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Detection of Viable Hookworm Ova from Wastewater and Sludge

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School of Public Health

Abstract

Infectious helminths are a worldwide major public health problem. In terms of morbidity, approximately 3×10^9 people are infected by soil-transmitted helminths (STHs) influencing rates of malnutrition and failure to thrive. All of this increases the global disease burden (GDB) by up to 5.9×10^6 Daily Adjusted Life Years (DALYs). Helminth infections are more often seen as a major public health issue for developing countries however, concerns have been expressed in many of the developed nations because of the increased use of treated wastewater and sludge with no clear way of knowing the infection potential that can be attributed from such water.

Guidelines have been established to minimise the potential public health risk associated with wastewater and sludge reuse. For example, the WHO guideline specified ≤ 1 ova (*Ascaris lumbricoides*) per L liquid (wastewater) or 4 *g* dry solid (sludge) for unrestricted use. Various wastewater treatment processes have been recommended to remove the helminths ova from wastewater depending upon its reuse. However, detection methods used to quantify viable helminths ova from wastewater has limitations. Therefore there is always a potential public health risk associated with reuse of wastewater and sludge.

In this research, a real-time PCR method was developed. The new PCR method is rapid and specific. The method was found to be able to detect less than one ovum in one litre treated wastewater and approximately four ova in one litre raw wastewater and ~ 4 *g* sludge. The real-time PCR method was modified to a quantitative PCR (qPCR) method and further used to quantify hookworm ova from wastewater and sludge. The qPCR had estimated an average of 1.1, 8.6 and 67.3 ova for treated wastewater that was seeded with 1, 10 and 100 ova, respectively. The gene copy numbers obtained for 1, 10 and 100 ova by qPCR varied significantly ($P < 0.05$) within the tested samples indicating that absolute quantification of ova may not be accurate. Despite the difficulty quantifying accurate numbers of hookworm ova, the lower limit of quantification (LLOQ) of the qPCR method was 30 gene copies. This was a lot less than the gene copies produced by one ovum. Therefore, the qPCR method has potential to use for complying with wastewater guidelines.

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Although the overall aim of this research was to develop a sensitive and specific method for quantitative detection of viable hookworm ova from wastewater, the importance of recovering the ova from wastewater and sludge samples for accurate detection was identified. While determining appropriate recovery rates was not an aim of this research, a suitable method that could be used to standardise research outcomes for further study was established. Therefore, ova recovery rate by different rapid methods was evaluated for further experiments. The result indicated that the ova recovery rate was higher for the treated wastewater (0.2 - 50%) than the raw wastewater (0.3 - 35%) and sludge (0.02 - 4.7%) samples. A significant difference ($P < 0.05$) was observed between the methods used to recover the ova from wastewater matrices. Therefore, using this newly developed detection method more research is needed in another study to improve the ova recovery rates from wastewater and sludge samples.

It is well known that the qPCR detection method cannot distinguish between viable and non viable microorganisms (cells, oocysts, and ova). Since only viable ova are capable of becoming infectious larvae and consequently causing infections in humans, it is important for health risk assessments to know what fraction of the PCR amplified ova are viable. Therefore, the developed qPCR method was combined with Propidium Monoazide (PMA) to develop a selective detection method for detecting viable hookworm ova from wastewater. Results confirmed that the PMA-qPCR method did not detect hookworm ova in samples that had been treated to kill. The performance of viability quantification of the newly developed PMA-qPCR method was then assessed against currently available (culture based and vital stain) methods. The percentage of *in vitro* viability assessed by PMA-qPCR was 19% lower than vital stain and 38% higher than culture based methods.

In general, the outcome of this research is an improved detection method which is sensitive and more specific than current method for detection of hookworm ova from wastewater. The method is rapid, cheap and does not require skilled personnel like microscopic methods. Therefore, it will be suitable for health regulators as well as wastewater utilities for quality control and risk assessment purposes. However, studies are required to determine the gene copy numbers in different cell stages of hookworm ova, in order to improve the accuracy of quantification using qPCR method.

Declaration by author

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Publications during candidature

Peer-reviewed publications relevant to this research

i. **Gyawali P,** Ahmed W, Sidhu JPS, Nery S, Clements A, Traub R, McCarthy J, Llewellyn S, Jagals P, Toze S (2016) Quantitative detection of viable helminth ova from wastewater, human feces and environmental soil samples using novel PMA-qPCR. *Environmental Science and Pollution Research,* **23**(18): 18639-18648.

ii. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2016) An approach to reduce false viability assessment of hookworm ova with vital stains. *Food and Waterborne Parasitology,* **3**: 9-12.

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iv. **Gyawali P,** Ahmed W, Sidhu JPS, Jagals P, Toze S (2015) Comparison of concentration methods for rapid detection of hookworm ova in wastewater matrices using quantitative PCR. *Experimental Parasitology*, **159**: 160-167.

v. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2015) Rapid concentration and sensitive detection of hookworm ova from wastewater matrices using a real-time PCR method. *Experimental Parasitology*, **159**: 5-12.

Other peer-reviewed publications published during candidature

i. Ahmed W, **Gyawali P,** Toze S (2016) Evaluation of glass wool filters and hollow-fibre ultrafiltration concentration methods for qPCR detection of human adenoviruses and polyomaviruses in river water. *Water Air and Soil Pollution,* **DOI: 10.1007/s11270-016-3026-5**.

ii. **Gyawali P**, Beale DJ, Ahmed W, Karpe AV, Soares Magalhaes RJ, Morrison PD, Palombo EA (2016) Determination of *Ancylostoma caninum* ova viability using metabolic profiling. *Parasitology Research,* **115**(9): 3485-3492.

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ix. Ahmed W, Harwood VJ, **Gyawali P**, Sidhu JPS, Toze S (2015) Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. *Applied and Environmental Microbiology*, **81**(6): 2042-2049.

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Conferences

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ii. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2015) Detection of viable hookworm ova using PMA-qPCR. Joint International Tropical Medicine Meeting, Bangkok, Thailand, **1st-4th December** [(Best poster award winner) Poster Presentation)].

iii. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2015) Selective detection of viable hookworm ova from wastewater. 18th International symposium on health-related water microbiology, Lisbon, Portugal, **13th-19th September** (Poster Presentation).

iv. Ahmed W, Staley C, Sadowsky MJ, **Gyawali P**, Sidhu JPS, Beale D, Toze S (2015) Application of genetic markers and sequencing for the identification of faecal pollution in an urban river. 18th International symposium on health-related water microbiology (HRWM), Lisbon, Portugal, **13th-19th September** (Poster Presentation).

v. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2015) Novel approach to detect hookworm ova from wastewater matrices. New Zealand Society of Parasitology and Australian Society of Parasitology conference, Auckland, New Zealand, **29th June-2nd July** (Oral presentation).

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vii. **Gyawali P,** Sidhu JPS, Ahmed W, Jagals P, Toze S (2014) Rapid detection of hookworm ova from wastewater and sludge. School of Population Health, Research Higher Degree Student Conference, Brisbane, Australia, **November 5th** (Oral Presentation).

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Keywords

Environmental health hazards, public health risks, wastewater, sludge, hookworm ova viability, quantitative detection methods, PCR/qPCR method, PMA-qPCR method, vital stain method, culture-based method

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Dedication

This thesis is dedicated to my family especially **SABITA KHANAL** who supported me throughout this journey (Ph.D.)

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List of abbreviations

ANOVA = Analysis of Variance

BLAST = Basic Local Alignment Search Tool

bp = Base Pair

CSIRO = Commonwealth Scientific and Industrial Research Organisation

C_T = Threshold Cycle

DALYs = Daily Adjusted Life Years

DNA = Deoxyribonucleic Acid

DW = Dry Weight

EMA = Ethidium Bromide Monoazide

fg = femtogram

g = gram

× g = gravity force

h = hectare

HFUF = Hollow Fiber Ultrafiltration

HRT = Hydraulic Retention Time

IDT = Integrated DNA Technologies

ITS = Internal Transcribed Spacer

L = Litre

LLOD = Lower Limit of Detection

LLOQ = Lower Limit of Quantification

MDA = Mass Drug Administration

min = minute

mL = Millilitre

mPCR = Multiplex Polymerase Chain Reaction

mRNA = Massager Ribonucleic Acid

NA = Not Applicable

NCBI = National Center for Biotechnology Information

nM = Nanomolar

NRMMC = Natural Resource Management Ministerial Council

PBS = Phosphate Buffer Saline

PCR = Polymerase Chain Reaction

pg = picogram

PMA = Propidium Monoazide

PPE = Personal Protective Equipment

QMRA = Quantitative Microbial Risk Assessment

qPCR = Quantitative Polymerase Chain Reaction

rDNA = Ribosomal Deoxyribonucleic Acid

RNA = Ribonucleic Acid

rRNA = Ribosomal Ribonucleic Acid

RT = Retention Time

SD = Standard Deviation

SLOD = Sample Limit of Detection

STHs = Soil-Transmitted Helminths

UN HABITAT = United Nations Human Settlement Program

US EPA = United State Environmental Protection Agency

vPCR = Viability Polymerase Chain Reaction

WASH = Water Sanitation and Hygiene

WHO = World Health Organisation

WWTP = Wastewater Treatment Plant

µL = Microliter

µm = Micrometer

Chapter: 1

Introduction

Introduction

1.1. General introduction

Soil-transmitted helminths (STHs) *Ascaris lumbricoides* (roundworm), *Ancylostoma duodenale* and *Necator americanus* (hookworm) and *Trichuris trichiura* (whipworm) are responsible for malnutrition, anemia and impaired cognitive development in humans (WHO, 2012a; WHO, 2015). It is reported that more than 2.0×10^9 people worldwide are suffering from single or multiple helminth infections at any given time (Bethony et al., 2006; Hotez et al., 2008; WHO, 2012a). Marginalized people with poor socio-economic conditions, especially in the developing regions such as Africa, Asia, and South America have the highest rate of infection (WHO, 2012a) (Table 1.1).

Table 1.1: Numbers of pre-school aged and school-aged children in need of mass drug administration against soil-transmitted helminths in different regions (source: WHO, 2012a)

Regions	Number of countries	No requiring mass drugs administration		
		Preschool-aged children	School-aged children	Total
African	42	9.6×10^7	1.9×10^8	2.9×10^8
Americas	30	1.4×10^7	3.1×10^7	4.5×10^7
South East Asia	8	1.1×10^8	2.7×10^8	3.7×10^8
European	11	1.2×10^6	3.0×10^6	4.3×10^6
Eastern Mediterranean	8	2.5×10^7	5.4×10^7	7.9×10^7
Western Pacific	13	3.3×10^7	6.6×10^7	9.9×10^7

The disease from STHs is mainly attributed to chronic and insidious impact on health wellbeing of individuals rather than the mortality. Therefore, Disability-Adjusted Life Years (DALYs) has been used to determine the impacts of STHs infections. DALYs are a measurement of the amount of time (in years) that is 'lost' due to imperfect health from infection, taking into account of both premature death and time lived in a state of ill-health (termed disability). The burden of disease associated with STHs infection is very high (5.9×10^6 DALYs), which then creates a vicious cycle of infection, poverty, failure to thrive and consequently reduced productivity (WHO, 2012a; Hotez et al., 2014).

In the last decade, mass drug administration (MDA) has been widely implemented for pre-school aged and school-aged children to reduce the infection rates of STHs infections

including hookworm (Anderson et al., 2013). However, MDA was not successful enough to control STHs infections especially hookworm in those endemic communities. This could be due to the fact that MDA is mainly focused on children, leaving the large numbers of infected adults. Those adults may have served as reservoirs for re-infection in children. Other interventions such as safe drinking water, improved sanitation, and health education also have been introduced in addition to the MDA program to control STH infections more effectively (WHO, 2012a; WHO, 2012b; WHO, 2015). However, increasing rates of re-infections have been reported in the literature (WHO, 2012a; WHO, 2015).

Land application of raw and partially treated wastewater and sludge can significantly increase the load of potentially infective ova/larvae of STH in the environment, which is one of the key routes of transmission to humans (Katakam et al., 2013; Karkashan et al., 2015). The land application of the raw wastewater and sludge are increasing due to the shortage of freshwater resources. It has been estimated that more than 2.0×10^7 h of land is being irrigated with partially treated and raw wastewater (Carr, 2005). Due to livelihood and food security need, poorest people in developing countries rely on this resource more than others. Negative public health impacts from the use of raw and partially treated wastewater and sludge have already been reported (Ensink et al., 2007; Do et al., 2007; Vuong et al., 2007; Ensink et al., 2008).

Among the STHs, hookworms pose a significant risk upon reuse of untreated wastewater and sludge because of their i) high prevalence rate (8.0×10^8) (Knopp et al., 2008), ii) ability to produce large numbers of ova ($10^3 – 10^4$) every day (Bethony et al 2006) iii) transmission potential (skin penetration through L_3 larvae and oral ingestion of viable ova) (Hotez, 2008a, Hotez, 2008b, Hotez and Gurwith, 2011), and iv) low dose (1-10 viable ova/larvae) to cause infection (WHO, 2006). In addition, the ova can remain viable for 9-12 months before hatching to infective larvae (L_3) which can survive up to another 3 months in the environment under favorable conditions (Abaidoo et al., 2010; Brooker, 2010).

Guidelines have been established to minimise the potential public health risk associated with wastewater and sludge reuse (US EPA, 2003; NRMMC, 2004; WHO, 2006). Depending on the potential for human exposures various levels of wastewater and sludge treatment processes such as ponding and deactivated sludge and incineration, have been recommended in the guideline to remove/inactivate the ova/larvae of STHs from wastewater (US EPA,

2003; NRMMC, 2004; WHO, 2006). These guidelines however, are based on a model organism (*Ascaris lumbricoides*). Since, the types of STHs and their numbers in wastewater and sludge depend on the prevalence rate of infections in the community that generates the wastewater (Mahvi and Kia, 2006; Sidhu and Toze, 2009). Therefore, using a model organism (*A. lumbricoides*) to determine the quality of treated wastewater and sludge may not be ideal.

Quantitative detection methods for the STHs in wastewater and sludge samples had two key fundamental issues including accurate quantification and viability assessment. Currently, detection and quantification of viable helminth ova from wastewater and sludge can be technologically difficult, labor intensive, time-consuming and expensive (Toze, 1999). Some of the existing methods considered as the more rapid and cheaper technologies to detect hookworm ova include the Kato-Katz, direct smear, thick smear and formalin-ethyl acetate methods (de Silva et al., 2006; Goodman et al., 2007; Cringoli et al., 2010; Habtamu et al., 2011; Bastos et al., 2013). These methods do not provide viability status of helminth ova. In view of this, culture and vital stain methods have been commonly used to quantify the viable helminth ova from wastewater and sludge (Bowman et al., 2003; de Victorica and Galván, 2003; US EPA, 2003; Trang et al., 2006; Do et al., 2007; Wen et al., 2009; Sharafi et al., 2012). However, there are some significant limitations of the culture and vital stain methods. Both methods require highly skilled personnel to accurately distinguish between ova/larvae of pathogenic and non-pathogenic species of helminth. For example, ova/larvae of *Ancylostoma duodenale*, *A. caninum* and *Necator americanus*, are morphologically similar and difficult to differentiate them to the species level using microscopic inspection alone (Cabaret et al., 2002; Verweij et al., 2007; Traub et al., 2008). In addition, the sensitivity of these methods depends on the detection limit of a microscope that can be very low (Weber et al., 1991).

Moreover, the culture method requires up to seven days to obtaining a result, which may not be practical for situations that require rapid results (Boehm et al., 2009). The vital stain method is relatively rapid compared to the culture method while assessing the viability of hookworm ova (de Victorica and Galván, 2003). However, the vital stain method has potential to overestimate the viability of the ova (Gyawali et al., 2016).

Sensitive and specific detection of helminth ova/larvae is essential to determine the accurate health risk assessment as well as providing information to formulate effective control measures. There is, therefore, a need for a rapid, sensitive and specific detection method that can quantify viable helminth ova from wastewater and sludge. Polymerase chain reaction (PCR/qPCR) assays have been developed and used for rapid, sensitive and specific detection of helminths from faecal samples (Pecson et al., 2006; Verweij et al., 2007; Traub et al., 2008; Taniuchi et al., 2011; Ngui et al., 2012a; Ngui et al., 2012b). PCR/qPCR methods can detect pathogens in a one-step closed-tube reaction within 2-4 h with much higher sensitivity and specificity by directly amplifying a specific gene from a target microorganism (Botes et al., 2013; Schar et al., 2013), overcoming the limitations of the culture and vital stain methods.

It has been demonstrated that Propidium Monoazide (PMA) (Biotium, Hayward, CA), a DNA intercalating dye, combined with PCR/qPCR methods can be used for selective detection/quantification of viable bacteria, protozoa and viruses from wastewater, sludge and environmental samples (Fittipaldi et al., 2011; Taskin et al., 2011; Nkuipou-Kenfack et al., 2013; Alonso et al., 2014; Li et al., 2014; Gensberger et al., 2014; Santiago et al., 2015). The working mechanism of PMA is that it penetrates non-viable cells or oo(cysts), and makes a covalent bond with DNA upon exposure to light preventing PCR amplification (Nocker et al., 2006; Nocker et al., 2007). Despite the successful application of PMA-qPCR for detection of viable viruses, bacteria, and protozoa from wastewater matrices, these methods have not been adapted for helminths. There is no information available in the literature that PMA/EMA is being used for selective detection of viable hookworm ova.

1.2. Knowledge Gaps

WHO and other regulatory bodies have set an acceptable limit of helminth ova for the use of treated wastewater and sludge for agriculture (unrestricted) considering the minimal detectable level of the existing, microscopic methods (Carr, 2005; IWMI and IWRC, 2010). However, quantitative microbial risk assessment (QMRA) indicated that the advised numbers of ova for agricultural use can still have a high risk of infection for the public (Navarro and Jimenez, 2011). Therefore, there is a growing interest in modifying the WHO guideline and lowering the threshold limit of helminth ova (0.1 ova per L) in treated wastewater for unrestricted use. In order to achieve the proposed threshold limit of ova in treated wastewater,

a sensitive method is essential for precise identification and quantification of viable hookworm ova/larvae in wastewater and sludge. Therefore, developing a consistent method for the detection, identification, and viability assessment of hookworm in wastewater and sludge is more necessary than ever before.

1.3. Research aims

The aims of the research presented in this thesis were to identify limitations of existing detection methods on detecting viable hookworm ova from environmental samples such as wastewater and sludge and therefore, to develop a rapid, specific and sensitive method to detect viable hookworm ova from wastewater and sludge.

1.4. Research questions

Several research questions were formulated to achieve the overall aim of this research. The research questions are listed below.

i. Can PCR methods be used to detect specific hookworm ova from wastewater and sludge samples?
ii. Can qPCR method be used to quantify hookworm ova accurately?
iii. Can PMA be used to distinguish between viable and non-viable hookworm ova?
iv. Can PMA-qPCR method be used for selective detection of viable hookworm ova from environmental samples such as wastewater?
v. Can PMA-qPCR method provide better viability assessment than culture-based and vital stain methods?

1.5. Hypothesis

To answer the research questions described in the earlier section, following hypothesis were tested in the course of this study.

i. That real-time PCR method can be used for sensitive and specific detection of hookworm ova from wastewater, sludge, and environmental samples.
ii. That quantitative PCR (qPCR) method can provide the numbers of hookworm ova in wastewater, sludge, and environmental samples.

iii. That PMA-qPCR method can be used for viability assessment of hookworm ova from wastewater and sludge samples.

iv. That PMA qPCR method can provide more accurate numbers of viable ova than culture and vital stain methods

1.6. Objectives

To achieve the overall aim of this research and answer the research questions, five specific objectives described below were formulated.

i. Develop a real-time polymerase chain reaction (PCR) method and evaluate the specificity and sensitivity of the method.

ii. Upgrade the PCR method to quantitative PCR (qPCR) method and evaluate the ova recovery rate of different methods from wastewater matrices.

iii. Develop and evaluate a PMA-qPCR method for selective detection of viable hookworm ova from wastewater.

iv. Evaluate the ability of the qPCR method for quantification of hookworm ova from wastewater matrices.

v. Compare the detection and quantification sensitivity of the newly developed PMA-qPCR method with currently used culture and vital stain methods.

1.7. Thesis structure/overview

A flow chart of thesis structure is shown in Figure 1.1. Chapter one is a general introduction which describes briefly about STHs and their medical importance, potential mode of transmission via wastewater reuse and currently available quantitative detection methods. Chapter two is a literature review. This section highlights the hookworm infection, a potential source of hookworm infection including wastewater reuse, wastewater treatment, and efficiency. Finally, this chapter described detection methods and their advantage and disadvantages.

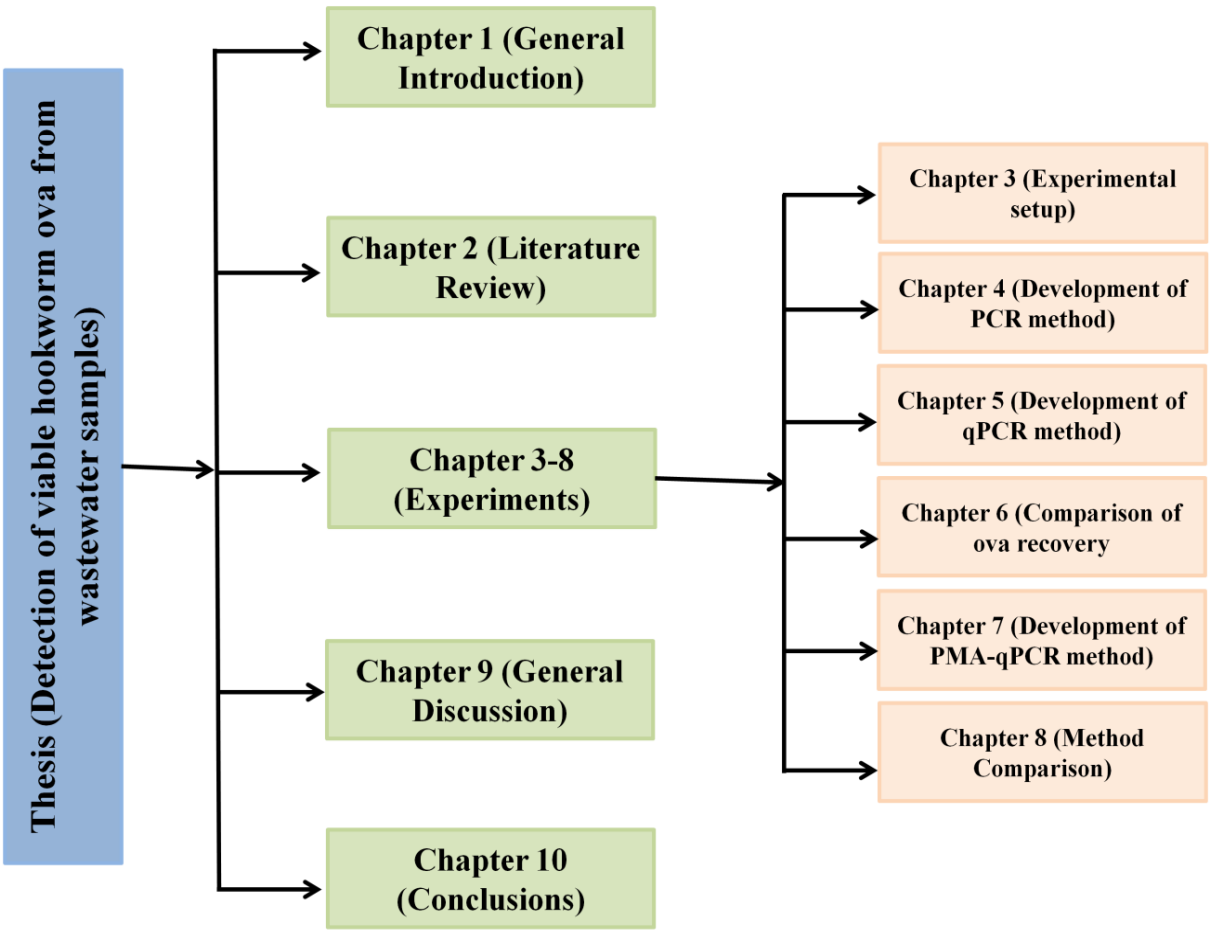

Figure 1.1: Flow chart showing structure of the thesis

Chapter 3 discusses ethical statement, the source of hookworm ova for experiments and QA/QC during experiments. In Chapter 4, a novel real-time PCR method was developed in the laboratory setting using dog hookworm (*Ancylostoma caninum*). The specificity and sensitivity of the method were evaluated. The sensitivity of the newly developed method was further validated using wastewater matrices. One paper was published from Chapter 4. ***Gyawali P, Sidhu JPS, Ahmed W, Jagals P and Toze S (2015) Rapid concentration and sensitive detection of hookworm ova from wastewater matrices using a real-time PCR method. Experimental Parasitology, 159: 5-12.***

However, the PCR method could not provide quantitative information which is necessary for assessing the potential health risks. Therefore, the PCR method was upgraded to the qPCR method and used for quantifying hookworm ova from ova seeded and unseeded wastewater samples. The finding of this study was presented in chapter 5. One article was published from this chapter.

Gyawali P, Ahmed W, Sidhu JPS, Jagals P and Toze S (2016) Quantification of hookworm ova from wastewater matrices using quantitative PCR. Journal of Environmental Sciences, accepted (see appendix G for acceptance letter).

Since the accuracy and rapidness of the qPCR method depend on the effective recovery of hookworm ova from wastewater and sludge samples, four different ova recovery methods for wastewater and two methods for sludge were evaluated for their performance. The result of this study was presented in chapter 6. The effective ova recovery method (filtration) for wastewater and (flotation) for sludge samples used for further experiments. One paper was published from this Chapter.

Gyawali P, Ahmed W, Sidhu JPS, Jagals P and Toze S (2015) Comparison of concentration methods for rapid detection of hookworm ova in wastewater matrices using the qPCR method. Experimental Parasitology, 159: 160-167.

The recently developed qPCR method can be used for quantitative detection of hookworm ova. The methods, however, is unable to distinguish between viable and non-viable ova. Since only viable ova are capable of hatching (L_3) larvae and consequently causing infections in humans. Therefore, quantification of viable hookworm ova is important to assess the public health risks. In Chapter 7 a PMA-qPCR method was developed for selective detection of viable hookworm ova from wastewater sample. The PMA-qPCR method for hookworm was further modified to quantify viable ova of other STHs. Two papers were published from this Chapter.

Gyawali P, Sidhu JPS, Ahmed W, Jagals P and Toze S (2016) An approach to reduce false viability assessment of hookworm ova with vital stains. Food and Waterborne Parasitology, 3: 9-12.

Gyawali P, Ahmed W, Sidhu JPS, Nery S, Clements A, Traub R, McCarthy J, Llewellyn S, Jagals P and Toze S (2016) Quantitative detection of viable helminth ova from wastewater, human feces and environmental soil samples using novel PMA-qPCR methods. Environmental Science and Pollution Research, 23(18): 18639-18648.

PMA-qPCR method was able to distinguish viable ova from non-viable ones. Therefore, the viability assessment performance of PMA-qPCR method was compared with previously available methods (culture-base and vital stain) in chapter 8. This method comparison study was conducted in the laboratory setting because all three methods require extensive isolation and concentration of hookworm ova from environmental and wastewater samples.

Chapter: 2

Literature review

Literature review

2.1. Background information

Hookworm infections in humans are a leading cause of malnutrition, anemia, physical and mental retardation (Hotez et al., 2005; Brooker et al., 2008; WHO, 2012). *Ancylostoma duodenale* and *Necator americanus* are the primary cause of human infections (Hotez et al., 2005; Traub et al., 2008; WHO, 2012). *N. americanus* is cosmopolitan in distribution, whereas *A. duodenale* is found to be more geographically restricted (Hotez et al., 2004). In addition, three species of zoonotic hookworm (*A. ceylanicum, A. caninum* and *A. braziliense*) also causes minor infections in humans (Traub et al., 2004; Traub et al., 2007).

An adult hookworm can survive 5-7 years in the intestine of its human host and produce 9.0×10^3-3.0×10^4 ova/day (Bethony et al., 2006). The ova are released into the environment through human defecation (Toze and Sidhu, 2011; Gyawali, 2012), which consequently increase the load of viable hookworm ova in the environment (WHO, 2006; Karkashan et al., 2014). In the receiving environment, the ova can remain viable for 9-12 months and can hatch into larvae under favourable conditions (Abaidoo et al., 2010). The infective larvae (L_3) are non-feeding organisms, therefore, they live in a state of developmental arrest up to three months in the environment (Brooker et al., 2006; Brooker, 2010); further development resumes after the L_3 larvae enter the human host via skin penetration (*N. americanus* and *A. duodenale*) or oral ingestion (*A. duodenale*). The life cycle of hookworm (*N. americanus* and *A. duodenale*) can be seen in Figure 2.1.

It has been estimated that approximately 8.0×10^8 people worldwide suffering from hookworm infection especially socioeconomically marginalised people in rural and areas of the tropical and subtropics of developing countries (WHO, 2012a; WHO, 2012b; WHO, 2015). The infections account for approximately 3.2×10^6 DALYs as reported in the global disease burden study in 2010 (Hotez et al., 2014). The higher hookworm infection rate in developing countries could be associated with poverty. Out of 8.0×10^8 hookworm infection cases, 6.0×10^8 cases are present in people earning less than $ 2.0 per day (Hotez et al., 2007). The mechanism of higher hookworm infection in people living with the poverty are inadequate sanitation, poor housing construction, lack of health education, lack of essential medicine and reuse of raw and partially treated wastewater.

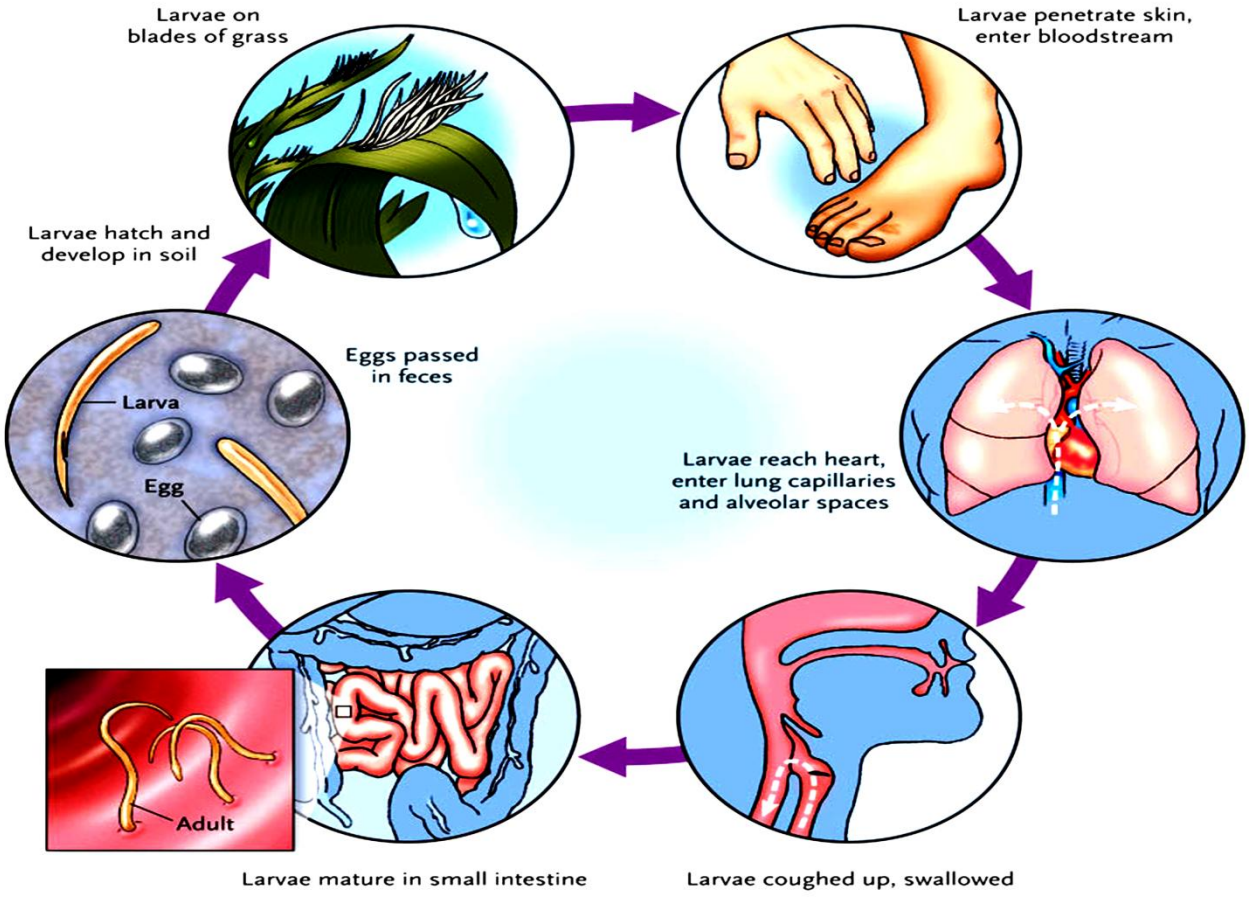

Figure 2.1: Life cycle of *N. americanus* and *A. duodenale* (Source: Hotez et al., 2004)

Due to the necessity of moist and sandy soil to complete the life cycle of hookworm and deposition of human faeces, people living without latrines access would be at high risk of hookworm infection. Wealthy families can easily afford anthelminthic drugs to treat hookworm infection on a frequent and periodic basis and therefore do not have to depend on sponsored deworming programs. The governmental funded deworming programs have mainly focused on pre-school aged and school-aged children rather than the entire community who serve as a reservoir for re-infection (Campbell et al., 2014). Another contributing factor for increased hookworm infections in developing countries could be poor housing construction. It has been reported that dirt floors, thatch roofs, and walls with cracks and crevices are also responsible for the transmission of hookworm infection (Hotez et al., 2007).

Furthermore, use of raw and partially treated wastewater is also responsible for increased hookworm infection in the rural community of developing countries (Vuong et al., 2007, Gupta et al., 2009, Trang et al., 2007). The use of treated and raw wastewater in broadacre agriculture have increased significantly worldwide (Carr, 2005; Sidhu and Toze, 2009; Pritchard et al., 2010; Hanjra et al., 2012). It has been estimated that 2.0×10^7 h of agricultural lands in developing countries are irrigated with raw wastewater (Carr, 2005; WHO; 2006;). However, the use of treated and raw wastewater is associated with socio-economic factors at the household level, such as poverty and a limited awareness of co-existed health risks (Carr, 2005; Jimenez, 2006). The households that depend on agriculture for their livelihood are forced to use partially treated or untreated wastewater for agriculture (Carr, 2005; Jimenez, 2006) (Figure 2.2). In addition, up to 42% of treated sludge produced globally from wastewater treatment process is being used as fertiliser (Oleszkiewicz et al., 2001; Wei et al., 2003; UN HABITA, 2008; ANZBP, 2009; Pepper et al., 2010; Kelessidis and Stasinakis, 2012).

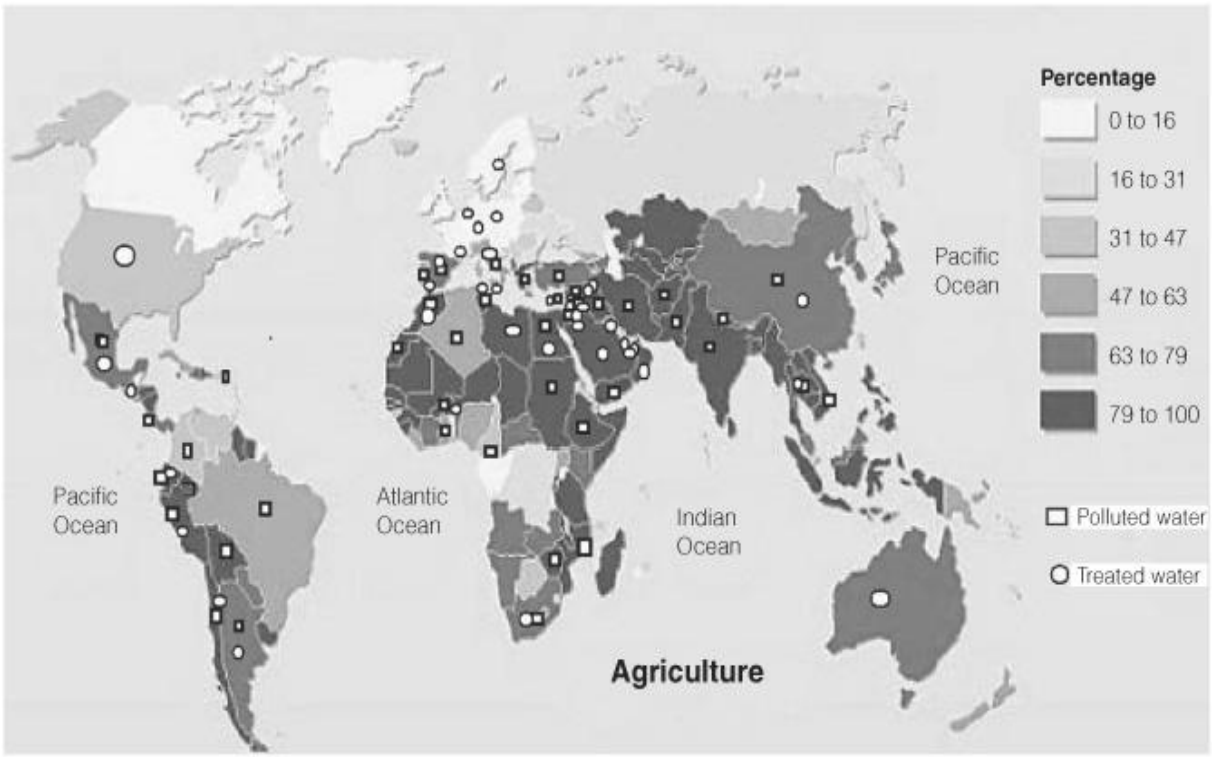

Figure 2.2: Global use of treated and raw wastewater for agriculture (Source: IWMI and IWRC, 2010)

Although the hookworm infection is predominantly found in developing nations (WHO, 2015), a serious concern has been expressed in many of the developed countries including Australia, due to the increased mobile population, and the influx of uncontrolled refugees from hookworm-endemic regions (Jensenius et al., 2008; Johnston and Conly, 2008; Cherian et al., 2009; Monge-Maillo et al., 2009; O'Brien, 2009; Abu-Madi et al., 2010; Baaten et al., 2011; Eslick and Kalantar, 2011). Due to their enteric source, hookworm ova excreted by infected individual reach to the wastewater and sludge (Sidhu and Toze, 2009; Toze and Sidhu, 2011). Since freshwater resources are under increasing pressure because of growing populations and climate change (Schwarzenbach et al., 2010; Hanjra et al., 2012), people are forced to use alternative water sources such as wastewater (Redcliffe, 2006; Redcliffe, 2010). One of the most significant issues in relation to wastewater reuse is the potential public health risks associated with viable hookworm ova (US EPA, 2003; WHO, 2006).

Stringent treatment is required before using wastewater for various (potable and non-potable) purposes where there is potential for human exposure. Several guidelines have been developed by national and international authorities and wastewater treatment methods have been put in place to eliminate the hookworm ova from treated wastewater and sludge (US EPA, 1999; US EPA, 2003; NRMMC, 2004; WHO, 2006). However, complete removal of hookworm ova may be difficult due to their protective cell wall (Toze and Sidhu, 2009).
A thorough understanding of the prevalence of viable hookworm ova in wastewater and sludge, potential health risk mechanism, the efficiency of various treatment methods and detection methods in terms accurate quantification is essential.

2.2. Literature search

An initial literature search was performed on the electronic database including PubMed, Google Scholar, ISI web of Knowledge to obtain published information and was regularly updated as information become available. Articles, reports, conference proceedings, guidelines published in English were taken into consideration for extracting the reverent information. The literature search was performed using keywords [Wastewater reuse, sludge reuse, biosolid reuse, wastewater reuse and public health risk, wastewater treatment methods, sludge treatment methods, wastewater reuse guidelines, hookworm in wastewater, hookworm in sludge].

Since *Ascaris lumbricoides* is being used as an indicator organism for helminth in wastewater and sludge, the search resulted in little information on the prevalence of hookworm in wastewater and sludge. Therefore, criteria for the literature search was broaden using keywords [helminths in wastewater, helminths in sludge, wastewater treatment and helminths reduction, wastewater reuse and helminth-associated health risk].

2.3. Prevalence of helminths in wastewater and sludge

The occurrence of helminth ova/larvae in wastewater depends on the prevalence of the helminth infection in the community (Mahvi and Kia, 2006). Due to the higher prevalence rate, and ability to produce a higher number of ova each day by a female (Bethony et al., 2006; Brooker et al., 2006; Knopp et al., 2012), ova and larvae of STHs (*Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, and *Trichuris trichiura*) are commonly found in wastewater and sludge around the world.

However, other helminths such as *Enterobius vermicularis*, *Strongyloides stercoralis*, *Toxocara* spp., *Taenia* spp., *Hymenolepsis nana*, *Echinococcus* spp., *Trichostrongylus* spp., *Dicrocoelium dendriticum* have also been reported to be present in wastewater and sludge (Gaspard and Schwartzbrod, 2003; Mahvi and Kia, 2006; Do et al., 2007; Wichuk and McCartney, 2007; Jimenez et al., 2007; Ben Ayed et al., 2009; Kelessidis and Stasinakis, 2012; Bastos et al., 2013; Sharafi et al., 2012; Hajjami et al., 2012; Konate et al., 2013a; Konate et al., 2013b). Commonly detected helminth ova and larvae in the wastewater and sludge around the world, their mode of transmission and the infective stage are presented in Table 2.1.

Table 2.1: Commonly detected helminths in wastewater and sludge, their mode of transmission and infective stage

Helminths	Numbers of ova production	Infective stage	Transmission mode
Ascaris spp.	10^5/day	Embryonated ova	Oral ingestion
Hookworm	10^4/day	Filariform larvae	Oral and skin penetration
T. trichiura	10^3/day	Embryonated ova	Oral ingestion
E. vermicularis	10^5/female	Embryonated ova	Oral ingestion
S. stercoralis	10^1 larvae/day	Filariform larvae	Skin penetration
Toxocara spp.	10^5/day	Embryonated ova	Oral ingestion
Taenia spp.	10^5/proglottid	Embryonated cyst	Oral ingestion
Hymenolepis spp.	-	Embryonated ova/cyst	Oral ingestion
Echinococcus spp.	-	Embryonated ova	Oral ingestion
Trichostrongylus spp	10^2/day	Filariform larvae	Oral ingestion
D. dendriticum	-	Metacercariae larvae	Oral ingestion
Fasciola spp.	10^5/day	Metacercariae larvae	Oral ingestion
Spirometra spp	-	Coracida larvae	Oral ingestion
Schistosoma spp	10^4	Metacercariae larvae	Oral ingestion

- = Data is not available

The numbers of STHs ova in raw wastewater can be as high as 10^3 per liter depending upon the rate of infection in the community (Gaspard and Schwartzbord, 2003; Sharafi et al., 2012; Hajjami et al., 2012; Bastos et al., 2013). Climatic conditions such as temperature, rainfall, relative humidity can also influence the numbers of ova and larvae in the wastewater and sludge (Sharafi et al., 2012; Hajjami et al., 2012; Bastos et al., 2013). This could be due to the fact that, STHs ova are known to develop faster at a temperature between 28-32ºC (Seamster, 1950; Beer, 1976; Smith and Schad, 1989; Brooker et al., 2006). In addition, soil moisture and relative humidity also influence the survival of viable ova and larvae (Nwosu and Anya, 1980; Udonis et al., 1980; Brooker et al., 2006). Therefore, a wider population is potentially infected, and eventually contributing more STHs ova into the wastewater system.

Helminths ova have high density and are large in size (55 -74 µm by 35 – 42 µm) depending on the species, therefore they can settle down rapidly in wastewater, and be concentrated in the sludge (Hotez et al., 2005; Do et al., 2007; Pecson et al., 2007; Yen Phi et al., 2010; Konate et al., 2010; Navarro and Jimenez, 2011; Konate et al., 2013a). However, the velocity of ova settling down depends on the density of wastewater. Sengupta and colleagues (2011) reported that helminth ova settled faster in raw wastewater than in tap water.

Table 2.2: Numbers of helminth ova in raw wastewater, treated wastewater and sludge in different countries

Countries	Numbers of helminth ova in wastewater matrices			References
	Raw wastewater (ova/L)	Treated wastewater (ova/L)	Sludge (ova/g) total solid	
Pakistan	46[a], 5[b], 3[c], 53[i]	1[a], 1[i]	*	(Sharafi et al., 2012)
Egypt	68[i]	4[i]	67[i]	(Stott et al., 2003; Jimenez, 2007)
Tunisia	455[a], 6[b], 51[d], 420[e], 932[i]	5[a], 6[d], 24[e], 35[i]	*	(Ben Ayed et al., 2009)
Mexico	6[i]	*	46[a], 4[c], 2[f], 52[i]	(Pecson et al., 2007; Navarro and Jimenez, 2011)
India	3[a], 1[c], 8[g], 47[i]	24[a], 5[g], 29[i]	*	(Gupta et al., 2009)
Iran	2[a], 3[d], 1[e], 2[g], 9[i]	1[a]	22[i]	(Bina et al., 2004; Mahvi and Kia, 2006)
Burkina Faso	7[a], 1[c], 6[g], 16[i]	*	536[i]	(Konate et al., 2010, Konate et al., 2013a)
France	9[i]	*	7[i]	(Navarro and Jimenez, 2011)
USA	8[i]	*	13[i]	(Navarro and Jimenez, 2011)
Vietnam	200[i]	*	450[i]	Do et al., 2006, Yen Phi et al., 2010)
Brazil	201[i]	*	101[a], 20[b], 11[d], 61[e], 5[g], 27[h], 229[i]	(Silvana et al., 2006; Navarro and Jimenez, 2011)
Oman	224[a], 69[c], 3[e], 18[f], 33[g], 6[h], 353[i]	45[a], 6[f], 6[h], 57[i]	*	(Rivera et al., 2012)
Australia	*	*41*[i]	*	(Water Corporation, 2012)

***[a]Ascaris, [b]Hymenolepsis, [c]Trichuris, [d]Taenia, [e]Enterobius, [f]Toxocara, [g]Hookworm, [h]Strongyloides,* [i]overall helminths , *numbers are not reported**

Table 2.2 shows the number of helminths ova present in treated wastewater, raw wastewater and sludge samples in different countries. The information presented in the table indicated that using the model organism (*A. lumbricoides*) to determine the treated wastewater quality may not be suitable. In addition, currently used microscopic detection methods may not be suitable for estimating the health risk associated with wastewater and sludge reuse because of lack of specificity and sensitivity.

2.4. Potential public health risks

Wastewater and sludge may contain a high number of helminth ova and larvae including hookworm that can pose significant public health risks either directly and indirectly (Figure 2.3). The extent of the health risk, however, depends on several factors such as numbers of viable ova/larvae present in the environment, infective dose, exposure routes and the susceptibility of the exposed individual (Navarro and Jemenez, 2011).

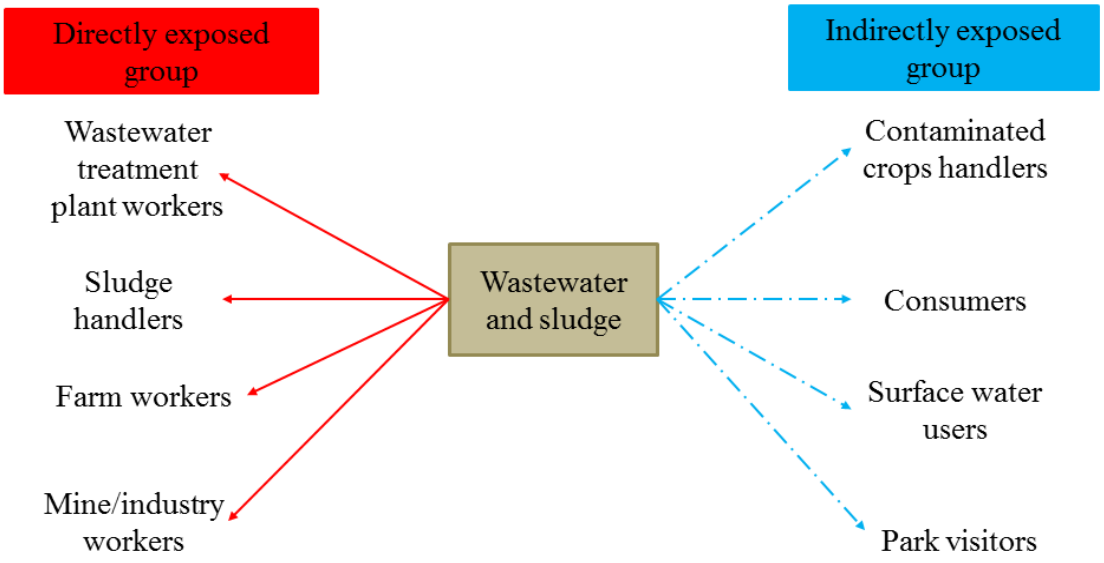

Figure 2.3: Potential path of public health risks associated with wastewater and sludge reuse

2.4.1. Health risks through direct exposure

People handaling wastewater and sludge including wastewater and sludge treatment plant workers, people transporting sludge as well as farmers applying wastewater and sludge to land and wastewater applied mine workers can be exposed to helminth ova and larvae directly during their work (Seidu et al., 2008; Ackerson and Awuah, 2012). This group has higher (40%) rate of helminth infections than indirectly exposed groups (Ensink et al., 2005; El Kettani and Azzouzi, 2006; Trang et al., 2007; The World Bank, 2010). In the directly exposed group, hookworm infection is found to be more common (Ensink et al., 2005; Ensink et al., 2008). This could be due to the transmission mode (oral ingestion and skin penetration) of hookworms.

The health risk is higher in developing countries compared to the developed countries (The World Bank, 2010). This could be the fact that, people in the developing countries do not use personal protective equipment during handling wastewater and sludge which increases the risk of infection (Gyawali et al., 2013). Studies have been suggested that the health risks in the directly exposed group (wastewater workers and farmers using partially treated wastewater) is directly related to working conditions, individual behavior, in particular, personal hygiene as well as use of personal protective equipment (El Kettani and Azzouzi, 2006; The World Bank, 2010). In addition, the prevalence of helminth infections is higher in developing countries compared to developed countries, which significantly increases the risk of infection to the directly exposed groups.

2.4.2. Health risks through indirect exposure

People can also acquire helminth infections via indirect exposure to helminths present in the wastewater and sludge by selling and consuming vegetables and aquaculture products grown on farms using wastewater and sludge (Gupta et al., 2009; Navarro and Jimenez, 2011; Ackerson and Awuah, 2012). Helminth ova contained in the treated wastewater and sludge can easily contaminate crops and aquaculture products. A study has reported that 90% of helminths found in the treated wastewater and sludge can be transferred onto green and leafy vegetables such as spinach and parsley when treated wastewater and sludge are applied (Navarro and Jimenez, 2011).

The contamination level of helminth ova on vegetables increases during the rainy season (Vuong et al., 2007). This could be due to the heavy rainfalls lifting the helminth ova/larvae from ground and depositing on the vegetables such as spinach, parsley, cabbage and cauliflower where they remain infective for a long period of time (Abaidoo et al., 2010). Helminth ova present in the treated wastewater and sludge also have the potential to contaminate subsurface vegetables such as carrots and radish. However, the magnitude of the contamination in the subsurface vegetable is lower (0.16 ova per 100 *g*) than in leafy vegetables (10 ova per 100 *g*) (Amahmid et al., 1999; Vuong et al., 2007).

Another way of indirect exposure to helminth ova/larvae is by using wastewater irrigated gardens, parks, and sporting venues for recreational activities (Moubarrad and Assobhei, 2007). For example, Moroccan children playing in a park had 18% higher rate of helminth infections when compared to children who never visited the park (Moubarrad and Assobhei, 2007). Moreover, pets especially dogs can carry helminths ova/larvae from park to home (Gyawali et al., 2013), and thereby be transmitted to humans. In addition, helminth ova can run off from agricultural land during rain and flood events. Those run off ova have the potential to contaminate environmental waterways such as rivers, creeks, ponds, and beaches. As a result, people can be infected with the helminths by using contaminated waterways for different activities including recreation and transport (Horweg et al., 2006; King, 2010).

2.5. Preventive measures used

To minimise the potential public health risk associated with the reuse of treated wastewater and sludge, various guidelines were developed by different national and international health regulatory bodies (US EPA, 2003; Gaspard and Schwartzbrod, 2003; NRMMC, 2004; WHO, 2006; DEC, 2012). These guidelines have established an acceptable limit of helminth ova in treated wastewater and sludge depending on their final use. For example, < 1 viable helminth ovum in 1L of treated wastewater or 4 *g* of dry sludge can be used without restriction (WHO, 2006). This number of helminth ova in treated wastewater and sludge for unrestricted use was established on the basis of that, less than one viable ovum in 1L treated wastewater or 4 *g* of sludge is difficult to detect using available detection methods (Carr, 2005).

The wastewater and sludge treatment process is the first and most important factor towards minimising the potential health risk associated with wastewater reuse. Various wastewater and sludge treatment methods also have been proposed to remove helminth ova from wastewater and sludge prior to reuse (US EPA, 2003; WHO, 2006). The guidelines have also identified the potential points of human health risks associated with treated wastewater and sludge reuse and designed multiple barrier approaches. The multiple barrier approaches include good safe agriculture, good manufacturing practice and good hygiene practices where wastewater and sludge treatment alone is not considered a sufficient pathogen barrier (WHO, 2006; IWMI AND IWRC, 2010) (Figure 2.4).

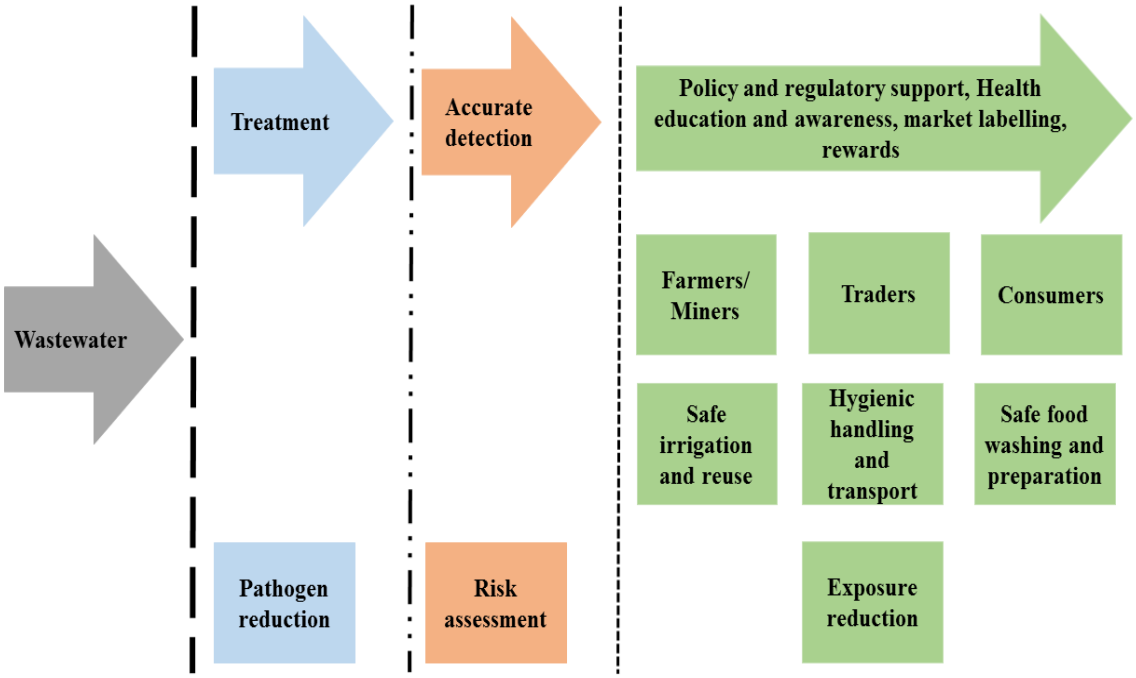

Figure 2.4: Flow chart showing multiple barrier approaches in order to minimise the public health risk associated with wastewater and sludge reuse

The main aim of wastewater treatment process is to remove/inactivate the numbers of helminth ova or larvae from wastewater and sludge. Since the density of helminth ova including hookworm is higher than raw wastewater, the ova settle down quickly in the raw wastewater (Sengupta et al., 2011). Therefore, the retention of wastewater (sedimentation) process effectively (2-3 $\log_{10}$ reduction) removes helminth ova from the wastewater (Toze, 2006; Reinoso et al., 2011; Konate et al., 2013a) (Table 2.3). These methods may remove helminth ova from the liquid, however, will concentrate the helminth ova in the solid phase (sludge) where they can remain viable for a long period of time (up to 20 months) (Sanguinetti et al., 2005). Therefore, sludge may require further treatment.

Different methods have been used to inactivate the helminths ova and other potential pathogens from sludge such as ponding, aerobic digestion, anaerobic digestion, lime stabilisation, heat treatment depending on the availability of resources and feasibility (Mendez et al., 2002; Bina et al., 2004; Capizzi-Banas et al., 2004; WHO, 2006; Mendez-Contreras et al., 2009; Maya et al., 2010; Maya et al., 2010; Navarro and Jimenez, 2011; Reinoso et al., 2011; Toze and Sidhu, 2011; Ruiz-Espinoza et al., 2012; Endale et al., 2012; Konate et al., 2013a) (Table 2.3). The helminth inactivation rate of these sludge treatment methods varied between studies. For example, a study reported that increasing the pH of sludge to 12 for 2 h can provide complete inactivation of helminth ova (Mendez et al., 2002) however, another study reported that only 84% helminth ova can be inactivated by increasing the pH of sludge to 12 with 120 h of holding time (Bina et al., 2004) (Table 2.3).

Similarly, WHO has made a recommendation that sludge must be treated at 45ºC for 1h to achieve the safe level (< 1 ova per 4 *g*) for unrestricted use (WHO, 2006). Conversely, other studies did not agree with the treatment conditions recommended by WHO (Capizzi-Banas et al., 2004; Maya et al., 2010; Navarro and Jimenez, 2011) (Table 2.3). This discrepancy between the calculated helminths ova removal rate could be the fact of inbuilt error of currently available detection methods. Other factors, such as climatic conditions may also influence the removal rate. The treatment method may provide optimum result in the tropical and subtropical climate but may not provide the same result in a temperate climate. Therefore, a generalisation efficacy of any sludge treatment method might not be appropriate. Another issue that requires considering is the reported removal rate from laboratory scale studies which may different in the real world scenario with a large volume of sludge.

Table 2.3: Efficacy of different sludge treatment methods on helminth ova inactivation

Treatment Methods	Treatment conditions	Helminth ova	Inactivation rate (%)	References
Lime stabilization	Ammonia 20% w/w for 2 h	*	83	(Mendez et al., 2004)
	Ammonia 20% w/w with 50ºC for 2 h	*	100	(Mendez et al., 2004)
	pH 10.2 for 40 days	*A. lumbricoides*	94	(Endale et al., 2012)
	pH 11 for 120 h	*	56	(Bina et al., 2004)
	pH 12 for 120 h	*	83	(Bina et al., 2004)
	pH 10 with humidity 90% for 8 months of RT	*A. lumbricoides*	100	(Maya et al., 2010)
	pH 12.5 with humidity 80% for 4 months of RT	*A. lumbricoides*	84-95	(Maya et al., 2010)
Heat treatment	50ºC-51.5ºC for 2 h at normal pH	*A. suum*	100	(Paulsrud et al., 2004)
	70ºC with humidity 80% for 2 h	*	100	(Maya et al., 2010)
	75ºC with 5% dryness for 3 h	*A. lumbricoides*	100	(Maya et al., 2012)
	78ºC with 5% dryness for 3 h	*A. suum*	100	(Maya et al., 2012)
	74ºC with 5% dryness for 3 h	*T. canis*	100	(Maya et al., 2012)
	73ºC with 5% dryness for 3 h	*T. trichiura and T. solium*	100	(Maya et al., 2012)
	72ºC with 5% dryness for 3 h	*H. nana*	100	(Maya et al., 2012)
Pond stabilization	Facultative pond for 9.5 days of HRT	*A. lumbricoides* and Hookworm	98	(Konaté et al., 2013a)
	Maturation pond for 5.5 days of HRT	*A. lumbricoides* and Hookworm	99	(Konaté et al., 2013a)
	Anaerobic pond for 0.4 days of HRT	*	90	(Reinoso et al., 2011)
	Facultative pond for 4.1 days of HRT	*	92	(Reinoso et al., 2011)
Anaerobic digestion	Mesophilic temperature (35ºC) for 31 days of HRT	*	0.3	(Méndez-Contreras et al., 2009)
	Thermophilic temperature (55ºC) for 21 days of HRT	*	85-100	(Méndez-Contreras et al., 2009)
	Thermophilic temperature (55ºC) for 120 min	*	94	(Ruiz-Espinoza et al., 2012)
	Thermophilic temperature (40ºC) for 120 min	*	74	(Ruiz-Espinoza et al., 2012)
	Thermophilic temperature (61ºC– 62.5^{0}C) for 45 min	*A. suum*	100	(Paulsrud et al., 2004)
Aerobic digestion	Sludge/40 days of RT	*A. lumbricoides*	73	(Endale et al., 2012)
	Mixed with ash/40 days of RT	*A. lumbricoides*	100	(Endale et al., 2012)
	Mixed with soil/40 days of RT	*A. lumbricoides*	76	(Endale et al., 2012)
	Mixed with smooth soil/28 days of RT	*	97	(Ferreira et al., 2002)
	Mixed with unsmooth soil/28 days of RT	*	89	(Ferreira et al., 2002)
Compression	Compressed for 7 min 42 sec	*A. suum*	86	(Buitrón and Galván, 1998)
	Compress for 15 min 47 sec	*A. suum*	90	(Buitrón and Galván, 1998)
	Compress for 29 min 53 sec	*A. suum*	100	(Buitrón and Galván, 1998)

*Helminths not specified, RT= Retention time, HRT= Hydraulic retention time

2.6. Isolation and quantitative detection of viable helminth ova

The efficiency of the wastewater and sludge treatment methods has been measured by quantifying the numbers of viable helminths ova in treated wastewater and sludge (US EPA, 2003; NRMMC, 2004; WHO, 2006). Currently, two quantitative detection methods have been used to quantify viable helminths ova from wastewater and sludge samples; culture-based (US EPA, 2003) and vital staining (de Victorica and Galven, 2003).

2.6.1. Isolation and concentration of ova

Numbers of helminths ova in treated wastewater and sludge is distributed heterogeneously (Gantzer et al., 2001). Therefore, detection and quantification of helminths ova from wastewater matrices require isolation and concentration of the 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 helminths ova from wastewater and sludge samples (US EPA, 1999). The recovery rate of this method can range 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 ultrafiltration (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 helminths ova from wastewater matrices.

2.6.2. Quantitative detection of viable helminths ova

In addition to culture-based and vital staining methods which are time consuming, laborious and non specific, other methods such as flow cytometry, PCR/qPCR have been used to detect helminths ova and larvae from faecal samples and have the potential to detect viable helminth ova from treated wastewater and sludge. The principle of different quantitative detection methods can be seen in Figure 2.5.

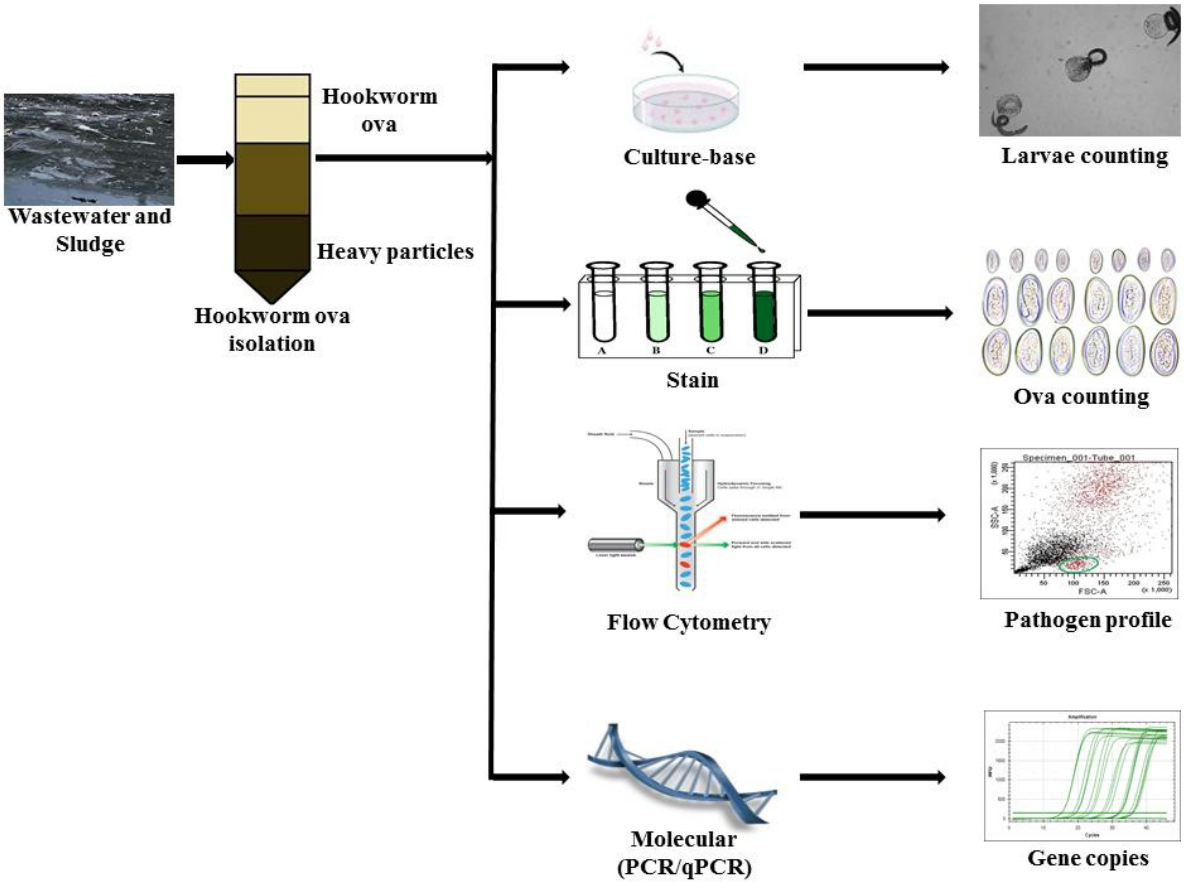

Figure 2.5: Principle of different method for quantifying viable helminth ova

2.6.2.1. Culture-based method

The culture-based method involves artificially hatching the ova in a laboratory. Helminth ova are incubated at 28ºC - 30ºC for up to 28 days depending on the helminths, to allow the viable ova to hatch and are observed microscopically (Bowman et al., 2003). Health regulators including the US EPA and WHO recommend this method because it has the ability to estimate viability of helminth ova recovered from treated wastewater and sludge.

The method, however, has limitations such as the requirement of highly skilled personnel to accurately distinguish between larvae of different species of helminths (Verweij et al., 2007). In addition, the detection limit of the method depends on detection sensitivity of a

microscope that may not be sensitive enough to detect low numbers of larvae in a sample (Weber et al., 1991). The most important limitation of the culture-based method is the lengthy wait for the result to be available which increases congestion in a laboratory. Therefore, the operational cost of the method can increase significantly. The main advantages and disadvantages of the culture-based method are listed in Table 2.4.

2.6.2.2. Vital stain method

The stain-based method is rapid, cheap and easy to use compared to the incubation method (de Victorica and Galvan, 2003; Dabrowaska et al., 2014; Karkashan et al., 2014). This method involves staining helminth ova with a stain such as Trypan blue, Congo red, Eosin Y, Hematoxylin, Methyl green, Safranin O, Methylene blue and Lugol's iodine and counting the ova under a microscopic (de Victorica and Galván, 2003).

The vital stain method takes advantage of different working mechanisms of the cell wall of viable and non-viable ova. A viable helminth ovum has three layers of intact cell walls that act as an alternative sieve and prevent the stain from entering into the cytoplasm (Matthews, 1986). Once the ovum becomes non-viable, the integrity of cell wall is compromised and it becomes permeable to stain (Bae and Wuertz, 2009). The cell wall, however, may not be permeable immediately after inactivation, and this can lead to over-estimation of viable ova in a sample.

The vital stain method also requires skilled personnel to prepare the samples and identify specific helminth ova under a microscope. Previous studies have noted that even skilled personnel may fail to distinguish hookworm ova from those of *Oesophagostomum bifurcum* due to highly similar morphologies (Cabaret et al., 2002, Traub et al., 2007, Verweij et al., 2007). Therefore, result obtained from stain method may not be accurate and reliable (Table 2.4).

The use of automated quantification of fluorescent microscope and double fluorescent dyes (PI and DAPI) may increase the sensitivity of the microscopic method (Rieger et al., 2010; Branco et al., 2012; Zotta et al., 2012), however, identification of hookworm ova may not be possible due to the similar morphology of some other helminth ova (Traub et al., 2007).

2.6.2.3. Flow cytometry

Flow cytometry combines advantages of microscopy and biochemical analysis in a single technique for rapid detection of pathogens (Hammes et al., 2008; Wang et al., 2010). In a flow cytometer, helminths ova in suspension intersect with a laser beam and produce a profile on the basis of scattered fluorescence. Therefore, detection can be rapid and sensitive. The principle of assessing the viability of helminths ova is similar to the staining method. The major issue of using flow cytometry for quantifying viable helminths ova is that the treated wastewater and sludge contain similar sized helminths ova of different species which can provide similar profiles during flow cytometry. As a result, the method may significantly over-estimate the numbers of specific helminths ova in treated wastewater and sludge (Table 4.3). In addition, treated wastewater or sludge can have a high level of background debris which may provide a similar profile to helminths ova during detection/quantification and compromise the sensitivity of the method (Barbosa et al., 2008).

Table 2.4: Advantages and disadvantages of different quantitative detection methods for helminths ova

Detection Methods	Advantages	Disadvantages	References
Culture-based	• Viability is possible • cheaper chemical and equipment • Can be done in a small scale laboratory	• Prolong time to culture larvae • Suitable conditions must maintain • Regular observation necessary • Low sensitivity and specificity	(Bowman et al., 2003; Nocker et al., 2007b; McCarthy et al., 2012)
Vital stain	• Cheap and easy process • Assessment can be done in few hours • Less chemical and equipment • Can be done in small scale laboratory	• Difficulty on viability assessment • Sensitivity depends on detection threshold of a microscope • Stain might not effective on recently inactivate ova • Lack specificity	(Weber et al., 1991; Nelson and Darby, 2001; Cabaret et al., 2002; Victorica and Galvan, 2003; Verweij et al., 2007; McCarthy et al., 2012; Gyawali et al., 2016b)
Flow cytometry	• Quick and easy process • Higher sensitivity than microscopy • Automated process • Quantification can be done	• Lack of specificity • Difficulty on viability assessment of ova • Difficulty distinguishing background debris from ova	(Hammes et al., 2008; Barbosa et al., 2008; Wang et al., 2010)
Molecular (PCR/qPCR)	• Quick and easy process • Higher sensitivity and specificity • Viability could be possible • Automated process • [a]Multiple species can be identified from single sample • [b]Quantification is possible	• Require advance laboratory and equipment • Genomic information is essential • Require right genomic target for viability • Possibility of providing false positive result by extracting DNA from inactivated ova • Possibility of false negative result via inhibitors present in the samples • [a]Multiple sets of primers require which can reduce the sensitivity • [b]Triplicate sample required	(Pecson et al., 2006; Verweij et al., 2007; Traub et al., 2007; Traub et al., 2008; Janwan et al., 2011; Jonker et al., 2012, Ngui et al., 2012b)

[a]= Multiplex PCR, [b]=Quantitative PCR

2.6.2.4. Molecular (PCR/mPCR/qPCR) methods

The molecular method can detect helminths ova through amplifying a target region of nucleic acid, which can be extracted from any samples (Queipo-Ortuno et al., 2008; Sołtysik et al., 2011). Recent development in PCR methods made detection faster, more accurate and more sensitive than culture-based and vital stain methods (Traub et al., 2004; Traub et al., 2007; Traub et al., 2008). Ribosomal rRNA or rDNA of Internal Transcribed Spacer (ITS-1 and ITS-2) regions contain high variability in a closely related species (Traub et al., 2007; Traub et al., 2008) and can be used to distinguish to species level.

Multiplex PCR (mPCR) method can be used to detect multiple parasites including different species of hookworm in a single assay (Verweij et al., 2007; Jonker et al., 2012; Saeki et al., 2013) and reduce detection time, costs and labor that is otherwise associated with running multiple assays (Toplak et al., 2012). However, it should be noted that multiple primers pairs must function under the same reaction conditions and do not form primer dimers during the reaction. Otherwise, the sensitivity of detection can be reduced (Kattenberg et al., 2011).

Quantification of pathogens including helminths is not possible through either binary PCR or mPCR methods. A quantitative PCR (qPCR) method, however, can quantify pathogens on the basis of amplified gene copy numbers present in the target nucleic acid (Botes et al., 2013). Therefore, it is imperative to know the exact gene copy number in a target nucleic acid (Pecson et al., 2006) for accurate quantification. The qPCR method has been widely used to quantify pathogens from different environmental samples (Bustin, 2010; Postollec et al., 2011; Rusiñol et al., 2013; Ahmed et al., 2014a; Ahmed et al., 2014b).

The qPCR method has improved detection and quantification of pathogens from various samples including treated wastewater and sludge. However, the viability of pathogens including helminth ova in a sample cannot be determined using a standard DNA target and there is a risk of overestimating the infectious pathogens when qPCR is used for environmental samples (Byappanahalli et al., 2010; Srinivasan et al., 2011). A high number of rDNA/rRNA copies in the ITS-1 and ITS-2 region makes detection more sensitive (Pecson et al., 2006; Raynal et al., 2012), caution however, is required interpreting the qPCR data to minimise the overestimation of helminth ova in environmental samples.

2.6.2.4.1. Dye based viability PCR

Dye based viability PCR has been developed to overcome the limitations of qPCR. In the dye based viability PCR, pathogens are incubated with a DNA intercalating dye such as Ethidium Bromide Monoazide (EMA) or Propidium Monoazide (PMA). These dyes bind to exposed DNA during photo-activation and interfere with PCR amplification. Morphologically damaged oocysts and cells have unprotected nucleic acid. Therefore, their amplification is restricted after EMA and PMA photo-activation (Nocker et al., 2006; Hein et al., 2007; Brescia et al., 2009; Chen and Chang, 2010; Byappanahalli et al., 2010; Taskin et al., 2011). In contrast, morphologically intact viable oocysts and ova exclude dyes (EMA and PMA), enabling strong PCR amplification. The operating mechanism is similar to the stain based detection method (Figure 2.6).

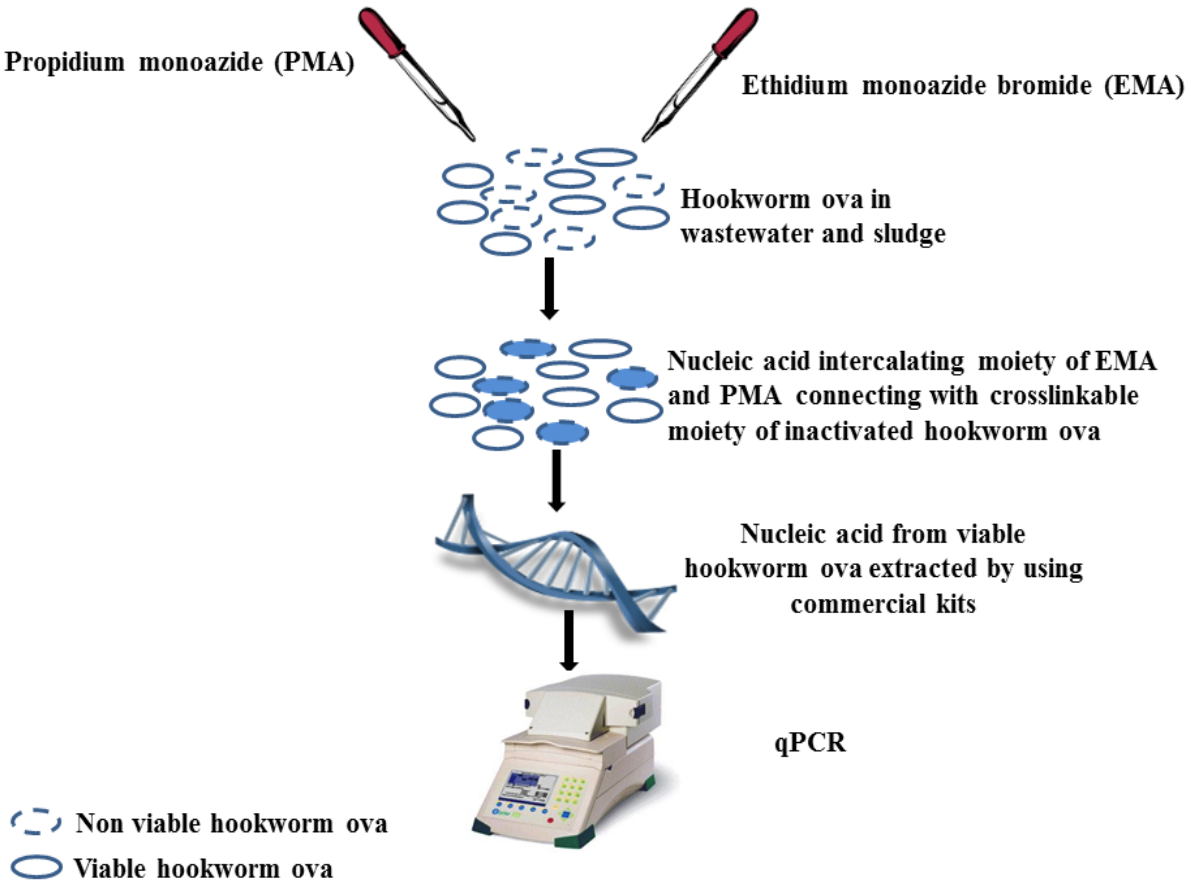

Figure 2.6: Operating mechanism and steps of stain based viability PCR (Source: Nocker et al., 2007)

The effectiveness of EMA and PMA is directly related to the concentration of stain, incubating time and light exposure (Rudi et al., 2005; Nocker et al., 2006; Wagner et al., 2008; Chang et al., 2010). Due to the chemical nature of EMA and PMA, the concentration of PMA must be 4-fold higher than EMA to achieve an effective result (Chang et al., 2010). However, there is no information available in the literature regarding the application of EMA and PMA for discriminating non-viable from viable hookworm ova.

2.6.2.4.2. Molecular viability PCR

Molecular viability PCR uses species-specific ribosomal RNA (rRNA) and messenger RNA (mRNA) precursors required for basic cell function or life within the cell, as indicators of viable ova (Widmer et al., 1999; Kobayashi et al., 2009; Vollmer et al., 2010; Sander et al., 2011; Vasconcelos et al., 2012). Since, rRNA and mRNA have a short life in the environment (Pecson et al., 2006; Sander et al., 2011; Vasconcelos et al., 2012), they can be used for detecting viable hookworm ova from treated wastewater and sludge samples. There is, however, no information regarding the status of rRNA and mRNA in ova inactivated during their incubating phase.

It should be noted that the sensitivity of molecular methods depends on the quantity and quality of DNA or RNA of the hookworm ova extracted using commercially available kits. Since, the hookworm ova contain hard and multiple cell walls, it is vital that these are lysed properly for optimal extraction efficiency. Freeze-thaw cycles with lysis buffer facilitate lysing hookworm ova (Traub et al., 2004; Sidhu and Toze, 2008; Gyawali et al., 2015a). Treated wastewater and sludge have different chemistries so different freeze-thaw cycles may be required for complete lysis the ova in those samples. Another factor that can influence the molecular detection method is the presence of PCR inhibitors in treated wastewater and sludge. Treated wastewater and sludge can have traces of heavy metals, fats, polyphenols, proteins, humic acid and fulvic acid (Rock et al., 2010; Xu et al., 2010; Schrader et al., 2012). Those compounds may not be completely removed during the nucleic acid extraction process. Small traces of those compounds in a nucleic acid can prevent PCR amplification. Therefore, a PCR inhibition test is important prior to detection and quantification. Skate 22 assay have been successfully used to evaluate the presence of PCR inhibitors in nucleic acid samples (Cao et al., 2012).

In conclusion, hookworm infection is leading cause of malnutrition, anaemia and impaired cognitive development in young children. Approximately 8.0×10^{6} million people worldwide suffer from hookworm infection. The majority of infections are found in economically marginalised population in Africa followed by Asia, and the Americas. This could be because people living in those areas expose themselves to soil contaminated with hookworm larvae (L_3) as a result of using raw wastewater or sludge on agricultural field as well as disposing night soil on open areas due to the lack of latrines. Despite the implementation of various interventions such as the improved sanitation, improved drinking water quality, health education, and MDA, it has been difficult to eradicate hookworm infections from an endemic region. Therefore, a rapid, sensitive and specific method for detecting viable hookworm ova from environmental samples is necessary in order to identify the potential source of hookworm reinfection.

The numbers of hookworm ova in environmental samples are generally detect or quantify by either US EPA recommended culture-based or vital staining methods. Both methods rely on the identification of ova and larvae using microscopic observation. Microscopic observation is not sensitive enough to detect low numbers of ova in wastewater samples. It has been recommended that molecular methods such as PCR and qPCR have the ability to detect hookworm ova with improved sensitivity and precision. The PCR/qPCR method in conjunction with PMA (DNA intercalating dye) can provide the viability of bacteria, protozoa, and viruses from environmental samples including wastewater and sludge. However, none of the published studies have investigated the ability of PCR/qPCR/PMA-qPCR for quantitative detection of viable hookworm ova from wastewater and sludge samples.

Chapter: 3

Experimental Setup

Experimental Setup

3.1. Ethical statements

As human and dog faecal samples were the source of hookworm (*A. caninum*, *A. duodenale and Necator americanus*) and roundworm *Ascaris lumbricoides* ova, ethical approvals were necessary. Human and dog faecal samples were collected after obtaining ethical approvals from the relevant ethics committees.

- The University of Queensland Animal Ethics Committee, Australia (Reference number: AEC/QU/12/2013) for collecting dog faecal samples.
- The University of Queensland Human Research Ethics Committee (Project number: 2011000734) for collecting human faecal samples.
- The Australian National University Human Ethics Committee (Protocol: 2014/311) for collecting human faecal samples.
- Timorese Ministry of Health Research and Ethics Committee (Reference number: 2011/51) for collecting human faecal samples.

While collecting human faecal samples, donors were informed about the use of their samples and written consent was obtained prior to collecting the samples.

3.2. Rationale of using *Ancylostoma caninum* as a surrogate

As human hookworm infection is rare in Australia it was, difficult to obtain large numbers of human hookworm ova required for the experiments. Dog hookworm (*A. caninum*), ova were therefore chosen as a surrogate because of their high prevalence in dog faeces in Australia and similar size (55-74 µm by 35-42 µm) of human hookworm ova. In addition, *A. caninum* ova have high up to 98% genetic and 100% morphological similarities with other hookworms of interest such as *A. duodenale* (Traub et al., 2004). It was, therefore, considered as a suitable surrogate to conduct the necessary laboratory experiments.

3.3. Obtaining and enumerating *A. caninum* ova

The *A. caninum* ova used in this study were collected from dog faecal samples that tested positive at the School of Veterinary Science in University of Queensland, Gatton, Australia using a standard microscopy. *A. caninum* ova were isolated from approximately 20 *g* of dog

faecal samples using the flotation method (Bowman et al., 2003). After isolation, ova were stored in 0.5% formalin. The ova in each sample were enumerated in triplicate by microscopic observation (10x × 10x) using a Sedgewick-Rafter Counting Chamber (Pyser-Sgi^{TM}). A photomicrograph of *A. caninum* ova used for the experiments can be seen in appendix A. The enumerated ova were aliquoted into 10 mL volumes in 50 mL tubes and stored at 4^{o}C for a week and to be used for subsequent seeding experiments. Dog faecal samples positive with hookworm ova were collected over the three years period to keep the ova fresh for different experiments. The number of ova in each stock solution is presented in appendix B.

3.4. Quality control

Various laboratory safety rules, hygiene, and quality control measures were undertaken during each experiment.

- All standard laboratory safety rules and hygiene controls were followed as described in Australian standards for the microbiological laboratory.
- Background levels of *A. caninum* DNA were tested in tap water, treated wastewater, raw wastewater, and sludge samples prior to every seeding experiment.
- To minimise qPCR contamination, DNA extraction and qPCR set-up was 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.
- Due to the light sensitivity of the PMA, all the PMA treatments were carried out in the dark room.
- For each PCR/qPCR experiment, positive control, standards (also served as a positive control) and triplicate negative controls (UltraPure^{TM} water) were included.
- For the PMA-qPCR experiment, positive (100% viable ova [n = 400 ± 40] without PMA) and negative (100% non-viable ova [n = 400 ± 40] without PMA) controls were used.

Chapter: 4

Development of PCR method

Development of real-time PCR method

4.1. Introduction

The use of wastewater and sludge in broadacre agriculture have increased significantly worldwide (Carr, 2005; Sidhu and Toze, 2009; Pritchard et al., 2010; Hanjra et al., 2012). It has been estimated that 5.0×10^6 ha of agricultural land is irrigated with raw wastewater (Carr, 2005), and up to 42% of sludge produced globally from wastewater treatment process is being used as fertilizer (Kelessidis and Stasinakis, 2012). Since a single viable ovum has the potential to cause infections in an individual, the health risks can be high for people coming into contact with wastewater and sludge in areas where helminth infections are endemic especially in the developing countries (Ensink et al., 2005; WHO, 2006).

Currently employed detection methods for hookworm are not sufficiently specific and sensitive to detect viable hookworm ova in wastewater matrices (liquid and solid). Culture-based and stain-based methods are the most commonly used methods to detect hookworm ova in wastewater matrices (US EPA, 2003; de Victorica and Galván, 2003; Bowman et al., 2003 Do et al., 2007; Wen et al., 2009; Sharafi et al., 2012). The major limitation of the culture-based method is that it requires up to seven days to obtain results, which may not be practical for situations that demand a large volume of work, fast turnover and rapid risk assessment (Boehm et al., 2009).

Vital stain method is relatively rapid compared to the culture-based method. Both methods however, require highly skilled personnel to accurately distinguish between ova/larvae of different helminths. Due to the similarities in morphological characters of ova/larvae of *A. duodenale*, *A. caninum* and *N. americanus*, it is difficult to differentiate them visually into species level (Cabaret et al., 2002; Verweij et al., 2007; Traub et al., 2008). In addition, hookworm ova recovered from raw wastewater and sludge can have a high level of foreign particles that are similar to the ova which makes quantification very difficult for trained staff (Weber et al., 1991; Barbosa et al., 2008). The detection limit of both methods depends on detection sensitivity of a microscope which may not be satisfactory particularly of low numbers of hookworm ova are present in a sample (Weber et al., 1991).

Specific detection of hookworm ova/larvae is essential to determine the accurate health risks associated with the use of wastewater and sludge as well as to establish mitigation plans. Therefore, a rapid more accurate and sensitive detection method is required. The developments in real-time PCR method has already enabled rapid, sensitive and specific detection of various microbial pathogens in faecal and wastewater samples (Verweij et al., 2007; ten Hove et al., 2009; Taniuchi, et al., 2011; Ahmed et al., 2015). PCR methods can quickly detect pathogens in a one-step closed-tube reaction within 2-4 h (Botes et al., 2013; Schar et al., 2013), thus overcoming the limitations of the incubation and staining methods. There is no PCR method currently available to detect hookworm ova from wastewater and sludge.

Therefore, the main objective of this chapter was to develop a rapid, specific and sensitive real-time PCR method to detect hookworm ova and to evaluate the applicability of the method for wastewater matrices.

4.2. Materials and methods

4.2.1. Real-time PCR positive control

A. caninum larvae were donated by Dr. Rebecca Traub from the University of Melbourne, Melbourne, Australia for the development of the real-time PCR assay. DNA was extracted from the larvae using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA was eluted in 100 µL AE buffer and concentration was measured using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technology) and found to be 39 ng per µL. The DNA was stored at -80^{o}C freezer until further used.

4.2.2. Primer and probe design

Nucleotide sequences of the 5.8S rRNA of Internal Transcribed Spacer (ITS) -1 region of *A. caninum* (NCBI accession no KC 755029.1) and *A. duodenale* (Accession no EU 344797.1) were obtained from GenBank. To identify the variation between the genes, the sequences were analysed using the NCBI Align Sequences Nucleotide Tool. A new set consisting of forward primer DHF (5´-TTT GCT AAC GTG CAC TGA ATG-3´), reverse primer DHR (5´-GAA ACA CCG TTG TCA TAC TAG CC-3´) and probe DHP (FAM-5´-AAC TCG TTG TTG CTG CTG AA-BHQ1-3´) was designed and used to amplify a 101 base pair (bp)

of the 5.8S rRNA gene of ITS-1 at the 626-726 region of *A. caninum*. The specificity of the newly designed primers was checked using NCBI Megablast as well as tested against human hookworm DNA.

4.2.3. Real-time PCR optimisation and conditions

Genomic DNA from *A. caninum* larvae was used for the optimisation of the real-time PCR assay. To ensure optimal performance of the PCR, primer concentrations ranging from 100 nM-400 nM and probe concentrations ranging from 300 nM-800 nM were titrated. The initial annealing temperature (T_m) value was chosen 2^0C lower than the Tm values provided on the primer sets (63^0C). Once the concentration of primer and probe were titrated, the annealing temperature was optimized by performing a gradient analysis ranging from 55- 61°C. The optimised real-time PCR amplifications were then performed in 25 µL reaction mixtures containing 12.5 µL iQ™ Supermix (Bio-Rad Laboratories, Calif), 250 nM of each primer, 400 nM of probe, 3 µL of template DNA and UltraPure™ DNase/RNase-free distilled water (Life Technologies, Australia). PCR cycling parameters were as follow: 95°C for 15 min, 45 cycles of 95°C for 15 s, 59°C for 1 min. For each PCR assay, positive (*A. caninum* DNA) and negative (UltraPure™ water) controls were included.

4.2.4. Real-time PCR lower limit of detection (LLOD)

The PCR LLOD was determined using the stored genomic DNA extracted earlier from the *A. caninum* larvae. Ten-fold serial dilutions (10^{-1} to 10^{-5}) of 5 ng of DNA were prepared in replicates (n = 6) and tested with PCR. The lowest quantity of DNA detected consistently in all replicate reactions within the cycle numbers used was considered as the PCR LLOD.

4.2.5. Sample limit of detection (SLOD)

To determine the SLOD, treated wastewater, raw wastewater and sludge samples were collected from two wastewater treatment plants (WWTPs) "A" (27°33'14.81"S; 152°59'29.26" E) and "B" (27°22'52.71"S; 153°08'52.33"E)] located in Brisbane, Qld, Australia. Fifteen liters of treated wastewater and raw wastewater were collected from each WWTP in sterile 20 L polypropylene carboy containers. Treated wastewater was collected prior to the chlorination stage whereas raw wastewater samples were collected from the primary influent. Dewatered sludge samples were also collected from the belt process from

each WWTP in 500 mL sterile polyethylene Zip Lock^{TM} bags. All samples were transported on ice to the CSIRO laboratory, Dutton Park, Queensland. Tap water was collected from a tap in the laboratory.

The samples (tap water, treated wastewater, raw wastewater, and sludge) were screened for the presence of *A. caninum* rRNA gene using the newly developed PCR method to obtain information on any background levels. Approximately 4.0×10^3 of *A. caninum* ova that were previously isolated from dog faeces and stored at 4°C (see section 3.3) added to 1 L tap water (control), treated wastewater and raw wastewater samples. Ten-fold serial dilutions (10^{-1} to 10^{-4}) were made for each sample. For the sludge experiment, 10-fold serial dilutions (10^{-1} to 10^{-4}) of 4.0×10^3 ova were made and added to approximately 4 *g* of samples. All samples were processed in triplicate according to the concentration method described below (in section 4.2.6). A method blank of the unseeded sample was included for each batch of tap water, treated and raw wastewater samples to check for cross contamination during sample processing.

4.2.6. Sample concentration and DNA extraction

Sample concentration varied depending on the solids present in the samples. A flow chart of sample concentrations is shown in Figure 4.1.

Initial concentration methods for tap water and treated wastewater samples involved filtering through 8 μm, 90 mm polycarbonate filters (Merck Millipore, Billerica, Massachusetts, USA) using glass funnels attached to a vacuum pump. The trapped ova were washed from the filters using 25 mL phosphate buffer saline (PBS) into a 50 mL polypropylene tube. The ova were then pelleted from the PBS suspension by centrifuging at $800 \times g$ for 15 min.

For raw wastewater, initial attempts to process 1L with the membrane filtration method failed due to rapid clogging of the filters by suspended solid materials in the wastewater. Subsequently, a centrifugation and flotation approach was used to concentrate the ova from raw wastewater samples. The final method involved transferring raw wastewater samples (1 L) into 700 mL centrifuge containers (Beckman Coulter Inc), and centrifuging at 5,200 × *g*

for 30 min. The remaining volume (300 mL) was added after discarding the supernatant and centrifuged again at 5,200 × *g* for 30 min.

The resulting supernatant was discarded and the pellet was resuspended in 20 mL MilliQ water and transferred to a 50 mL polypropylene centrifuge tube and centrifuged at 5,200 × *g* for 10 min to obtain a pellet. Ova were then separated from the pellet with the $MgSO_4$ (specific gravity 1.2) floatation method outlined previously by Bowman et al., (2003). In brief, the pellet was suspended in 45 mL of $MgSO_4$ solution by vortexing in a centrifuge tube. The suspension was then centrifuged at 800 × *g* for 3 min and the supernatant was transferred to a 15 mL polypropylene tube. This was then further centrifuged at 800 × *g* for 10 min to obtain a pellet containing the *A. caninum* ova.

A similar approach was also followed for the sludge. In brief, sludge samples (4 *g* dry weight) were placed into a 50 mL polypropylene tube and 45 mL of $MgSO_4$ solution was added. The tube was vortexed followed by centrifugation at 800 × *g* for 3 min and the supernatant was transferred into a 15 mL polypropylene tube, and further centrifuged at 800 × *g* for 10 min to obtain a pellet.

DNA from the concentrated hookworm ova (pellet) in the tap water and treated wastewater (WWTP-A) samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with a slight modification. In brief, pellets from the centrifugation step were mixed with 180 µL of lysis buffer ATL followed by five freeze -thaw cycles of 10 min each at -80ºC (standard freezer) and 95ºC (hot water bath). Due to the presence of high suspended solid contents and initially observed PCR inhibition in the treated wastewater from WWTP-A, the Mo Bio Power Soil DNA Kit (Mo Bio, Carlsbad, CA, USA) was used to extract DNA from all subsequent WWTP-A and WWTP-B samples including sludge samples. In this case, pellets from the centrifugation step were mixed with 60 µL of lysis buffer C1 followed by five freeze-thaw cycles of 10 min each. A reagent blank was also included during DNA extraction.

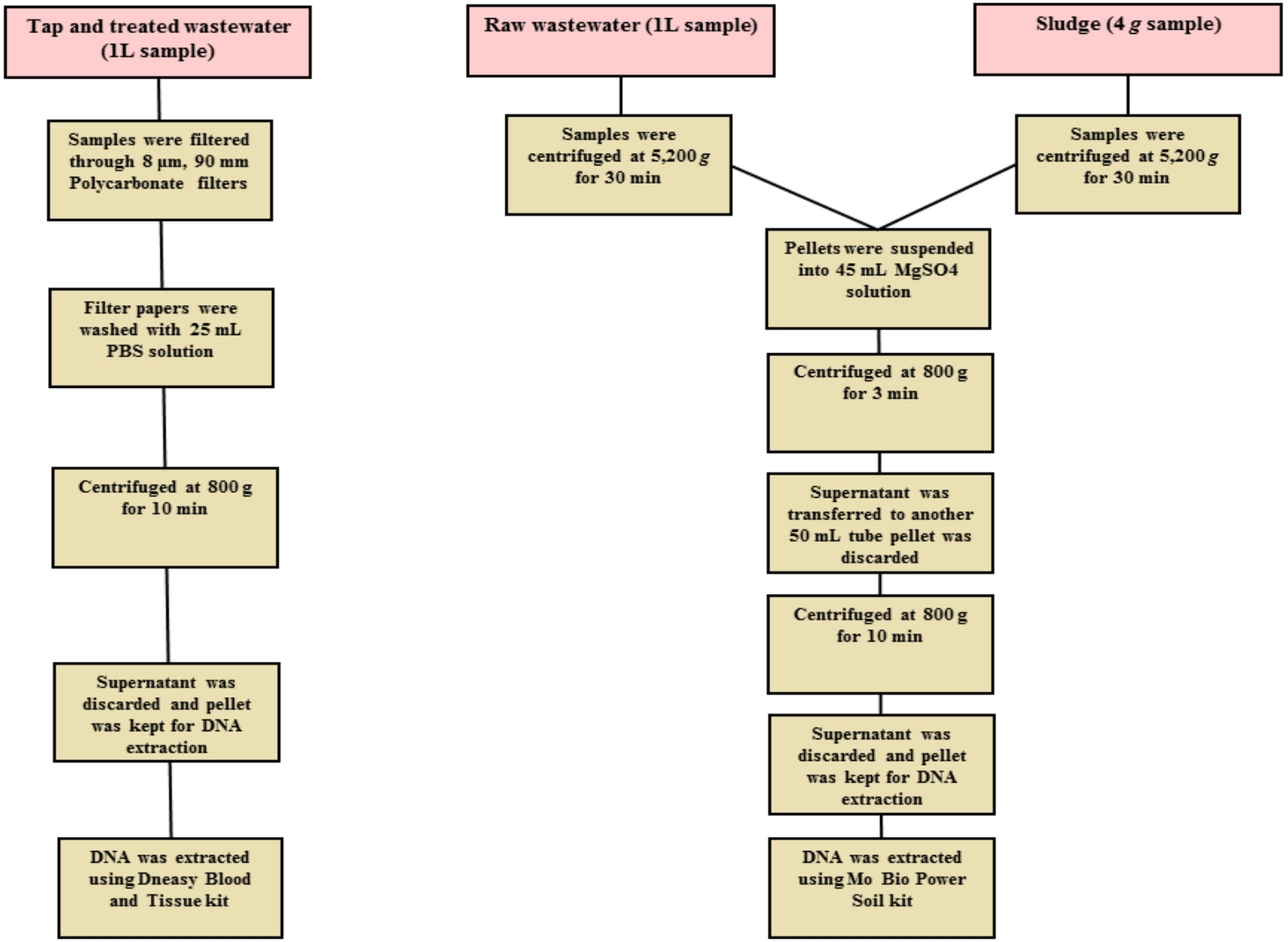

Figure 4.1: Sample concentration methods for tap water, treated wastewater, raw wastewater, and sludge samples

4.2.7. Application of the real-time PCR method

In addition to the seeded samples, the applicability of the developed real-time PCR method was tested for unseeded tap water (n = 3), treated wastewater (n = 12), raw wastewater (n = 18), and sludge (n = 6) samples. Tap water samples were collected for a tap located into the laboratory. Treated wastewater, raw wastewater, and sludge samples were collected from two different WWTPs in different time intervals. Sample concentration and DNA extraction were conducted as described above.

4.2.8. PCR inhibition test

A sketa22 real-time PCR assay with previously published primers and the probe was used to determine the presence of inhibitors in the DNA samples extracted from tap water, treated wastewater, raw wastewater and sludge samples (Haugland et al., 2005). All DNA samples were seeded with 10 pg of chum salmon (*Oncorhynchus keta*) DNA (Ahmed et al., 2015). The threshold cycle (C_T) values of the *O. keta* seeded DNA samples were compared to the equivalent quantities of *O. keta* DNA suspended in UltraPure™ water (Life Technologies). Where inhibition was detected, the DNA extracts were diluted 10 fold and retested. The sketa22 PCR assay was performed in 25 µL reaction mixtures using iQ™ Supermix (Bio-Raid Laboratories). The sketa22 PCR assay mixture contained 12.5 µL of Supermix, 300 nM of each primer, 400 nM of probe, 10 pg of *O. keta* DNA and 3 µL of template DNA sample.

4.2.9. Statistical analysis

GraphPad Prism 6 (GraphPad Software) 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* contaminated DNA samples extracted from tap water and wastewater matrices samples. ANOVA was also used to determine whether the C_T values obtained for seeded and unseeded treated wastewater, raw wastewater and sludge samples varied significantly within and between WWTPs as well as tap water samples.

4.3. Results

4.3.1. Real-time PCR optimisation and LLOD

The specificity of the newly designed primers was checked using NCBI Megablast. The results of the BLAST search showed that the sequences of selected primers matched with the rRNA of the ITS-1 region of *A. caninum*. The PCR result indicated that the newly developed primer sets does not cross react with human hookworm DNA.

Among the series of primer and probe concentrations tested at an initial annealing temperature of 61°C, 250 nM of each primer and 400 nM of the probe provided the earliest C_T value with the smallest standard deviation (29.3 ± 0.1). The mean and standard deviation of C_T values for different concentrations tested primers and probes can be seen in appendix C. The optimized primer and probe concentration was found to produce the best C_T value (22.2 ± 0.07) at the optimised annealing temperature of 59°C. The LLOD of the real-time PCR assay was performed using genomic DNA from *A. caninum* larvae. The PCR assay was able to detect *A. caninum* DNA up to a dilution 10^{-4} (equivalent to 500 fg DNA) (Table 4.1).

Table 4.1: Real-time PCR lower limit of detection (LLOD) of serially diluted *A. caninum* genomic DNA (*n* = 6)

DNA dilutions	Positive samples (%)	Mean ± SD of C_T values
10^{-1}	100	25.6 ± 0.04
10^{-2}	100	29.1 ± 0.04
10^{-3}	100	32.4 ± 0.09
10^{-4}	100	36.6 ± 0.10
10^{-5}	0	ND

C_T: Threshold cycle
ND: Real-time PCR not detected
SD: Standard deviation

4.3.2. PCR inhibitions

The mean C_T value and standard deviation for the *O. keta* seeded UltraPure™ water was 27.8 ± 0.36. The mean C_T values and standard deviations values for *O. keta* seeded tap water, treated wastewater, raw wastewater, and sludge DNA samples were similar to *O. keta* seeded UltraPure™ water (Table 4.2). The treated wastewater DNA samples from WWTP-A, and sludge DNA samples from both WWTPs did not amplify *O. Keta* DNA, indicating the

presence of PCR inhibitors. These DNA samples were serially diluted to reduce any PCR inhibitors then re-analysed. The mean C_T values and standard deviations value for *O. keta* seeded diluted (10 fold) treated wastewater and sludge samples were 27.9 ± 0.21 and 28.5 ± 0.54 (WWTP-A) and 28.1 ± 0.11 (WWTP-B), respectively. An ANOVA analysis on the C_T values obtained for *O. keta* seeded UltraPure™ water and all the undiluted DNA samples that were free of PCR inhibitors as well as the 10 fold diluted samples showed that there was no significant ($P > 0.05$) difference suggesting the absence of PCR inhibitors. Based on these results, all the samples without PCR inhibition (undiluted and 10-fold diluted samples) were used for the PCR assays.

Table 4.2: Sketa22 PCR assay for the evaluation of PCR inhibition in *A. caninum* ova seeded samples

Sample sources	Sample types	DNA extraction kit used	Mean ± SD of C_T values	
			Undiluted DNA	Diluted DNA (10^{-1})
WWTP-A	Treated wastewater	DNeasy Blood and Tissue	NPA	27.9 ± 0.21
	Raw Wastewater	Mo Bio Power Soil	27.7 ± 0.45	N/A
	Sludge	Mo Bio Power Soil	NPA	28.5 ± 0.54
WWTP-B	Treated wastewater	Mo Bio Power Soil	27.5 ± 0.30	N/A
	Raw Wastewater	Mo Bio Power Soil	27.5 ± 0.23	N/A
	Sludge	Mo Bio Power Soil	NPA	28.1 ± 0.11
	Tap water	DNeasy Blood and Tissue	28.5 ± 0.12	N/A
	Distilled water	N/A	27.8 ± 0.36	N/A

NPA: No PCR amplification
N/A: Not applicable
C_T: Threshold cycle
SD: Standard deviation

4.3.3. Sample limit of detection (SLOD)

The real-time PCR method indicated that the background of the tap water treated wastewater, raw wastewater and sludge samples used for seeding experiments were free from *A. caninum* rRNA. The method was able to detect *A. caninum* ova at a dilution of 10^{-4} (< 1 ovum) for ova seeded tap water samples (Table 4.3). Similar results were also obtained for ova seeded treated wastewater from both WWTPs. The SLOD of *A. caninum* ova for the seeded raw wastewater and sludge samples from both WWTPs was at a dilution of 10^{-3} (4 ova) indicating a lower detection limit of *A. caninum* ova in these matrices compared to treated wastewater.

Table 4.3: Sample limit of detection (SLOD) of real-time PCR method in samples seeded with *A. caninum* ova

Source of samples	Wastewater matrices	Triplicate PCR results at the dilutions tested			
		10^{-1} (400 ova)	10^{-2} (40 ova)	10^{-3} (4 ova)	10^{-4} (< 1 ova)
CSIRO Lab	Tap water	+	+	+	+
WWTP-A	Treated wastewater	+	+	+	+
	Raw wastewater	+	+	+	-
	Sludge	+	+	+	-
WWTP-B	Treated wastewater	+	+	+	+
	Raw wastewater	+	+	+	-
	Sludge	+	+	+	-

+: Real-time positive results
-: Real-time negative results

The range of mean C_T values obtained for the *A. caninum* ova seeded tap water samples was < treated wastewater < raw wastewater < sludge samples from both WWTPs (Figure 4.2a and b). The C_T values of tap water samples were significantly different ($P < 0.05$) than treated wastewater from WWTP-A and raw wastewater and sludge samples from both WWTPs. Significant differences in C_T values were also observed for the treated wastewater with raw wastewater and sludge samples for both WWTPs. However, the C_T values of raw wastewater and sludge did not differ significantly ($P > 0.05$) from each other for both WWTPs. The C_T values of the treated wastewater, raw wastewater and sludge samples (for WWTP-A) were higher than those obtained for WWTP-B. However, these differences were not statistically significant ($P > 0.05$).

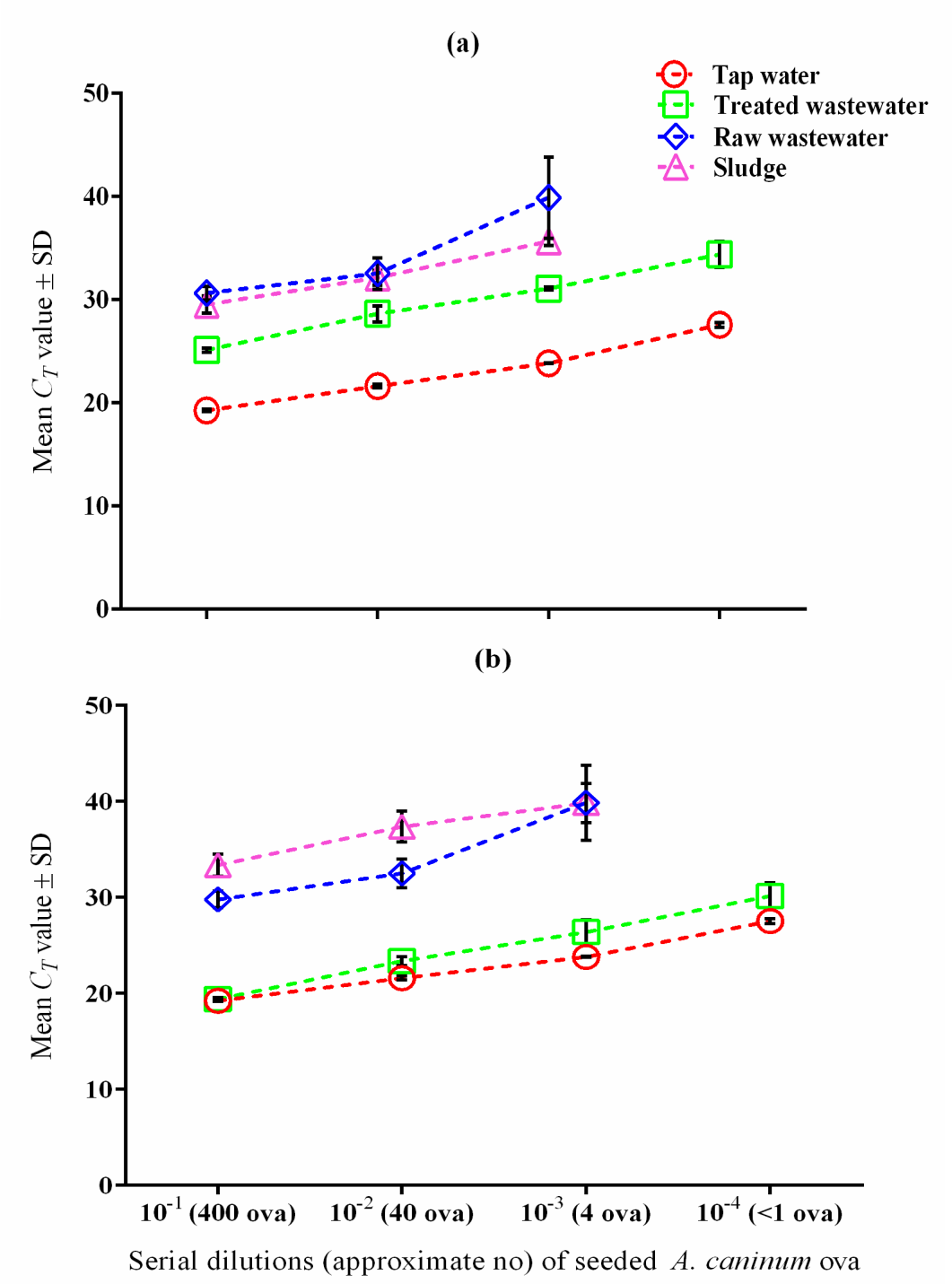

Figure 4.2: Mean and standard deviation of C_T value of serially diluted *A. caninum* ova seeded into tap water and different wastewater samples collected from treatment plants A (a) and B (b)

4.3.4. Application of the real-time PCR method to detect *A. caninum* ova in unseeded samples

Tap water samples were free from *A. caninum* DNA. *A. caninum* DNA was detected in treated wastewater (50%), raw wastewater (38%) and sludge (33.3%) samples (Figure 4.3). Although more treated wastewater was positive for *A. caninum* DNA, the treated wastewater had the higher C_T values (C_T = 36.9 ± 0.80) followed by raw wastewater (C_T = 35.6 ± 2.43) and sludge samples (C_T = 34.9 ± 2.43). This means that the raw wastewater and sludge samples had higher variations in numbers of ova than treated wastewater samples.

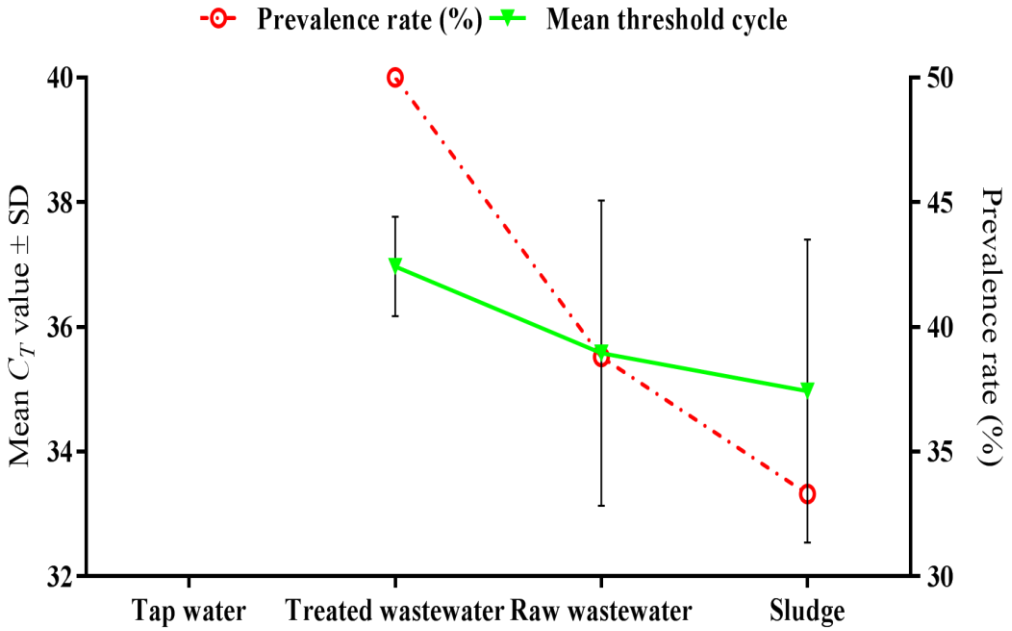

Figure 4.3: Prevalence of *A. caninum* DNA in unseeded tap water (*n* = 3), treated wastewater (*n* = 12), raw wastewater (*n* = 18) and sludge (*n* = 6) samples collected from different wastewater treatment plants (WWTPs)

4.4. Discussion

The application of PCR-based methods has generated interest for the direct monitoring of parasites in faecal samples (Verweij et al., 2007; Yong et al., 2007; Traub et al., 2008; Ngui et al., 2012; Schar et al., 2013). PCR methods are rapid and can be used to detect specific parasites of interest with high sensitivity. Detection of helminths ova from wastewater matrices, however, requires isolation and concentration of the ova, which is often challenging due to the presence of high suspended solids and PCR inhibitors. In this study, a probe-based

real-time PCR method was developed for the rapid, sensitive and specific detection of canine hookworm (*A. caninum)* ova from wastewater matrices.

The sensitivity of the real-time PCR assay was thoroughly tested by amplifying a known concentration (500 pg to 50 fg) of genomic DNA obtained from *A. caninum* larvae. The PCR LLOD of the newly developed assay was determined to be 500 fg of genomic DNA for all replicate samples which is similar or one order of magnitude lower than the LLOD values for different helminths reported in previous studies (Thaenkham et al., 2007; Traub et al., 2009; Rahman et al., 2011; Taniuchi et al., 2011; Ngui et al., 2012).

This study also determined the effects of PCR inhibitors on the detection of *A. caninum* ova in wastewater matrices. Our results indicated that DNA samples from the treated wastewater of one wastewater treatment plant (from WWTP-A) had PCR inhibitors present. The DNeasy Blood and Tissue Kit was used to extract DNA from these samples, and it is possible that the kit was unable to remove the inhibitors effectively. In view of this, Mo Bio Power Soil DNA Kit was used for DNA isolation from the remaining wastewater and sludge samples. The main advantage of the Mo Bio Power Soil DNA Kit over the DNeasy Blood and Tissue Kit is the ability to remove humic substances and other inhibitors. This is supported by the fact that no PCR inhibitors were detected in wastewater samples from WWTP-B and raw wastewater samples from both WWTPs when DNA was extracted by using this kit. However, sludge DNA samples from both WWTPs indicated the presence of PCR inhibitors despite the use of Mo Bio Power Soil DNA Kit. This suggests that the concentration of PCR inhibitors in sludge samples was higher than raw wastewater samples as reported by (Schrader et al., 2012).

Serial dilution of DNA is the strategy commonly applied in environmental, clinical, food samples to overcome PCR inhibition (Drosten et al., 2002; Audemard et al., 2004; Van Doorn et al., 2009). Our results indicated that a 10 fold dilution of those samples showing PCR inhibition was adequate to remove the inhibitors. Based on the result, it is suggested that DNA samples extracted from large volumes of wastewater matrices or complex environmental samples should be checked for the presence of PCR inhibitors prior to use for PCR amplification.

The PCR method used in this study was capable of detecting *A. caninum* ova in a range of <1 in tap water and treated wastewater (1L) and < 4 in raw wastewater (1L) and sludge (4 g) across all wastewater matrices from both WWTPs unless PCR inhibition is present. These results were consistent for all dilutions across the both WWTPs. The earlier C_T values for the treated wastewater indicated the better recovery of ova from treated wastewater than raw wastewater and sludge samples. This could be attributed to the low suspended solid content of treated wastewater making it possible to pass through the membrane using the membrane filtration method. It has been previously reported that ova were better retained on the membranes during filtering of water samples compared to a potential loss of ova during floatation (Nieminski et al., 1995; Ferguson et al., 2004). Because of the high solid contents and turbidity, the membrane filtration method was not suitable for processing raw wastewater and sludge samples. In view of this, the flotation technique was used for concentrating ova from raw wastewater and sludge samples. Another important point to consider is that the flotation technique involves multiple steps of centrifugation, flotation and concentration with the potential loss of ova in each step compared to the membrane filtration method, which involves a single step for recovering the ova directly from the filters.

The newly developed real-time PCR method was validated by testing of tap water, treated wastewater, raw wastewater and sludge samples from two different WWTPs. The method was able to detect low levels of *A. caninum* DNA from all three matrices. The earlier C_T values (Figure 4.3) obtained for the sludge and raw wastewater samples than treated wastewater suggest the presence of relatively more DNA in the sludge and raw wastewater samples despite the potential poor recovery of the flotation method. This indicates that the developed method could be easily adapted by designing species-specific primers to detect other pathogenic helminth ova that have a public health concern such as *A. duodenale, N. americanus, Ascaris lumbricoides* from wastewater matrices and other environmental samples.

A significant challenge still remains to develop a rapid and effective ova recovery method for helminths from raw wastewater and sludge. This requires further research to develop and validate new rapid concentration methods. For instance, hollow-fibre ultrafiltration has been shown to recover up to 83% *Giardia* and *Cryptosporidium* oocysts from environmental waters (Kuhn and Oshima, 2002; Hill et al., 2009). It is, therefore, recommended that concentration methods such as hollow-fibre ultrafiltration need to be compared with

membrane filtration and flotation methods for ova recovery efficiency. Although improvement of ova recovery rate was not a part of this thesis, it was noted the importance of rapid ova recovery method to be the success of PCR method. Therefore, the performance characteristic of various recovery methods was evaluated in Chapter 6.

4.5. Conclusions

In conclusion, a real-time PCR method was successfully developed for rapid, sensitive and specific detection of canine hookworm (*Ancylostoma caninum)* ova from wastewater matrices (liquid and solid). The newly developed PCR method has high detection sensitivity with the ability to detect less than one *A. caninum* ova from 1 L of treated wastewater. The method is also able to detect four *A. caninum* ova from 1 L of raw wastewater and from ~ 4 *g* of treated sludge, respectively. The better detection sensitivity obtained for treated wastewater compared to raw wastewater and sludge samples could be a matrix issue given the higher concentration of suspended solid particles in raw wastewater and sludge compared to treated wastewater. The developed method is rapid, sensitive and specific compared to traditional methods and has the potential to aid in the public health risk assessment associated with land application of wastewater matrices. Since the morphology of STHs ova is similar to the *A. caninum*, it is anticipated that the method could be adapted to detect other pathogenic helminth ova such as *A. duodenale, N. americanus* and *A. lumbricoides* from wastewater matrices. Despite the rapidness, specificity, and high sensitivity, the real-time PCR method is unable to quantify the numbers of hookworm ova from treated wastewater and sludge which is necessary to assess public health risks. Quantitative (q)PCR however, has been used to quantify various pathogens from wastewater and sludge. Therefore, real-time PCR method requires upgrading to qPCR and attempt to quantify hookworm ova from wastewater matrices.

Chapter: 5

Quantification of hookworm ova

Quantification of hookworm ova using qPCR

5.1. Introduction

Recent development in real-time PCR method can enable rapid, specific and sensitive detection of *Ancylostoma caninum* (dog hookworm) ova from wastewater matrices (Gyawali et al., 2015a). The detection sensitivity of the method was determined to be < 1 ova per L of treated wastewater, < 4 ova per L of raw wastewater and < 4 ova per 4 *g* of sludge. The results can be obtained within few hours compared to the incubation method that requires up to few weeks. However, to assess the magnitude of the public health risks from hookworm ova present in the wastewater and sludge need to quantify the targeted gene.

Quantitative PCR (qPCR) has already been used to quantify different types of pathogens from environmental samples (Shanks et al., 2008; Ahmed et al., 2014; Ahmed et al., 2015). However, no information is available on the application of qPCR-based methods to quantify hookworm ova from such samples. A handful of studies attempted to quantify *Ascaris* ova using qPCR in the laboratory setting (Pecson et al., 2006; Raynal et al., 2012). For instance, Pecson and colleagues (2006) created profiles of the ITS-1 rDNA and rRNA levels during the development of *Ascaris* ova from single cells to mature larvae.

However, hookworm ova present in the faecal samples may contain 4 to 8 cell stages, and the ova multiply rapidly under suitable conditions. In addition, it is highly likely that wastewater and sludge samples may contain mixed population (early to late cell staged) of hookworm ova. Therefore, estimating gene copy numbers according to the Pecson et al., (2006) and Raynal et al., (2012) may not yield an accurate result. Therefore, determining the average gene copy numbers for an ovum from a mixed population may be appropriate.

In view of this, work was undertaken to convert the real-time PCR method developed in Chapter 4 to a qPCR method. The successful development of a qPCR method will assist to determine the magnitude of health risks associated with the use of treated wastewater, raw wastewater and sludge for agricultural purposes.

5.2. Materials and methods

5.2.1. Development of qPCR method

A DNA standard involving 101 base pairs of the 5.8S rRNA gene from the ITS-1 region of *A. caninum* plasmid DNA sequence (TTTGCTAACGTGCACTGAATGACAGCAAACTCG TTGTTGCTGCTGAATCGTTTACCGACTATAAAACGTTTTGGCAGTGGCTAGTAT GACAACGGTGTTTC) was designed. This DNA segment was synthesised by Integrated DNA Technologies (IDT) (IDT Technology, USA). Of on receipt, 100 µL UltraPure™ water was added to the tube to obtain 40 ng per/µL of plasmid DNA. Gene copy numbers were calculated by multiplying the DNA concentration by Avogadro's number and dividing by the product of the plasmid size (bp) and an average weight of a base pair (Yun et al., 2006). Serial dilutions were prepared ranging from 10^5 to 10^0 gene copies per µL and served as the standards for the qPCR method. The qPCR method performance criteria such as efficiency (E), slope, intercept, R^2 and lower limit of quantification (LLOQ) were determined by analysing standard curves over the course of the study. The qPCR assay was performed using previously designed and optimized primers and probe (Chapter 4). A Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA) was used to run the thermal cycler program described in Chapter 4. All qPCR reactions were performed in triplicate.

5.2.2. qPCR reproducibility and lower limit of quantification (LLOQ)

The reproducibility of the qPCR assay was assessed by determining the intra-assay repeatability and inter-assay reproducibility. The coefficient of variation (CV) of the assay was calculated by analysing the DNA standards described above. The intra-assay repeatability was calculated based on the quantification cycle (C_q) value by testing the DNA standard series in the same experiment (n = 6). The inter-assay reproducibility was calculated based on the C_q value of standard series on six different days (n = 6). The qPCR lower limit of quantification (LLOQ) value was determined from the DNA standard series in replicates (n = 6) at 95% confidence level. The DNA detected in the lowest dilution consistently for all DNA standard series ($3 \times 10^5 – 3 \times 10^0$ gene copies) were considered as the qPCR LLOQ.

5.2.3. Estimation of gene copy numbers per ova

To estimate the gene copy numbers in a single *A. caninum* ovum, known numbers of ova (5 ± 3, 50 ± 18 and 500 ± 27 ova) were used in replicates (n = 10). DNA was extracted directly from these 30 samples using a Mo Bio Power Soil DNA Extraction Kit (Mo Bio, Carlsbad, CA) as described in Chapter 4. The number of gene copies in each DNA sample was determined using the qPCR assay described above (section 5.2.1). The qPCR estimated gene copy numbers were then divided by corresponding ova numbers to obtain gene copy numbers per ovum. Based on the information available in the literature (Pecson et al., 2006; Raynal et al., 2012) that different cell staged ova produced different gene copy numbers and *A. caninum* ova are excreted in the dog faeces present in the fresh dog faeces may contain 4 to 8 cell staged a minimum, average and maximum numbers of gene copies for an ovum were determined.

5.2.4. Seeding experiment for qPCR accuracy

A seeding experiment was conducted to determine the accuracy of the qPCR method. To conduct the seeding experiment, 20 L treated wastewater (before chlorination) was collected in sterile 2 × 10 L polypropylene carboys from a wastewater treatment plant (WWTP A) located in Brisbane, Queensland, Australia. The samples were transported on ice to the laboratory. The sample (1 L) was screened for the presence of background *A. caninum* ova using the real-time PCR assay described in Chapter 4. As no *A. caninum* DNA was detected in the treated wastewater sample, approximately, 100 ± 21, 10 ± 2 and 1 ± 1 *A. caninum* ova were seeded into 1 L of treated wastewater samples in triplicate. Ova from treated wastewater samples were concentrated using the filtration method as described in Chapter 6 and DNA was extracted from each concentrated sample using a Mo Bio Power Soil DNA Kit described in Chapter 4. All the extracted DNA samples were evaluated for PCR inhibitions using Sketa 22 PCR described in Chapter 4. Since the DNA samples were free from PCR inhibitions (appendix D), the qPCR analysis was performed.

5.2.5. Conversion of qPCR estimated gene copies to ova numbers

The gene copy numbers estimated by qPCR for ova seeded wastewater samples were converted to ova by using equation 1.

$$\mathrm{N}(O) = \frac{\mathrm{N}(GC_S)}{\mathrm{N}(GC_O)} \qquad (1)$$

N (O) = Numbers of ova

N (GC_s) = Gene copy numbers for wastewater samples estimated by qPCR

N (GC_O) = Gene copy numbers for an ovum (minimum, average and maximum) estimated by qPCR

5.2.6. Validation of qPCR method on unseeded wastewater samples

The qPCR method was also applied to quantify the *A. caninum* ova in unseeded treated wastewater (n = 4), raw wastewater (n = 10) and sludge (n = 6) samples collected from three different WWTPs (A, B, and C) across Southeast Queensland, Australia. One L of treated wastewater and raw wastewater samples were collected from each WWTP in sterile 1 L Schott Glass Bottles. Treated wastewater was collected at the point of discharge into the environment, whereas, raw wastewater sample was collected from the influent. Sludge samples were collected in a 50 mL sterile polyethylene tube from the belt (dewatering) press as well as the sludge holding pond. All samples were transported on ice to the laboratory and processed within 48 h.

Ova from treated wastewater samples were concentrated using the filtration method, however, raw wastewater and sludge samples were processed using the flotation method described in Chapter 6. DNA was extracted from all the concentrated samples using a Mo Bio Power Soil DNA Kit described in Chapter 4. All the extracted DNA samples were evaluated for PCR inhibitions using Sketa 22 PCR described in Chapter 4. Since the DNA samples were free from PCR inhibitions (appendix D), the qPCR analysis was performed. The qPCR estimated gene copy numbers were converted to the ova numbers as described in earlier (5.2.5) section.

5.2.7. Statistical analysis

GraphPad Prism 6 (GraphPad Software, USA) was used to perform the statistical analysis and produce graphs. An analysis of variance (ANOVA) was performed to determine the minimum, average and maximum numbers of ova estimated by qPCR for 1 ± 1, 10 ± 2 and 100 ± 21ova. A t-test for equal means was performed to determine the differences between ova estimated by qPCR and seeded ova in treated wastewater.

5.3. Results

5.3.1. qPCR standard, reproducibility, and LLOQ

qPCR DNA standards were analysed to determine the reaction efficiencies. The DNA standards had a linear range of quantification from 3×10^5 - 3×10^1gene copies per 3 µL of plasmid DNA. The efficiency, slope of the DNA standards, correlation coefficient (r^2), and intercept ranged from 97.3% to 102.2%, -3.28 to -3.39, 0.993 to 0.998 and 33.96 to 35.23, respectively. The qPCR amplification output for *A. caninum* standards can be seen in Figure 5.1. 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 (Table 5.1).

Table 5.1: The intra - assay and inter - assay coefficient of variation (CV) for the qPCR method of *Ancylostoma caninum* ITS-1 rDNA gene

Dilution series of DNA standards	Coefficient of variation (%)	
	Inter - assay	Intra - assay
10^5	0.30	0.05
10^4	0.50	0.30
10^3	0.30	0.30
10^2	0.70	0.20
10^1	0.60	0.30

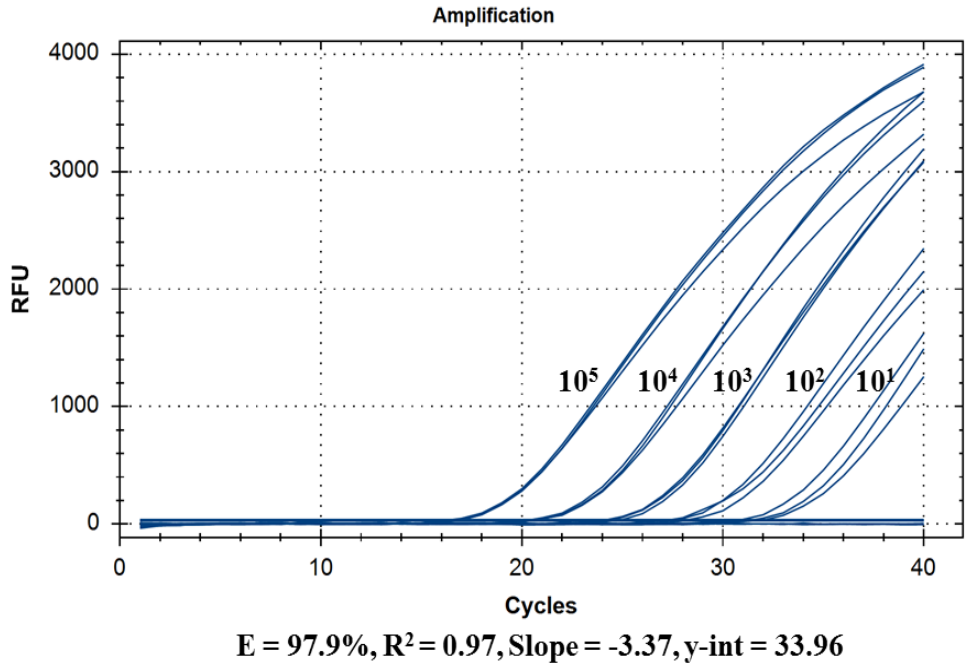

Figure 5.1: qPCR amplification output for *A. caninum* DNA standards ($3 \times 10^5 - 3 \times 10^1$) gene copy numbers

5.3.2. qPCR estimation of gene copy numbers for an ovum

The qPCR estimated gene copy numbers for 5 ± 3 *A. caninum* ova ranged from 2.8×10^3 to 1.9×10^4. Similarly, the gene copy numbers for 50 ± 18 and 500 ± 27 ova ranged from 1.7×10^5 to 7.6×10^5 and 1.4×10^6 to 5.1×10^6 (Figure 5.2). The gene copy numbers obtained for 5 ± 3 ova had observed high variations in gene copy numbers within the replicate samples compared to samples containing 50 ± 18 and 500 ± 27 ova. The qPCR estimated gene copy numbers for an ovum range from 5.6×10^2 to 1.0×10^4 with an average of 3.7×10^3.

5.3.3. qPCR estimated *A. caninum* ova in seeded wastewater samples

The qPCR estimated gene copy numbers for 1 ± 1, 10 ± 2 and 100 ± 21 ova that were seeded in wastewater ranged from 0.4 - 7.4, 2.5 - 45 and 24 - 440 with an average of 1.1, 8.6 and 67.3 ova, respectively (Figure 5.3). The difference between minimum, average and maximum ova that were estimated by qPCR was statistically significant ($P < 0.05$). The t-test for equal means indicated that there was a significant difference between seeded ova and qPCR estimated ova for 100 ± 21 and 1 ± 1 ova ($P < 0.05$). However, for 10 ± 2 ova, no significant ($P > 0.05$) difference was observed.

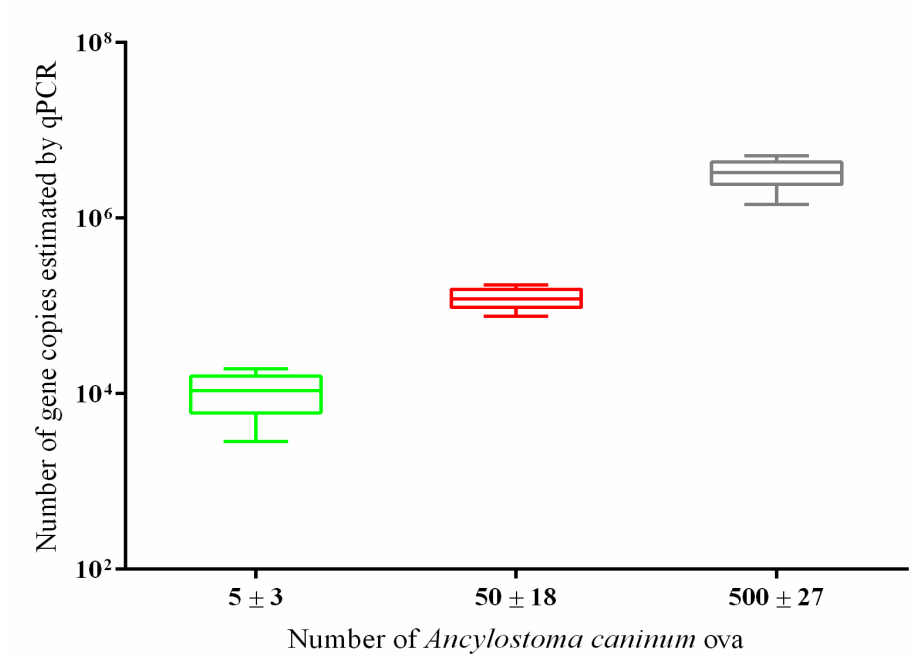

Figure 5.2: Box and whisker plots of the number of ITS-1 rRNA gene copy estimated by qPCR from 5 ± 3, 50 ± 18 and 500 ± 27 *A. caninum* ova. The inner box lines represent the mean while the outer box lines represent 5th and 95th percentiles (*n* = 10)

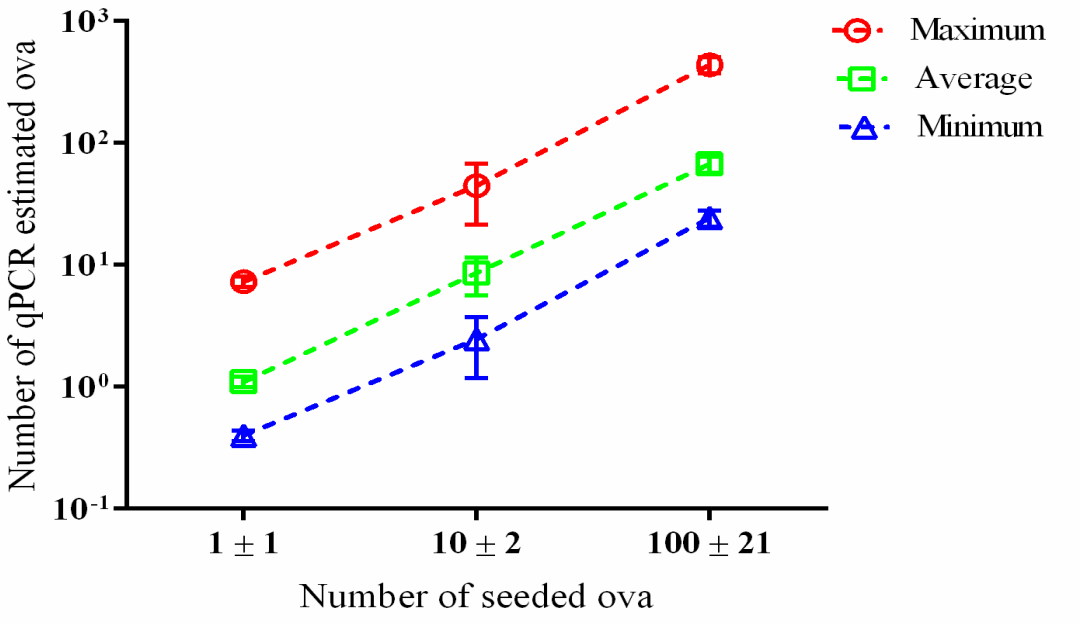

Figure 5.3: Mean and standard deviation of maximum, average and minimum ranges of qPCR estimated ova from 1 ± 1, 10 ± 2 and 100 ± 21 *A. caninum* ova seeded into treated wastewater (1L) (*n* = 10)

5.3.4. Numbers of ova in non-seeded wastewater samples

The qPCR method indicated that *A. caninum* DNA was present in 50% treated wastewater, 90% raw wastewater, and 67% sludge samples (Table 5.2). Converting the qPCR estimated gene copies to the ova numbers indicated that treated wastewater (1 L), raw wastewater (1 L) and sludge samples (4 *g*) had an average of 0.02, 1.24 and 67.0 ova, respectively. The minimum and maximum range of qPCR estimated ova in treated wastewater, raw wastewater, and sludge samples were 0.007 - 0.14, 0.45 - 8.16 and 24.2 - 438, respectively (Table 5.2).

Table 5.2: Mean and standard deviation of minimum, average and maximum range of *Ancylostoma caninum* ova in non-seeded treated wastewater (*n* = 4), raw wastewater (*n* = 10) and sludge (*n* = 6) samples from different wastewater treatment plants across Brisbane.

Sample matrices	No of positive/ tested samples	Ova recovery rate (%)	Mean and standard deviation of qPCR estimated ova		
			Minimum	Average	Maximum
Treated wastewater	2/4	43.4	0.007 ± 0.001	0.02 ± 0.01	0.14 ± 0.07
Raw wastewater	9/10	7.2	0.45 ± 0.84	1.24 ± 2.32	8.16 ± 15.2
Sludge	4/6	0.02	24.2 ± 41.7	67.0 ± 115	438 ± 754

5.4. Discussion

Hookworm ova have been detected frequently in wastewater and sludge samples around the world (Mahvi and Kia, 2006; Jimenez et al., 2007; Wichuk and McCartney, 2007; Konate et al., 2013). Guidelines have been developed to minimise the risk of hookworm infections associated with wastewater and sludge reuse (US EPA, 2003; NRMMC, 2004; WHO, 2006). Due to the lack of a universal protocol for the quantification, hookworm ova have been quantified using either culture-based (US EPA, 2003) or vital stain (de Victorica and Galvan, 2003) methods. Both quantification methods rely on microscopic observation, which is not sensitive enough to detect low numbers of ova in wastewater and sludge samples (Weber et al., 1991).

A real-time PCR detection method was developed (Chapter 4). The method is specific and sensitive for the detection of hookworm ova compared to microscopy (Gyawali et al., 2015a). However, real-time PCR method provides only presence and absence information, which is not sufficient to estimate the magnitude of health risks. A qPCR method on the other hand

simultaneously detects and quantifies pathogens on the basis of amplified gene copy numbers present in the target DNA/RNA (Botes et al., 2013). However, these qPCR generated gene copy numbers need to be converted to the numbers of pathogen to determine the health risks. This information is critical for the successful application of qPCR for utility providers and health regulators. However, this information for hookworm ova was not available in the literature. Therefore, direct DNA was extracted from different numbers of *A. caninum* ova (5 ± 3, 50 ± 18 and 500 ± 27) previously isolated from various dog faecal samples in different time. This was done to capture the variability of different cell staged ova that may be present in different dog faecal samples. In this study, the estimated gene copy numbers for a single ovum ranged from 5.6×10^2 to 1.0×10^4 with an average of 3.7×10^3. Such a high variation in gene copy numbers has also been reported for the *Ascaris suum* (Pecson et al., 2006; Raynal et al., 2012). According to these authors, a single *A. suum* ovum can have 1 to 600 cells depending on the development stage which provides 32 to 42 (1 cell stage) - 1.9×10^4 to 2.5×10^4 (600 cells stage) gene copies.

Apart from variations in gene copy numbers associated with cell numbers per ovum, there are other potential sources of this variation. For example, DNA extraction process, that can be a source of variation in gene copy numbers since commercially available DNA extraction kits may not yield 100% DNA. Loss of DNA during extraction is common, and may cause inability to capture all potential PCR signals from a sample that has low numbers of ova (Kishore et al., 2006; Colussi et al., 2009). Another factor that can have an impact on qPCR results is that the structure of ova. The cell wall of an ovum contains three layers that may be difficult to lysis during the DNA extraction process, especially when extracting DNA from a large number of ova compacted in an extraction tube. Therefore, further study should investigate the DNA extraction efficiency of the DNA extraction kit prior to adopting a qPCR method for monitoring purposes.

Such high variations in gene copy number per ovum indicated that conversion of qPCR estimated gene copy numbers to hookworm ova may not be accurate. To circumvent this issue, a range (minimum, average and maximum) of gene copies per ovum was determined which may yield meaningful semi-quantitative results. The minimum (5.6×10^2 gene copies), average (3.8×10^3 gene copies) and maximum (1.0×10^4 gene copies) values indeed suggested the presence of variable stages of *A. caninum* ova in our stock ova solution. This approach

taken in this study mimics the real world scenario, where it is highly likely that ova containing different numbers of cells may be present in a wastewater sample.

Despite the difficulty in estimating the gene copy number for one ovum, the qPCR assay estimated ova for 1 ± 1, 10 ± 2 and 100 ± 21 were 1.1 ± 0.1, 8.6 ± 2.9 and 67.3 ± 10.4, respectively. The qPCR estimated ova were reasonably close to the numbers of seeded ova, however there were a small variations. These variations could be due to factors such as the numbers of ova seeded had some standard deviations associated with them. Since qPCR can generate a variable number of gene copies for a single ovum, it is likely that qPCR estimated ova numbers would also be variable. For accurate estimation of ova numbers from qPCR generated gene copies, a kinetic assessment would assist in determining the gene copy numbers in ova isolated from fresh faeces until just before the larvae are hatched. This work still remains to be done.

In this study, the qPCR method was used to quantify hookworm ova from unseeded wastewater samples. The results indicated that the treated wastewater (1 L), raw wastewater (1 L) and sludge (4 g) contained *A. caninum* ova ranged from 0.007- 0.14, 0.45- 8.2 and 24.2-438 ova, respectively (Table 5.2). Since, flushing dog faeces down the toilet is considered as an eco-friendly method for dog waste disposal in Australia, large numbers *A. caninum* ova could be released in wastewater streams. Once raw wastewater is retained in the treatment plant, the hookworm ova will begin to settle into the sludge because of their high settling velocity (Sengupta et al., 2011; Sengupta et al., 2012). As a result, their numbers could be higher in sludge than raw wastewater samples. This is also supported by the data presented in this study where the numbers of *A. caninum* ova were higher in sludge samples than treated and raw wastewater samples. The range of *A. caninum* ova in the sludge samples was almost similar to those (70-735 human hookworm ova) reported for developing countries (Jimenez et al., 2007). This could be the fact that, qPCR is more sensitive (Traub et al., 2004; Verweij et al., 2007; Gyawali et al., 2015a), than culture based method which Jimenez and her colleagues used in their study. Additionally, the numbers of *A. caninum* ova in the sludge samples might contain more cells than the one isolated from dog faecal samples in this study. Therefore, the maximum range of gene copy numbers may be appropriate while converting qPCR estimated gene copy numbers for hookworm ova isolated from environmental samples.

Treated wastewater contained less than one *A. caninum* ovum which could be the qPCR method amplifies DNA from non-viable ova. Since non-viable ova cannot pose any risk to humans, further studies (Chapter 6) will investigate use of Propidium Monoazide (PMA) to discriminate between non-viable ova from viable ova.

5.5. Conclusions

qPCR method has the potential to provide information on the magnitude of hookworm gene copies in wastewater and sludge samples. However, the utility of the qPCR method may be limited for interpreting health risks, which requires conversion of qPCR estimated gene copy numbers to ova. In this chapter, an attempt was undertaken to quantify hookworm ova using qPCR method. The results indicated that gene copy numbers estimated by qPCR are proportionally related to the numbers of ova present in wastewater samples. This means that qPCR can be used to quantify hookworm ova. However, there was variation in gene copy numbers for an ovum due to various factors such as numbers of cells in an ovum. Therefore, quantification of hookworm ova from environmental samples using qPCR may not be accurate.

Further studies are required in order to determine gene copy numbers in a kinetic fashion according to the ova cell stage and also investigate a new target that is not variable with the cell development. Another factor influencing the qPCR estimated gene copy numbers is the recovery of ova from complex matrices such as wastewater and sludge, which is not easy and consistent. It was also determined to be important to have a rapid ova recovery method that could provide consistency in the future development of this method. Therefore, further study was conducted to evaluate the recovery rate of hookworm ova from wastewater and sludge using various recovery methods in order to standardise the qPCR method.

Chapter: 6

Development of a standardised ova recovery method

Development of a standardised of ova recovery method

6.1. Introduction

The distribution of hookworm ova in wastewater and sludge samples is not homogeneous. Quantifying unevenly distributed ova using microscopy and qPCR methods may result in significantly under or over estimation of ova. Therefore, it is essential to recover and concentrate them prior to quantification. Several methods have also been used to recover various microorganisms including ova from water and soil samples. Those include centrifugation (Whitmore and Carrington, 1993; Higgins et al., 2003), hollow-fiber ultrafiltration (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 (US EPA, 1999; Bowman et al., 2003; de Victorica and Galván, 2003; Bastos et al., 2013). Some of these methods are rapid and could potentially shorten the detection time of hookworm ova from wastewater matrices.

Despite the availability of different concentration methods, the flotation method proposed by the US EPA has been the most commonly used to recover hookworm ova from wastewater and sludge samples (US EPA, 1999). This method, however, is laborious and time-consuming due to the requirement of multiple steps of blending, washing, settling, filtering, floating and centrifuging (Ferguson et al., 2004). The recovery rate of this method is reported as being suboptimal and variable depending on the matrices (65-74% and 26-82% from raw wastewater and sludge) (Bowman et al., 2003; Maya et al., 2006; Bastos et al., 2013).

Although improving hookworm ova concentration was not the main aim of this research, it was recognised that a rapid and reliable standardised concentration method is necessary to improve the qPCR method. Therefore, in this chapter, the performance of various rapid concentration methods was evaluated using the qPCR method developed in chapter 5. For wastewater samples, (A) centrifugation, (B) HFUF, (C) filtration, and (D) flotation, and for sludge samples, (E) flotation, and (F) direct DNA extraction methods were chosen for their efficiency on recovering hookworm ova. Known numbers of *A. caninum* ova were seeded into the wastewater and sludge samples in order to identify the best performing method(s).

6.2. Materials and methods

6.2.1. Sample collection

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

Sludge samples were collected from the dewatering belt from WWTP-A, and from the facultative pond from WWTP-B 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 the dark until processing (2-4 days).

The background levels of *A. caninum* ITS-1 rRNA gene copies in all samples (treated wastewater, raw wastewater, and sludge) were determined using the developed qPCR method. All samples were determined to be free of *A. caninum* ITS-1 rRNA.

6.2.2. Sample preparation

Approximately, 400 ± 40 *A. caninum* ova were seeded into 1 L of treated wastewater, raw wastewater, and sludge (~ 4 *g* dry weight) samples. Three repeat trials were undertaken, and all samples were tested in triplicate in each trial.

6.2.3. Ova recovery from wastewater

Ova concentration methods flow chart is shown in Figure 6.1. 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 (700 mL first followed by 300 mL). The pellet was then transferred into a 50 mL polypropylene tube, further centrifuged at 5,200 × *g* for 10 min to obtain a pellet, which was 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.). The 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 the sample 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. The 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, larger to smaller pore size) (Rowe scientific Pty Ltd, Australia) with the help of a stream of tap water. Particles including ova retained in the smallest pore size (38 µm) sieve were collected in a 50 mL polypropylene 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 sediments of treated and raw wastewater samples (1 L) at 5,200 × *g* for 10 mins to achieve a pellet. The pellet was then transferred into a 50 mL polypropylene 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 centrifuged for 3 min at 800 × *g* and the materials present in the top 10 mL were transferred into a 15 mL polypropylene 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. The pellet was then stored at -20ºC until DNA was extracted.

6.2.4. Ova recovery from sludge

Ova from sludge samples were concentrated using Methods E and F. Method E began with centrifugation of ova seeded sludge (~ 4 *g* dry weight) samples at 800 *g* for 10 min. The supernatant was discarded, and 40-45 mL flotation solution was added to each sample. The mixture was then centrifuged for 3 min at 800 × *g* and floated materials were transferred into 15 mL polypropylene 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. The pellet was then stored at -20ºC until DNA was extracted. For Method F, direct DNA extraction was performed from ova seeded sludge samples (~ 4 *g* dry weight).

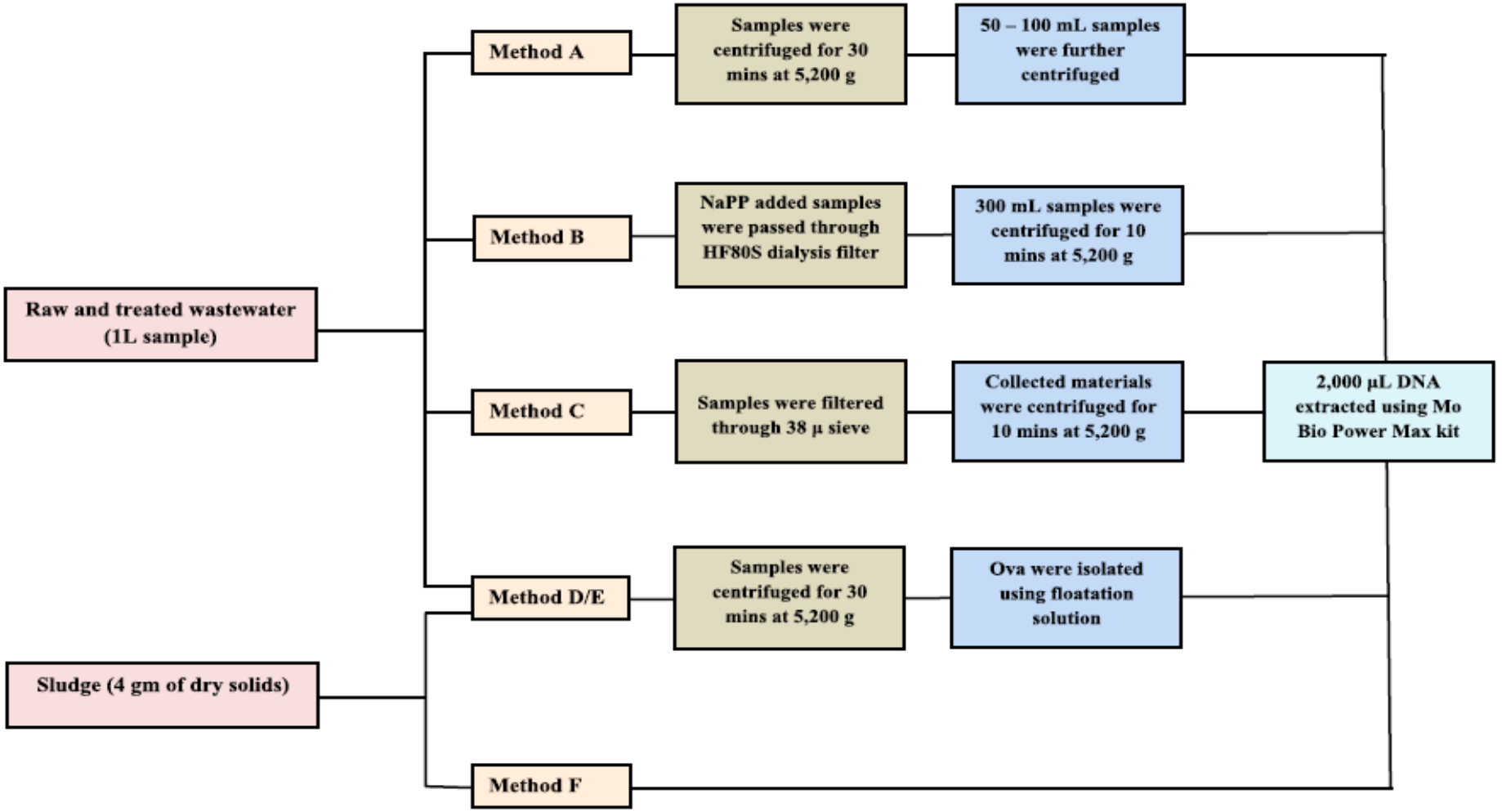

Figure 6.1: Hookworm ova concentration methods for 400 ± 40 ova seeded raw wastewater, treated wastewater, and sludge samples. Method A = centrifugation, Method B = HUFU, Method C = Filtration, Method D = Floatation (for wastewater samples), Method E = Floatation (for sludge samples) and Method F = Direct DNA extraction

6.2.5. DNA extraction and inhibition test

DNA was extracted from each pellet obtained through all concentration methods (A, B, C, D, E) and sample from method F using a Mo Bio Power Max DNA Extraction Kit (Mo Bio, Carlsbad, CA) with minor modifications. All samples were mixed with lysis buffer C1, and freeze-thawed for 10 min -80ºC (standard freezer) and 95ºC (hot water bath) (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 2 mL of Solution C6. All the DNA samples were tested for potential PCR inhibition as described in Chapter 4. Some samples (35%) contained PCR inhibition (appendix E). Samples that showed PCR inhibition were then serially diluted (10-fold) and re-analysed to remove the PCR inhibition. Once the DNA samples were free from PCR inhibitions the qPCR analysis was performed.

6.2.6. Determination of seeded gene copies numbers

To determine the seeded gene copy numbers, DNA was extracted from approximately 400 ± 40 ova in replicates (n = 6) using a Mo Bio Power Max DNA Extraction Kit as described in section 6.2.5. The numbers of ITS-1 rRNA gene copies in *A. caninum* DNA samples were determined using a qPCR method described in Chapter 5.

6.2.7. qPCR method for the quantification of ITS-1 rRNA

The qPCR method was performed using previously designed and optimized primers and probe (Chapter 4). All qPCR reactions were performed in triplicate. The qPCR assay performance criteria such as efficiency (E), slope and intercept, R^2 were determined by analysing the standard curves as described in Chapter 5.

6.2.8. Recovery rate determination

The recovery rate of hookworm ova in the wastewater and sludge samples by the different concentration methods was calculated using equation 1.

$$R = \frac{N(Q_{GC})}{N(S_{GC})} \times 100 \qquad (2)$$

Where, R is recovery efficiency, $N\ (Q_{GC})$ is quantified gene copies numbers and $N\ (S_{GC})$ is seeded gene copies numbers.

6.2.9. 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 numbers 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 significances were determined at $\alpha = 0.05$.

6.3. Results

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

The standards had a linear range of quantification from $3 \times 10^5 – 3 \times 10^1$ gene copies per 3 µL of plasmid DNA. The slope of the standards ranged from -3.31 to -3.38. The amplification efficiencies ranged from 97.8% to 100.7%, and the correlation coefficient (R^2) ranged from 0.98-0.99. The intercepts for the qPCR standards were 35.8 to 38.4 (appendix F).

6.3.2. Recovery rate of *A. caninum* ova from wastewater and sludge

To obtain the recovery rates for each method, 400 ± 40 viable ova (equivalent to $3.3\times10^7 \pm 8.5 \times 10^6$ gene copies) were seeded into each wastewater and sludge samples. The mean number of *A. caninum* gene copies recovered from treated wastewater collected from WWTP-A did not vary significantly ($P > 0.05$) among the methods tested. The numbers ranged from 4.6×10^5 (Method A) to 1.3×10^6 (Method D) for wastewater sample collected from WWTP-A (Figure 6.2a). Similar results were also obtained for WWTP-B except when using Method D. The mean gene copy numbers (3.5×10^3) recovered through Method D was 2-3 orders of magnitude lower than recovered using the Methods (A-C). Furthermore, this difference was significant ($P < 0.05$).

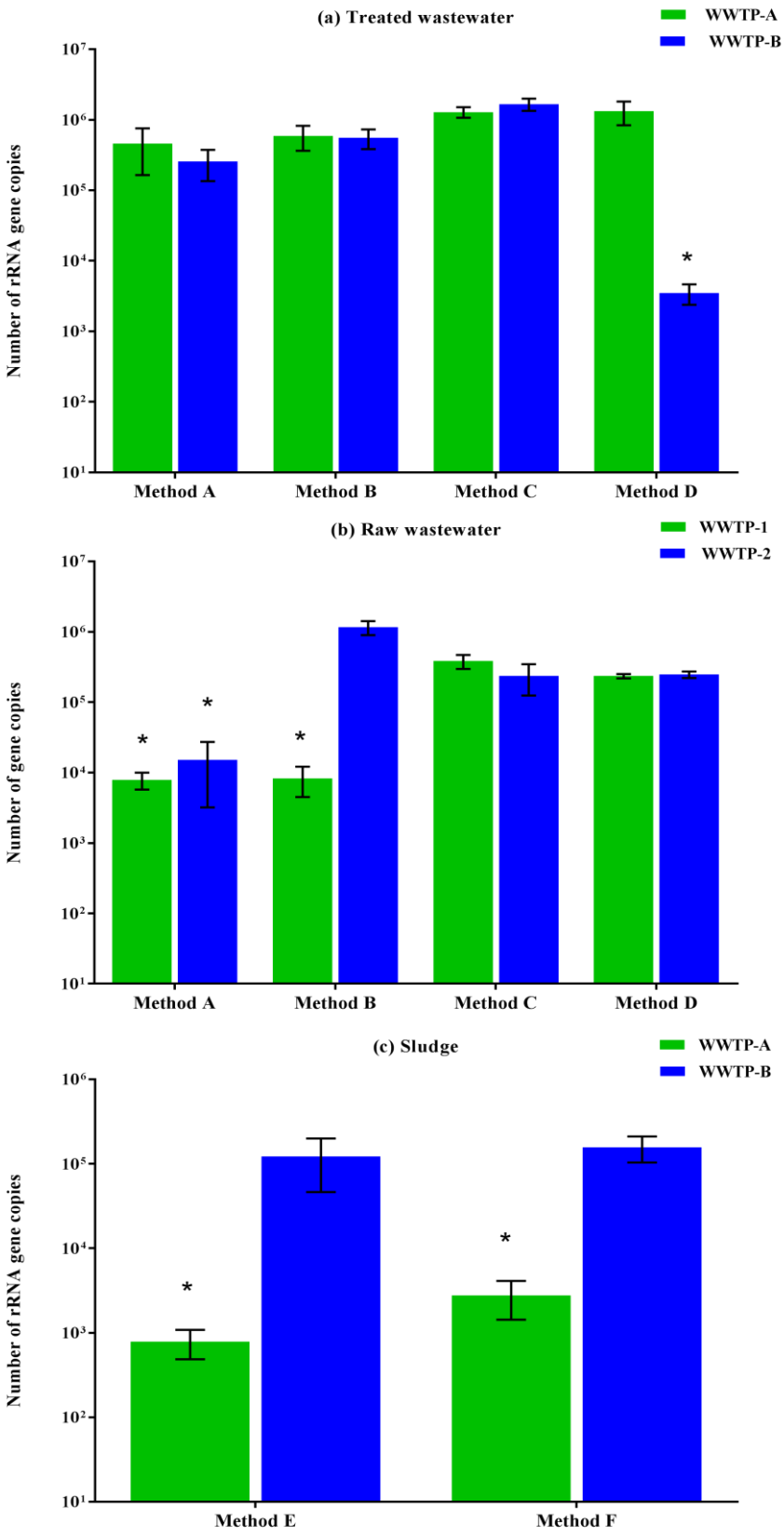

Figure 6.2: Mean and standard deviation of the number of gene copies recovered through different methods [(Method A = centrifugation, Method B = HUFU, Method C = Filtration, Method D = Floatation (for wastewater samples), Method E = Floatation (for sludge samples) and Method F = Direct DNA extraction)] tested from *A. caninum* ova seeded into (a) treated wastewater, (b) raw wastewater, and (c) sludge samples. Statistically significant differences were representing using star (*).

For raw wastewater samples, the mean number 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-A (Figure 6.2b). However, Methods A and B yielded 2 orders of magnitude lower gene copy numbers, and this difference was found to be statistically significant ($P < 0.05$). For WWTP-B, Method B yielded the highest number (1.1×10^6) of gene copies followed by Methods D and C, although these results did not differ significantly ($P > 0.05$). The mean number 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-A, Methods E (7.8×10^2) and F (2.7×10^3) yielded a similar number of gene copies (Figure 6.2c), and were not significantly different ($P > 0.05$). Sludge samples collected from WWTP-B also yielded a similar number of gene copies for Method E (1.2×10^5) and F (1.5×10^5), and again the difference was not statistically significant ($P > 0.05$). Both methods (D and E) were able to recover ~ 2 orders of magnitude higher gene copies from WWTP-B samples compared to WWTP-A samples ($P < 0.05$).

The recovery rate was calculated from the qPCR estimated gene copy numbers (Table 6.1). For treated wastewater, Method D outperformed all other methods closely followed by Method C for WWTP-A (Table 6.1). However, for WWTP-B, Method C performed better than the other methods.

For raw wastewater, Methods C had much better recovery rate followed by Method D for WWTP-A (Table 6.1) For WWTP-B, the recovery rate of Method B outperformed all other methods.

For sludge samples, the recovery rate of ova was 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-A; 4.7 ± 6.2%, WWTP-B) recovery rate than Method E (Table 6.1).

Table 6.1: Evaluation of recovery rate of *A. caninum* ova from raw wastewater, treated wastewater, and sludge samples from six concentration methods [(Method A = centrifugation, Method B = HUFU, Method C = Filtration, Method D = Floatation (for wastewater samples), Method E = Floatation (for sludge samples) and Method F = Direct DNA extraction)] using qPCR

Concentration methods	Wastewater Sample types	Mean and standard deviation of recovery rate (%)	
		WWTP-A	WWTP-B
Method A	Treated	14 ± 35	7.6 ±14
	Raw	0.3 ± 0.2	0.5 ±1.4
Method B	Treated	18 ± 26	**17 ± 20**
	Raw	0.3 ± 0.4	**35 ± 30**
Method C	Treated	**39 ± 26**	**50 ± 39**
	Raw	**12 ± 10**	7.1 ± 13
Method D	Treated	**40 ± 57**	0.2 ± 0.1
	Raw	**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

6.4. Discussion

The research described in Chapter 7 required a reliable and rapid ova recovery method that will consistently recover the low numbers of hookworm ova from complex matrices such as wastewater, sludge, and environmental soil samples. Various methods have been used previously 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, several rapid concentration methods for the recovery of hookworm ova from wastewater matrices including sludge samples were evaluated. For the methods evaluation, wastewater and sludge samples were collected from two WWTPs with very different characteristics.

Method A (centrifugation) was originally developed to separate helminth ova from environmental water samples that have 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 a large amount of heavy particles and grease that may bind to ova (Kuczynska and Shelton, 1999), potentially leading to inefficient DNA extraction.

Method B (HFUF) has been widely used to simultaneously concentrate bacterial, viral and protozoan pathogens from environmental water samples (Hill et al., 2005; Hill et al., 2007). The recovery rates of ova using HFUF from treated wastewater samples were better than the centrifugation method (Method A). However, the recovery rates of ova using HFUF from raw wastewater were found to be 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 (Simmons et al., 2001; Ferguson et al., 2004). The turbidity of raw wastewater collected from both the WWTPs were much higher (246-286 NTU) than the treated wastewater (86-197 NTU). The result obtained in this ova concentration study suggested that the HFUF method is more suitable for concentrating ova when the turbidity of the water samples is low. Mull and Hill (2012) and Ferguson and lleagcoues (2004) have also demonstrated that the turbidity of water samples is inversely proportional to the recovery rates.

Method C (filtration) tested in this study is based on retaining hookworm ova by filtering through a series of sieves. This method is simple, involves only a few steps, and therefore, has the potential to recover higher numbers of ova from wastewater samples. The 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 variable recovery rates (12%-32%) from wastewater samples (Maya et al., 2006). This is in agreement with the findings of this study. The recovery rate obtained through Method D for the treated wastewater collected from WWTP-A was high, although the result was not consistent for both WWTPs. Treated wastewater

samples from the WWTP-B contained a large amount of blue-green algae, which may have attached to hookworm ova, therefore may be discarded during the process (Jakubowski et al., 1996; Ferguson et al., 2004).

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

It has been reported that direct DNA extraction from water samples may yield better recovery of viruses as it bypasses the concentration procedure (Ahmed et al., 2015). In view of this, DNA was directly extracted from sludge samples (Method F). Method F was indeed able to recover higher numbers of ova from sludge samples than Method E. However, the DNA samples obtained through this method had PCR inhibitors present despite the DNA extraction kit used in this study being equipped with inhibitor removal technology. PCR inhibitors are known to be matrix associated, and a wide array of PCR inhibitors with varying concentration could be present in sludge samples (Schrader et al., 2012). Results of this study also indicated that the 35% of DNA samples extracted from wastewater matrices had PCR inhibitors. This problem associated with PCR inhibition could still, however, be overcome by a serial dilution of DNA.

6.5. Conclusions

In conclusion, this study showed 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, the filtration (Method C) was able to recover higher

numbers of *A. caninum* ova consistently from treated wastewater and raw wastewater samples collected from both WWTPs. Both methods (Methods E and F) failed to recover *A. caninum* ova efficiently from sludge samples. The best performing recovery method (Method C, filtration) was therefore used to concentrate the ova from treated wastewater and raw wastewater, respectively for the experiments listed in Chapter 7. Despite the poor recovery rate of the (Method E, flotation) method was used for sludge, soil and faecal samples in Chapter 7. This method was selected over direct DNA extraction method (Method F) due to the lower processing costs.

While improving the hookworm ova recovery rate was not a major focus of this research, further study is needed to develop more reliable recovery method is to be used with the developed qPCR method to ensure that comparable, consistent results can be obtained from different types of samples. Despite the better sensitivity of qPCR method, the method is unable to distinguish between viable and non-viable hookworm ova present in a sample. Selective detection of viable hookworm ova is important to assess the potential public health risk accurately. Therefore, further study was undertaken to develop a PMA-qPCR for selective detection of viable hookworm ova.

Chapter: 7

Quantitative Detection of viable helminths ova

Quantitative detection of viable helminths ova using PMA-qPCR

7.1. Introduction

Detection of viable ova is critical to evaluate the potential human health risks from hookworm because the viable hookworm ova have the potential to hatch infectious (L_3) larvae and infect human. Since the dose of hookworm infection is quite low (1-10 ova) (WHO, 2006), it is vital that the detection method used is sensitive enough to detect small numbers of viable ova in environmental samples such as wastewater and sludge which could be the source of environmental transmission (US EPA, 2003; WHO, 2006). Traditional detection methods (microscopy) lack sensitivity and specificity and require extended time to obtain results (Traub et al., 2004; Traub et al., 2007; McCarthy et al., 2012). Some of these limitations such as sensitivity and specificity can be overcome by using PCR/qPCR methods (Chapter 4 and 5). However, PCR/qPCR does not discriminate between viable and non-viable cells in a sample (Rudi et al., 2005), resulting in a potential overestimation of infectious ova. Since only viable ova are capable of hatching infectious (L_3) larvae and consequently causing infections in humans, it is important to know what fraction of the PCR amplified ova are viable for the accurate assessment of public health risks.

It has been demonstrated that Propidium Monoazide (PMA), a DNA intercalating dye, combined with PCR/qPCR methods can be used for selective detection and quantification of viable bacteria, protozoa and viruses from wastewater, sludge and environmental samples (Fittipaldi et al., 2011; Taskin et al., 2011; Nkuipou-Kenfack et al., 2013; Alonso et al., 2014; Li et al., 2014; Gensberger et al., 2014; Santiago et al., 2015). The working mechanism of PMA is that it penetrates non-viable cells, and makes a covalent bond with DNA upon photoactivation. This covalent bonding causes the formation of a stable DNA-PMA complex, which prevents the DNA from being to amplify during the PCR reaction (Nocker et al., 2006; Nocker et al., 2007). However, there have been no published studies that have investigated the ability of PMA to discriminate between viable and non-viable hookworm ova.

As a proof-of-concept, the primary aim of this chapter was to evaluate the use of PMA to discriminate between viable and non-viable hookworm ova by developing a PMA-qPCR method using *A. caninum* ova. Finally, the newly developed PMA-qPCR method was to be

tested for applicability to detect viable *Ancylostoma duodenale*, *Ascaris lumbricoides* and *Necator americanus* ova from complex matrices such as raw wastewater, human faeces, and contaminated soil samples.

7.2. Materials and methods

7.2.1. Optimisation of method for producing non-viable *A. caninum* ova

Incubation temperature and time was optimised to produce non-viable *A. caninum* ova that were used to develop PMA-qPCR method. Approximately 400 ova were suspended in 230 µL phosphate buffer saline (PBS) in 1.5 mL tubes and incubated at temperatures ranging from 50 to 80°C at 10°C intervals for 15, 30 and 60 min. This procedure was performed in triplicate. The heat inactivated ova were kept at room temperature for 12 h to allow the cell wall to become permeable (Gyawali et al., 2016a). After incubation, the tubes were centrifuged at 10,000 *g* for 2 min. The supernatant was discarded and ova were stained with the vital stain (Methylene blue 0.05%) and observed under a microscope (Olympus, Japan). Incubation at 80°C for 15 min was found to be suitable for consistently producing non-viable ova and was further used for all PMA-qPCR experiments (Figure 7.1).

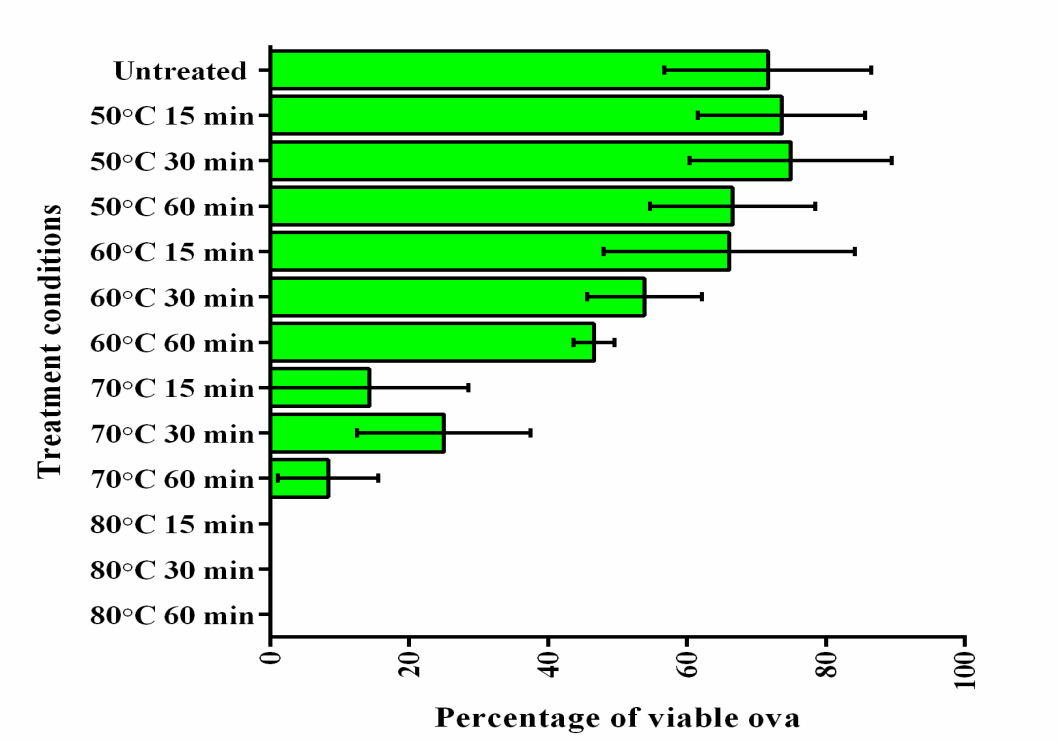

Figure 7.1: Percentage of viable ova identified using microscope before and after heating to different temperatures for different time (*n* = 3)

7.2.2. Optimisation of PMA concentration

To optimise the PMA ($C_{27}H_{33}Cl_2N_6$; Biotium, Inc., USA) concentration, 2 mM working solution was prepared by dissolving 1 mg PMA with 20% dimethyl sulfoxide (978 µL) (Sigma-Aldrich, Australia).

Approximately 400 non-viable ova were transferred into 1.5 mL transparent centrifuge tubes (GeniUL, Spain) and treated with varying concentrations of PMA (50, 100, 200, 300 and 400 µM). PMA treated non-viable ova were then incubated in the dark for 5 min to allow PMA to penetrate the damaged ova. Photo-induced cross-linking of PMA in all samples was achieved using PhAST Blue (GeniUL, Spain) light for 15 min. The tubes were then incubated on ice for 5 min. Triplicate samples were used to ensure the reproducibility of the assay.

DNA was extracted from the ova by using Mo Bio Power Soil Kit (Mo Bio, Carlsbad) and tested with qPCR described elsewhere (Gyawali et al., 2015a; Gyawali et al., 2015b). Non-viable ova treated with PMA at a concentration of 50 µM permitted qPCR amplification suggesting that this concentration is not sufficient to distinguish between viable and non-viable ova. However, other concentrations (100, 200, 300 and 400 µM) did not permit qPCR amplification. Based on these results, the lowest concentration (100 µM PMA) which did not permit qPCR amplification was chosen to distinguish viable from non-viable *A. caninum* ova using the PMA-qPCR method.

7.2.3. Primer and probe design

Nucleotide sequences of *A. duodenale* (accession No EU 344797.1) and *A. lumbricoides* (accession No KL872896) were obtained from National Centre for Biotechnology Information (NCBI). New sets of primers and probes were designed using Integrated DNA Technology (IDT) Primer Quest software (IDT, USA) and used to amplify a 75 bp (*A. duodenale*) and 104 bp (*A. lumbricoides*) of the rRNA gene of Internal Transcribed Spacer (ITS-1 and ITS-2) region (Table 7.1). For *N. americanus*, and *A. caninum* previously published 190 and 101 bp primer sets were used (Wang et al., 2012, Chapter 4). However, for *N. americanus*, a new probe was designed and tested in this study (Table 7.1). The specificity of the newly designed primers and probes were tested using NCBI Megablast. The results of the BLAST search showed that the sequences of selected primers matched with the rRNA of the ITS-1 region of *A. duodenale*, *A. lumbricoides* and *N. ameroicanus.*

7.2.4. qPCR standards and optimisation

A. duodenale, *N. americanus* and *A. lumbricoides* gene fragments matching the PCR target region were synthesized by Integrated DNA Technologies (IDT) and cloned into a vector followed by plasmid extraction (IDTDNA.com). Procedure of obtaining A. caninum plasmid was described previously in Chapter 5. The standards were prepared as described in chapter 5. Serial dilutions were prepared from 10^6 to 10^0 gene copies per µL. Each qPCR method was optimised for primer and probes concentrations and annealing temperature (Table 7.1).

7.2.5. qPCR methods and lower limit of quantification (LLOQ)

qPCR amplifications for each of the standards listed in section 7.2.4. were performed in 25 µL reaction mixtures containing 12.5 µL iQ™ Supermix (Bio-Rad Laboratories, CA, USA), 3 µL of template DNA, appropriate concentrations of primers and probes (Table 7.2) and UltraPure™ water (Invitrogen, USA). The qPCR cycling parameters are shown in Table 7.1. qPCR assays were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, CA, USA). Each qPCR lower limit of quantification (LLOQ) value was determined from the standard series in replicates (n = 6) at 95% confidence level. The lowest quantity of DNA detected consistently in all replicate reactions was considered as the qPCR LLOQ.

Table 7.1: Primer sequences, cycling parameters and concentrations of primers and probes for qPCR methods

Species	Primer and probe sequences[a]	PCR cycling parameters	Primer/probe concentrations (nM)	References
A. caninum	F: (5'- TTT GCT AAC GTG CAC TGA ATG -3')	15 min at 95^0C, 35	250	Chapter 4
	R: (5'- GAA ACA CCG TTG TCA TAC TAG CC -3')	cycles of 15 sec at	250	
	P: (FAM-5'- AAC TCG TTG TTG CTG CTG AA -3'TAMRA)	95^0C, 60 sec at 57^0C	300	
A. duodenale	F: (5'-ATA GCC CTA CGT AAG GTG TCT ATG T-3')	15 min at 95^0C, 35	250	This study
	R: (5'-CGA ACT TCG CAC AGC AAT CAC-3')	cycles of 15 sec at 95^0C,	250	
	P: (FAM-5'-CAA GAG TCG TTA CTG GGT GAC GGC-3'TAMRA)	60 sec at 58^0C	300	
A. lumbricoides	F: (5'- GAG CCA CAT AGT AAA TTG CAC A -3')	10 min at 95^0C, 40	300	This study
	R: (5'- CTC CCT CAA CAC ATA GCA AAT C-3')	cycles of 15 sec at	300	
	P: (FAM-5'- ACC GCC GAC TGC TAT TAC ATC ACC-3'TAMRA)	95^0C, 60 sec at 60^0C	300	
N. americanus	F: (5'-TGT TCA GCA ATT CCC GTT TA-3')	10 min at 95^0C, 35	300	(Wang et al., 2012)
	R: (5'- GTC CTT CAC ATT GTC TCC GT-3')	cycles of 15 sec at	300	
	P: (FAM-5'-ATT CGC TCT CGC GAC TTA TGA GCG-3BHQ1)	95^0C, 60 sec at 60^0C	400	

[a] F: forward primer; R: reverse primer; P: probe

7.2.6. PMA-qPCR method development and validation

To develop a method for quantification of viable *A. caninum* ova, 100% viable (*n* = 400), 50% viable (*n* = 200) + 50% non-viable (*n* = 200) and 100% non-viable (*n* = 400) ova were transferred into 1.5 mL transparent centrifuge tubes (GeniUL, Spain). The ova were treated in triplicate with 100 μM PMA (see section 7.2.2).

To validate the newly developed PMA-qPCR method for the quantification of viable *A. caninum* ova in raw wastewater, 10 L samples was separately collected from two metropolitan wastewater treatment plants (WWTPs A and B) located in Brisbane, QLD, Australia. Triplicate wastewater samples (1 L) were seeded with 100% viable, 50% viable + 50% non-viable and 100% non-viable ova.

Each of the seeded samples was filtered through a series of sieves (800-38 μm pore size, larger to smaller pore size) (Rowe scientific Pty Ltd, Australia) with the help of a stream of tap water. Particles including ova retained in the smallest pore size (38 μm) sieve were collected in a 50 mL polypropylene tube and centrifuged at 5,200 *g* for 15 min to obtain a pellet. Ova were separated from the pellet using the flotation method (Bowman et al., 2003; Gyawali et al., 2015b). Separated ova were then treated with 100 μM PMA (see section 7.2.2. for detail).

7.2.7. Application of PMA-qPCR method in real world samples

The optimized parameters for PMA-qPCR method for *A. caninum* were adapted to develop *A. duodenale*, *N. americanus* and *A. lumbricoides* PMA-qPCR methods.

All PMA-qPCR assays were used to determine the numbers of helminth ova in raw wastewater samples (*n* = 5) collected from a WWTP located at Sunshine Coast, Australia. A 10 L raw wastewater sample was collected in sterile 20 L polypropylene carboys and transported to the laboratory with an ice pack. In addition, human faecal (*n* = 10) and environmental soil samples assumed to be contaminated with STHs (*n* = 24) were collected from STHs endemic communities in East Timor. Approximately, 5 g of individual human faecal and 50 g of soil sample were collected in sterile 50 mL polypropylene tubes. The samples were mixed with potassium carbonate (K_2CO_3), and transported on ice to the CSIRO

laboratory, Australia for analysis. Upon arrival, ova were recovered from human faecal and soil samples using the flotation method (Bowman et al., 2003).

Each pellet containing STHs ova recovered by the flotation method was suspended in 1 mL PBS and divided into two aliquots (500 µl each). Both tubes containing aliquots were centrifuged at 1,000 *g* for 5 min to obtain pellets. One of the pellets was treated with 100 µM PMA and the remaining pellet was not treated with PMA.

7.2.8. DNA extraction and inhibition test

DNA from each PMA treated and untreated concentrated samples were extracted using Mo Bio Power Soil DNA Isolation Kit (Mo Bio, CA, USA) (Chapter 4). A Sketa22 real-time PCR assay was used to determine the presence of inhibitors in the DNA samples (Chapter 4). All samples indicated the absence of PCR inhibitors and were used for further analysis.

7.2.9. Ova recovery rate and DNA extraction efficiency

The ova recovery rate from raw wastewater (1 L), human faeces (1 *g*) and environmental soil (20 *g*) was conducted as described in Chapter 6. To determine the DNA recovery efficiency of the Mo Bio Power Soil DNA kit, 15 ng of *Oncorhynchus keta* DNA was seeded into the raw wastewater, human faecal and environmental soil samples prior to extract DNA. A Sketa22 qPCR was performed on extracted DNA samples (Haugland et al. 2005). The DNA recovery efficiency was determined according to Rogers et al., (2011).

7.2.10. Statistical analysis

Microsoft excel ver. 2010 (Microsoft, USA) was used to conduct the statistical analysis. One-way ANOVA was performed to determine the significant difference between numbers of gene copies determined by qPCR and PMA-qPCR in laboratory condition as well as ova seeded raw wastewater samples collected from WWTP-A and B (section 7.2.6).

A paired T-test for means was performed to determine the significant difference between numbers of gene copies determined by qPCR and PMA-qPCR for *N. americanus* in human faecal samples and *A. lumbricoides* for environmental soil samples (section 7.2.7).

7.3. Results

7.3.1. qPCR standards and lower limit of quantitation (LLOQ)

qPCR standard curves for target hookworms (*A. caninum, A.duodenale,* and *N. americanus*) and the roundworm (*A. lumbricoides*) had a linear range of quantification from 10^6 to 10^1 gene copies per µL of DNA. The qPCR amplification efficiencies ranged from 90.0% to 98.3%, and the correlation coefficients (r^2) were 0.99 for all qPCR assays (Table 7.2). The slope of the line ranged from -3.36 to -3.59. The qPCR LLOQ were 3×10^1 gene copies for all four methods tested in this study which is equivalent to approximately one ovum (Table 7.3).

Table 7.2: Performance characteristics of qPCR assays of *Ancylostoma duodenale, Necator americanus, Ancylostoma caninum and Ascaris lumbricoides*

qPCR assays	Amplification efficiency (*E*)	Correlation coefficient (r^2)	Slope	Intercepts
A. caninum	98.3	0.99	-3.36	36.89
A. duodenale	94.4	0.99	-3.46	38.26
A. lumbricoides	94.5	0.99	-3.46	38.48
N. americanus	90.0	0.99	-3.59	37.51

7.3.2. Ova recovery rate

The *A. caninum* qPCR result indicated that the average ova recovery rate was highest for human faecal samples (50%) followed by raw wastewater (12%) and environmental soil (4%) samples. The ova recovery rate was factored in for all calculation of gene copy numbers determined by qPCR and PMA-qPCR methods.

7.3.3. DNA recovery efficiency

The Sketa22 qPCR indicated that the average DNA recovery efficiency rate of the Mo Bio Power Soil DNA Isolation Kit for soil, human faecal and raw wastewater samples were 59%, 47%, and 30%, respectively. The DNA extraction efficiency rate was also factored in while calculating gene copy numbers determined by qPCR and PMA-qPCR methods.

Table 7.3: qPCR lower limit of quantification (LLOQ) of serially diluted standards of *Ancylostoma duodenale, Necator americanus, Ancylostoma caninum and Ascaris lumbricoides* (*n* = 6)

Serial dilutions of gene copies	*A. caninum*		*A. duodenale*		*A. lumbricoides*		*N. americanus*	
	Positive Samples	Mean ± SD of C_T values	Positive Samples	Mean ± SD of C_T values	Positive Samples	Mean ± SD of C_T values	Positive Samples	Mean ± SD of C_T values
3×10^6	6	15.5 ± 0.11	6	15.8 ± 0.10	6	15.8 ± 0.14	6	14.4 ± 0.17
3×10^5	6	18.7 ± 0.10	6	19.3 ± 0.15	6	19.3 ± 0.07	6	17.5 ± 0.16
3×10^4	6	23.1 ± 0.01	6	22.9 ± 0.11	6	22.7 ± 0.37	6	21.6 ± 0.33
3×10^3	6	26.6 ± 0.40	6	26.3 ± 0.06	6	27.0 ± 0.30	6	25.3 ± 0.22
3×10^2	6	30.7 ± 0.18	6	29.5 ± 0.07	6	30.3 ± 0.06	6	28.7 ± 0.14
3×10^1	6	33.6 ± 0.20	6	33.2 ± 0.25	6	32.6 ± 0.13	6	32.4 ± 0.31
3×10^0	0	-	0	-	0	-	0	-

-: No amplification
SD: Standard deviation
C_T = Threshold cycle

7.3.4. Detection of viable *A. caninum* ova by PMA-qPCR

For 100% viable ova, the mean number of gene copies determined by PMA-qPCR was 4.6 × 10^5 Figure 7.2. For 50% viable + 50% non-viable ova, the number of gene copies was (5.4 × 10^4) which was approximately one order of magnitude lower than 100% viable ova. For 100% non-viable ova a sample produced detectable gene copy numbers (6.7 × 10^1). The reduction in gene copy numbers was four orders of magnitude compared to 100% viable ova.

One-way ANOVA indicated that the numbers of gene copies for the three mixtures were significantly different ($P < 0.05$) from each other. On the other hand, the qPCR results for 100% viable and 100% non-viable control ova samples (without PMA treatment) were 5.8 × 10^5 and 4.7 × 10^5 gene copies, respectively.

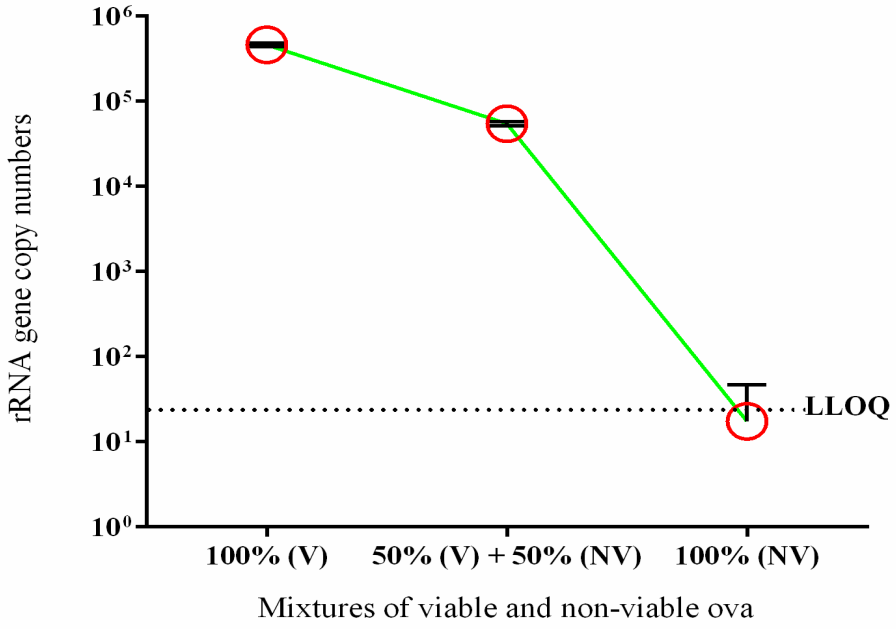

Figure 7.2: Box and whisker plots of the numbers of gene copy detected from ~ 400 *A. caninum* ova mixed in a ratio of 100% viable (V), 50% viable + 50% non-viable and 100% non-viable (N-V) under laboratory conditions. The inner box lines represent the medians while the outer box lines represent 5th and 95th percentiles (*n* = 9)

7.3.5. Validation of PMA-qPCR methods for ova seeded wastewater

For 100% viable ova, the mean numbers of gene copies were 1.1×10^6 in raw wastewater samples from WWTP-A and 1.8×10^6 in raw wastewater from WWTP-B, respectively (Figure 7.3a and b). For 50% viable + 50% non-viable ova, the mean numbers of gene copies were 2.3×10^5 in samples from WWTP-A and 3.7×10^5 in samples from WWTP-B. Overall, 79-80% reductions in gene copies were observed for 50% viable + 50% non-viable ova seeded wastewater samples compared to 100% viable ova.

The reductions of gene copies were statistically significant ($P < 0.05$). For 100% non-viable ova, the gene copies were not quantifiable (< LLOQ) for both raw wastewater samples. On the other hand, the qPCR results for 100% viable and 100% non-viable control ova samples (without PMA treatment) were 5.1×10^6 and 4.5×10^6 for WWTP-A and 2.5×10^6 and 2.0×10^6 gene copies for WWTP-B, respectively.

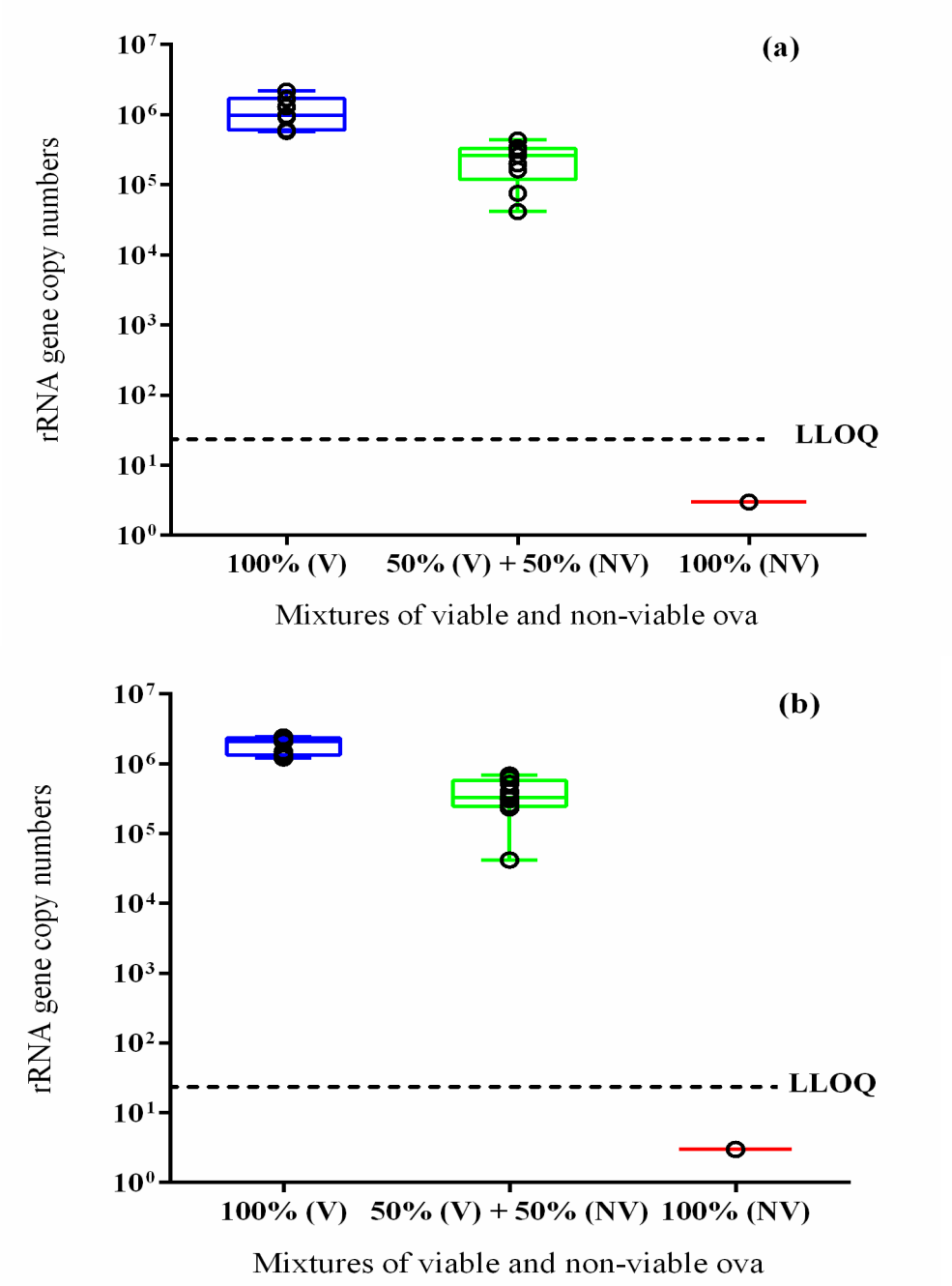

Figure 7.3: Box and whisker plots of the gene copy numbers detected from ~ 400 *A. caninum* ova mixed in a ratio of 100% viable, 50% viable + 50% non-viable and 100% non-viable seeded into raw wastewater (a) WWTP-A (b) WWTP-B. The inner box lines represent the medians while the outer box lines represent 5th and 95th percentiles (*n* = 9)

7.3.6. Application of PMA-qPCR methods in real world samples

None of the unseeded raw wastewater samples from the Australian WWTPs were positive for helminth ova (*A. caninum, A.duodenale, N. americanus and A. lumbricoides*). Of the 10 unseeded human faecal samples collected from East Timor, six were positive for *N. americanus* using qPCR with an average of $6.8 \times 10^5 \pm 6.4 \times 10^5$ gene copy numbers per *g* of faeces. However, using the PMA-qPCR method only five out of the six qPCR positive samples were positive with an average of $6.3 \times 10^5 \pm 4.7 \times 10^5$ gene copies per *g* of faeces (Figure 7.4). A paired T-test for means indicated that the numbers of gene copies determined by qPCR were not statistically significantly ($P > 0.05$) different than those gene copies determined using PMA-qPCR.

Out of 24 environmental soil samples collected from East Timor, all samples were negative for all STHs apart from only one sample which was positive for *A. lumbricoides*. The mean gene copy number in the environmental soil sample that tested positive for *A. lumbrocides* was $1.0 \times 10^5 \pm 1.5 \times 10^4$ (determined by qPCR) compared to $4.9 \times 10^4 \pm 3.7 \times 10^3$ (determined by PMA-qPCR). The number of *A. lumbricoides* gene copies in the environmental soil sample determined by qPCR was significantly ($P = 0.02$) different from the gene copies determined using PMA-qPCR.

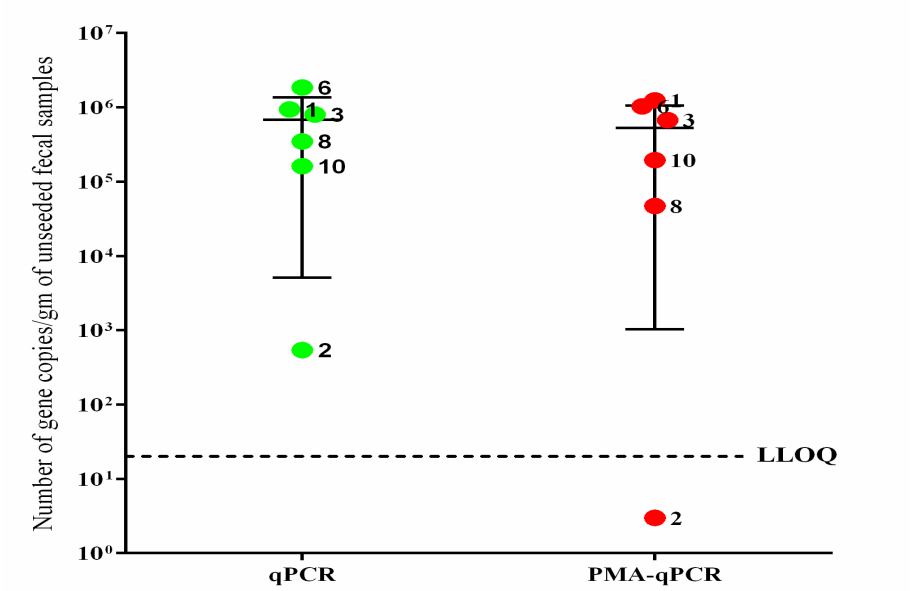

Figure 7.4: Mean and standard deviation of *Necator americanus* gene copies per gram of unseeded human faeces determined by qPCR and PMA-qPCR. Sample numbers were plotted in the graph and for each sample, a mean value was used

7.4. Discussion

Differentiation between viable and non-viable hookworm ova in environmental samples such as wastewater and sludge is necessary in order to identify hookworm contamination and implement strategies to mitigate re-infections in endemic regions (Gyawali et al., 2016b). The traditional detection method (microscopy) lacks sensitivity and specificity and requires extended time to obtain results (Traub et al. 2004; Traub et al. 2007; McCarthy et al. 2012). Some of these limitations can be overcome by using molecular detection methods such as qPCR, which has better sensitivity and specificity than the microscopy method (Gyawali et al. 2015a; Gyawali et al., 2015b). However, qPCR does not discriminate between viable and non-viable cells in a sample (Rudi et al. 2005), resulting in an overestimation of the total number of target gene copies. Information on the viability of cells is important for accurate risk assessment and mitigation (Li et al. 2014; Gensberger et al. 2014).

The preliminary results indicated that PMA-qPCR is able to differentiate between mixtures of viable and non-viable ova (Figure 7.2). In this study, PMA-qPCR did not completely (100%) prevent amplification for 100% non-viable ova. Only a small fraction (0.01%) gene copies were amplified using PMA-qPCR. This could be due to the fact that, heat inactivated ova may attach with each other and prevent PMA from entering in the cytoplasm. Another factor that should be noted is that heat inactivated ova require up to 12 h to become permeable to high molecular weight stains (Gyawali et al., 2016a). Nonetheless, this would not affect the application of PMA-qPCR to distinguish between viable and non-viable ova in environmental samples or human faeces.

Based on these results, the newly developed PMA-qPCR method was further evaluated to determine the numbers of gene copies in viable and non-viable hookworm ova seeded into raw wastewater samples. As with any laboratory procedure, PMA-qPCR has some limitations in detecting viable pathogens in wastewater matrices especially raw wastewater. Sample turbidity can prevent photoactivation; therefore, samples must be diluted in a state that will enable light to reach the PMA (Varma et al., 2009; Li et al., 2014). Previous studies reported that the performance of PMA treatment on the enumeration of viable faecal indicator bacteria was poor for wastewater matrices (Varma et al., 2009; Li et al., 2014). This could be attributed to the sample processing procedures where authors used a centrifugation method to concentrate faecal indicator bacteria, which may have co-concentrated PCR inhibitors and

other unwanted debris. However, in this study, when mixtures of viable and non-viable ova were seeded into wastewater samples, the PMA-qPCR was able to measure signals from viable hookworm only. It is possible that the flotation method used to recover the hookworm ova from wastewater samples effectively eliminated unwanted debris, PCR inhibitors and chemical compounds from the wastewater samples (Schrader et al., 2012).

The percentage of gene copy reduction for ova seeded wastewater samples in different trials was not always consistent. The numbers of gene copies of helminth ova can vary depending on the target region and development stage, with embryonated ova having higher gene copies compared to unembryonated ova (Pecson et al., 2006; Raynal et al., 2012). The ova seeded into the wastewater samples may have had variable numbers of gene copies and may have produced variable results using qPCR and PMA-qPCR. Therefore, more studies would be required to determine the numbers of gene copies for each cell development stage. This may provide a threshold value of gene copy numbers for different stages of ova.

The PMA-qPCR method was adapted to determine the viable helminth ova in human faecal and soil samples presumed to be contaminated with helminths. The results indicated the presence of *A. lumbricoides* ova in one out of 24 environmental unseeded soil samples. The numbers of gene copies determined by qPCR and PMA-qPCR was significantly different suggesting presence of some non-viable ova in the environmental soil samples. This indicates that the PMA-qPCR method may serve as an appropriate diagnostic tool for undertaking interventions to control soil-transmitted helminth infections in developing countries.

The results also indicated the presence of viable *N. americanus* ova in five out of 10 unseeded human faecal samples. The number of *N. americanus* gene copies in faecal samples measured with qPCR and PMA-qPCR were slightly different suggesting majority of viable ova in faecal samples.

Concentration of PMA and light exposure time may affect accurate measurements of viable microorganisms in wastewater and environmental samples (van Frankenhuyzen et al., 2011). Unwanted debris and compromised cells in wastewater and environmental samples may absorb PMA. As a result, low concentration of PMA may not be sufficient to bind extracellular DNA or those contained in dead cells. On the other hand, high PMA concentration may cause infiltration to live cells as well as PCR inhibition. Several studies

have recommended using 100 μM PMA to detect viable pathogens from wastewater and other environmental samples (Varma et al., 2009; Chen and Chang, 2010; Alonso et al., 2014; Li et al., 2014). The PMA optimization result in this study indicated that PMA concentrations > 100 μM did not permit qPCR amplification.

Finally, the variation in DNA recovery from DNA extraction kit may have influenced the numbers of gene copies. In this study, the recovery efficiency of the Mo Bio Power Soil DNA Kit was determined. The results indicated that better recovery efficiency for soil samples than wastewater and faecal samples. Organic matters and fatty acids present in wastewater and faecal samples can have a negative impact on DNA recovery efficiency (Dineen et al., 2010). Further studies would be required to improve the DNA recovery efficiency by comparing multiple kits.

7.5. Conclusions

In conclusion, a PMA-qPCR method has been successfully developed. The method was demonstrated to be able to distinguish between viable and non-viable *A. caninum* ova under laboratory condition. The PMA-qPCR method was also able to distinguish between viable and non viable *A. caninum*, *N. americanus* and *A. lumbricoides* ova present in wastewater, environmental soil and human faeces.

This new method will aid in identifying the potential environmental source of STHs re-infections in endemic regions. In addition, more accurate risk assessments of wastewater reuse for agriculture can be assessed; therefore, a practical risk management plan can be formulated. Since treated wastewater and sludge guidelines specify ≥ 1 viable ovum per L (liquid) or 4 *g* (solid) for unrestricted use, any PMA-qPCR amplification can suggest the potential public health risks; therefore, appropriate wastewater and sludge treatment plans can be put in place.

The variations in the number of detectable gene copies within trials (~ 400 ova) indicate that there is a need for further study to determine the gene copy numbers according to the cell development stage of helminth ova. The suitability of the PMA-qPCR method, however still requires comparison with culture-based and vital stain methods prior to recommending for regular monitoring proposes.

Chapter: 8

Comparison of quantitative detection methods

Comparison of PMA-qPCR, culture based and vital stain methods

8.1. Introduction

The numbers of viable hookworm ova in environmental samples including wastewater and sludge are generally quantified by the culture based method, which is the standard method and involves incubating the ova at 28-30ºC for up to a week (US EPA, 2003; WHO, 2006). The viable ova become larvae (L1), and then they are enumerated by microscopic observation. The method, however, has limitations such as prolonged time to obtain results (de Victorica and Galván, 2003; Nocker and Camper, 2008; Gyawali et al., 2015a), which not only increases the operating cost, but also may not be ideal for a scenario where rapid monitoring is required (Boehm et al., 2009). Another issue is that the culture based method requires highly skilled personnel to differentiate the larvae of different species of hookworm (Traub et al., 2004; Verweij et al., 2007).

To overcome the limitations of the culture-based method, a rapid, simple and a relatively cheaper vital staining method has been developed to quantify STHs ova including hookworm (de Victorica and Galven, 2003). This method relies on the difference in structural integrity of viable and non-viable ova. The cell wall of a viable ovum has three layers that act as an alternative barrier and prevents the vital stains from entering into the cytoplasm (Matthews, 1986; Gregori et al., 2001). Once the ovum becomes non-viable, the integrity of cell wall is compromised and becomes permeable to the vital stain (Bae and Wuertz, 2009). The cell wall, however, requires 12 h to become permeable to vital stains after becoming non viable (Gyawali et al., 2016a). Therefore, this method has the potential to overestimate the numbers of viable ova in a sample. Moreover, this method also relies on skilled personnel to differentiate hookworm ova to the species level, which may introduce bias (Cabaret et al., 2002; Traub et al., 2004; Verweij et al., 2007).

PMA-qPCR methods can quantify viable hookworm ova in a one-step closed-tube reaction with much higher sensitivity and specificity by directly amplifying a specific gene (Gyawali et al., 2016c). In this chapter, the viability assessment performance of the PMA-qPCR method was compared with the culture based and vital stain methods. Due to the unavailability of sufficient fresh human hookworm ova in Australia, the method comparison experiment was conducted using *A. caninum* ova.

8.2. Materials and methods

8.2.1. Ova collection and enumeration

Since the viability of *A. caninum* ova can decrease during storage at 4ºC, fresh dog faecal samples were collected from the School of Veterinary Science University of Queensland, Gatton, Australia. *A. caninum* ova were isolated from approximately 20 *g* of dog faecal samples using the flotation method described elsewhere (Bowmen et al., 2003). The ova were enumerated using a Sedgewick-Rafter Counting Chamber (PYSER – SGI, UK) as described in Chapter 4.

8.2.2. Methods used for quantification of hookworm ova

8.2.2.1. Culture based method

The methods used for quantifying of viable hookworm ova are shown in Figure 8.1. The procedure used in this study has been described elsewhere (Bowmen et al., 2003). In brief, approximately 1,000 ± 50 ova were transferred into a 5 mL tube. Four mL of 0.1 N H_2SO_4 (cultural fluid) was added into the tube. The level of liquid in the tubes was marked on the outside using a permanent marker. Replicate uncapped tubes (n = 6) containing ova were incubated at 30ºC in an incubator for one week. The liquid in the tubes was observed daily for loss due to evaporation, and culture fluid was added to compensate the evaporated volume as required. After a week of incubation, the tubes were centrifuged at 800 × *g* for 5 min and 3.5 mL supernatants discarded from each tube. The remaining volume of liquid was vortexed and 20 µL transferred onto a slide.

Embryonated ova/larvae and unhatched ova were enumerated using microscopic observation (Figure 8.2). Each sample was enumerated three times and the total numbers of viable ova were estimated using the equation 3.

$$N_{VO} = \frac{O_{EO}}{O_V} \times T_V \qquad (3)$$

Where, N_{VO} is numbers of embryonated ova/larvae in a sample, O_{EO} is observed embryonated ova/larvae on a slide, T_V is the total volume of sample and O_V is observed volume of sample. Similarly, the total numbers of non-viable ova were estimated using equation 4.

$$N_{NVO} = \frac{O_{NVO}}{O_V} \times T_V \qquad (4)$$

Where, N_{NVO} is numbers of non-viable ova in a sample, O_{NVO} is observed non-viable ova on a slide, T_V is the total volume of sample and O_V is observed volume of sample.

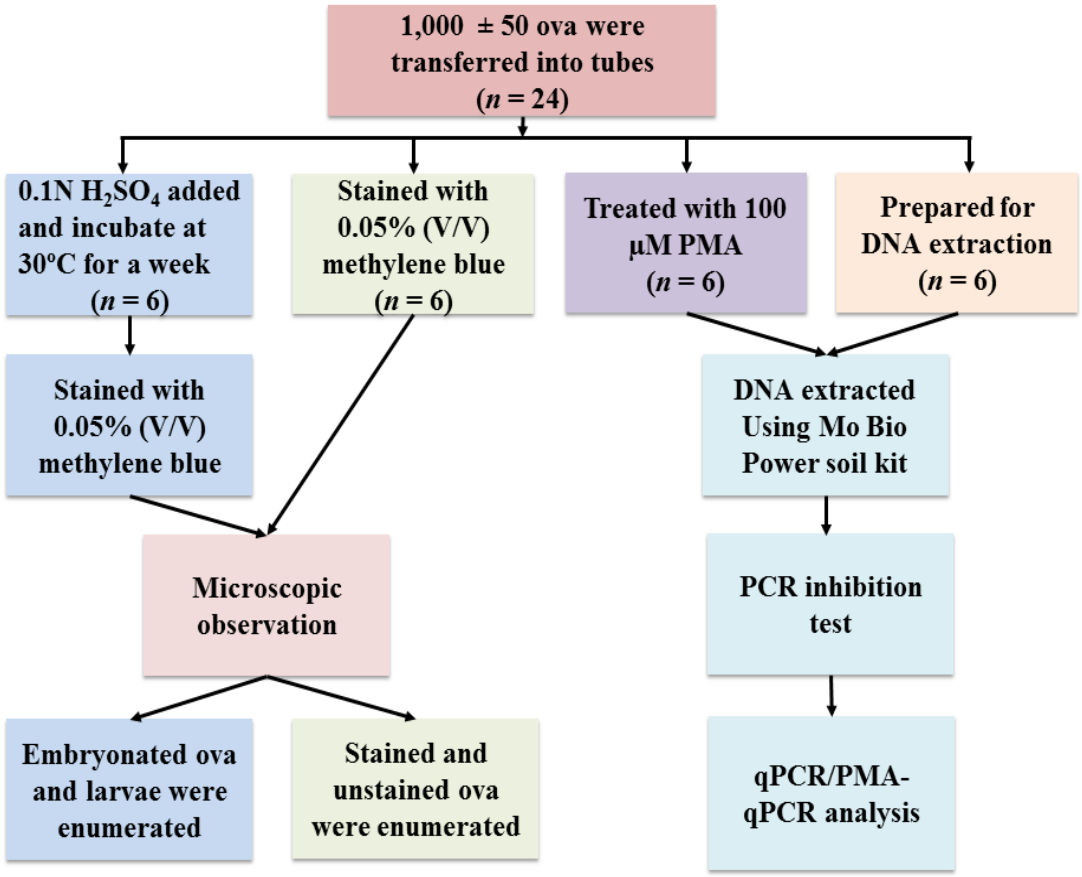

Figure 8.1: Flow chart showing the procedure for quantitative detection of hookworm ova and larva

8.2.2.2. Vital stain method

The vital stain method used in this study has been described elsewhere (de Victorica and Galvan, 2003). In brief, approximately 1,000 ± 50 ova were transferred into a 1.5 mL centrifuge tube (n = 6). The ova were stained with 0.05% (v/v) methylene blue for 5 min at room temperature. The tube was vortexed, and 20 µL of liquid was transferred onto a slide and viable (unstained) and non-viable (stained) ova were recorded (Figure 8.2). Each sample was enumerated three times and the total numbers of viable ova were estimated using the equation 3. Similarly, the total numbers of non-viable ova were estimated using equation 4.

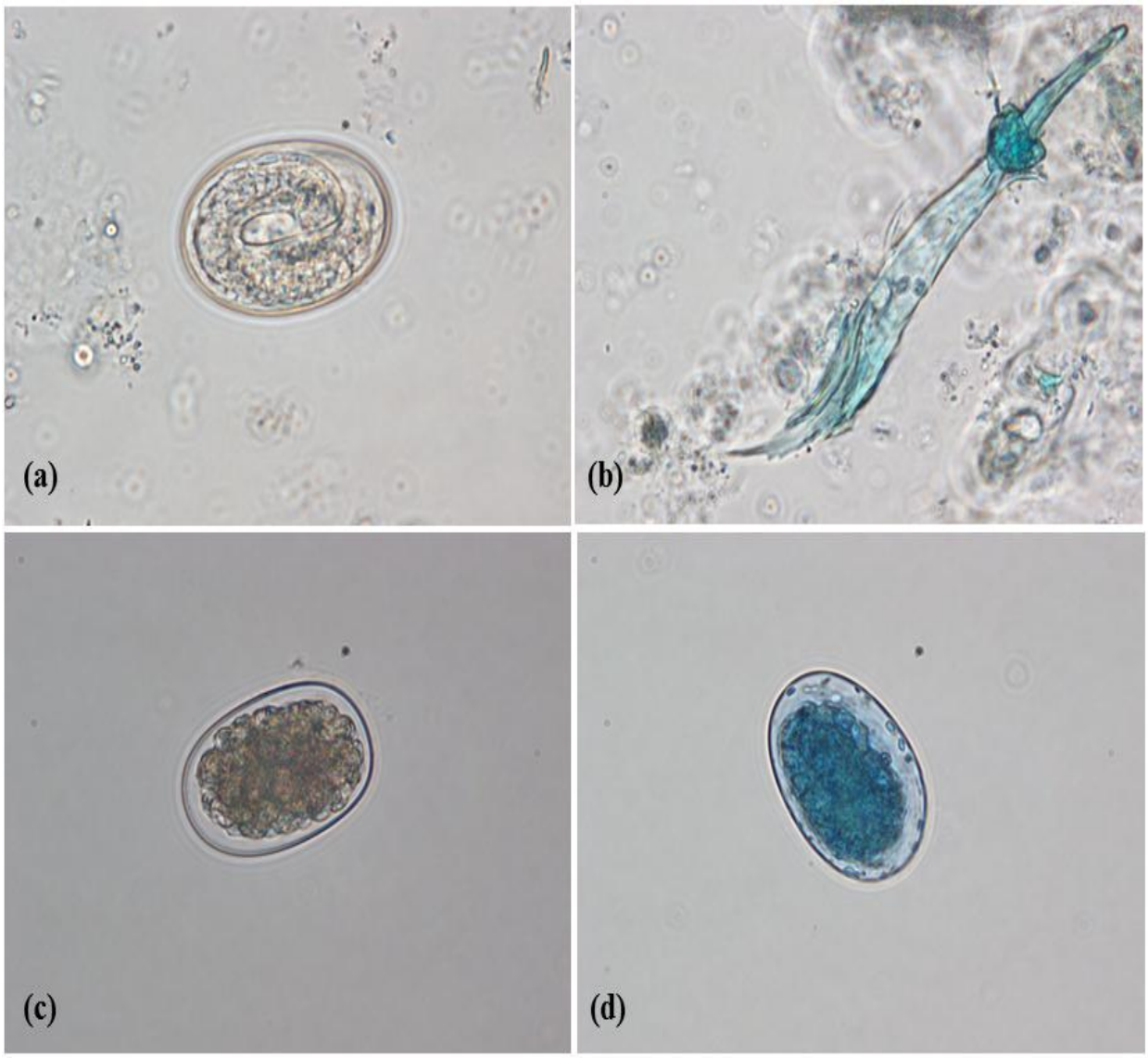

Figure 8.2: Photomicrographs of (a) embryonated ova, (b) larva, (c) viable ova and (d) non-viable ova of hookworm (*A. caninum*) obtained using culture-based and vital stain methods

8.2.2.3. PMA-qPCR method

Approximately 1,000 ± 50 ova were transferred into 2 mL transparent centrifuge tube (n = 6) and treated with 100 µM PMA (Figure 8.1). The ova were incubated in the dark for 5 min to allow PMA to penetrate non-viable ova. Photo-induced cross-linking of PMA to DNA was achieved by using a PhAST Blue light (GeniUL, Spain) for 15 min. The tubes were further incubated on ice for 5 min. DNA from each sample was extracted using a Mo Bio Power Soil DNA Extraction Kit (Mo Bio, Carlsbad, USA) as described in Chapter 4.

To determine the gene copy numbers in the *A. caninum* ova (1,000 ± 50) used for PMA-qPCR, DNA was extracted directly from 1,000 ± 50 ova using a Mo Bio Power Soil DNA Extraction Kit (Mo Bio, USA) in replicates (n = 6) samples without PMA treatment and quantified using qPCR described in Chapter 5.

8.2.2.3.1. qPCR standard and cycling parameters

Previously published primer and probe sets were used for the qPCR/PMA-qPCR assay (Chapter 7). The qPCR amplifications were performed in 25 µL reaction mixtures containing 12.5 µL iQ™ Supermix (Bio-Rad Laboratories, Calif), 3 µL of template DNA, 250 nM of each primer, 400 nM of probe and UltraPure™ DNase/RNase-free distilled water (Life Technologies, Australia). For each qPCR/PMA-qPCR assay, a standard series (developed in the previous chapter) and a negative (UltraPure™ water) control (in triplicate) were included. The PCR cycling parameters cycling parameters were as follows: 95°C for 15 min, 95°C for 15 s, 59°C for 1 min for 40 cycles. The qPCR analysis was performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA). All qPCR reactions were performed in triplicate.

8.2.2.3.2. Conversion to numbers of ova from PMA-qPCR estimated gene copy numbers

The PMA-qPCR estimated gene copies numbers were converted to viable ova numbers by using the equation 5.

$$N_{VO} = \frac{NG_{PMA(qPCR)}}{NG_{qPCR}} \times N_O \qquad (5)$$

*W*here, N_{VO} is numbers of viable ova, $NG_{PMA(qPCR)}$ is average gene copy numbers estimated by PMA-qPCR, NG_{qPCR} is average gene copy numbers estimated by qPCR and N_O is total numbers of ova. Similarly, the numbers of non-viable ova were obtained by using the equation 6.

$$N_{NVO} = \frac{NG_{qPCR} - NG_{PMA(qPCR)}}{NG_{qPCR}} \times N_O \qquad (6)$$

*W*here, N_{NVO} is numbers of non-viable ova, $NG_{PMA(qPCR)}$ is average gene copy numbers estimated by PMA-qPCR, NG_{qPCR} is average gene copy numbers estimated by qPCR and N_O is total numbers of ova.

8.2.3. Statistical analysis

Microsoft excel ver. 2010 (Microsoft, USA) was used to conduct the statistical analysis. A T-test for equal means was performed to determine the significant difference between the numbers of viable ova.

8.3. Results

8.3.1. Numbers of viable and non-viable ova using different methods

Out of the 1,000 ± 50 ova analysed by the culture based method, it was determined that an average of 694 ± 27 ova were quantifiable (viable and non viable), and the 306 ± 27 ova were unaccounted for due to inbuilt analytical errors. Among the quantified ova, 397 ± 59 were found to be viable and 296 ± 52 non viable (Figure 8.3).

Similarly, the vital stain method quantified an average of 751 ± 50 ova (viable and non viable), with 249 ± 50 ova unaccounted for due to analytical errors. Among the quantified ova using the vital staining, 644 ± 57 were viable and 107 ± 31 were non viable (Figure 8.3). Of the 1,000 ± 50, the PMA-qPCR quantified an average of 595 ± 74 viable and 405 ± 75 non viable ova (Figure 8.3). Due to the PCR method, no ova were unaccounted for.

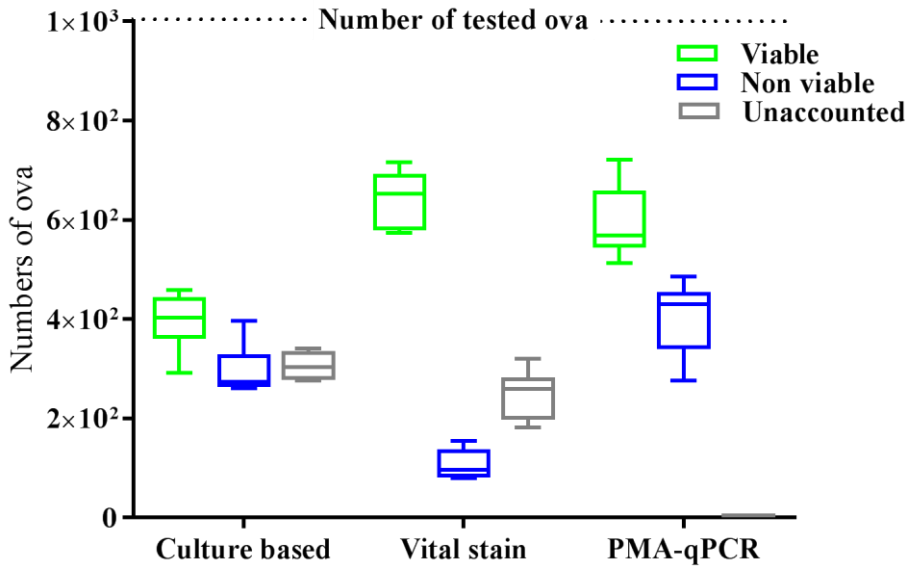

Figure 8.3: Numbers of viable, non viable and unaccounted ova estimated by three different quantitative methods

8.3.2. Comparison of viability assessment

The PMA-qPCR method estimated an average of 54 ± 6% viability of *A. caninum* ova in the tested samples (Figure 8.4). The PMA-qPCR estimated the viability of ova in the test samples was 19% lower than the percentage of viability of ova (64 ± 5%) estimated by the vital stain method. In comparison, the percentage of viability estimated by the PMA-qPCR was 38% higher than the viability of ova estimated by the culture based method (40 ± 6%).

The viability assessed by the culture based method were significantly ($P < 0.05$) lower than that estimated by both PMA-qPCR and vital stain methods. T-test for equal means indicated that the viability assessed by vital stain method and PMA-qPCR method were not statistically significant ($P > 0.05$).

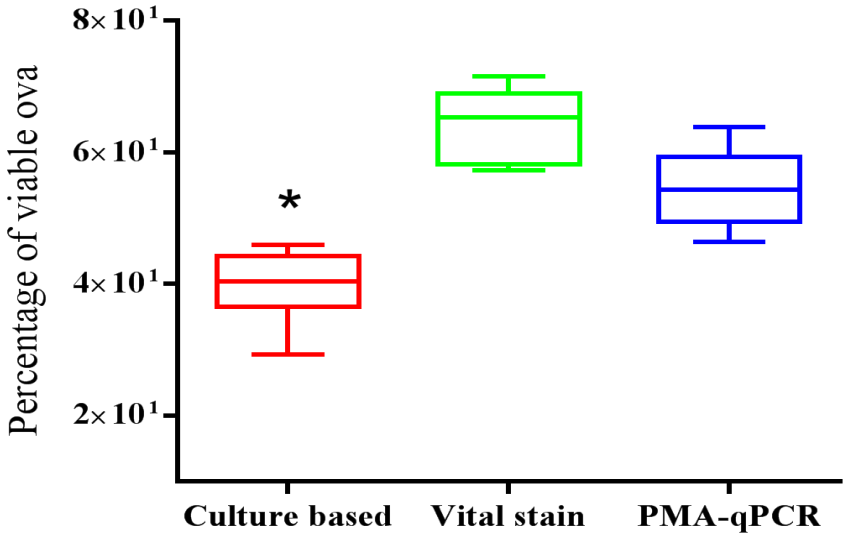

Figure 8.4: Box and Whisker plots of an average percentage of viability assessed by three different detection methods for *A. caninum* ova. The inner box lines represent the medians while the outer box lines represent 5th and 95th percentiles (n = 6). Statistically significant result is indicated with star (*).

8.4. Discussion

Quantitative detection and differentiation between viable and non-viable hookworm ova in point source samples such as environmental soil, wastewater, sludge and contaminated foods are necessary in order to implement strategies to eliminate re-infections in endemic regions (McCarthy et al., 2012; Gyawali et al., 2016b). Despite the advancements of PCR/qPCR methods, the viability of hookworm ova has been determined by either culture-based or vital stain methods. This might be because PCR/qPCR methods are unable to differentiate between viable and non-viable ova. The recent development of PMA-qPCR method overcomes such as limitations of qPCR method and allows quantitative detection of viable hookworm ova from environmental samples (Chapter 7). In this study, viability assessment performance of PMA-qPCR method was compared against culture-based and vital stain methods in the laboratory settings.

Among the three methods compared, the culture based method has estimated the lowest percentages of viable ova in the samples compared to the PMA-qPCR and vital stain methods. Such discrepancy could be the fact that, the hookworm ova tested in this study were recovered from dog faecal samples using a flotation method described by Bowmen et al.,

(2003) and stored in 0.5% formalin for a day. The recovery method involves various steps and chemicals that can compromise the structural integrity of the cell wall of ova (Nelson and Darby, 2001). Incubating those cell wall-compromised ova at 28-30ºC for a week might result in the loss of ova prior to microscopic observation. In addition, microscopic observation always has inbuilt errors which significantly reduce the sensitivity of the method (Weber et al., 1999; Verweij et al., 2007; Gyawali et al., 2015a).

Despite inbuilt errors such as (low detection threshold of a microscope, ova need to be observed within 30 min) associated with the microscopic observation, the vital stain method has estimated highest percentages of viable ova in the samples compared to the culture based and PMA-qPCR methods. This could be because the cell wall of inactivated ova may act as an alternative filter (Matthews, 1986; Bae and Wuertz, 2009) and prevent stains from reaching the cytoplasm of the ova. Gyawali et al., (2016a) reported that the inactivated ova require up to 12 h to become permeable to the stains. Similar results of overestimation of viable ova using vital stain methods have already been reported by others (Nelson and Darby, 2001; Kato et al., 2001; Schlosser et al., 2001; O'Grady and Smith, 2002). In this study, the ova were stained for 5 min prior to the microscopic observation, which is acceptable for the vital stain method. Although PMA is a dye (DNA binding), the process of PMA treatment involves incubating the ova in a bright light for 15 min followed by ice for 5 min (Nocker et al., 2006; Nocker et al., 2007; Nocker et al., 2009). This incubation process may help PMA to enter to the cytoplasm of recently inactivated ova and make a covalent bond with DNA. This could be reason for lower percentages of viable ova estimated by PMA-qPCR than the vital stain method. Therefore, further study would focus identifying whether staining the ova for more than 5 min would reduce the numbers of viable ova in a sample using vital stain method.

The major disadvantage of culture based and vital stain methods identified in this study was a loss of significant numbers of ova. This might be associated with either inbuilt errors of microscopy or individual judgement while identifying hookworm ova during visualisation. This phenomenon, however, was not observed while using PMA-qPCR method because the method quantify ova on the basis of amplified gene copy numbers with no inbuilt error and less individual judgement. However, the PMA-qPCR generated gene copy numbers need to be converted to ova. There is a lack of clear information regarding the accurate gene copy

numbers per ovum. Therefore, total gene copy numbers for 1,000 ± 50 ova needed to be estimated using qPCR method in parallel with PMA-qPCR method.

Despite difficulty estimating accurate gene copy numbers per ovum, the result indicated that the approach taken in this study could be used for assessing the in-vitro viability of hookworm ova. The percentage of viability assessed by PMA-qPCR method was 19% lower than vital stain and 38% higher than culture based methods. Since the culture based and vital stain, the methods can underestimate and overestimate the viability of ova, respectively (Maya et al., 2006; Katakam et al., 2014), the viability assessed by PMA-qPCR could be more reliable than that of the other two methods. In addition, PMA-qPCR method is specific and more sensitive than both methods (Botes et al., 2013; Schar et al., 2013; Gyawali et al., 2016c) and the result can be obtained within 4-6 h, the method could be an alternative option for detecting viable hookworm ova from environmental samples including wastewater and sludge. Further study using environmental samples however, would be required to validate the results obtained in this study.

8.5. Conclusions

In conclusion, specific detection/quantification of viable hookworm ova from environmental samples is fundamental to assess the potential human health risks. Culture-based and vital stain methods rely on the sensitivity of a microscope. The sensitivity of a microscope associates with the inbuilt error, such as loss of ova during observation. The PMA-qPCR method has provided more viable hookworm ova in the tested samples than culture-based and less viable ova than vital stain method. Since the vital stain method has a tendency of overestimating viable ova in a particular sample, the viability of hookworm ova estimated by PMA-qPCR could be applicable for assessing the potential health risks. The viability of hookworm ova assessed by vital stain method was not significantly different from the PMA-qPCR, therefore, vital stain method can still be a cheaper option for quantifying viable hookworm ova unless there is a need for specific detection.

Chapter: 9
General discussion

9.1. Discussion

With an estimation of 8.0×10^8 million infections, hookworm is a major public health problem in developing countries (Hotez et al., 2005; Brooker et al., 2008; WHO, 2012; Knopp et al., 2012). Various interventions such as mass drug administration (MDA), water sanitation and hygiene (WASH) programs combined with public health education such as, always wearing shoes, washing your hands before eating, and staying away from water/area contaminated by human faeces have been introduced to control the hookworm infection in the developing countries (WHO, 2012a; WHO, 2012b; Campbell et al., 2015).

Regardless the combined efforts to control hookworm infection in poverty-stricken communities, it has proven difficult (Brooker et al., 2010; Hotez et al., 2014). This could be because of multiple reasons such as i) people living in poverty may not be able to afford shoes to wear, ii) may not have access to clean water, iii) live in environments without proper sanitation (Hotez et al., 2005; WHO, 2006; Gyawali, 2012), and, iv) their livelihood depends on agriculture (Carr, 2005) where partially treated wastewater, raw wastewater and sludge is widely used (Carr, 2005; Vuong et al., 2007; Gupta et al., 2009) that significantly increase a load of viable hookworm ova on the environment and infects human (Vuong et al., 2007; Gupta et al., 2009; Trang et al., 2007; Karkashan et al., 2012).

Since WHO established the relationship between diarrhoeal disease and reuse of wastewater and sludge for agriculture in 1989, safe use of wastewater and sludge reuse guideline has been developed (WHO, 2006). The guideline suggests ≤ 1 helminth ova (*Ascaris lumbricoides*) per L treated wastewater. This lower limit of helminth ova in wastewater was recommended because the available detection methods were unable to detect less than that. A QMRA conducted by Navarro and his colleagues in 2011 indicated that, treated wastewater and sludge treated to WHO standards can have public health consequences while using for crop production.

Therefore, there was a strong interest to modify the WHO guidelines and lower, the limit of helminth ova in treated wastewater from ≤ 1 ova to ≤ 0.1 ova per L (IWMI, 2010). This proposal was not adopted because there was not a method for detecting less than an ovum from treated wastewater. Despite the little importance has been given to develop a standardised sensitive detection method that can quantify the low numbers of ova from

wastewater and sludge. In this study, a real time PCR method has been successfully developed using *A. caninum* ova as a surrogate of human hookworm, for detection of viable hookworm ova from environmental samples such as wastewater, sludge, and soil. The sensitivity of the method was found to be less than one ovum for treated wastewater (1L). Therefore, with this newly developed PCR method, WHO guideline can be modified with purposed limit of helminth ova (< 0.1) in treated wastewater (1L). In addition, the PCR method does not require skill personal identify helminth ova. Therefore this method has the potential to be a standardised method for monitoring treated wastewater quality across the world.

The detection sensitivity of the PCR method however, decreased one order of magnitude for raw wastewater and treated sludge. The decreased sensitivity obtained for raw wastewater and sludge samples could be because the samples were more turbid than treated wastewater. Therefore, further study is needed developing a reliable, rapid and improved method for recovering hookworm ova from raw wastewater and sludge samples.

Specific detection hookworm ova to the species level is another advantage of PCR method (Traub et al., 2007; Ye et al., 2012) over culture based and vital stain methods while detecting hookworm ova from environmental samples, which is important to determine the potential public health risk because some species of hookworms are only accidental parasite for human species (Traub et al, 2007; Traub et al., 2008). Another benefit of the specific detection of hookworm is that, it potentially can assist to answer whether canine are reservoir host for human hookworm. Due to the morphological similarity between canine and human hookworm ova, microscopic methods (culture based and vital stain) are unable to distinguish ova to the species level (Verweij et al., 2007; Traub et al., 2007; Traub et al., 2008; Ngui et al., 2012). This could be a reason for hookworm re-infection to baseline levels soon after MDA programmes are ceased in endemic regions (Campbell et al., 2014). In conjunction with MDA and other interventions, the newly developed method can assist to eliminate hookworm infection from endemic regions.

Detection of viable hookworm ova in the sources such as food, soil and water of hookworm reinfection is fundamental to eradicate the hookworm infection from an endemic region. Currently used detection methods to detect viable hookworm ova/larvae from point of sources have unsatisfactory performance and are not well suited for use in the parasite

control programmes (Traub et al., 2007; Traub et al., 2008; McCarthy et al., 2015; Gordon et al., 2015; Gyawali et al., 2015a); because culture based method potentially underestimate and vital stain method overestimate the viability (Maya et al., 2006; Katakam et al., 2014). Since the viability of hookworm ova assessed by PMA-qPCR was 30% higher than the culture based method and 18% lower than the vital stain method, the viability assessed by PMA-qPCR method could be more accurate than other two methods. Therefore, the PMA-qPCR method can be a tool to detect viable hookworm ova more accurately from the point of source samples and fight against hookworm infection.

Although *A. caninum* ova were used in this study to develop the PMA-qPCR method, the general structure of the ova is similar to other pathogenic STHs. This means that the method can be customised to detect other STHs ova such as *A. duodenale, N. americanus,* and *A. lumbricoides* from environmental samples including wastewater matrices by designing the species-specific primers and probes (Traub et al., 2007; Ye et al., 2012). The future outcome of this study could be the development of gene-based oligonucleotide microarray that can detects multiple pathogenic helminths from environmental and faecal samples, similar to the array that has already been used to monitor bacterial community in the environment (Sergeev et al., 2004; Dugat-Bony et al., 2012).

Another advantage of the new PMA-qPCR method is its ability to quantify the number of viable hookworm ova from environmental samples which is fundamental for assessing the magnitude of risks (Navarro and Jimenez, 2011). The PMA-qPCR method quantifies viable hookworm ova on the basis of amplified target gene copies. Since the targeted gene (rRNA) from ITS-1 region has multiple copies, a kinetic study starting with an early stage of ova and ending just before larvae would be appropriate to estimate the numbers of gene copies per ovum. However, there is a problem of conducting such kinetic study for hookworm ova because ova excreted in the host faecal sample may not be at the same cell stage. In addition, the ova are three-dimensional and it is difficult to estimate the actual cell number using simple microscope. Since the infectious dose of hookworm is low (1-10 larvae), any PMA-qPCR positive signals should be considered as health risks.

PCR inhibitors could be an issue using this method for regular monitoring hookworm ova in wastewater and sludge samples (Rock et al., 2010; Schrader et al., 2012; Xu et al., 2010). Therefore, a PCR inhibition test should be conducted prior to analysing the samples. Commercially available DNA extraction kits may not always eliminate the PCR inhibitors, especially from sludge samples. This issue, however, can be resolved by applying common strategy of diluting the DNA (10 to 100 fold) depending on the concentration of PCR inhibition in the DNA samples (Drosten et al., 2002; Audemard et al., 2004; Van Doorn et al., 2009).

Chapter: 10

Conclusion

10.1. Conclusion

The increasing use of the wastewater and sludge for crop production or the direct discharge into surface water can have negative public health implications due to the presence of viable hookworm ova. Several guidelines have been developed to set the standard and set the limit of numbers of helminth ova in the treated wastewater and sludge. This limit was established on the basis of the minimum level of detection using currently available methods. However, there is evidence of infections occurring when the WHO standard wastewater and sludge is applied for agriculture. Therefore, there was a strong expression to improve the guideline and lower the threshold limit of helminth ova to 0.1 per L in treated wastewater for unrestricted use. Detection of helminth ova into the treated wastewater and sludge using currently used method is complicated by the presence of multiple species. Furthermore, the sensitivity of those methods can be low because of the low detection limit of a microscope. Since a hookworm ovum contains large numbers of gene copy PCR method can detect low numbers (0.1 per L) of ova from wastewater and sludge samples. The main findings of this study, potential impact, limitations and future directions are as follow.

10.2. Major findings

i. The PCR/qPCR method developed in this study offers specific detection of hookworm ova from wastewater and sludge.
ii. The sensitivity of the PCR method is ~ 0.4 ova in treated wastewater (1 L) and ~ 4 ova in raw wastewater (1 L) and sludge (4 g) samples.
iii. The PCR method is rapid and the result can be obtained within 6 h.
iv. Since the detection of hookworm ova from treated wastewater, raw wastewater, and sludge samples require isolation and concentration of ova, the rapidness of the method depends on the time taken to isolate and concentrate the ova from the wastewater and sludge samples.
v. The detection sensitivity of the PCR method depends on the effective recovery of hookworm ova from wastewater and sludge.
vi. The sieve filtration (series of sieves) provides consistency and good recovery rate of the hookworm ova from treated wastewater (39 ± 26 – 50 ± 38%) and raw wastewater (12 ± 10 – 7 ± 13%) samples.

vii. The recovery rate of hookworm ova from sludge samples using the flotation method is poor (3.7 ± 9%).

viii. Wastewater and sludge samples may contain PCR inhibitors, therefore, a PCR inhibition test on the DNA samples should be mandatory.

ix. A significant amount of DNA can be lost during the DNA extraction process therefore, DNA recovery efficiency of the kit should be mandatory.

x. Since the qPCR quantifies the numbers of ova on the basis of gene copy numbers present in the DNA samples, an accurate quantitation of hookworm ova using qPCR method is not possible when targeting ITS-1 rRNA or rDNA gene.

xi. Despite the difficulties quantifying hookworm ova using qPCR, when combined with photoactive dye (PMA), the method can be used for selective detection of viable hookworm ova from wastewater and environmental samples.

xii. The viability assessment performance of the developed PMA-qPCR method was 30% more than culture based method and 20% lower than vital stain method.

10.3. Potential impacts relating to health risk assessments

i. Since the PCR method is specific the method can be used to distinguish human hookworm with animal ones.

ii. Since the sensitivity of the method is ~ 0.4 ova from treated wastewater (1 L) samples, this method can be used to purposed limit of helminth ova in wastewater and sludge.

iii. Since the infectious dose of hookworm ova is low (1-10 viable ova/larvae), the PMA-qPCR has potential to measure the public health risk associated with treated wastewater and sludge use for agriculture.

iv. Since the structures of the soil-transmitted helminth ova are similar, the PMA-qPCR would be useful for detecting viable ova for other helminths of interest.

v. Given the ability to discriminate non viable ova from viable ones in a short period of time, the PMA-qPCR has the potential to test more samples with reduced labor cost.

vi. Since the results can be obtained in a short period of time, the health regulators and utility operators can assess the efficiency of the wastewater and sludge treatment methods in real time.

vii. The method can be a useful tool for identifying the source of re-infection of hookworm as well other STHs in MDA and water sanitation and hygiene (WASH) implemented communities.

10.4. Future study

i. Future study would focus on improving ova isolation and recovery from raw wastewater and sludge samples. This would improve the sensitivity of the qPCR method.

ii. Since the ITS-1 rRNA/.rDNA gene copy numbers in the ova vary according to the cell development stage, further kinetic studies would require estimating the gene copy numbers in the each cell development stage of the hookworm ova.

iii. Further study should focus on identifying a stable target that would improve the accuracy of quantification of hookworm ova using qPCR method.

iv. The PMA exploits the structural integrity of the cell wall of the inactivated ova, which requires 12 h of incubation at room temperature to become permeable to the stains. Therefore, ova isolated from the samples should be incubated at room temperature for 12 h prior to PMA treatment.

v. Although the PMA-qPCR method is rapid, sensitive and specific, it requires advanced equipment, reagents, and facilities. This might restrict the application of PMA-qPCR method in poor countries where the hookworm infection is endemic.

vi. Multi-omics (metagenomics, metabolomics, and proteomics) are the most rapidly growing technologies that might identify low-cost biomarkers from infectious helminths, including hookworm. Further study would focus on identifying biomarkers from individual helminths and developing a library of biomarkers, which can be used to assist in detecting, identifying and quantifying helminths in the future.

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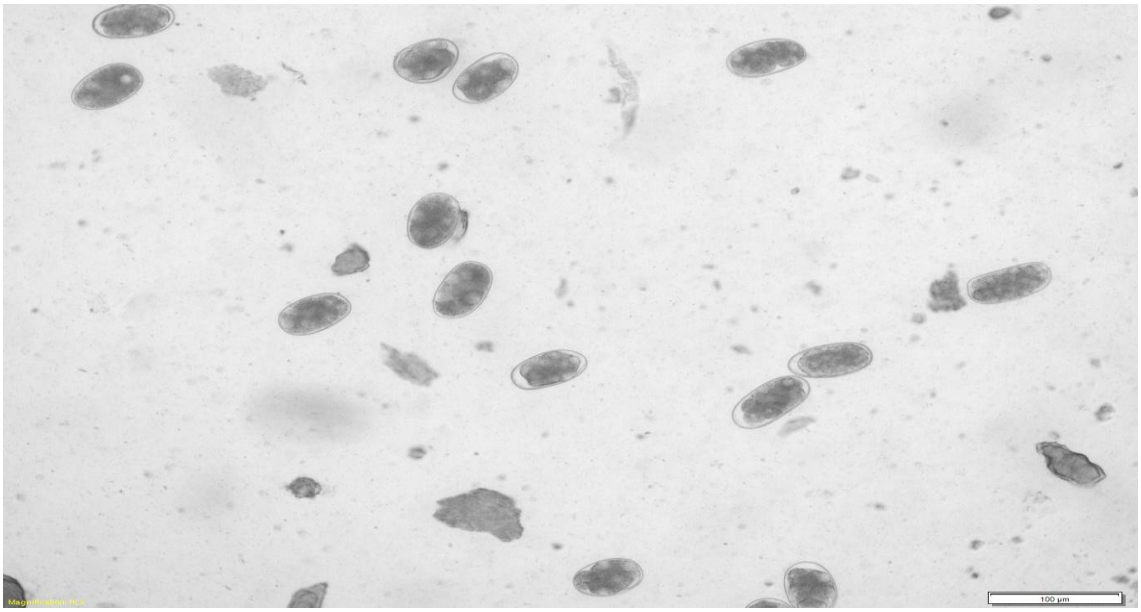
Appendices

Appendix-A



Photomicrograph (100x) of *A. caninum* ova isolated from dog faecal samples and further used for the experiments

Appendix-B

Numbers of *A. caninum* ova in each stock solution

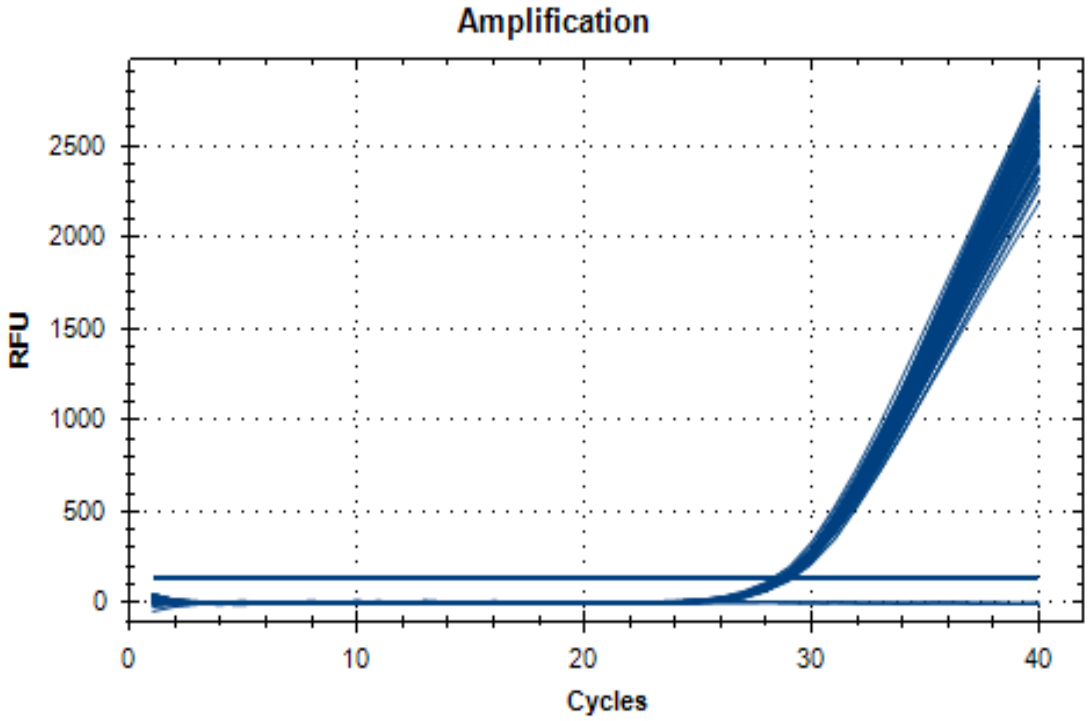
Stock No	Volume of stock (mL)	Stock use for enumeration (µL)	Mean ± SD No of ova/20 µL
1	10	20	$4.1 \times 10^2 \pm 16$
2	10	20	$3.9 \times 10^2 \pm 18$
3	10	20	$4.8 \times 10^2 \pm 19$
4	10	20	$4.6 \times 10^2 \pm 21$
5	10	20	$4.0 \times 10^2 \pm 18$
6	10	20	$7.5 \times 10^2 \pm 27$

Appendix-C

Mean and standard deviation (SD) of C_T values for different concentration of primer and probe concentration

Primer concentration (nM)	Probe concentration (nM)					
	300	400	500	600	700	800
100	30.3 ± 1.4	29.3 ± 0.7	29.6 ± 0.5	31.7 ± 0.8	29.6 ± 1.4	30.8 ± 1.9
200	30.6 ± 1.2	29.7 ± 0.4	30.4 ± 0.4	31.0 ± 1.2	30.1 ± 1.1	30.6 ± 1.4
250	30.3 ± 0.5	**29.3 ± 0.1**	30.3 ± 0.7	31.8 ± 0.6	30.6 ± 1.6	29.8 ± 1.5
300	31.8 ± 0.4	30.8 ± 0.2	30.8 ± 0.5	30.7 ± 1.6	31.5 ± 1.7	29.7 ± 1.2
350	30.9 ± 0.3	31.2 ± 0.2	31.6 ± 0.9	31.1 ± 0.9	29.7 ± 0.6	30.3 ± 1.5
400	32.1 ± 0.7	31.2 ± 0.5	31.5 ± 0.7	31.2 ± 0.5	31.1 ± 1.4	30.4 ± 0.8

Appendix-D



PCR amplification of Sketa22 PCR assay for the evaluation of PCR inhibition in *A. caninum* ova seeded secondary treated wastewater and unseeded treated wastewater, raw wastewater and sludge DNA samples as opposed to *O. keta* DNA (10 pg) seeded UltraPure™ water samples

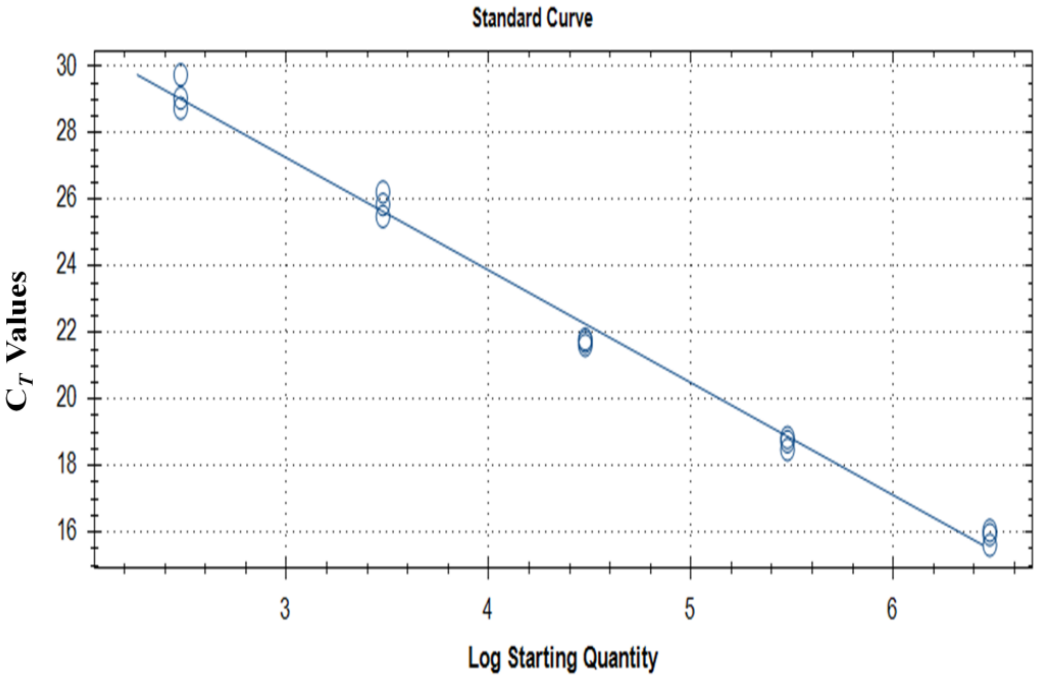
Appendix-E

Sketa22 real-time PCR assay for the evaluation of PCR inhibition in ova seeded DNA samples as opposed to distilled water samples. Mean ± standard deviation of C_T values for distilled water samples = 28.5 ± 0.2

Concentrations methods	Wastewater Sample types	Mean ± standard deviation of threshold cycle (C_T) values for Sketa22 PCR assay			
		Undiluted DNA samples		10-fold diluted DNA samples	
		WWTP-A	WWTP-B	WWTP-A	WWTP-B
Method A	Treated	27.8 ± 0.2	31.2 ± 1.9	NA	29.0 ± 1.7
	Raw	28.0 ± 0.1	31.4 ± 1.2	NA	27.0 ± 0.2
Method B	Treated	27.7 ± 0.1	30.5 ± 0.2	NA	29.2 ± 1.8
	Raw	28.0 ± 0.1	30.0 ± 0.1	NA	NA
Method C	Treated	28.1 ± 0.1	29.9 ± 0.1	NA	NA
	Raw	28.3 ± 0.4	33.0 ± 1.5	NA	27.0 ± 0.1
Method D	Treated	28.1 ± 0.2	29.8 ± 0.1	NA	NA
	Raw	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
Method F	Sludge	NAP	NAP	29.2 ± 0.1	27.1 ± 0.1

NA: Not applicable
NAP: No amplification

Appendix-F



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

A standard curves generated using the plasmid DNA. The numbers of gene copies are plotted against C_T values. The C_T is the cycle number at which the fluorescence signal increased above the defined threshold value, calculated by the real-time PCR software.

Appendix-G

Fwd: Your Submission is accepted by JES (2)

To: pradep033@yahoo.com
Subject: Your Submission is accepted by JES

Ms. Ref. No.: JES-D-16-00962R1
Title: Quantification of hookworm ova from wastewater matrices using quantitative PCR
Journal of Environmental Sciences

Dear Mr. Gyawali,

I am pleased to inform you that your paper "Quantification of hookworm ova from wastewater matrices using quantitative PCR" has been accepted for publication in Journal of Environmental Sciences.

Below are comments from the editor and reviewers.

Thank you for submitting your work to Journal of Environmental Sciences.

Yours sincerely,

Journal of Environmental Sciences

Comments from the editors and reviewers:

Reviewer #2: After review the manuscript titled "Quantification of hookworm ova from wastewater matrices using quantitative PCR" (MS#JES-D-16-00962R1), I feel that the authors have carefully addressed the issues raised by the reviewers and they have made some changes in their manuscript accordingly. I have no further comments for the revised manuscript, and the draft may be ready for publication.

A letter from the Journal of Environmental Sciences regarding accepting article "Quantification of hookworm ova from wastewater matrices using quantitative PCR" came out from this thesis.