized sieve (38 µm) were collected in a 50 mL polycarbonate tube and centrifuged at 5,200 *g* for 15 min to obtain a pellet. The pellet was then stored at -20ºC until DNA was extracted.

Method D began with centrifuging treated and raw wastewater samples (1 L) to achieve a pellet. The pellet was then transferred into a 50 mL polycarbonate tube and approximately 40-45 mL flotation solution ($MgSO_4$) was added. The pellet was mixed with the flotation solution by vortexing. The mixture was centrifuge for 3 min at 800 *g* and the materials present in the top 10 mL were

transferred into a 15 mL polycarbonate tube. Water was added to make up the volume to 15 mL and further centrifuged at 800 *g* for 10 min to obtain a pellet.

2.5. Ova recovery from sludge

Ova from sludge samples were concentrated using Methods E and F. Method E began with centrifugation of ova spiked sludge (~ 4 gm dry weight) samples at 800 *g* for 10 min. The supernatant was discarded, and 40-45 mL flotation solution was added in each samples. The mixture was then centrifuged for 3 min at 800 *g* and floated materials were transferred into 15 mL polycarbonate tube. Water was added to make up the volume to 15 mL and further centrifuged at 800 *g* for 10 min to obtain a pellet. For Method F, direct DNA extraction was performed from ova spiked sludge samples (~ 4 gm dry weight) using a MO Bio Power Max® Soil DNA Extraction Kit as described below.

2.6. DNA extraction

DNA was extracted from each pellet obtained through all Methods (A, B, C and D) using the MO Bio Power Max® Soil DNA Extraction Kit with minor modification. In brief, pellets were mixed with lysis buffer C1 and freeze-thawed for 10 min (repeated 5 times). Extracted DNA samples were eluted through the spin filter membranes by adding 2 mL solution C6 and stored at -80ºC until processed. DNA was extracted from each pellet using a MO Bio Power Max® Soil DNA Extraction Kit with minor modification.

2.7. PCR inhibition

Previously published assay (Sketa22) was used to determine the presence of PCR inhibitors in the extracted DNA samples from treated wastewater, raw wastewater and sludge samples (Ahmed et al., 2015).

2.8. Preparation of standard curves

DNA was extracted from the larvae using DNeasy Blood and Tissue® Kit (Qiagen, Valencia, CA). qPCR standards were prepared by cloning the purified amplicons into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Plasmid DNA was extracted using Plasmid Mini Kit (Qiagen). Standards were prepared from the plasmid DNA (Yun et al. 2006; Ahmed et al. 2014). Serial dilutions were prepared ranging from 10^5-10^0 gene copies per µL and used as standard curves.

2.9. qPCR assay for the quantification of ITS-1 rDNA

For qPCR assay, newly designed primers (F: 5'-TTT GCT AAC GTG CAC TGA ATG-3' and R: 5'-GAA ACA CCG TTG TCA TAC TAG CC-3'), and a probe (P: FAM-5'-AAC TCG TTG TTG CTG CTG AA-3'-TAMRA) targeting the 5.8S ITS-1 rDNA genes were used. The qPCR amplification was performed in 25 μL reaction mixtures containing 12.5 μL iQ™ Supermix (Bio-Rad Laboratories, CA, USA), 250 nM of each primer, 400 nM of probe, 3 μL of template DNA and UltraPure™ DNase/RNase-free distilled water (Life Technologies, Australia). The thermal cycler program consisted of 15 min at 95°C, 15s at 95°C and 1 min at 59°C. The qPCR assays were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA). All qPCR reactions were performed in triplicate. The qPCR assay performance criteria such as efficiency (E), slope, intercept, R^2 and lower limit of quantification (LLOQ) were determined by analyzing the standard curves over the course of the study.

2.10. qPCR lower limit of quantification

The qPCR lower limit of quantification (LLOQ) was determined from the Ct values obtained for standards range (3×10^5 – 3 gene copies). The lowest amount of diluted standards detected in 100% triplicates assays was considered as qPCR LLOQ.

2.11. Recovery rate determination

The recovery rate of hookworm ova in the wastewater and sludge samples by the different concentration methods was calculated as follows:

Recovery rate (%) = (Quantified gene copies/spiked gene copies) ×100.

2.12. Quality control

To minimize qPCR contamination, DNA extraction and qPCR set up were performed in separate laboratories. A method blank was included for each batch of treated wastewater, raw wastewater, and sludge samples. A reagent blank was also included during DNA extraction to account for any contamination during extraction. For each qPCR experiment, standards (also served as a positive control) and triplicate negative controls (UltraPure™ water) were included.

2.13. Statistical analysis

GraphPad Prism 6 (GraphPad Software, CA, USA) was used to conduct the statistical analysis. A one-way ANOVA was performed to determine the differences between the C_T values obtained for *O. keta* DNA suspended in UltraPure™ water and *O. keta* seeded DNA samples extracted from wastewater matrices. ANOVA was also used to assess whether the concentration of *A. caninum* gene copies obtained through Methods (A-D) for treated and raw wastewater samples were statistically different within and between WWTPs. A paired T- test was used to assess the significant difference between Methods (E and F) for sludge samples within and between WWTPs. Statistical significance was determined at $\alpha = 0.05$.

3. Results

3.1. qPCR standards and lower limit of quantification (LLOQ)

qPCR standards were analysed to determine the reaction efficiencies. The standards had a linear range of quantification from 10^5 - 10^1 gene copies per µL of plasmid DNA. The slope of the standards ranged from -3.31 to -3.38. The amplification efficiencies ranged from 100.7% to 108.2%, and the correlation coefficient (R^2) ranged from 0.96-0.98. The intercepts for the qPCR standards were 35.8 to 38.4 (Fig 2). LLOQ of qPCR assays were determined using the standards. The qPCR LLOQ was 30 gene copies for all triplicate samples. The intra-assay and inter-assay Coefficient of Variation (CV) of the standards were also determined. These values were less than 1% and 3% respectively, indicating high reproducibility of the qPCR assay.

3.2. PCR inhibition

Sketa22 assay was used to determine the presence of PCR inhibitors in the extracted DNA samples. The mean C_T value and standard deviation for the *Oncorhynchus keta* seeded UltraPure™ water was 28.5 ± 0.2. The C_T values for *O. keta* seeded treated and raw wastewater DNA samples from WWTP-1 processed through all methods (A-D) were similar to *O. keta* seeded UltraPure™ water, indicating the DNA samples were free of PCR inhibitors (Table 1). However, PCR inhibition was observed in DNA samples extracted from treated wastewater (WWTP-2) processed through Methods A and B. Raw wastewater DNA samples from WWTP-2 processed through Methods A and C also had PCR

inhibitors. Sludge DNA samples (WWTP-1) processed through Method E had no PCR inhibitors. In contrast, sludge DNA samples from WWTP-2 processed through Method E had PCR inhibitors. None of the sludge DNA samples (both WWTPs) processed using Method F showed PCR amplification.

Samples that showed the sign of PCR inhibitors were then serially diluted (10-fold) to relieve PCR inhibitors, and re-analysed by seeding *O. keta* DNA. The mean C_T values and standard deviations of *O. keta* for the 10-fold diluted treated wastewater, raw wastewater and sludge samples indicated the removal of PCR inhibition (Table 1). Further ANOVA analysis on the C_T values for *O. keta* seeded UltraPure™ water, undiluted DNA and those 10-fold diluted DNA samples did not differ significantly. Based on the results, all the samples that passed PCR inhibition test were used for qPCR assays.

3.3. Recovery rate of *A. caninum* from wastewater matrices

To obtain the recovery rates for each method, 400 ± 40 ova (corresponds to $3.3\times10^7 \pm 8.5 \times 10^6$ gene copies as determined by the qPCR) were seeded into each wastewater and sludge samples. The mean concentration of *A. caninum* gene copies recovered from treated wastewater did not vary significantly ($P > 0.05$) among the methods. The concentrations ranged from 4.6×10^5 (Method A) to 1.3×10^6 (Method D) for wastewater sample collected from WWTP-1 (Fig. 3a). Similar results were also obtained for WWTP-2. However, the mean concentration of gene copies (3.5×10^3) recovered through Method D was 2-3 orders of magnitude lower than the other Methods (A-C). Furthermore, this difference was significant ($P < 0.05$).

For raw wastewater samples, the mean concentration of *A. caninum* gene copies recovered using Method C was the highest (3.8×10^5) followed by Method D (2.3×10^5) for WWTP-1 (Fig. 3b). However, Methods A and B yielded 2 orders of magnitude lower concentrations of gene copies compared to Methods C and D, and this difference was significant ($P < 0.05$). For WWTP-2, Method B yielded the highest concentration (1.1×10^6) of gene copies followed by Methods D and C, although Methods B, C and D did not differ significantly ($P > 0.05$). However, the mean concentration of gene copies (1.5×10^4) recovered through Method A was 1-2 orders of magnitude lower than the other methods ($P < 0.05$).

For the sludge samples collected from WWTP-1, Methods E (7.8×10^{2}) and F (2.7×10^{3}) yielded similar concentrations of gene copies (Fig. 3c), that were not significantly ($P > 0.05$) different. Sludge samples collected from WWTP-2 also yielded similar concentrations of gene copies for Method E (1.2×10^{5}) and F (1.5×10^{5}), and the difference was not statistically significant ($P > 0.05$). Both Methods were able to recover ~ 2 orders of magnitude higher gene copies from WWTP-2 samples compared to WWTP-1 samples ($P < 0.05$).

For treated wastewater, Method D outperformed all other methods except Method C, yielding a recovery rate of 40 ± 57% for WWTP-1 (Table 2). Interestingly, for WWTP-2, Method C performed better than the others, yielding a recovery rate of 50 ± 39%. For raw wastewater, Method C (12 ± 10%) and D (7.1 ± 2.0%) had much better recovery rate than Methods A (0.3 ± 0.2%) and B (0.3 ± 0.4%) for WWTP-1. For WWTP-2, the recovery rate of Method B outperformed all other methods. For sludge samples, the recovery rates of hookworm ova were poor compared to treated and raw wastewater samples. For both WWTPs Method F yielded 1-2 orders of magnitude higher (3.7 ± 9.0%, WWTP-1; 4.7 ± 6.2%, WWTP-2) recovery rate than Method E (0.02 ±0.03%, WWTP-1; 0.10 ±0.15%, WWTP-2).

4. Discussion

A reliable, sensitive and rapid method is needed in order to detect low concentrations (1-10 ova) of helminth ova in the wastewater matrices. Various methods have been used to recover hookworm ova from wastewater matrices with variable degrees of success (Bowman et al., 2003; McCuin and Clancy, 2005; Maya et al., 2006; Ensink et al., 2008;). In light of this, we have evaluated several rapid concentration methods for the recovery of hookworm ova from wastewater matrices including sludge samples. For the methods evaluation, wastewater and sludge samples were collected from two WWTPs with variable characteristics. Method A (centrifugation) used in this study was originally developed to separate helminth ova from environmental water samples with low turbidity (Whitmore and Carrington, 1993). The results obtained in this study suggest that the recovery rate of the Method A was 1-2 orders of magnitude higher for treated wastewater than raw wastewater samples. Raw wastewater samples generally contain large amount of heavy particles and grease that may potentially

bind to ova (Kuczynska and Shelton, 1999). As a result it is possible that DNA extraction lysis buffer may not have penetrated the cell wall, which may have led to inefficient DNA extraction.

Method B (HFUF) has been widely used to concentrate bacterial, viral and protozoa pathogens simultaneously from environmental water samples (Hill et al., 2005; Hill et al., 2007). The recovery rates of the HFUF from treated wastewater samples were slightly better than the centrifugation (Method A). However, the recovery rates from raw wastewater were highly variable (0.3-35%) between the WWTPs. Such discrepancy again could be attributed to the variable solid contents present in wastewater samples in time and space. The turbidity of raw wastewater collected from both the WWTPs were much higher (246-286 NTU) than the treated wastewater (86-197 NTU). Several studies have demonstrated the efficacy of the HFUF system to recover higher concentrations (up to 86%) of *Giardia* cysts and *Cryptosporidium* oocysts from surface water samples (Simmons et al., 2001; Ferguson et al., 2004). Perhaps, HFUF method is suitable for concentrating protozoa when the turbidity of the water samples is low. Mull and Hill (2012) and Ferguson and collegues (2004) demonstrated that the turbidity of water samples is inversely proportional with the recovery rates.

Method C (filtration) used in this study is based on retaining hookworm ova on a filter through a series of sieves. This method is simple, involves only few steps, and because of that, has the potential to recover higher concentrations of ova from wastewater samples. Our results indicated that the recovery rate of Method C was as high as 50% for treated wastewater and 12% for raw wastewater samples. This is comparable to a 26% recovery rate of *Ascaris* from treated wastewater reported by Maya et al. (2006), and 9-49% recovery rate of *Cryptosporidium* oocysts and *Giardia* cysts from environmental waters reported by Nieminski et al. (1995) using a similar methodology. One drawback of this method is the potential clogging of the sieve with large solid wastewater particles. This may leave behind a portion of ova attached to the solid particles on the sieve (Nieminski et al., 1995; Zarlenga and Trout, 2004).

The flotation method (Method D) separates helminth ova by selecting their specific gravity while other denser particles present in a sample sink to the bottom for removal (Dryden et al., 2005; Goodman et al., 2007). Thus, this method is more suitable to recover helminth ova from highly turbid samples like raw wastewater and sludge. Studies have shown that the flotation method can provide

328 variable recovery rates (12%-32%) from wastewater samples (Maya et al., 2006). This is in agreement
329 with the findings of this study. The recovery rate obtained through Method D for the treated
330 wastewater collected from WWTP-1 was high, although the result was not consistent for both
331 WWTPs. Treated wastewater samples from the WWTP-2 contained large amount of blue green algae,
332 which may have affected the recovery rate. However, more studies would be required to determining
333 the effect of blue green algae on ova recovery rate possibly from large number of samples from
334 different ponding facilities.

335 The flotation method (Method E) has also been used to recover hookworm ova from sludge
336 samples. The result of this study indicated that the recovery rates of this method were very poor (0.02-
337 3.7%). McCuin and Clancy (2005) could not recover any *Cryptosporidium* oocysts from lime
338 stabilized sludge samples using flotation method. In contrast, several studies reported 26-82%
339 recovery rate of helminth ova from different sludge samples using flotation method (Bowman et al.,
340 2003; Maya et al., 2006). Several factors such as sample matrix, sample volume and the
341 concentrations of ova present in samples may influence the recovery rate, therefore, making direct
342 comparison among the studies is difficult.

343 It has been reported that direct DNA extraction from water samples may yield better recovery of
344 viruses as it bypasses the concentration procedure (Ahmed et al., 2015). In view of this, we used
345 Method F, which involved direct DNA extraction from sludge samples. Method F was indeed able to
346 recover higher numbers of ova from sludge samples than Method E. However, the DNA samples
347 obtained through this method had PCR inhibitors present, despite the DNA extraction kit used in this
348 study being equipped with inhibitor removal technology. PCR inhibitors are known to be matrix
349 associated, and a wide array of PCR inhibitors with varying concentration could be present in sludge
350 samples (Schrader et al., 2012). Our results also indicated that the 35% of DNA samples extracted
351 from wastewater matrices had PCR inhibitors. This is a formidable barrier for downstream PCR
352 detection or quantification of hookworm ova. Based on our data, we recommend that DNA samples
353 extracted from wastewater matrices should be checked for the presence of PCR inhibitors prior to
354 PCR/qPCR analysis. In the present study, we simply assumed that the DNA extraction efficiency of
355 the MO Bio Power Max® Soil DNA Extraction Kit was 100% in order to calculate the concentrations

of seeded ova in wastewater matrices. Further work would be required to determine the extraction efficiency of the DNA extraction kit.

5. Conclusions

- From the results obtained in this study, it appears that the recovery rates of *A. caninum* ova from wastewater matrices can be highly variable and matrix-specific.
- The results indicated that centrifugation (Method A), HFUF (Method B), filtration (Method C), and flotation (Method D) were able to yield better recovery rates from treated wastewater samples than raw wastewater. The recovery rates obtained through flotation (Method E) and direct DNA extraction (Method F) from sludge samples were low compared to treated and raw wastewater samples.
- Among the four concentration methods tested, filtration (Method C) was able to recover higher concentrations of *A. caninum* ova consistently from treated wastewater and raw wastewater samples collected from both WWTPs. The performances of Methods B (HFUF) and D (flotation) were reasonable, although, the results were not consistent for both WWTPs.
- Both methods (Methods E and F) failed to recover *A. caninum* ova efficiently from sludge samples. Further method development would be required in order to improve the recovery rate of hookworm ova from sludge samples.

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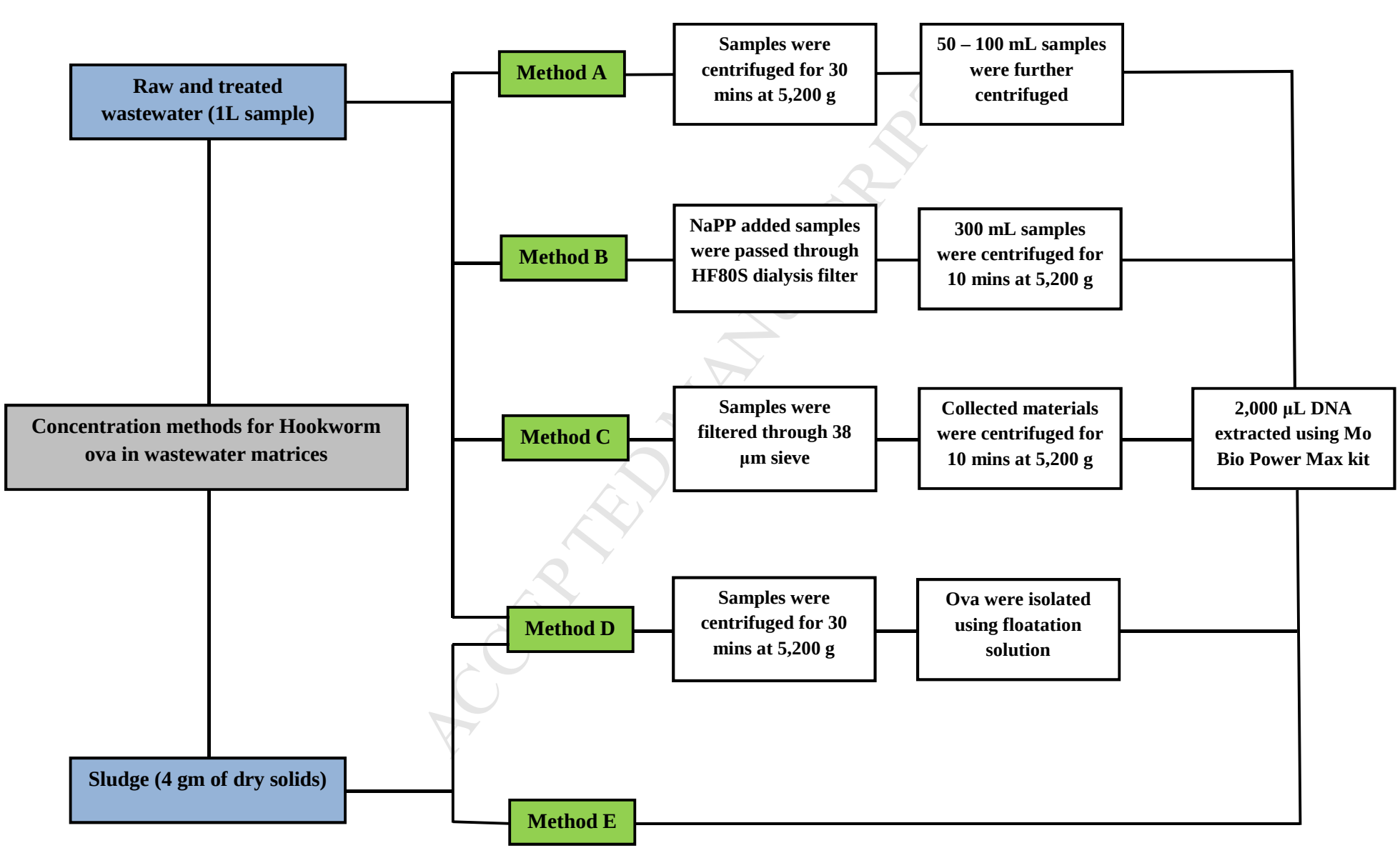
506 **Table 1:** Sketa22 real-time PCR assay for the evaluation of PCR inhibition in ova spiked raw wastewater, treated wastewater, and sludge DNA samples as
507 opposed to UltraPure™ water samples. UltraPure™ water samples, undiluted and diluted DNA samples were spiked with 10 pg of *Oncorhynchus keta* DNA
508

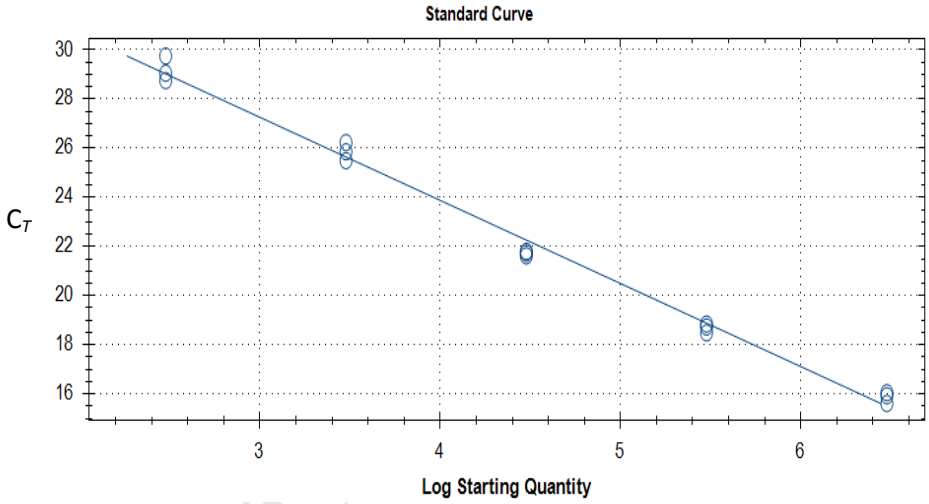
Concentrations methods	Sample types	Mean ± standard deviation of threshold cycle (C_T) values for Sketa22 PCR assay			
		Undiluted DNA samples		10-fold diluted DNA samples	
		WWTP-1	WWTP-2	WWTP-1	WWTP-2
Method A	Treated wastewater	27.8 ± 0.2	31.2 ± 1.9	NA	29.0 ± 1.7 [b]
	Raw wastewater	28.0 ± 0.1	31.4 ± 1.2	NA	27.0 ± 0.2 [b]
Method B	Treated wastewater	27.7 ± 0.1	30.5 ± 0.2	NA	29.2 ± 1.8 [b]
	Raw wastewater	28.0 ± 0.1	30.0 ± 0.1	NA	NA
Method C	Treated wastewater	28.1 ± 0.1	29.9 ± 0.1	NA	NA
	Raw wastewater	28.3 ± 0.4	33.0 ± 1.5	NA	27.0 ± 0.1 [b]
Method D	Treated wastewater	28.1 ± 0.2	29.8 ± 0.1	NA	NA
	Raw wastewater	28.2 ± 0.1	29.8 ± 0.1	NA	NA
Method E	Sludge	28.2 ± 0.2	31.3 ± 2.0	NA	27.9 ± 1.7 [b]
Method F	Sludge	No amplification	No amplification	29.2 ± 0.1[a],	27.1 ± 0.1 [b]

509 Mean ± standard deviation of C_T values for UltraPure™ water samples = 28.5 ± 0.2
510 NA: Not applicable.
511
512
513

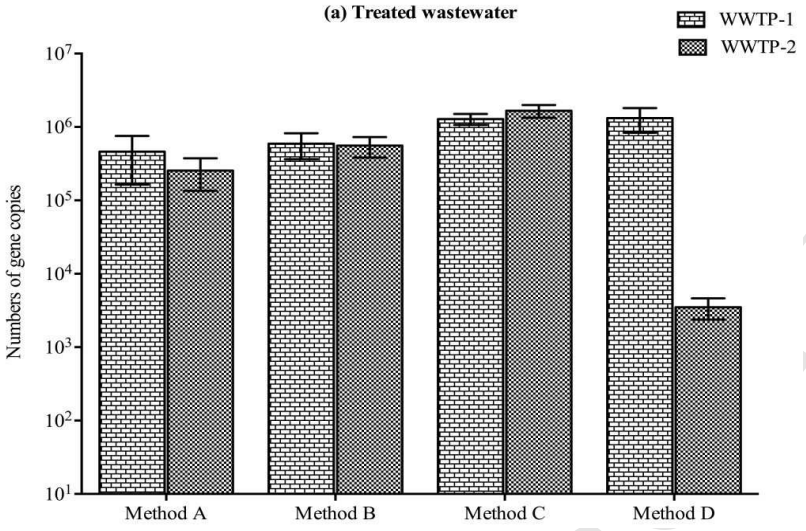
Table 2: Evaluation of recovery rate of *A. caninum* ova from raw wastewater, treated wastewater, and sludge samples from six concentration methods (A-F)

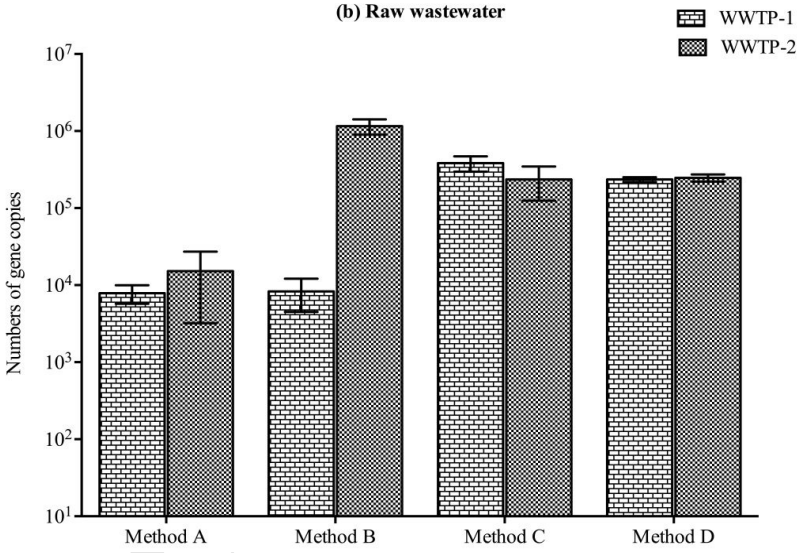
Concentration methods	Sample types	Mean and standard deviation of recovery rate (%)	
		WWTP-1	WWTP-2
Method A	Treated wastewater	14 ± 35	7.6 ±14
	Raw wastewater	0.3 ± 0.2	0.5 ±1.4
Method B	Treated wastewater	18 ± 26	17 ± 20
	Raw wastewater	0.3 ± 0.4	35 ± 30
Method C	Treated wastewater	39 ± 26	50 ± 39
	Raw wastewater	12 ± 10	7.1 ± 13
Method D	Treated wastewater	40 ± 57	0.2 ± 0.1
	Raw wastewater	7.1 ± 2.0	7.4 ± 31
Method E	Sludge	0.02 ± 0.03	3.7 ± 9.0
Method F	Sludge	0.10 ± 0.15	4.7 ± 6.2

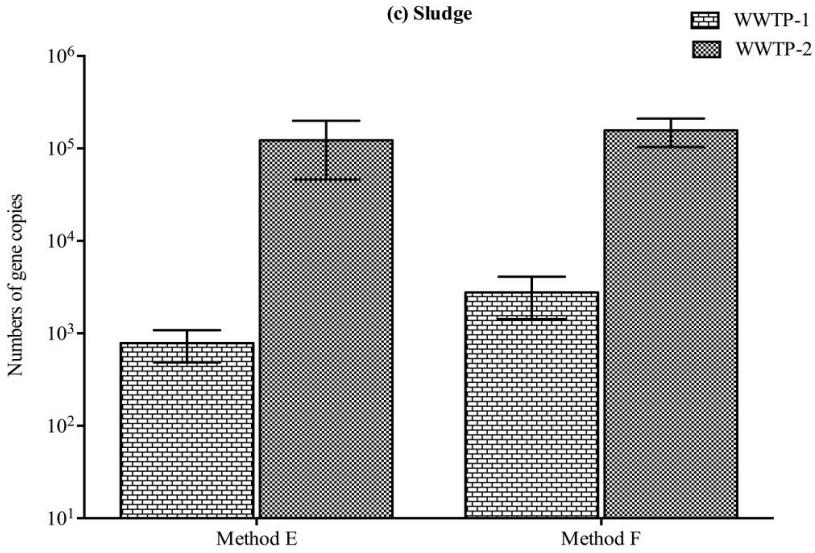

Raw and treated wastewater (1L sample)
Concentration methods for Hookworm ova in wastewater matrices
Sludge (4 gm of dry solids)
Method A
Samples were centrifuged for 30 mins at 5,200 g
50 – 100 mL samples were further centrifuged
Method B
NaPP added samples were passed through HF80S dialysis filter
300 mL samples were centrifuged for 10 mins at 5,200 g
Method C
Samples were filtered through 38 µm sieve
Collected materials were centrifuged for 10 mins at 5,200 g
Method D
Samples were centrifuged for 30 mins at 5,200 g
Ova were isolated using floatation solution
Method E
2,000 µL DNA extracted using Mo Bio Power Max kit



E = 97.8%, R^2 = 0.992, Slope = -3.380, y-int=37.398


(a) Treated wastewater
WWTP-1
WWTP-2
Numbers of gene copies
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Method A
Method B
Method C
Method D


(b) Raw wastewater
WWTP-1
WWTP-2
Numbers of gene copies
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Method A
Method B
Method C
Method D


(c) Sludge
WWTP-1
WWTP-2
Numbers of gene copies
10^6
10^5
10^4
10^3
10^2
10^1
Method E
Method F

668 **Fig 1:** Hookworm ova concentration methods for raw wastewater, secondary treated wastewater and sludge
669 samples. Method A = centrifugation, Method B = HUFU, Method C = Filtration, Method D = Floatation (for
670 wastewater samples), Method E = Floatation (for sludge samples) and Method F = Direct DNA extraction.

671 **Fig 2:** A standard curves generated using the plasmid DNA. The concentrations of gene copies are plotted
672 against C_T values. The C_T is the cycle number at which the fluorescence signal increased above the defined
673 threshold value, calculated by the real-time PCR software.
674
675 **Fig 3:** Mean and standard deviation of the concentrations of gene copies recovered through different
676 methods tested from *A. caninum* ova seeded into (a) treated wastewater, (b) raw wastewater, and (c) sludge
677 samples
678
679
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Highlights:

- The distribution of hookworm ova in wastewater matrices could be patchy.
- A rapid concentration method is required for the detection of ova from wastewater matrices.
- Six rapid methods were compared to identify the best performing method to recover ova from wastewater matrices.
- Recovery rates of *A. caninum* ova from wastewater matrices especially sludge samples can be highly variable.
- Further method development would be required in order to improve the recovery rate of hookworm ova from sludge samples.