TECHNISCHE UNIVERSITÄT MUNCHEN

Lehrstuhl für Bodenökologie

Quantification of *Cryptosporidium parvum* and enteroviruses by quantitative Real-Time PCR (qPCR) in environmental samples - Methodological developments for monitoring anaerobic systems

Gabriela Garcés Sanchez

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Dr. h.c. J. Bauer

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. Ch. Munch
2. Univ.-Prof. Dr. H. Horn (Karlsruhe Institute of Technology)

Die Dissertation wurde am 15.10.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 06.05.2013 angenommen.

Abstract

Manure and wastewater are valuable sources of nutrients that are used in agriculture. Besides the nutrient content and their economic value, these matrices can also be carriers of pathogenic microorganisms and viruses that can affect the health of living beings. *Cryptosporidium* strains and enteroviruses are human and animal pathogens that can be present. Both show resistance against various environmental stresses and are very infective and of high significance for public health.
Effective hygiene control of manure and wastewater as well as control of the sanitizing efficiency of treatment systems is only possible when suitable detection methods are available. This work aimed at advancing the application of molecular detection tools based on real-time qPCR and RT-qPCR in this field.

A DNA extraction procedure for *Cryptosporidium* oocysts in manure and for detection of the *hsp70* gene by real-time qPCR was developed and optimized. Emphasis was on the oocyst lysis and nucleic acid extraction steps, as these are of high importance for application with complex matrices and oocysts with strong walls. The DNA based qPCR detection method was evaluated with soil from agricultural land and manure and digested manure from anaerobic fermenters to assess the screening potential for oocysts and the treatment efficiencies.
Furthermore, optimized lysis and extraction procedures for RNA and mRNA were developed and evaluated in RT-qPCR based assays. RNA enteroviruses and mRNA production in oocysts were quantified in complex samples within this context. The optimized methods for *C. parvum* were applied to different environmental matrices and oocysts with different viability in order to assess their viability by targeting *hsp70* mRNA. The procedures were used to evaluate the inactivation and degradation of *C. parvum* in anaerobic digesters treating manure.

This work shows that lysis by cumulative bead beating for 165 s (performed in time intervals) allows rapid and efficient recovery of nucleic acids from oocysts. The optimized DNA extraction and qPCR detection method can be applied to complex substrates like manure and soil, provides specific detection in short time, and allows the quantification of *Cryptosporidium* pathogens. The DNA based method applied to manure yielded a detection limit of $2.4 \times 10^2 - 8.3 \times 10^2$ oocysts / ml sample with a DNA recovery of 83 %. The analyses of manure and digested manure from anaerobic bioreactors show that the DNA measurement

alone underestimates the reduction of pathogen viability and the sanitizing efficiency of the treatment system.

Measuring the *hsp70* mRNA content, however, showed a positive relation with viability. This was found for oocysts subjected to thermophilic anaerobic treatment, as well as for oocysts aged over a period of 12 months. An indication of the viability of *Cryptosporidium* oocysts contained in environmental substrates can be given by measuring the *hsp70* mRNA content.
In addition, the measurement of the *hsp70* mRNA production response to a short heat shock trigger was shown to provide an indication of the vitality of oocysts present in a sample, even when this response was small. The production of *hsp70* mRNA after heat shock for fully viable and infectious oocysts was increased by a mean factor of 1.6 times. For samples containing a reduced fraction of viable oocysts, the factor was lower than 1.2 fold.
The optimized (m)RNA based methods provided reliable and sensitive detection for manure and digested manure in the RT-qPCR assay. The direct extraction and purification of mRNA from these matrices using cumulative bead beating as lysis step and oligo $(dT)_{25}$-magnetic beads for mRNA extraction in combination with a specific purification procedure yielded a detection limit of *hsp70* mRNA from 5.5×10^3 - 8.1×10^3 viable oocysts / ml sample and occasionally even from a lower oocyst concentration. However, qPCR results obtained for manure could not be reproduced for spiked agricultural soil samples with relatively high clay content.

The optimized DNA and RNA based (RT)-qPCR detection systems allow the quantification of *C. parvum* oocysts and enteroviruses in complex environmental samples. For enteroviruses in manure, the method extraction and detection efficiency was 37 %.

For sanitizing purposes, thermophilic (anaerobic) treatment at 55°C can ensure the inactivation of oocysts in a short time (1 or 4 h), as seen by infectivity test results. At this temperature and retention times, a reduction in *hsp70* mRNA content of $\geq$ 1.5 log units was obtained. The reduction of mRNA content can therefore be associated to the inactivation of oocysts in hygiene tests of treatment systems, e.g. using sentinel chambers.

The developed molecular tools can be useful for the rapid, sensitive and specific screening of *Cryptosporidium* and enteroviruses in complex environmental samples in order to identify contaminated sources from which these pathogens can disseminate in the environment.

Zusammenfassung

Gülle und Abwasser sind wertvolle Reststoffe mit Nährstoffen, die in der Landwirtschaft Verwendung finden. Beide Substrate sind allerdings auch potentielle Träger von pathogenen Mikroorganismen und Viren, die die Gesundheit von Mensch und Tier beeinträchtigen können. Kryptosporidien und Enteroviren gehören zu diesen Pathogenen. Beide zeigen Resistenz gegen verschiedenste Umwelteinflüsse, sind sehr infektiös und haben eine hohe Relevanz für die öffentliche Gesundheit.
Effektive Hygienekontrolle für Gülle und Abwasser sowie die Steuerung der Hygienisierung von Behandlungssystemen ist nur möglich, wenn geeignete Methoden zum Nachweis von Pathogenen zur Verfügung stehen. Ziel dieser Arbeit war die Untersuchung, Entwicklung und Anwendung von auf Real-Time qPCR und RT-qPCR basierenden molekularen Detektionsmethoden für die gennanten Krankheitserreger.

Ein DNA-Extraktionsverfahren für *Cryptosporidium*-Oozysten in Gülle und die Detektion des *hsp70*-Gens durch quantitative Real-Time qPCR wurde entwickelt und optimiert. Ein Schwerpunkt lag auf der Oozysten-Lysis und der Nukleinsäureextraktion, da diese von hoher Bedeutung für die Anwendung in komplexen Matrizen und mit dickwandigen Oozysten sind. Um das Potenzial für den Einsatz im Screening nach Oozysten und die Untersuchung der Effizienz von Behandlungssystemen zu beurteilen, wurde die DNA-basierte qPCR-Nachweismethode anhand von Bodenproben aus landwirtschaftlichen Flächen, Gülle und Gärresten aus der anaeroben Behandlung evaluiert.
Weiterhin wurden optimierte Lysis- und Extraktionsverfahren für RNA und mRNA entwickelt und mit RT-qPCR-basierten Methoden evaluiert. In diesem Zusammenhang wurden RNA-Enteroviren und mRNA-Produktion in Oozysten in komplexen Proben quantifiziert. Die optimierten Methoden für *C. parvum* wurden auf verschiedene Umweltproben und Oozysten unterschiedlichen Alters mit dem Ziel angewendet, die Lebensfähigkeit der Pathogene mittels RT-qPCR mit *hsp70*-mRNA als Zielmolekül zu beurteilen. Die Verfahren wurden verwendet, um die Inaktivierung von *C. parvum* während der anaeroben Behandlung von Gülle auszuwerten.

Diese Arbeit zeigt, dass die Lysis durch kumulatives Bead-Beating für 165 s (durchgeführt in Zeitintervallen) eine schnelle und effiziente Extraktion von Nukleinsäuren aus Oozysten ermöglicht. Die optimierte DNA-Extraktion und qPCR-Nachweismethode kann auf komplexe Substrate wie Gülle und Boden angewendet werden. Sie bietet einen spezifischen Nachweis in

kurzer Zeit und ermöglicht die Quantifizierung von *Cryptosporidium*-Pathogenen. Die Nachweisgrenze der DNA-basierten Methode für Gülle lag bei 2,4 x 10^2 – 8,3 x10^2 Oozysten / ml Probe mit einer DNA-Wiederfindungsrate von 83 %. Die Analysen der Proben aus den anaeroben Bioreaktoren haben gezeigt, dass die DNA-Messung keine direkte Beurteilung der Verringerung der Erreger und der Hygienizierungseffizienz des Behandlungsverfahrens erlaubt.

Die gemessene *hsp70*-mRNA-Produktion zeigte andererseits eine positive Korrelation mit der Lebensfähigkeit. Dies ergab die Untersuchung von Oozysten die einer thermophilen anaeroben Behandlung unterzogen worden sind und von Oozysten die über 12 Monate gelagert wurden. Eine Indikation für die Lebensfähigkeit von C*ryptosporidium*-Oozysten in Umweltproben kann durch die Messung des *hsp70*-mRNA-Gehaltes gegeben werden.
Darüber hinaus wurde gezeigt, dass die Messung der *hsp70*-mRNA-Produktion als Reaktion auf einen induzierten Hitzeschock ebenfalls die Vitalität der Oozysten indiziert, auch wenn diese Reaktion relativ gering war. Die *hsp70*-mRNA-Produktion für voll lebensfähige und infektiöse Oozystem erhöhte sich nach dem induzierten Hitzeschock um einen mittleren Faktor von 1,6. Für Proben, die einen reduzierten Anteil von lebensfähigen Oozysten enthielten, war der Faktor kleiner als 1,2.
Die optimierten (m)RNA-basierten Methoden erlauben einen zuverlässigen und sensitiven RT-qPCR-basierten Nachweis in Gülle und Gärresten. Für die Methode mit direkter mRNA-Extraktion unter Verwendung des kumulativen Bead-Beating für den Lysis-Schritt und mit Oligo$(dT)_{25}$-magnetischen Partikeln in Verbindung mit den speziellen Reinigungsverfahren für die Extraktion ergab sich für die mRNA eine Detektionsgrenze von 5,5 x 10^3 bis 8,1 x 10^3 vitalen Oozysten / ml Probe. Gelegentlich konnten sogar noch niedrigere Oozysten-Konzentration nachgewiesen werden.
Für mit Oozysten dotierte landwirtschaftliche Bodenproben mit relativ hohem Tongehalt konnten die (m)RNA-basierten Methode jedoch nicht zur quantitativen Bestimmung herangezogen werden, was auf eine verstärkte Inhibition zurückgeführt wurde.

Die optimierten DNA- und RNA-basierten (RT)-qPCR-Nachweismethoden erlauben die Quantifizierung von *C. Parvum*-Oozysten und Enteroviren in umweltrelevanten Matrizen. Für Enteroviren in Gülle lag die Extraktions- und Detektions-Effizienz bei 37%.

Die Ergebnisse der Infektivitätstests haben gezeigt, dass mittels thermophiler anaerober Behandlung bei 55°C eine Hygienisierung und Inaktivierung von Oozysten in einer kurzen Zeit (1 bzw. 4 h) erreicht werden kann. Bei dieser Temperatur und Verweilzeit konnte eine

Reduktion der *hsp70*-mRNA-Gehalte von ≥ 1,5 log-Stufen nachgewiesen werden. Die Messung der mRNA-Reduktion, z.B. unter Verwendung von Diffusions-Keimträgern, kann damit die Inaktivierung der Oozysten bei Hygienetests von Behandlungssystemen nachweisen.

Die entwickelten molekularbiologischen Methoden erlauben ein schnelles, sensitives und spezifisches Screening nach *Cryptosporidium*-Oozysten und Enteroviren in komplexen Umweltproben. Sie können mithelfen, Kontaminationen zu identifizieren und analysieren, von denen aus sich diese Erreger in der Umwelt verbreiten können.

Acknowledgements

This work was initiated in the frame of an inter-institutional project at the Institute of Water Quality Control, Technische Universität München.
I would like to express my profound gratitude to my advisor, Prof. Jean Charles Munch, Director of the Institute of Soil Ecology, Helmholtz Zentrum München, for his continuous support, positiveness and advises. His support has been invaluable during this project, and it would not have been possible to complete it without it. Thank you for the valuable discussions. It has been an honour to me. I am very thankful for your encouragements, especially in the difficult times.

My special gratitude is also to Prof. Harald Horn from the Karlsruhe Institute of Technology, Germany, for his co-advise and support. I thank him very much for the opportunity he gave me to conduct this research at the Institute of Water Quality Control, while he was chairing the institute. Thank you for your support in completion of this work, the open attitude and the helpful discussions.

I would like to thank deeply my project advisor Dr. Michael Lebuhn, from the Bayerische Landesanstalt fur Landwirtschaft, LfL, Germany, for the very valuable talks about this research, for his suggestions, and for critical and positive comments that helped to shape this work. Many thanks for the strong encouragement at every stage! Working with him and in his team, has been a great experience. Thank you for your friendship.

My gratitude I also would like to express to Prof. Peter A.Wilderer, Institute of Water Quality Control, for his motivating words and the inspiration that he gave me to work on topics related to waste and wastewater management. Thank you for the great opportunity that you gave me to learn and work in this field in Munich.

It is specially acknowledged the scholarships and financial support from: the TUM HWP-Program "Chancengleichheit für Frauen in Forschung und Lehre"; the Institute for Advanced Studies of Sustainability, Munich; and the STIBET-Program from the TUM Graduate School that supported this work.
Ms. Petra Ritter from the International office, TUM: thank you for your support at the times it was needed.

I thank my colleagues and friends Dr. Christoph Bauer and Dr. Mathias Effenberger from the LfL, for their comments and review of parts of this thesis. Besides, it has been always fun the moments we shared. Mathias, it was an interesting and positive time we had working with the model biogas plant in Garching.

I would like to thank Dr. Michael Najdrowski, formerly at the Universität Leipzig, for his technical comments and contribution to this work, and for his friendship and humour. I was always happy to receive the post with the little but hazardous living samples.
My thanks also to Dr. Martin Haslbeck, Institute of Biotechnology, TUM, for providing facilities and samples for experiments at his laboratory.

I thank my friends and colleagues from the Institute of Water Quality Control for the positive environment and their friendship: Claudia Behle, Andrea Hille, Manfred Lübken, Hoa Kieu, Danial Taherzadeh, Susanne Wießler, and many others that remain unforgettable. Thank you to Ursula Wallentits for her support during lab work and nice working days. Thanks to my friends Nhgia and Viviane from the Institute of Soil Ecology for your cheerfulness.

I also thank my good friends Tobias K. and Robert L. for being there, and for the positive daily life and nice after work dinners. Rob, thanks for your support and the daily green tea.

This has been a great learning and working experience. I thank very much to my parents, Laura and Francisco, for every word of encouragement at the best and at the most difficult times. *Muchas gracias por todo!* To my brothers Benjamin and Marko, thanks for being there!

For Laura

List of Publications

Papers in refereed publications

Garcés G., Effenberger M., Najdrowski M., Wackwitz C., Gronauer A., Wilderer P.A., Lebuhn M. (2006) Quantification of *Cryptosporidium parvum* in anaerobic digesters treating manure by (reverse-transcription) quantitative real-time PCR, infectivity and excystation tests. Water Sci. Technol. 53, 195-202.

Garcés-Sanchez G., Wilderer P.A., Munch J.C., Horn H., Lebuhn M. (2009) Evaluation of two methods for quantification of *hsp70* mRNA from the waterborne pathogen *Cryptosporidium parvum* by reverse transcription real-time PCR in environmental samples. Water Research 43, 2669-2678.

Garcés-Sanchez G., Wilderer P.A., Horn H., Munch J.C., Lebuhn M. (2013) Assessment of the viability of *Cryptosporidium parvum* oocysts with the induction ratio of *hsp70* mRNA production in manure. J. Microbiol. Methods 94, 280-289.

Conference papers

Garcés G., Effenberger M., Najdrowski M., Gronauer A., Wilderer P.A. and Lebuhn M. (2005). Quantitative real-time PCR for detecting *Cryptosporidium parvum* in cattle manure and anaerobic digester samples - Methodological advances in DNA extraction. Proc. 8th Latin American Workshop and Symposium on Anaerobic Digestion, Oct. 2-5, Punta del Este, Uruguay. ISBN 9974-7699-3-0, pp.68-73.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations and Acronyms

(m)RNA	Messenger RNA and RNA
AD	Anaerobic digestion
Anon.	Anonymous
bb	Bead beating
BSA	Bovine serum albumin
CAFO	Concentrated animal feeding operations
cDNA	Complementary DNA
CFR	Code of Federal Regulations
COWP	*Cryptosporidium* oocyst wall protein
Ct	Cycle threshold (qPCR)
CV	Coefficient of variation
DAPI	4' 6-Diamidono-2-Phenylindole
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide
ds	Double strand
dscDNA	Double strand complementary DNA
DTT	Dithiothreitol
DVGW	Deutscher Verein des Gas- und Wasserfaches
EC	European Community
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency US
FAO	Food and Agricultural Organization
FISH	Fluorescence *in situ* hybridization
FT	Freezing thawing
GITC	Guanidinium isothiocyanate
GITC-PC	Guanidinium isothiocyanate-phenol/chloroform
HS	Heat shock
HSP70	Heat shock protein 70
IF	Immunofluorescence
IMS	Immunomagnetic separation
KCl	Potassium chloride
LiCl	Lithium chloride
LiDS	Lithium dodecyl sulfate
LNA	Locked nucleic acid probe
MPN	Most probable number
mRNA	Messenger RNA
NASBA	Nucleic acid sequence based amplification
nd	Not detected
NH3	Ammonia
NH3-N	Ammonia nitrogen
NH4	Ammonium
p	P-value
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PLG	Phase-lock gel

PVP	Polyvinylpyrrolidone
qPCR	Quantitative PCR
R^2	Coefficient of determination
RKI	Robert Koch Institut
Rn	Normalized reporter (Fluorescence emission of reporter dye divided by emission of reference dye)
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	6-carboxy-X-rhodamine
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription qPCR
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SoTa	Sodium taurocholate
Tris	Tris(hydroxymethyl)aminomethane
u	unit
US	United States
UV	Ultraviolet
WHO	World Health Organization

Introduction

Food demand worldwide is leading to increased animal farming and intensive agriculture, and many farms do not follow best practices. Water scarcity is leading more and more to the use of treated or untreated wastewater for irrigation (Scheierling et al., 2011).

Manure from animal farms is a valuable product as fertilizer and soil conditioner in agriculture. Also wastewater constitutes a nutrient source, particularly of nitrogen and phosphorus. However, manure and wastewater contain a variety of pathogenic microorganisms, such as bacteria, viruses and protozoa (Bicudo et al., 2000), which can directly contaminate soil, pasture land and crops. Pathogens can percolate through soil and reach groundwater or can be transported with the runoff to surface water (Fayer et al., 2004). Microbial contamination can occur if manure or wastewater are not sufficiently sanitized and are discharged to the environment in an uncontrolled way.

There is increasing concern on the risk of infection of human and animals with pathogens by contact with contaminated substrates in affected areas. Manure from animal farms is a potential source of microbial pollution (EC 2010; FAO 2006), and animal waste from large scale farming operations with inadequate manure management practices poses major problems (EPA 2005). The economic cost of microbial contamination of drinking water sources, water resources and crops is high, not counting for the health expenses arising in case of infections.

Countries continue with efforts to regulate the use of manure and to promote good management practices at farm level. In some countries like Germany, spreading manure in areas close to water protection zones is prohibited to prevent contamination with pathogens (DVGW, 1995). In any case, manure discharge should be avoided close to any drinking water source or bathing water, and their reuse in agriculture should guarantee no threat to natural resources. Good animal husbandry and manure management practices may include different measures to prevent and reduce microbial contamination of water and land, such as suitable storage conditions and / or treatment options for manure and slurry. Under this framework, control points may be identified by farm operators or the corresponding authorities to carry out hygiene evaluations if health risks are identified (Kirchmayr et al., 2003; EPA 2005; EC 2009).

Unlike manure management guidelines that are followed at voluntary basis, strict hygiene regulations and standards exist for reusing wastewater and sludge in agriculture (WHO, 2006). Treatment options for manure, slurry and wastewater include anaerobic digestion (Effenberger et al., 2006). It produces biogas as renewable energy, improves the availability of nutrients

contained in manure, reduces substrate volume and odour pollution, and it reduces the number of pathogens in the slurry. In anaerobic digestion, efficient hygienization may be achieved only under certain conditions (thermophilic, 55°C or higher) and a defined retention time of the substrate in the digester (Effenberger et al., 2006; Lebuhn and Wilderer, 2006; Paavola et al., 2006). Variations in process parameters, such as temperature, pH, or effective retention time, and chemical composition of the substrate can affect the sanitizing potential of the system (Burton and Turner, 2003; Clancy et al. 2008). Thus, the efficiency of the anaerobic treatment for pathogen reduction should be monitored to ensure good quality of effluents before reuse.

Two health-relevant pathogens, *Cryptosporidium* protozoa and enteroviruses can be present in animal manure and wastewater, from which they can spread to the environment. Both pathogenic agents are of high significance for public health (WHO, 2011). *Cryptosporidium parvum* and *C. hominis* are worldwide one of the major causes of waterborne illness in humans, and foodborne infections can also occur through contaminated crops (Smith and Rose, 1998; Duffy and Moriarty, 2003). High prevalence of *Cryptosporidium* infections has been reported in developing countries (Snelling et al., 2007; Georgiev, 2009). Grazing animals can also be affected and re-infected. *Cryptosporidium* strains are of major concern for public health because the oocysts, the infective form, are very resistant to different environmental stresses and water disinfection treatments (e.g. desiccation, heat, chlorine). They can remain viable and infectious in water for months and can survive in pasture land and in soil for many weeks (Robertson et al., 1992; Kato et al., 2004). The risk of contamination of water, crops and forage is high because oocysts are shed in high numbers. Oocysts were found, for example, in groundwater (Hancock et al., 1998) and vegetables (Fayer et al., 2008).

The inactivation of this protozoa pathogen at mesophilic conditions in manure treatment systems does not seem to be sufficient (Burton and Turner, 2003). Poorly operated systems or operating failures during the treatment can increase the survival of oocysts.

Enteroviruses and norwalk-like viruses (RNA viruses) can also cause a wide spectrum of illnesses to humans by contact with contaminated sources. They have been detected in sewage, crops, surface and drinking water, and can be present in manure. Enteroviruses can survive in the environment long enough to represent a risk for humans (Gerba and Smith, 2005). Blumenthal et al. (2000) reported on records of infections with enteroviruses after consumption of vegetables irrigated with partially treated wastewater. According to hygiene standards, drinking water must be free of enteroviruses to ensure negligible risk of transmitting viral infection (WHO, 2006).

Therefore controlling for the presence of these pathogens in end-products of treatment systems can ensure that reusing the substrates do not represent a risk of contamination and infections.

Technical problems and limitations

Failures or low performance of wastewater treatment systems and anaerobic digesters can occur (Mara and Horan, 2003) and result in insufficient sanitation of the substrates. A critical point is that controlling for the level of indicator organisms may not guarantee adequate reduction of the number of other, more resistant pathogens such as some protozoa or enteroviruses (Rose et al., 2004; Ottoson et al., 2006; WHO, 2006; WHO, 2009). Hygiene evaluation of *C. parvum* and *C. hominis* oocysts in manure and / or other complex matrices have not been regularly performed mainly due to the lack of rapid, cost effective and simplified methods suitable for routine tests. The rapid detection of living, potentially infectious, oocysts has been hindered by the impractical use of traditional detection methods with those substrates.

Standard techniques for *Cryptosporidium* oocysts based on isolation and identification of oocysts by microscopy (e.g. vital staining, immunofluorescence assay) are optimized for water samples (EPA, 2005b), but they are difficult to apply with turbid and complex matrices and cannot identify species. Species identification is needed to determine the risk for human infections and to trace back the source of contamination for subsequent implementation of control measures. While there are procedures to purify oocysts, they are time consuming, can produce loss of organisms and reduce the sensitivity of the detection method, and can affect the viability of the organism (Bukhari et al., 1995; Kuczynska and Shelton, 1999).

Infectivity test in cell culture commonly employed to determine infectious *Cryptosporidium* oocysts is laborious and requires purified organisms and microscopy. It can take 2 – 3 days to assess infectivity in the cell cultures. The infectivity test for viruses can take longer to assess infectious particles. Therefore, infectivity tests are less suitable for routine applications with environmental matrices, particularly when rapid results are needed for decision making.

Simple, rapid and cost effective methods can help to overcome technical limitations for pathogen control. PCR (Polymerase Chain Reaction), the molecular technique based on detection of nucleic acids (DNA or cDNA), is used mainly due to its speed, specificity to identify target species and for its potential to detect low amount of organisms in a sample. Nowadays, real-time quantitative PCR (qPCR) provides a much faster application, and the possibility of automation and quantification (Holland et al., 2001). In order to detect viable organisms, reverse transcription PCR (RT-PCR) that is based on detection of RNA or mRNA

molecules is used. A detection method based on RT-qPCR can be a very powerful tool for the rapid and specific detection of viable pathogens in environmental matrices.

PCR or RT-PCR have been mainly employed for *Cryptosporidium* protozoa in clean and simple matrices, such as water or purified oocysts (Stinear et al., 1996; Baeumner et al., 2001; Hallier-Soulier et al., 2003; Kato et al., 2003b), and in many cases studies focused on DNA detection (e.g. Wiedenmann et al., 1998). The use of real-time qPCR with complex substrates is till now very limited, and showed the need for optimization of the technique (Higgins et al., 2001; Stroup et al., 2006). Detection of viable pathogens by RT-qPCR in complex matrices has been as well limited. The major challenge in the application of qPCR based techniques for such samples is the extraction and purification of nucleic acids, steps that have to be done before qPCR and RT-qPCR. Basically, (RT)-qPCR requires nucleic acids in sufficient amount and virtually free of inhibitory substances (Wilson, 1997; Bustin, 2000). This may be difficult to achieve when nucleic acid extractions are done directly from the environmental matrix. Particularly mRNA poses problems because the molecule is much more prone to degradation than DNA. Particulate material and humic compounds that are typically present in environmental samples, can greatly reduce the recovery of nucleic acids and / or inhibit the (RT)-qPCR detection system (Stults et al., 2001; Matheson et al., 2010). Lower specificity to target organisms, insufficient method sensitivity or inhibition of enzymatic steps can limit the application of RT-qPCR based assays as molecular tools for environmental analysis. Therefore, advances are needed in this respect.

Suitable methods based on qPCR or RT-qPCR have not been previously reported for *C. parvum* oocysts in manure. Neither RT-qPCR has been employed to assess the viability of oocysts in effluents from manure treatment processes. Detection of enteroviruses in turbid samples by RT-qPCR may provide high specificity, sensitivity and simplicity, and may be more suitable than methods such as cell culture for routine analysis. Moreover, some enteroviruses are difficult to grow in cell cultures.

About this research

This work intended to develop detection and quantification tools for *C. parvum* oocysts and enteroviruses in environmental matrices, and to advance in the application of RT-qPCR methods in hygiene control. Suitable procedures should be applicable for samples with complex structural and chemical composition such as manure, provide specific and sensitive detection, deliver results rapidly and be able to detect viable, potentially infectious *C. parvum* oocysts.

This research started from an interdisciplinary project that evaluated the performance of an anaerobic biogas system treating manure and its sanitizing efficiency (Lebuhn and Wilderer, 2006; Effenberger, 2008). Optimized detection methods ought to be applicable to assess the presence of *Cryptosporidium* oocysts and enteroviruses in manure, and to determine oocysts inactivation in anaerobic digesters.

Structure of the study

Chapter 1 reviews manure and wastewater reuse in agriculture, and the health and environmental implications when poorly sanitized matrices are reused or discharged into the environment. It describes the occurrence of *Cryptosporidium* parasites and enteroviruses in the environment. Current detection tools for these organisms and their limitations are described.

Chapter 2 describes the material and general procedures that were employed in this study, and outlines the overall experimental work.

The following chapters report results and discuss the findings of each investigation.

Chapter 3 reports the optimization of DNA extraction procedures for *C. parvum* oocysts to detect genomic DNA by qPCR in manure and soil samples. The method was employed to determine the fate of oocysts after anaerobic digestion.

Chapter 4 presents the development and optimization of procedures for RNA and mRNA extraction of *C. parvum* oocysts in manure and for detection by RT-qPCR. It includes optimization for oocyst lysis, purification of nucleic acids and reverse transcription. In addition, the evaluation of extraction methods for RNA enteroviruses in environmental samples is presented.

In **Chapter 5,** the RNA and mRNA extraction and RT-qPCR methods for *C. parvum* are applied to detect viable oocyst in manure, water and soil samples. The procedures were evaluated to indicate living oocysts.

Chapter 6 deals with the application of the optimized procedures to evaluate the degradation and inactivation of *C. parvum* in anaerobic digesters treating manure.

Chapter 7 is concerned with the evaluation of the induction rate of *hsp70* mRNA in oocysts and the usefulness of *hsp70* mRNA production as a marker of oocyst viability. The assessment was also done in comparison to another mRNA molecule that is known to be constitutively produced.

General Discussion and Conclusions

CHAPTER 1

State of knowledge

Pathogens in the environment, their relevance for public health, and their behaviour in manure and wastewater treatment

1.1 Manure Management and Reuse in Agriculture

Animal manure has always been applied to agricultural land as natural fertilizer. Manure is rich in nutrients such as phosphorus, nitrogen and trace elements. However, poor animal husbandry and manure management practices can also have detrimental effects. Too high amounts of nutrients brought to the land can result in eutrophication of soils and water bodies. Manure further can contain high amounts of pathogens (bacteria, protozoa and viruses) that can be responsible for human and animal illness. Animal farms, particularly those with large operations, are nowadays of concern for health authorities because in many cases the manure produced exceeds the needs in agriculture, and unregulated manure discharge or spills often occur (EPA, 2005; FAO, 2006).

The application of manure to the land should be better controlled for balanced nutrient cycles and in order to avoid contamination of agricultural soils, plants and water with pathogenic microorganisms. Besides land and crops, groundwater is also at risk because pathogens can be transferred to deeper soil layers carrying water (Hancock et al., 1998; Borchardt et al., 2007). Furthermore, surface water can also be contaminated by runoff, especially after heavy raining events.

For manure to be adequate and safe as natural soil conditioner without the risk of disseminating pathogenic microorganisms into the environment, it should be treated or conditioned through temporary storage or by composting or anaerobic digestion. Monitoring of the sanitizing treatment processes and their products contributes to ensure the nominal operation of the processes and minimize the risk of contamination. This is especially important if treated manure is applied in proximity to risk areas, such as water protection zones. The protection of water resources and land from contamination is generally more effective when setting up multiple measures as also promoted in the frame of a Multiple Barrier Approach (WHO, 2006). This can

include improved protection of water resources, securing adequate facility management, and treatment of manure and wastewater (including monitoring) and minimizing the risk of pathogen entry into the water flow.

1.2 Wastewater and Sludge Reuse

The WHO estimated that more than a tenth of the earth's population consumes food that is irrigated by wastewater (WHO, 2006). Sludge is produced as a byproduct of wastewater treatment. It is applied to agricultural land either with previous or even without treatment. Sludge contains all the compounds removed from the wastewater and microbes grown in organic compounds, including pathogenic microorganisms. Depending on the type of treatment, it contains nutrients that are useful for soil conditioning.

There is a trend to promote the reuse of wastewater and sludge for agricultural practices in order to make use of the nutrients contained in the substrates. Drivers for the reuse of wastewater are the increasing water scarcity in many regions and the increasing urbanization (Scheierling et al., 2011). These practices represent an economic advantage as they reduce investment needs for chemical fertilizers, buffer land degradation, and can balance water demand for food production. As these practices are implemented, there is an increasing concern about the risk of transmission of pathogenic microorganisms (WHO, 2006).

In developing countries, the lack of regulations or the absence of controlling institutional bodies to regulate the discharge of wastewater, sludge and manure to the fields carries a high risk of contamination for agricultural land and water sources (Jimenez and Asano, 2008). In some countries (like Germany), the reuse of (treated) sludge and manure in agricultural fields is not allowed in water protection areas.

1.3 Manure, Wastewater and Sludge as Carrier of Microbial Contamination

In addition to chemical contaminants, wastewater, sludge and manure can carry significant amounts of a wide range of pathogenic bacteria (e.g. *Vibrio cholerae*, *E. coli*, *Salmonella* spp., *Clostridium* spp.), fungi, helminths (e.g. *Ascaris*, hookworms), protozoa (e.g. *Cryptosporidium*, *Giardia*, *Cyclospora*) and viruses (e.g. hepatitis virus, rotavirus, norovirus and enteroviruses such as poliovirus or coxsackievirus) (Burton and Turner, 2003; Mara and Horan, 2003; Gerba and Smith, 2005; WHO, 2006). The variety of the different pathogens makes it necessary to focus analysis, risk assessment and quality control on important indicator organisms and pathogens. Traditional indicators of faecal contamination, such as faecal coliforms are not always suitable for virus detection (Skraber et al., 2004; Ottoson et al., 2006), and are also very

poor indicators for protozoa pathogens for which no satisfactory surrogate exists (Mara and Horan, 2003; Rose et al., 2004). This is mainly due to differences in the resistance of the organisms and their survival capabilities.

Two pathogens of high health significance are *Cryptosporidium* and enteroviruses (WHO, 2011). The order of magnitude of occurrence of some pathogens and indicators is presented in the following Table 1.1.

Table 1.1 – Selected indicators and pathogens in faeces, wastewater and raw water

Microorganism / group	Number per gram of faeces	Number per liter in untreated wastewater	Number per liter in raw water
Faecal coliforms	(human) 10^8 - 10^9 (cow) 5 x 10^9 / day	10^6 -10^9	10^2 - 10^5
Campylobacter spp.	10^6	10^2 - 10^6	10^2 - 10^4
Vibrio cholerae	10^6	10^2 -10^6	10^2 - 10^8
Rotaviruses	10^9	50-5000	0.01 - 10^2
Enteroviruses	10^6 / 10^7	1-10^3 / 10^8 *	0.01 - 10
Cryptosporidium spp.	10^7	1 - 10^4	0 - 10^3
Giardia intestinalis	10^7	1 - 10^4	0 - 10^3

Sources: Shuval and Fattal, 2003; Godfree, 2003; EPA, 2009; La Rosa et al., 2010; WHO, 2011.
*: numbers can be as high as that.

1.4 Public health importance of *Cryptosporidium* and enteroviruses

Cryptosporidium parvum is considered one of the most common causes for diarrhea worldwide, and in some studies from developing countries it is identified as the organism found with the greatest frequency in diarrheal stool (Georgiev, 2009). The World Heatlh Organization has estimated the mortality due to diarrhea to about 1.798 million victims per year (WHO, 2006). Cases of cryptosporidiosis are required to be reported to public authorities in countries like Germany, Sweden or Irleand (Chalmers, 2009), but in other countries many cases are not reported.

Enteroviruses are considered one of the major sources of infections in humans worldwide and have been estimated to cause yearly about 30 million infections alone within the US (WHO, 2011). In 2010 for example, there have been more than 1,000,000 cases of hand-foot-and-mouth disease registered in China (RKI, 2010).

Cryptosporidium species and enteroviruses are very infective. Reports on the infectious dose for *Cryptosporidium* range between 10 – 2,000 oocysts (DuPont et al., 1995; Chappell et al., 1999;

Godfree, 2003). The infection and its severity depend on the actual health status of the individual. For enteroviruses, the infectious dose can be below 100 particles (WHO, 2011). Infections with enteroviruses can cause a broad range of medical conditions and symptoms, such as myocarditis, meningoencephalitis, gastroenteritis and poliomyelitis (WHO, 2011).

Cryptosporidiosis results most commonly in self-limiting diarrhea. For immuno-compromised persons and especially children, the infection can be severe and life threatening.

There are various reports of *Cryptosporidium* outbreaks. The most famous being the Milwaukee incident in the Unites States in 1993, where more than 400,000 people contracted cryptosporidiosis from the Milwaukee public water supply, despite purification according to federal standards (Goldstein et al., 1996). The total cost associated with this outbreak has been estimated at 96.2 million US dollars (WHO, 2011). For the United States, the yearly number of infections with *Cryptosporidium* has been estimated as high as 300,000 (Mead et al., 1999). Cases of infections are also reported for Europe. In the United Kingdom, there was 5,561 cases registered in 2005 (Semenza et al., 2007). For Germany about 1,000 infections are reported per year (RKI, 2011).

1.5 The *Cryptosporidium* pathogen

Cryptosporidium is an intercellular protozoan parasite of the phylum *Apicomplexa*. It infects the microvillous region of the epithelial cells in the digestive and respiratory tract of vertebrates (WHO, 2009). Multiple species of *Cryptosporidium* exist and they are found worldwide. Many vertebrates can host this parasite, which range from sea lion to koala and guinea pig and includes cattle, swine, goat, sheep, horse, dog, cat and humans (Fayer, 2008).

Two species of this pathogen are the main cause of human diseases: *C. hominis* is specific to humans, while *C. parvum* infects humans, cattle and a range of other mammals (Semenza et al., 2007; WHO, 2009). For instance, in England and Wales, they accounted together for 95.9 % of all human *Cryptosporidium* infections in the years of 1989 to 2005 (Nichols et al., 2009). Although the pathogenicity of *Cryptosporidium* species seems to be mainly restricted to specific hosts, cross-infections have been reported in humans (Leoni et al., 2006). For instance, *C. meleagridis* which is found in turkeys may infect humans.

1.5.1 Characteristics of cryptosporidia infecting humans

Cryptosporidium parvum and *C. hominis* are excreted with the faeces of the host in the form of oocysts, which are the infectious stage. The oocyst size is about 4 - 5 µm and contains 4 sporozoites (cells). The oocyst wall is particularly strong and composed by an outer layer of glycoproteins, a complex lipidic central layer and an inner proteinic layer which are regarded to

provide the oocyst's rigidity and impermeability (Jenkins et al., 2010). This strength may however sligthly differ between different strains (Nichols and Smith, 2004).

Oocysts are very resistant to different environmental stresses, such as desiccation and temperature, and they are not susceptible to conventional water treatment procedures such as chlorination (Robertson et al., 1992). They can remain viable and infectious in various matrices for many months (Robertson et al., 1992, Fayer et al., 2000). Oocysts stored in manure seem to acquire extended resistance to environmental stresses and are reported to resist even temperatures as low as -22°C (Robertson et al., 1992).

1.5.2 Sources and occurrence of *Cryptosporidium*

An overview of the primary paths of transmission of *Cryptosporidium parvum* and *C. hominis* is given in Fig. 1.1. Cryptosporidiosis is transmitted by the faecal-oral route directly from person to person, from animal to person and by ingesting contaminated food or water.

Manure from animal farms is the major source of *C. parvum* oocysts. Inadequate manure management practices, such as excessive and uncontrolled load of insufficiently sanitized manure to the land, can lead to contamination of water, crops and forage with this pathogen. In many cases of waterborne disease outbreaks where *C. parvum* was identified, manure was pointed out as the most likely source of contamination (Gerba and Smith, 2005; EPA, 2009). Infected calves can shed high numbers of oocysts, up to 10,000 million oocysts / day (WHO, 2011). Prevalence of cryptosporidiosis in calves is high. Additionally, a lower number of oocysts are shed by asymptomatic animals.

Cryptosporidium oocysts are also found in municipal wastewater and sewage sludge. They are one of the principle pathogens of concern in these matrices (Gerba and Smith, 2005). Oocyst concentrations of up to 14,000 per liter in raw sewage have been reported (WHO, 2011). A study in Norway found values ranging between 100 and 44,500 oocysts per liter of effluents from sewage treatment facilities (Robertson et al., 2006).

Oocysts in animal fecal pads and manures can be transported with the runoff after rain events, particularly in soils with little or no vegetation (Davies et al., 2004). Oocysts release from manure can also leach through soil layers particularly in karst soils (Boyer et al., 2009). Viable *Cryptosporidium* oocyst have been registered in surface waters with concentrations of up to 5,800 oocysts per liter (WHO, 2011), and even in groundwater (Hancock et al., 1998; Lobo et al., 2009).

Relevant amounts of oocysts can be present in recreational waters like pools, hot springs, fresh water lakes and rivers. Related disease outbreaks have been presented by Beach and

collaborators (Beach, 2008). *Cryptosporidium* oocysts have been isolated from foods and beverages including vegetables, fruits, tap water, milk, apple-cider and even shellfish or meat (Ortega and Cama, 2008; Fayer, 2004 ; Dixon, 2009).

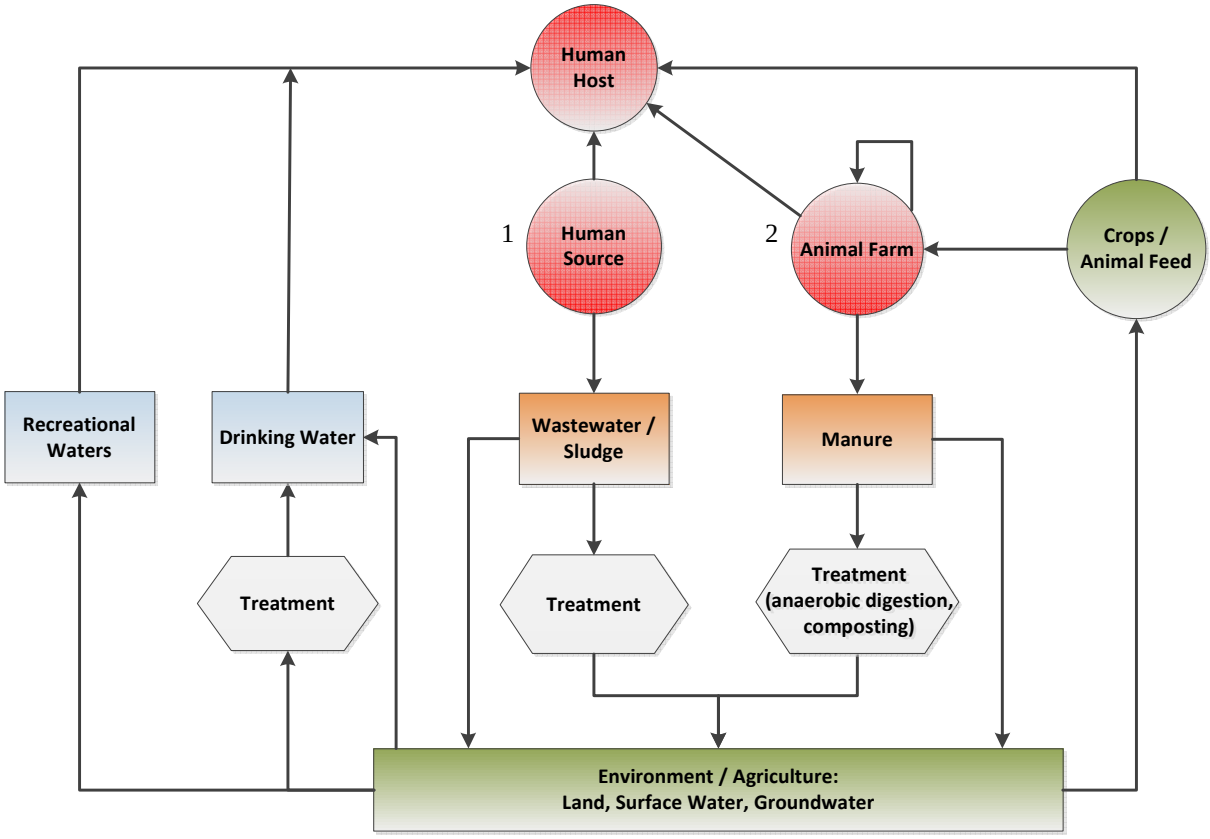


Fig. 1.1 – Transmission routes of *C. parvum* and *C. hominis* oocysts. Numbers indicate the primary sources of oocysts.

1.6 Enteroviruses

Enteroviruses are a large family of viruses causing a wide range of illnesses, most of them with non-enteric symptoms. They are one of the most common viruses found in contaminated waters. Together with noroviruses and adenoviruses, they are pathogens of health concern that can be found in water bodies (Fong and Lipp, 2005; Sinclair et al., 2009). The genus *Enterovirus* is part of the family *Picornaviridae* and consists of more than 80 serotypes (Fong and Lipp, 2005). Most of these are human pathogens while others can infect animals (e.g. bovine and porcine enteroviruses). Important enteroviruses associated with human disease are polioviruses, echoviruses, coxsackieviruses, and enterovirus types 68 to 73 (WHO, 2011).

1.6.1 Characteristics

With overall dimensions of only 20 – 30 nm, enteroviruses are very small (Fong and Lipp, 2005). They consist of a non-enveloped icosahedral capsid (protein coat) containing a single-strand RNA genome. Enteroviruses are very resistant and can remain infective for months in the environment. They are resistance against lipid solvents, conventional chlorination, wastewater treatment conditions, can be stable at pH 3 – 10 and have a high persistence in water supplies (Payment et al., 1985; Fong and Lipp, 2005; WHO, 2011). An average environmental lifetime of 3 months has been reported (Shuval and Fattal, 2003). With an infectious dose below 100 particles their infectivity is high (WHO, 2011). The amount excreted by an infected individual can be as high as 10^7 particles per gram of faeces (Shuval and Fattal, 2003), and this can last for several weeks.

1.6.2 Sources and occurrence

Enteroviruses are excreted in relatively large numbers with the faeces of infected hosts (Ley et al., 2002; Rose et al., 2004). The transmission of enteroviruses can occur via the fecal-oral (e.g. water, food) and respiratory routes, inoculation and blood (Morens and Pallansch, 1995).

They are found in raw and treated wastewater, surface water and groundwater, and even in treated drinking water (Borchardt et al., 2007; Katayama et al., 2008; Sinclair et al., 2009; Aw and Gin, 2010). Surveillances of effluents of municipal wastewater systems revealed varying numbers of enteroviruses and noroviruses from 10^2 to 10^6 particles / ml (Katayama et al., 2008; Aw and Gin, 2010; La Rosa et al., 2010).

Bovine enteroviruses are common in cattle in many cases as asymptomatic infections, but they can produce diarrhea and abortion (Ley et al., 2002). Enteroviruses have been detected with high frequency in animal faeces, pastures, runoff streams, and in the surrounding waters, including rivers, close to animal farms (Ley et al., 2002; Jimenez et al., 2005). The virus was also detected in faeces of wild animals visiting farming areas (Ley et al., 2002).

The presence of enteroviruses in wastewater and animal manure can result in contamination of various water sources and represent a potential risk for causing human and animal infections. For example, a study reported a strong correlation of infection with enteroviruses due to groundwater contamination and heavy rain events (Jean et al., 2006). Enteroviruses can also be be found in shellfish (Mara and Horan, 2003; Jothikumar et al., 2005).

1.7 Survival of pathogens in the environment

Once brought to the environment, *Cryptosporidium* and enteroviruses can survive a significant amount of time. Oocysts can remain viable and infectious in various matrices for many months

(Robertson et al., 1992). In agricultural soils, survival times of 120 days have been found with no effect of moisture content at temperatures of 0 - 1°C (Kato et al., 2004). Also survival of oocyst in soils has been reported by Jenkins et al. (1999).

In water, the typical die-off rate of *Cryptosporidium* oocysts ranges between 0.005 and 0.037 log units per day (WHO, 2009). Jenkins et al. (2003) found that oocysts remained infective for 7 months in water at 15°C. Survival of oocysts can be extended at cool temperatures. They can survive for 300 days at temperatures near the freezing point in fecal slurry (Jenkins et al., 1997), but they are inactivated more rapidly by freeze–thaw cycles (Fayer, 2008). Oocysts can survive longer in slurry, faeces, manure and moist conditions (Ortega and Cama, 2008). For example, the survival of oocysts in manure piles was reported to be about 40 % after 100 days at temperatures between 20 – 32 °C (Jenkins et al., 1999).

In seawater (at 10°C), *C. parvum* oocysts have been shown to be infectious after 12 weeks (Fayer et al., 1998). Other reports suggest viability after more than a year in seawater (Tamburini and Pozio, 1999).

Enteroviruses can also remain infective for months in the environment. Survival times reported for relevant mediums are: up to 120 days in fresh water and sewage, up to 100 days in soil, and up to 60 days on plants (WHO, 2006). In seawater mixed with sewage that was exposed to sunlight, infectious enteroviruses persisted longer than fecal indicator bacteria such as enterococci (Walters et al., 2009). Wei et al. (2010) studied the survival of hepatitis A virus (a picornavirus) in different animal manures and biosolids. The authors found that although viruses were rapidly inactivated in high pH biosolids, for other type of manures and biosolids viral particles were first significantly reduced after 60 days storage at 20°C, and persisted in all type of manures at 4°C.

1.8 Inactivation of pathogens and treatment systems

Treatment of pathogen carrying matrices can and should be performed in order to inactivate potentially pathogenic agents and to protect the environment and public health. Hygiene treatment of manure and slurry can be performed by prolonged storage, composting, anaerobic digestion, aerobic treatment, chemical disinfection and physical treatment involving heat, incineration or irradiation, while in practice the first three are most commonly used (Burton and Turner, 2003). *Cryptosporidium* strains and enteroviruses, however, are very resistant. *Cryptosporidium* can be viable more than 90 days in cattle slurries stored at cold temperatures (4°C), while aeration of slurry at 15°C can render oocysts inactive after about 4 days (Svoboda et al., 1997).

In agricultural farms, treatment of manure and slurry in anaerobic digesters is done for biogas production, and also to reduce the solids content and the number of pathogens in the susbtrate. Anaerobic digestion is the biological degradation of organic matter carried out by a consortium of microorganisms in the absence of oxygen. End products are methane and a liquid organic residue rich in nutrients like nitrogen and phosphorus. A very effective treatment for pathogens reduction can be thermophilic anaerobic digestion. In laboratory studies Whitmore et al. (1995) showed that oocysts in sludge at 50°C could be completely inactivated within the first 24 h, while Meyer (2002) found viable oocysts in manure treated at that temperature. Kato et al. (2003a) reported reduction of oocysts by 2 log units after 2 days at 55°C during sludge digestion. Temperature is considered as the primary factor for oocysts inactivation, but also other parameters such as the time of treatment and the chemical composition of the substrate (e.g. ammonia) can affect their inactivation rate (Jenkins et al., 1998; Burton and Turner, 2003). In mesophilic anaerobic batch digesters, Meyer (2002) found that about 60 % of oocysts were viable after 24 h. Whitmore et al. (1995) reported 10 % of viable oocysts in treated sludge (35°C) after 18 days.

In other systems such as anaerobic lagoons, oocysts in dairy wastewater were reduced by 4 log units after 60 days (Karpiscak et al., 2001). However, overflows from storage ponds can often occur (Rahman et al., 2011), and in such cases viable oocysts may be released from the system.

Bovine enteroviruses that underwent anaerobic digestion at 54°C have been reported to be inactivated in less than 2 hours (Burton and Turner, 2003). Lund and collaborators have shown that a 4 log reduction of bovine enterovirus during thermophilic anaerobic digestion (55°C) can be reached in less than 0.5 hours, while Böhm (2004) reported > 4 log units reduction in less than 6 h. For mesophilic conditions, in comparison, 23 h were needed to reach the 4 log units (Lund et al., 1996). Other studies showed reduction of enteroviruses by 1 log after 11 h in mesophilic anaerobic co-digestion of manure and biowaste (Böhm, 2004), and even after 11 - 13 days, infectious enteroviruses were found in mesophilic treated liquid manure (Monteith, 1986). Popat et al. (2010) investigated the inactivation of poliovirus type 1 during thermophilic anaerobic digestion of sludge and found also more than 3 log reduction within 2 h at temperatures between 51.1 and 55.5°C. In activated sludge systems enteric viruses could be reduced almost 2 log units (Rose et al., 2004). Removal efficiency of enteric viruses in wastewater treatment plants in Italy was 38 % for enteroviruses and 78 % for noroviruses, while this was up to 99 % for bacterial indicators (La Rosa et al., 2010).

Cryptosporidium oocysts show resistance against routine chlorination, as used for drinking water disinfection and reclaimed waters (Bowman, 2008). UV irradiation and ozonation can be

very effective for oocyst inactivation but also impeded by turbidity of the treated matrix (Bowman, 2008). For ozonated apple cider for example, Blackburn et al. (2006) reported an associated outbreak.

In a study performed on six wastewater treatment plants in Scotland, removal efficiencies between 5 and 91 % have been found for *Cryptosporidium* (Robertson et al., 2000). In that study, the averaged oocyst viability in the effluents was 46 %. This shows that well operated treatment systems can remove oocysts, however they do not guarantee that they are completely removed or inactivated.

Overall, the inactivation efficiency of the different treatments can vary depending on a number of factors like temperature, effective retention time, pH or substrate composition (Burton and Turner, 2003; Clancy and Hargy, 2008) and poorly operated or impeded treatment processes achieve eventually very little inactivation (Burton and Turner, 2003). It is therefore important to monitor the treatment processes in order to ensure that the final products are free of viable pathogenic microorganisms or that at least acceptable levels for disposal or reuse are reached.

The use of surrogate organisms may not always indicate the reduction of pathogenic oocysts or enteroviruses (Mara and Horan, 2003; Rose et al., 2004; Skraber et al., 2004; WHO, 2006). For example, spores of *Clostridium perfringens* are not suitable because spores are highly resistance to heat. They are also of smaller size than oocysts and will be differently removed during the treatments. Moreover, traditional indicators have shown not to fully correlate with the reduction of oocysts during biological wastewater treatment systems (Rose et al., 2004), thus requiring validation testing to verify reduction of *Cryptosporidium* oocysts (WHO, 2006).

Bacteria fecal indicators can re-grow in the environment, as found for sediments at river banks, and this can make it also difficult to relate the results for the indicators to the presence of viruses and protozoa (Desmarais et al., 2002).

1.9 Hygiene standards

Significant pathogenic risks can be related to the unregulated and uncontrolled reuse of manure, wastewater and sewage sludge. For wastewater and sludge reuse, there have been several standards and regulations established at various levels. The WHO has established guidelines for the safe use of wastewater, excreta and greywater (WHO, 2006). The guidelines follow a risk assessment strategy and promote a multi barrier approach to protect from wastewater and sludge related hazards (e.g. treatment, handling precautions, application restrictions, withholding periods). They define critical control points for storage and collection, treatment, transport, application and agricultural products. As primary hygiene indicator organisms *E. coli* and

helminths have been adopted and associated with threshold values while *Cryptosporidium* and enteroviruses are recognized as important microbial hazards.

The multi barrier approach is also implemented in other important reference regulations like the United States EPA 40 CFR 503 (EPA, 1993; standards for the use or disposal of sewage sludge) that classifies sludges into two categories, according to their pathogen profile and defines hygiene limits for enteric viruses, fecal coliforms, helminths and *Salmonella*. In 2004, the EPA issued guidelines for water reuse that recognized the importance of *Cryptosporidium* (EPA, 2004). At state level, for example, the state of Florida have develop their own guidelines that requires monitoring *Giardia* and *Cryptosporidium* for reclaimed water treatment plants exceeding certain size (FAC, 2007). Federal regulations concerning pathogen reduction in manure do not exist at this stage in the US. However a big concern is manure from animal feeding operations and the importance of *C. parvum* has been reflected in guidelines for manure management (EPA, 2005).

In Europe, the European Commission Sewage Sludge Directive 86/278/EEC (EC, 1986) is governing the hygiene and land application of sewage sludge. It's monitoring mechanism focuses on chemical pollutants but it requires biological, chemical, heat or other appropriate treatment to significantly reduce health hazards (EC, 1986).

The European Commission has also defined directives that relate to the reuse and application of manure. The primary concerns of the Nitrate Directive (EC, 1991) and Industrial Emissions Directive (EC, 2010) are the associated chemical substances like nitrates, heavy metals and dioxins while the latter directive foresees to review the need for controlling emissions from the intensive rearing of cattle and the spreading of manure by the end of 2012.

Currently in place and addressing hygiene and handling of manure are the directives EC 1069/2009 (EC, 2009; on the handling of animal by-products not for human consumption) and EC 142/2011 corresponding implementing regulation. Here, manure is registered as category 2 material that might be used directly in biogas and composting plants or applied to land, if not considered as risk by the competent authorities. Hygiene control thresholds for processed manure to be placed on the market are defined for *E. coli* or *Enterococcaceae* and *Salmonella*. Within these directives also the concept of critical control points has been adopted.

In Germany, the agricultural reuse of manure and digestion residues is governed primarily by the Regulations on Fertilizers and Fertilization (Anon., 2006, 2008), which restrict the application with respect to amounts of nitrogen, phosphate and other chemical substances (e.g. heavy metals) to the proximity to surface waters and water protection zones. Numbers of salmonellae must be indicated if manure and digestion residues are to be placed on the market.

If manure is mixed with biowaste or other animal by-products, hygiene regulations apply (Anon., 1998; EC, 2009). In Germany, the biowaste regulation requires hygiene treatment (> 50°C for a defined retention time or pasteurization for at least 1 h) before application within agriculture, and defines *Salmonella seftenberg* as test organism for process verification (Anon., 1998).

Control points have been introduced also for the monitoring of water bodies. The WHO guidelines for bathing water refer to intestinal enterococci for assessment of the microbiological quality of sea and fresh recreational waters while acknowledging the health importance of enteroviruses and *Cryptosporidium* (WHO, 2003).

Another aspect of the multi barrier approach is the designation of water protection zones as implemented in Germany. The German groundwater regulation defines water protection zone II as areas that should be protected from contamination by pathogenic microorganisms (bacteria, parasites, viruses) (DVGW, 1995). It has been suggested that for these areas, restrictions on the application of manure and sewage sludge could be reduced in case of advanced treatment (EC, 2003). Such treatment needs to be closely controlled and monitored to exclude the risk of spreading infectious pathogens with the effluents.

Overall, there exist several standards affecting the hygiene of wastewater, sludge and manure foreseen to be reused within agriculture. Many of these are focused on chemical pollutants. In terms of pathogens, *E. coli*, enterococci, helminths and salmonellae dominate as hygiene indicators. Enteric viruses have been considered for monitoring of sewage sludge treatment in the United States (EPA, 1993). *Cryptosporidium* has not been generally adopted yet, while its health significance is clearly recognized. For the time it is to be included within future regulations, efficient and rapid methods will be needed for detection of pathogens and implementation of monitoring programs.

1.10 Detection methods for *Cryptosporidium* in environmental samples

Suitable detection methods are needed to determine the degree of contamination of environmental matrices, the risk of microbial pollution with viable potentially infectious pathogens, and to verify the performance of sanitizing systems. So far, the existent standard detection techniques and improvements for *Cryptosporidium* have focused on processing environmental water samples. These methodologies, however, cannot be simply applied on more complex matrices like manure, wastewater and soil.

Currently applied techniques include assays based on immunoflorescence (IF) and microscopy determination, viability tests (e.g. excystation test), animal and cell culture infectivity tests,

molecular assays based on PCR, and molecular hybridization assays, such as fluorescence *in situ* hybridization. These techniques are described in this section.

1.10.1 Standard techniques and limitations

Immunofluorescence (IF) based assays are widely applied to detect intact oocysts in environmental water samples. The steps for processing water consists of oocysts concentration and purification (e.g. through filtration, gradient centrifugation), staining with fluorescently labelled antibodies directed towards the oocysts wall, and detection by epifluorescence microscopy. The US Environmental Protection Agency has established the method 1622 as standard for detecting oocysts in water (EPA, 2005b). It uses filtration and elution steps, immunomagnetic separation (IMS) of the oocysts, and a direct IF assay. Purified oocysts are needed for the IF assay to avoid unspecific hybridization of the antibodies and fluorescence background signals from debris and particles (Nieminski et. al., 1995; Kuczynska and Shelton, 1999). Although immunomagnetic beads are employed for the separation of oocysts in water, low recovery efficiency has also been reported, particularly with turbid water samples (Ramadan et al., 2010). The recovery of oocysts from fecal substrates after a pre-dispersion treatment and IMS showed a variable range from 0 - 83 % (Davies et al., 2003). The IF assay has also been employed for the detection of oocysts in wastewater and fecal samples (e.g. Johnston et al., 2003; Castro-Hermida et al., 2008).

Although IF-based methods have improved in the last years and IF is currently used for water quality monitoring (e.g. Lobo et al., 2009), the method does not discriminate among species, particularly of those that cause infections to humans. The IF assay does not provide information on viability, thus its application is only useful to indicate the presence of oocysts.

Viability tests

Two tests based on microscopy are commonly used to indicate the metabolic state of the organism. The *in vitro* excystation test is based on the ability of living oocysts to excyst after treatment with a bile salt solution at 37°C. The vital staining assay is based on the inclusion or exclusion of fluorescent dyes (DAPI and PI) which depends on the permeability of the oocyst wall and sporozoite membrane. DAPI dye blue all oocysts whereas PI dye red only dead oocysts (Campbell et al., 1992). The dye permeability test has been employed to indicate changes in membrane integrity after disinfection or ammonia treatment (Robertson et al., 1992; Jenkins et al., 1997). Both, the excystation test and the dye permeability assay were shown to correlate with each other (Campbell et al., 1992; Robertson et al., 1992).

The assays are mainly applied with purified oocysts or not turbid water samples. Although they may be also used with turbid matrices (Jenkins et al., 1997), this is less suitable due to difficulties in determination of oocysts by microscopy. For the screening of oocysts in lagoons of swine facilities, Jenkins and collaborators (2010) purified oocysts by flotation techniques before the dye permeability test.

Techniques based on microscopy require the expert identification of oocysts, especially for samples containing debris or particulate material. This aspect can be a bottleneck in the routine test of multiple samples. Also the application of *in vitro* viability tests in environmental analysis can be limited by the high concentration of oocysts needed for the analysis.

1.10.2 Infectivity tests

The animal (mouse) infectivity test has been the decisive method to determine the infectious potential of *Cryptosporidium* oocysts (Widmer et al., 1999), however its application has been limited due to the high costs for analysis, long procedure, and for ethical considerations. *In vitro* infectivity tests with cell cultures have been used as an alternative method. They consist of infecting cell culture monolayers with oocysts, antibody labelling (IF assay) of the culture, and identification of the parasite development stages (foci of infection) by epifluorescence microscopy (Slifko et al., 1997; Rochelle et al., 2002). Various cell lines has been evaluated to improve the cell culture infectivity test (Rochelle et. al., 2002), and various technical improvements have been made (e.g. Najdrowski et al., 2007a). The method provides information about the infectivity potential of the organism, but results can be variable with different oocyst strains and cell lines, and due to variation in oocyst preparation (Rochelle et al., 2002; Quintero-Betancourt, 2003; Shets et al., 2005). *In vitro* infectivity tests alone cannot identify species. Thus, for the assessment of public health risks due to *Cryptosporidium* spp. pathogenic for humans, specie identification should be done using molecular tools.

In vitro infectivity tests require pre-isolation of oocysts from the matrix, as contaminants can affect cell cultures. Various procedures can be used for purification and concentration of oocysts from environmental water samples, reclaimed water and animal faeces,(Quintero- Betancourt, 2003; Schets et al., 2005; Najdrowski et al., 2007a). These include density gradient centrifugation steps (e.g. percoll, sucrose gradients) or filtration steps and IMS (for samples with less particulate material). These pre-steps, however, can result in varying recovery rates (Bukhari et al., 1995; Nieminski et al., 1995¸ Quintero-Betancourt, 2003).

Advances to a more rapid and specific processing includes the use of cell culture in an integrated approach with PCR to detect DNA from infectious organisms in the cell culture (Di

Giovanni et al., 1999; Rochelle et al., 2002; Najdrowski et al., 2007b). It avoids microscopy and can be designed to detect specie.

Despite the new developments and the advantages of the cell culture test to indicate the infectivity potential, overall the technique is time consuming and requires many days to generate results, therefore it does not fully suit for routine use.

1.10.3 Molecular techniques based on PCR

Molecular tools are increasingly used for the specific detection of pathogenic microorganisms in environmental samples. PCR based techniques can be designed to identify target organisms by using species specific oligonucleotide primers that bind to genomic DNA or to cDNA. The principle of amplification of nucleic acids by PCR provides the possibility to detect a low amount of organisms in a sample. Conventional end-point PCR is a qualitative test used to identify organisms. PCR is used in combination with an upstream extraction step to release and purify nucleic acids.

There are two general approaches for nucleic acid extraction: i) the first one starts with the isolation and purification of oocysts from the matrix through various purification steps; or ii) the second one consists of the direct extraction of nucleic acids from the organisms in the environmental matrix.

Cryptosporidium species have been detected by PCR from a number of samples including clinical material, animal faeces and water, using pre-isolated and purified oocysts (see Wiedenmann et al., 1998 for a review; Lobo et al., 2009). Also using purified organisms, PCR in combination with genotyping procedures has been employed for the genetic differentiation among species (Leoni et al., 2006).

Direct nucleic acid extractions may be preferred in environmental analysis because procedures are shorter, less expensive, avoid possible losses of organisms, and permit the unbiased analysis of living organisms. Direct extractions avoid the risk of reducing the viability of *Cryptosporidium* oocysts, which may occur during purification steps.

Nucleic acid extraction directly from environmental matrices (e.g. wastewater, manure or soil) is a crucial step in PCR based analysis. Complex matrices, usually with particles, fibers, and a particular chemical composition, can reduce the recovery rate of nucleic acids and / or affect the quality of the extracts that is needed for PCR detection (Lemarchand et al., 2005). Common substances in environmental matrices known to impede amplification or detection by PCR, if co-extracted with nucleic acids, are humic acids, complex polysaccharides, phenols and polyphenols (Tebbe et al., 1993; Wilson, 1997). A specific suitable purification procedure for

nucleic acids from the given substrate is hence needed to produce pure extracts for PCR. For organisms with strong walls (i.e. spores, oocysts), nucleic acid extraction procedures should also include suitable lysis steps to disrupt strong walls.

Analyses of environmental matrices have been mainly done to detect genomic DNA by PCR. For instance, Sturbaum et al. (2002) used PCR to detect DNA of oocysts that were purified by IMS from river water samples. Castro-Hermida et al. (2008) employed IMS to separate oocysts from wastewater concentrates and used PCR with genotyping tools to identify species. The recovery efficiency of IMS from wastewater was not reported in that study. Ramirez et al. (2006) evaluated methods for extraction of DNA from *C. parvum* oocysts that were isolated from manure and soil by density gradients. The authors reported good method sensitivity using a PCR-hybridization technique, but their protocol was long, particularly due to the hybridization step.

PCR directed to DNA molecules is useful to determine species and the presence of pathogens, but does not provide information about viability because DNA is generally stable after the death of the organisms. However, the decay rate of DNA molecules in dead organisms may also be influenced by the environment in which the organism is present (Nielsen, 2007).

A proposed procedure to determine viable oocysts include DNA extraction and PCR from samples that were previously exposed to excystation trigger (Wagner-Wiening et al., 1995; Meyer et al., 2002). Excystation of viable oocysts in bile salts results in the release of sporozoites. This integrated approach, however, may not be accurate enough if oocysts are unable to excyst even if they are fully viable (Neumann et al., 2000).

The major limitation of conventional PCR is that is not quantitative. More recently, real-time qPCR has been advancing for the specific quantification of organisms.

1.10.4 Quantitative real-time PCR application in environmental samples

Quantitative real-time qPCR is a powerful technique that allows specific, fast and high throughput analysis for the quantification of nucleic acids from the target organism. Real-time qPCR allows detection of amplicons as they accumulate during cycling in real time using real time PCR instrumentation. It does not need gel electrophoresis as does conventional PCR. Real-time qPCR using target-specific probes is based on the 5' - 3' exonuclease activity of the enzyme DNA *Taq* polymerase (Holland, 1991). Target-specific probes are labelled with a fluorescent dye and a quencher, and bind specifically to the DNA. During each PCR cycling, the polymerase, which acts to extend new DNA strands, hydrolyze the dual labelled probe attached to the DNA. Probe hydrolysis produces a quantifiable signal that is detected by the qPCR

instrument. The fluorescent signal generated during each PCR cycling is related to the amount of amplicons synthesized.

qPCR results are dependent on optimal chemistry, including reaction components (Bustin, 2000; Edwards et al., 2004), and on the quality of nucleic acids which is dependent on the extraction / purification procedure. In recent years, the assay has been applied extensively in the clinical practice, and now a days it is advancing in environmental analysis. For example, bacterial pathogens were detected in manure and soil using DNA based qPCR assays (Lebuhn et al., 2003; Klerk et al., 2006). DNA extraction procedures and qPCR has been evaluated with different matrices, such as soil for the analysis of microbial communities, marine sediments, and wastewater (Bach et al., 2002; Shannon et al., 2007; Lloyd et al., 2010; Töwe et al., 2010).

For *Cryptosporidium*, Higgins et al. (2001) used qPCR to detect DNA in diarrhea and manure samples, and they found qPCR inhibition when processing manure. The effect of qPCR inhibitory substances in enviromental samples was also observed by Guy and collaborators (2003). The author's procedure was useful for DNA detection from water and wastewater samples using a multiplex qPCR assay, but inhibition was also found for some environmental water samples.

As for conventional PCR, real-time qPCR requires nucleic acids free of inhibitory substances. In addition, co-extracted impurities such as minerals can affect qPCR results by quenching of the fluorophor dye (Stults et al., 2001; Matheson et al., 2010).

1.10.5 Determination of living pathogens using RNA molecules as indicator

Assays based on the detection of mRNA can serve for the identification of living organisms. mRNA molecules are transcribed only in living organisms for protein synthesis. In contrast to DNA, the shorter half-life of mRNA molecules that can be a few minutes (e.g. 3 to > 90 min in yeast; Wang et al., 2000), and their lower stability makes mRNA more suitable to assess the viability of *Cryptosporidium* and of other organisms.

qPCR assays directed to RNA or mRNA includes a reverse transcription step (RT) prior to qPCR to produce a complementary DNA strand (cDNA) from a single stranded (m)RNA. The use of mRNA as target molecule in RT-qPCR is a promissing and powerful tool for the rapid, specific and sensitive determination of living pathogens in environmental matrices, as well as for studies of functional ecosystems and specific microbial activities in the environment. However, the application of RT-qPCR in environmental matrices is still limited, mainly due to difficulties in the extraction of mRNA from those matrices and its processing (Saleh-Lakha et al., 2005). The challenge to produce pure and intact mRNA extracts that can be used for RT-

qPCR is because mRNA can be rapidly degraded during extraction and storage. mRNA molecules are very labile and prone to degradation by RNases (enzymes), and may be affected by other physical and chemical factors during the extraction steps (Bustin, 2000). Limited studies have employed RT-qPCR to quantify mRNA from (pathogenic) microorganisms in environmental matrices. They include water (Gonzalez-Escalona et al., 2006; Fey et al., 2004), and more complex substrates, such as estuarine sediments (Smith et al., 2007), soil (Bürgmann et al., 2003), and the detection of bacterial pathogens in manure (Jacobsen et al., 2007).
Previous studies with *Cryptosporidium* used RT and conventional (end point) PCR to identify living oocysts in water or pure samples (Stinear et al., 1996; Jenkins et al., 2000; Hallier-Soulier and Guillot, 2003). RT-qPCR has been more recently evaluated in water samples and pure suspensions (Fontaine and Guillot, 2003; Lee et al., 2008) showing a good transcript detection in these matrices. Moreover, not all mRNA molecules may be adequate as indicator of viability because of different stability and production rates.

Another molecular tool that targets RNA molecules is Fluorescence *in situ* hybridization (FISH). The assay, which is not a PCR based method and does not require the extraction of nucleic acids, uses fluorescently labeled probes that bind to ribosomal RNA (rRNA). The assay uses pre-isolated oocysts from the substrate and identification of oocysts is done by fluorescent microcopy. The assay has been evaluated to detect living *Cryptosporidium* oocysts (Vesey et al., 1998; Davies et al., 2005). In order to avoid overestimation of viability when targeting rRNA, improvements on the assay has included the addition of RNAses to degrade rRNA from dead organisms (Smith et al., 2004). However, for inactive oocysts that maintain their wall intact, RNase treatment may not be sufficient (Smith et al., 2004).

The *hsp70* mRNA

Eukaryotic organisms and prokaryotes can respond to elevated or a sudden increase of temperature or other triggers (stresses) by inducing the transcription of heat shock protein (HSP) mRNAs and translating them to proteins in order to protect the organism against cellular damage. This reaction is known as the heat shock response (Petersen and Lindquist, 1989). *Hsp70* mRNA codes for major protein chaperones that are essential in maintaining the structure of vital proteins, preventing protein denaturation during stress events, but also without it. HSP70 proteins also function in folding and translocation of polypeptides across membranes and protein formation (Lindquist and Craig, 1988; Beckmann et al., 1990; Dalley and Golomb, 1992). The induction of *hsp70* mRNA was extensively studied in the fruit fly *Drosophila melanogaster* (Lindquist and Craig, 1988; Petersen and Lindquist, 1989). In this organism,

hsp70 mRNA is rapidly produced during heat shock, and can be induced by a factor of 1,000 times and be stable under stress conditions (i.e. exposure to elevated temperature, 37°C; Velasquez et al., 1983). Maresca and Kobayashi (1994) reviewed the induction of *hsp70* mRNA in some eukaryotic parasites before an infection, whereby organisms can accumulate HSP70 proteins as a result of entering into their infective developmental stage to prepare against detrimental factors within a host (e.g. redox potential, lower pH and oxidative products). This may be also the case for the resistant forms in parasitic organisms like *Cryptosporidium*.

1.11 Detection methods for enteroviruses

1.11.1 Standard techniques

In vitro cell culture has been generally employed for the detection of infectious enteroviruses. It uses concentrated and purified viral particles to infect specific cell lines, according to the type of virus (Ley et al., 2002; Sedmak et al., 2005). Cytophatic effects in the cells are then determined by microscopy. However there are other viruses, such as noroviruses, which is another RNA enteric virus from the family *Caliciviridae*, that cannot be grown in traditional cell culture systems. Also some enteroviruses, such as coxackieviruses may grow only poorly in cultures (Lipson et al., 1988). In general, the cell culture procedure is labor intensive and can take 3 - 12 days depending on the type of virus (Skraber et al., 2004; Sedmak et al., 2005; WHO, 2011). Thus, its application is not suitable for rapid routine tests.

A recent standard method for enteroviruses and noroviruses in water samples is Method 1615 (EPA, 2010). It consists of cell culture (for the culturable viruses) and identification of cytopathic effects in the cultures over a 2 weeks period, with a subsequent confirmation step that involves additional time. Method 1615 also includes an RT-qPCR assay for the detection of the non-culturable viruses, and it has been optimized for water samples.

Various filtration systems and concentration steps have been employed for the processing of water. Fong and Lipp (2005) presented a review of methods for virus concentration. They include the use of cartridge filters, glass fiber filters, and acid floculation for virus concentration. Since filtration can be difficult with particulate and more complex matrices, alternative procedures that include chemical based elution of viruses and precipitation techniques for concentration (Monpoeho et al., 2001) or ultracentrifugation were preferred (La Rosa et al., 2010).

1.11.2 RT-PCR and RT-qPCR for virus detection in complex substrates

RT-PCR and recently RT-qPCR are more rapid assays that allow the specific detection of viral particles that have an RNA genome. There has been a number of investigations that applied RT-(q)PCR for the monitoring of viruses in environmental water samples (e.g. Fong and Lipp, 2005; Fuhrman et al., 2005). Because environmental (water) samples can contain many inhibitory substances for RT-qPCR, nucleic acid purification procedures should be able to virtually eliminate such inhibitors. For example, including chemical facilitators, such as T34 gene protein and / or BSA in (q)PCR, or PVP during purification of RNA or qPCR have shown to be useful in reducing (q)PCR interferences, as these components can bind to phenolic or lipid compounds (Kreader et al., 1996; Monpoeho et al., 2000; Fuhrman et al., 2005). However, such chemical facilitators were not needed in other studies (Ley et al., 2002; Jothikumar et al., 2005), or their use was even contraproductive (Lebuhn et al., 2003).

The application of RT-(q)PCR in more complex matrices includes, for example, sludge material (Monpoeho et al., 2000), shellfish (Jothikumar et al., 2005) or animal faeces (Ley et al., 2002). More recently, a number of surveillance studies of viruses in wastewater treatment facilities has been performed using RT-qPCR based assays (Katayama et al., 2008; Aw and Gin, 2010; La Rosa et al., 2010).

The application of RT-qPCR to detect genomic RNA from enteric viruses is useful to indicate the presence of viral agents and to quantify them in the matrices evaluated, but does not necessarily indicate infectivity. Nevertheless, single stranded RNA from inactivated viral particles may not survive for a long time in the environment. There were also indications that RT-qPCR results were related with community health (EPA, 2010). Although, the detection of genomic RNA from enteroviruses in river water samples was longer than their infectivity, both measurements correlated positively (Skraber et al., 2004).

Another approach to identify infectious viral particles is the use of integrated cell culture (RT)-PCR (Chapron et al., 2000; Greening et al., 2002). Besides the possibility to identify infectious particles, this approach was shown to be faster (about 3 - 5 days; Greening et al., 2002) than conventional cell culture, and to provide a higher sensitivity (Chapron et al., 2000). The procedure consists of RT-PCR detection of viruses from the infected cells.

Overall, the use of RT-qPCR based assays as monitoring tool for RNA viruses allows for rapid analysis, the processing of multiple samples in one qPCR run, and the identification of target species. Viral contamination with enteroviruses in water samples were identified using RT-qPCR (Skraber et al., 2004; Fuhrman et al., 2005). Surveillance studies of enteroviruses in manure, slurry or sludge samples by real-time RT-qPCR have been limited (e.g. Jimenez et al.,

2005; Costantini et al., 2007). This may however advance quickly as pathogen control at the source is intensified with the aim to prevent dissemination of pathogenic agents in the environment.

This investigation

Aim and objectives

This project aimed to establish rapid, sensitive and pathogen-specific methods based on direct extraction of DNA or (m)RNA and real-time quantitative PCR to detect and quantify *Cryptosporidium parvum* oocysts and enteroviruses in complex environmental substrates such as manure, wastewater and soil. The optimized methods should be useful for screening of these pathogens in hygiene controls in order to identify contaminated sources and prevent microbial / viral dissemination in the environment. The methods ought to be useful to detect viable *C. parvum* oocysts by targeting *hsp70* mRNA in RT-qPCR, and to control the inactivation of oocysts during, e.g., anaerobic digestion of cattle manure.

These investigations were intended to advance these molecular tools for application in environmental assessments and hygiene evaluations.

The objectives of this investigation were the following:

1. Optimize a DNA extraction procedure from *C. parvum* oocysts in manure and soil for detection and quantification of *hsp70* DNA by real-time qPCR. Evaluate the usefulness of the optimized DNA based approach to determine the inactivation of oocysts in effluents from anaerobic digesters.
2. Develop and optimize mRNA and RNA extraction and purification procedures for maximum recovery of *C. parvum* (m)RNA strands from manure, and optimize the RT-qPCR assay to obtain specific and sensitive detection of *hsp70* mRNA.
3. Optimize RNA extraction procedures for the quantification of enteroviruses by RT-qPCR in complex environmental substrates.
4. Evaluate the production of *hsp70* mRNA in oocysts and assess the transcript as target molecule to indicate the presence of viable, potentially infectious oocysts in environmental samples.
5. Evaluate and assess the inactivation of *Cryptosporidium* strains during anaerobic treatment of manure by quantification of *hsp70* mRNA.

CHAPTER 2

General Materials and Methods

2.1 Collection and processing of environmental samples

Samples of 500 ml manure and digested manure (content of a mesophilic and a thermophilic digester) were collected from an anaerobic model biogas plant treating semi-liquid cattle manure (7.5 % total solids) (Effenberger et al., 2006). Samples were homogenized with an Ultra-Turrax® disperser for about 1 min. Aliquots of homogenized manure (2 - 10 ml) were washed two times with 1x PBS buffer and once with 0.85 % KCl in order to reduce the concentration of qPCR inhibitory substances present in the substrate (Lebuhn et al., 2003). Sample washing consisted of addition of 1x PBS buffer or KCl to the sample, mix by short vortex, centrifugation at 5,000 x g for 3 min and discarding the supernatant containing dissolved inhibitory substances. The final pellet after washing was resuspended in 0.85 % KCl to an equal volume as initially in the sample. Samples were stored at 4°C.

Soil samples from an agricultural land under conventional, integrated farming from the Research Station Scheyern, Pfaffenhofen (north of Munich) (Fuß et al., 2011) were used to evaluate the nucleic extraction methods, and were kindly provided by the Institute of Soil Ecology, Helmholtz Zentrum München. Samples of two management systems were maintained at 4°C until analysis. Soil texture was classified as silty-loam, with an average of 16.7 % sand, 56.7 % silt, and 27 % clay for the 8 - 22 cm depth.

Table 2.1 – Characteristics of soils (depth 0 - 8 cm) used in Chapters 3 and 6. Research Station Scheyern, Pfaffenhofen. Data provided by Institute of Soil Ecology, Helmholtz Zentrum München.

Parameter	Unit	Minimum-tilled soil	Tilled soil
Texture - clay content	w %	26.2	27.9
Soil pores (> 50 µm)	vol %	3.11	14.02
Soil pores (10 - 0.2 µm)	vol %	24.6	20.6
pH	-	5.12	5.71
C	w %	1.78	1.36
N	w %	0.18	0.15
C/N	-	9.81	9.25

Sludge samples from a wastewater treatment plant in Berlin (Pusch et al., 2005) and secondary-treated wastewater samples from an activated sludge plant in Garching near Munich were used for experiments with enteroviruses. Wastewater samples (0.5 l) were collected, and aliquots (10 ml) were centrifuged at 5,000 x g for 10 min. Pellets were resuspended in about 1 ml 1x PBS buffer, and 90 µl were used for seeding enteroviruses.

Solutions were prepared with DNAse/RNase-free pure water (DEPC treated, 0.1 %). Labware (e.g. bottles, pipette tips and microcentrifuge Safe-Lock tubes, Eppendorf) was autoclaved before used. Stringent conditions were maintained during sample processing by disinfecting surfaces, racks and pipettes with alcohol and DNAse/RNase solutions.

2.2 *Cryptosporidium parvum* and enteroviruses

2.2.1 Organisms

Various pure suspensions of *C. parvum* oocysts from artificially infected cattle and from wild infections were kindly provided by Dr. Najdrowski, Institute of Parasitology, Universität Leipzig, Germany. Oocysts were purified from animal faeces by washing in diethyl ether and by centrifugation in saturated sodium chloride solution at 2,500 x g for 10 min as described by Najdrowski et al. (2007a). Oocysts were stored at 4°C until used in 1x PBS buffer supplemented with antibiotics (Penicillin, 100 u/ml and Streptomycin, 100 µg/ml) and antimycoticum (Amphotericin B, 2.5 µg/ml; Sigma-Aldrich®). Oocyst suspensions were used for spiking environmental samples. Purification of oocysts from manure in sentinel chamber experiments (Chapters 3 and 6) was done by percoll gradient centrifugation according to Peeters and Villacorta (1995) (see section 2.7.3).

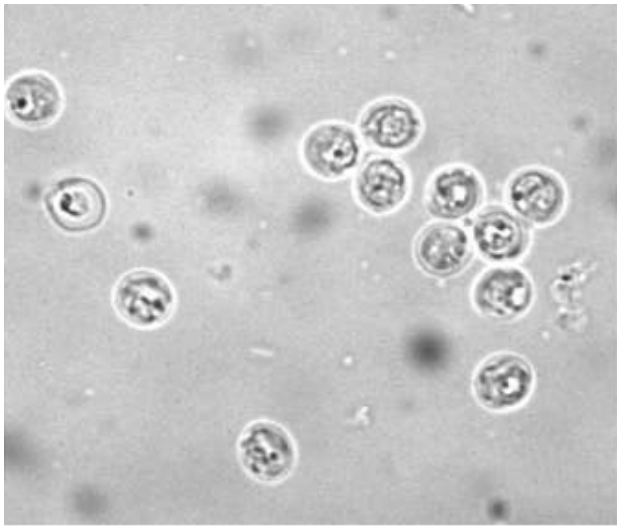


Fig. 2.1 - *Cryptosporidium parvum* oocysts (4-5 µm), provided by M. Najdrowski (project communications).

Inactivated *Enterovirus* suspensions were used for experimentations. Human poliovirus 1 strain Sabin was kindly provided by Dr. S. Ihle, Umweltbundesamt, Berlin, Germany. Human Echovirus type 6 samples were from stock suspensions from the Institute of Water Quality Control, Technische Universität München. Known concentrations of Sabin 1 and Echovirus type 6 were used for spiking environmental samples. Suspension aliquots were stored at -80°C until used.

2.2.2 Microscopy counts and oocyst identification

Oocysts from pure suspensions were enumerated in a Neubauer counting chamber in an epifluorescence phase contrast microscope (Carl Zeiss) at 100x or 600x magnification. Oocysts under the microscope appeared round and bright with inner content (four sporozoites that were not always well noticed). The mean number of oocysts of 4 counting grids in the counting chamber was then calculated per ml sample. Oocysts spiked to manure or digested manure were identified microscopically in sample smears on microscope slides. For this, a small volume (about 10 µl) from diluted or undiluted samples was transferred to the slide and covered with a coverslip by making pressure to obtain a thin layer.

2.3 Optimization experiments based on DNA and (m)RNA – *C. parvum*

The following Figs. 2.2 and 2.3 give an overview of the steps that were optimized in this study. Evaluations were done with environmental samples seeded with known amount of *C. parvum* oocysts. Lysis of oocyst was done with different procedures based on freeze-thaw, pre-excystation, and bead beating (Chapters 3 and 4). Lysis by bead beating, which combines a physical and chemical treatment, was done in a FastPrep® Instrument (Qbiogene, MP Biomedicals), with the lysing matrix E tubes (BIO 101® Systems, Qbiogene), unless otherwise stated. The E tube contains a mixture of beads: 1.4 mm ceramic beads, 0.1 mm silica beads and one 4 mm glass bead. Other bead beating tubes were also evaluated and are reported in Chapter 4.

DNA extraction / purification (Fig. 2.2) consisted of lysis with a buffer containing sodium phosphate-SDS-EDTA, and purification with guanidinium isothiocyanate (GITC) using a silica matrix in spin columns (FastDNA® SPIN Kit for Soil, Qbiogene, MP Biomedicals). This kit based system showed to be adequate for the purification of bacterial DNA in manure (Lebuhn et al., 2003; 2004). The kit was used mainly following the manufacturer suggestions and including modifications for the lysis step (Chapter 3).

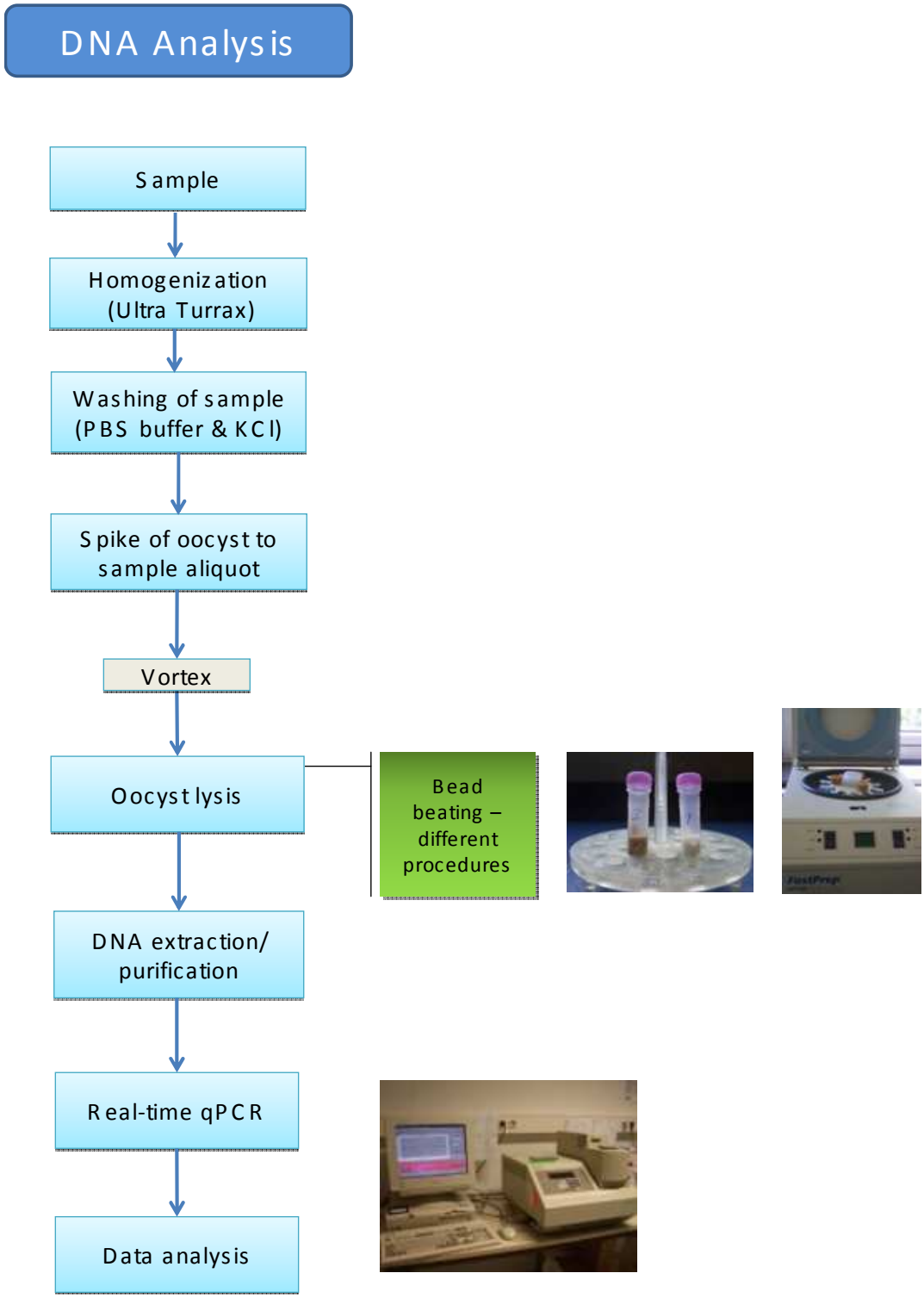


Fig. 2.2 - Experimental procedure for DNA based experiments. The green box indicates the step that was evaluated using different procedures (evaluations are reported in Chapter 3).

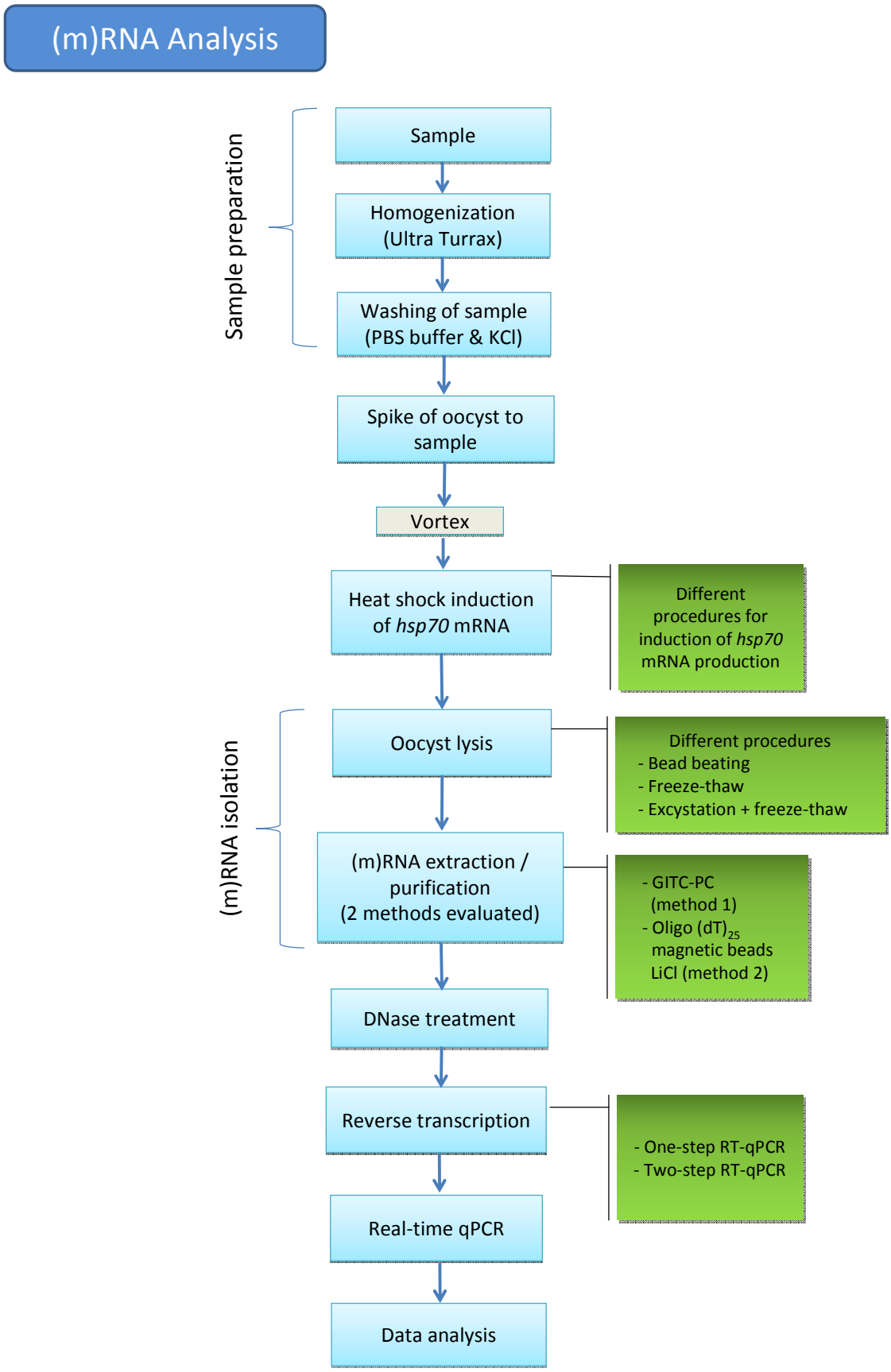


Fig. 2.3 - Experimental procedure for RNA and mRNA based experiments. Green boxes indicate the steps that were optimized (evaluations are reported in Chapters 4 and 5).

Total RNA extraction (Fig. 2.3), here referred as GITC-PC method 1, used a chemistry based on GITC, phenol-chloroform (PC) and isopropanol included in the FastRNA® Pro Soil-Direct Kit (Qbiogene, MP Biomedicals). The procedure was done following the manufacturer's suggestions and incorporating optimized steps for oocysts lysis and purification, as described in Chapter 4.

Direct extraction of mRNA molecules (Fig. 2.3) was done with oligo $(dT)_{25}$-coated magnetic beads, here referred as method 2 (Dynabeads® mRNA DIRECT™ Micro Kit, Dynal Biotech). The method is based on binding of the mRNA poly A tail at its 3' end to complementary oligo $(dT)_{25}$-magnetic beads. The system chemistry includes lithium chloride (LiCl) in the lysing and binding buffers. Extractions were done following the manufacturer's notes and the optimized steps developed and described in Chapter 4. Other buffers were also evaluated (Chapter 4), and the complete method was optimized to process complex matrices.

DNAse treatment of RNA and mRNA extracts was done to eliminate co-extracted DNA with the system TURBO DNA-*free*™ (Ambion®, Applied Biosystems).

2.4 Optimization experiments based on RNA – Enteroviruses

Three extraction methods were evaluated for enteroviruses in manure, sludge and wastewater samples: The direct extraction of mRNA strands with oligo $(dT)_{25}$ - magnetic beads, method 2 (Dynabeads® mRNA DIRECT™ Micro Kit, Dynal Biotech); the total RNA extraction based on GITC-PC and silica matrix (FastRNA® Pro Soil-Direct Kit, Qbiogene, MP Biomedicals), and a method that uses Streptavidin coated plates (Strep-thermo-Fast®, AB gene). The latter consisted on hybridization of a biotinylated oligonucleotide probe to a complementary RNA site, with subsequent binding of the biotin to streptavidin. This system used a lysing buffer containing GITC and β-mercaptoethanol (RNeasy® Mini kit, Qiagen). The methods were used following the manufacturer's instructions or unless otherwise stated in Chapter 4.

2.5 Reverse transcription and qPCR

Extracts of mRNA and RNA were reversed transcribed to obtain cDNA for subsequent qPCR. RT and qPCR can be done either sequentially in the same tube (one-step RT-qPCR) or separately (two-step RT-qPCR). These two systems were evaluated for *Cryptosporidium parvum* in Chapter 4. One-step RT-qPCR was done with the Real MasterMix RT Probe qRT-PCR Kit (Eppendorf) following the manufacturer's instructions. The two-step system was generally used in this study. The RT in this system used reagents from ThermoScript™ (Invitrogen). For enteroviruses, the two-step system was used.

2.5.1 Reverse transcription

The RT reaction separately from the qPCR (in the two-step system) was done with 5 µl of extracted (m)RNA. The reaction was performed at 57.5°C for 30 min, followed by 5 min at 85°C, and a 4°C hold time in a Mastercycler® (Eppendorf). The reaction volume (25 µl) included 1x synthesis buffer (Invitrogen), RNase inhibitor (30 u) (Eppendorf or Fermentas), reverse primer (see later) specific for the gene sequence of the target organism (500 nM), Dithiothreitol (4 mM), dNTPs mix (1 mM), and 9.3 units of the enzyme reverse transcriptase (ThermoScript™, Invitrogen). The enzyme and the (m)RNA aliquot were added to the RT tube after all reagents were pipetted. RT product was immediately processed for qPCR.

The RT reaction for enteroviruses with the streptavidin system was done in a 60 µl volume with RNase inhibitor (100 u), 1000 nM reverse primer, 12.5 µl (1x synthesis buffer), dNTPs mix (1mM), Dithiothreitol (5 mM) and 22.5 units of enzyme.

In order to check for the presence of co-extracted DNA, RT reactions were also done with (m)RNA extracts that were not pre-treated with DNAse, and including or not the RT enzyme. RT negative controls consisted of reactions with DNase treated (m)RNA minus the RT enzyme.

The one-step RT-qPCR system is described in the next section.

2.5.2 Real-time qPCR

Real-time qPCR used the hot-start Taq DNA polymerase system which allows an automatic reaction. The enzyme is activated at the elevated temperature during the start of the PCR. Sample processing was done maintaining stringent conditions in a biosafety cabinet (Applied Biosystems). The cabinet and lab utensils were sterilized by UV radiation for 15 min.

The qPCR reaction in the two-step RT-qPCR system contained 2.5 µl of extracted DNA or cDNA (transcribed from mRNA or RNA) and 0.75 units Platinum® Taq DNA polymerase (Invitrogen) in a reaction volume of 25 µl. Higher volume of the extracts per qPCR reaction was reported to reduce the sensitivity of the assay (Lebuhn et al., 2003). First, a qPCR master mix was prepared for a number of qPCR reactions (see an example in Table 2.2). The mixture contained 1x synthesis buffer (Invitrogen), 600 nM primers and 300 nM hydrolysis probe (see sequences in Table 2.3), 6 mM of $MgCl_2$ to increase Taq performance in the presence of inhibitors (Lebuhn et al., 2003), 1 µM passive reference dye (ROX), 200 µM dNTPs mix, and pure sterile water. The mixture was briefly mixed by vortex. Subsequently, the DNA polymerase was added, the mixture was briefly vortex, and kept on an ice bucket. A 22.5 µl of the master mix was pipetted to a qPCR tube, and 2.5 µl of DNA or cDNA was added. Reactions were done using qPCR thin tubes (Applied Biosystems) or 96 well twin.tec PCR plates (semi-skirted, Eppendorf). Plates were closed with ABI PRISM™ Optical Adhesive Covers (AB gene,

Applied Biosystems). Real-time PCR amplification was done in a Gene Amp® 5700 Sequence Detection System (AB Applied Biosystems). For *Cryptosporidium and C. elegans* nematode, the thermal protocol consisted of 3 min polymerase activation at 95°C, and 45 or 50 cycles of denaturation (15 s at 95°C) and 1 min combined stringent annealing/extension at 63.5°C. For enteroviruses, the qPCR reaction included 1200 nM reverse primer, and the annealing/extension temperature was 57.5°C. qPCR reactions were measured in duplicates or triplicates. For negative controls, pure sterile water was used instead of nucleic acids.

Table 2.2 – Example of chemical composition for a qPCR reaction in the two-step system.

Organism: *Cryptosporidium parvum* (In parenthesis: concentration of stocks).	For 1 sample:	example calculated for:
	µl	43 samples
Buffer (10x contains no $MgCl_2$)	2.5	107.5
MgCl (50 mM)	3	129
dNTPs (10 mM each)	0.5	21.5
FP (10 µM)	1.5	64.5
RP (10 µM)	1.5	64.5
Sonde (10 µM)	0.75	32.25
Rox (100 µM)	0.25	10.75
Platinum Taq-Pol. (5 u / µl)	0.15	6.45
Pure H_2O	12.35	531.05
dispatch	22.5	967.5
DNA or cDNA	2.5	107.5
Total vol	25	1075

The one-step RT-qPCR system was performed as follow: one reaction was done in a 25 µl volume (22.5 µl of master mixture and 2.5 µl of mRNA). The mixture was prepared with primers and probe (same concentration as for qPCR, see above), RNase-free pure water, 0.25 µl of RNase inhibitor (30 u, Eppendorf), Real Mastermix RT (5 u, Eppendorf) and 10 µl of Real MasterMix Probe (containing dNTPs, reaction buffer, Taq-polymerase and 12.5 mM Mg^{+2}; Eppendorf). The mixture was briefly vortex, 2.5 µl of mRNA template was added, mixed shortly, and placed on ice. The thermal cycling for the RT-qPCR included the following steps: reverse transcription at 42°C for 30 min, initial denaturation / RT inactivation at 90°C for 5 min, and subsequently 50 cycles of 15 s at 95°C and annealing at 63.5°C for 1 min.

2.5.3 Primers and Probes

Primers and probes were synthesized and purified with high performance liquid chromatography by Thermo Hybrid or Biopolymers (Biomers). Specific probes were dual labelled with the fluorogenic dyes FAM and TAMRA. *Cryptosporidium* species that represent a risk of infection

to humans and cattle were the targets in this study. Sequences of primers/probe set 1PS were specifically designed to target sequences of the *hsp70* gene of *C. parvum* and *C. hominis*. The sequences also match to the respective gene in *C. wrari*, and *C. meleagridis*. To improve readability all four will be referred as *C. parvum* when specificity is mentioned. Specific oligonucleotide sequences were designed for the β-tubulin gene (system 3PS) in *C. parvum*, *C. hominis and C. meleagridis*. Specific primers/probe that target the *hsp4* gene, member of the *hsp70* family in *Caenorhabditis elegans*, were also developed. This nematode was used as positive control organism in Chapter 7. Primers and probes for viruses were specific for the genus *Enterovirus* (Pusch et al., 2005). The specificity of all primers/probe was verified *in silico* by FASTA and BLAST N2 analysis against nucleotide data base (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 2.3 – Primers and probes used in RT and qPCR, specific for the *hsp70* and β-tubulin genes in *Cryptosporidium parvum*, *hsp4* gene (*hsp70* family) in *Caenorhabditis elegans*, and the genus *Enterovirus*. fp: forward primer; rp: reverse primer.

Organism	**Oligonucleotide name**	**Sequence**
C.parvum hsp70	1PS Cr_*hsp70*-1005-fp	5'-AACTTT AGCTCCAGTTGAGAAAGTACTC-3'
	1PS Cr_*hsp70*-1095-rp	5'-CATGGCTCTTTACCGTTAAAGAATTCC-3'
	1PS Cr_*hsp70*-1062-probe	5'FAM-AATACGTGTAGAACCACCAACCAATACAACATC-TAMRA 3'
C.parvum β-tubulin	3PS CrBT-1026 fp	5'-GAATGGATTCCAAATAATATCAAGTCATCAGTT-3'
	3PS CrBT-1136 rp	5'- GAAGTGAATTGCTCAGCAACACGTC-3'
	3PS CrBT-1076-probe	5'FAM-CCAACAAAGGTTGAAGCCATCTTCAATCCTTT-TAMRA 3'
C. elegans	4PS Cel-hsp4-1438-fp	5'-GTTCAGAAAGTTTTGGAAGATGCTGACA-3'
	4PS Cel-hsp4-1530-rp	5'-CCGTTGAAGTAGTCCTTGATAAGTTGTT-3'
	4PS Cel-hsp4-1489-probe	5' FAM-AATTCTGGTTGATCCTCCTACCAGTACGAT-TAMRA 3'
Enterovirus	5PS 450-fp	5'- GCCCCTGAATGCGGCTAAT-3'
	5PS 577-rp	5'- RATTGTCACCATAAGCAGCCA -3'
	5PS 532-probe	5' FAM- CACCCAAAGTAGTCGGTTCCG-TAMRA 3'

Numbers are the position in the respective gene, or in Poliovirus Sabin 1 genome.

2.6 Analysis of qPCR measurements and statistical analysis

Ct values generated from the qPCR instrumentation represent the PCR cycle number at which the amplification curve of one reaction crosses a defined threshold. Ct values are a function of the target DNA or cDNA template concentration (Lie and Petropoulos, 1998). The threshold in the qPCR amplification plot was set in the exponential phase of the amplification curves and above the noise background fluorescence. The crossing point defines the Ct value. The same threshold was used for samples that were compared. Copy numbers of DNA or cDNA in the analyzed samples were measured with Ct values by interpolating the Ct in a standard curve.

For preparation of the standard curves, known amount of organisms were spiked to an environmental sample. Subsequently, DNA and (m)RNA were extracted. The extracted DNA or the cDNA transcribed from the extracted (m)RNA was used for qPCR. Complementary DNA (cDNA) is obtained from (m)RNA after a reverse transcription step.

The standard spiking approach allows the quantification of DNA or cDNA in a sample, considering the efficiency of extraction and detection of the nucleic acids (Lebuhn et al., 2003).

For each organism and gene analyzed, standard curves were prepared by plotting known copy numbers of DNA or cDNA in a 25 µl qPCR volume versus the corresponding measured Ct value.

The efficiency of the qPCR, which depends on the slope of the regression curve, was calculated with the following equation (Bernard and Wittwer, 2002).

$$E = \left[10^{\left(-1/\text{slope}\right)}\right] - 1 \qquad \text{Equation (1)}$$

For *Cryptosporidium parvum*, cDNA copies per 25 µl qPCR reaction were calculated as double stranded DNA (dscDNA). For this, 1 Ct value was subtracted from the measured Ct value. dscDNA is theoretically equivalent to one DNA. The DNA copies present in each qPCR volume were derived from the number of oocysts spiked to the sample. The measured dscDNA from mRNA was quantified with reference to the number of oocysts initially seeded to the sample. Calculations consider that one oocyst contains 4 sporozoites, each with one copy of the target sequence.

Genomic DNA copies in a qPCR volume were also calculated by the most probably number (MPN) statistics using Ct values from serial dilutions of DNA extracts (Fredslund et al., 2001; Lebuhn et al., 2005). Subsequently, the efficiency of the DNA extraction and qPCR was determined with respect to the copies spiked to the sample before extraction.

Relative quantification of cDNA copies in a sample was also done by comparison to a reference sample taken as control using Ct values, with simplified models after Pfaffl (2001), and considering optimum qPCR efficiencies (E=2).

$$ratio = E^{(Ct\ control - Ct\ sample)} \qquad \text{Equation (2)}$$

For comparison of the procedures evaluated and to assess the influence of treatments, statistical analysis was performed using SPSS® software version 15. Significance was tested by t-tests with a 5 % level of significance, whereby $p \leq 0.05$ indicates significant differences. Multiple treatments were compared by analysis of variance (ANOVA) with the Gabriel Post-Hoc test since the variance of samples was equal, as identified in the Levene test.

2.7 Viability and infectivity tests for *C. parvum* oocysts

2.7.1 Excystation test

The method is based on differentiation of intact and broken oocysts after treatment with a bile solution (i.e. sodium taurocholate, SoTa). The solution triggers the excystation (breaking) of viable oocyst and the release of sporozoites. The test was done in pure oocysts suspensions or with oocysts in fermented manure. Fermented manure (50 µl) spiked with oocysts and mixed with 50 µl 1x PBS buffer, or pure oocyst suspensions (50 - 100 µl) was mixed with 0.8 % of sodium taurocholate (prepared with 1x PBS buffer, Sigma-Aldrich®, T 4009) in a proportion of 2:1 v/v (sample to SoTa) (Najdrowski, 2007b). Samples were mixed and incubated for 2 hours at 37°C at mild shaking conditions. Afterwards, samples were mixed and kept on ice water. Small aliquots (about a drop) were observed in a phase contrast microscope at 100x or 600x of magnification. The following were registered: intact oocysts identified as bright and round, and excysted oocysts identified as dark oocysts with ruptured walls (totally or partially empty oocysts). The viability of oocysts (their ability to excyst) was determined as follow:

$$Excystation\ (\%) = \frac{number\ \ empty\ \ oocysts}{number\ \ empty\ \ oocysts + number\ \ intact\ \ oocysts} \qquad \text{Equation (3)}$$

2.7.2 Dye permeability assay

The assessment of viability with the membrane permeability assay was done with pure oocyst suspensions. A 100 µl aliquot was mixed with 10 µl DAPI solution (2 mg / ml methanol) and incubated at 37°C for 2 h in the dark. Then, 10 µl of propidium iodide (PI) (1 mg / ml in 0.1 M PBS buffer, pH 7.2) was added, the sample was mixed, and incubated for 5 min. Samples were washed with 1x PBS buffer at 2,500 x g for 5 min, the supernatant was discarded, and the pellet

resuspended in 100 µl of PBS buffer. An aliquot (7 - 10 µl) was placed on a microscopic slide and observed for viability by fluorescence microscopy using UV filter for DAPI and a green filter for PI at 100x and 600x of magnification. DAPI dye blue dead and living organisms. Oocysts that take PI were non-viable and were dyed red. Oocysts that take DAPI but not PI were considered viable. The proportion of non-viable oocysts was calculated as the percentage of PI.

2.7.3 Infectivity test for *C. parvum*

In vitro infectivity test was performed at the Laboratory of Parasitology, Universität Leipzig, in collaboration with Dr. Najdrowski. The test consisted of infection in monolayers of HCT-8 human adenocarcinoma cells with purified oocysts, and detection of infections by immunofluorescence assay using monoclonal antibodies, according to Najdrowski et al. (2007a). Oocysts spiked to (digested) manure in sentinel chamber experiments were purified from manure content by percoll gradient centrifugation. Subsequently, oocysts were recovered and washed 3 times in PBS buffer at 1,500 x g for 10 min (Peeters and Villacorta, 1995).

Infectivity test was done as follows: cell monolayers were grown in Lab-Tek™ II (Nunc) eight-well chamber slides for 2 days. Cells were inoculated with 100 µl aliquots of serial dilutions of oocysts and 0.4 % sodium taurocholate solution (prepared with cell culture medium RPMI-1640 containing 2 mM L-Glutamine). After 48 hours, cell cultures were washed 3 times with PBS, fixed in cold methanol (70 %) for 10 min, washed 3 times in PBS buffer for 2 min, seeded with specific monoclonal antibodies and incubated at 37°C for 45 min. Afterwards, cells were washed 3 times in PBS buffer, secondary antibodies were seeded, cells were incubated at 37°C for 45 min and washed again in PBS buffer. Infected cells and foci (intracellular development stage of *C. parvum*) were observed by epifluorescence microscopy. Total foci fluorescence area in 20 randomly chosen views were calculated, and extrapolated for one well area. The number of infectious oocysts in a given sample was calculated with reference to the foci fluorescence in control oocysts.

2.8 Sentinel chamber experiments in anaerobic digesters

Sentinel chamber experiments were performed to evaluate the inactivation of oocysts in anaerobic digesters treating manure. This was evaluated by measuring DNA (Chapter 3) and mRNA (Chapter 6). Oocysts seeded to manure and digested manure were subjected to anaerobic treatment at different temperatures and retention times, as specified in Chapters 3 and 6.

2.8.1 Description of model biogas plant

The model biogas plant consisted of three anaerobic digesters treating semi-liquid cattle manure (7.5 ± 0.5 % total solids) operated at mesophilic (set point 38°C) and thermophilic conditions

(50°C and 55°C). The model digesters resembled pilot-scale digesters in an animal farm in southern Bavaria, Germany (Effenberger, 2008). Digesters had working volumes of 200 l, 240 l and 600 l for the mesophilic 1, thermophilic and mesophilic 2 digesters, respectively (Fig. 2.4). Operating and process parameters were recorded to control for digester performance and the stability of the process. Temperature in the mesophilic digester fluctuated slightly from the set point to a mean of 36.2°C during experimentations with the RNA based approach (Chapter 6).

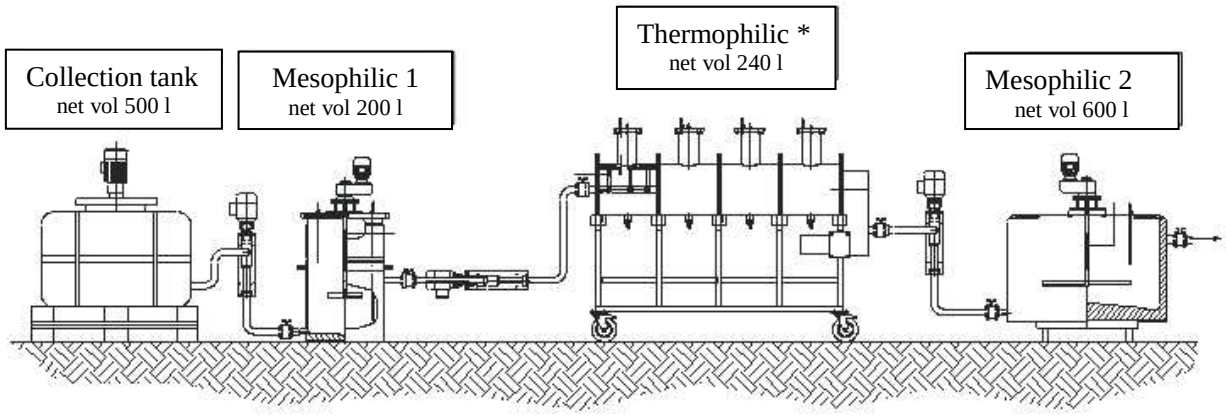


Fig. 2.4 - Schematic representation of the model biogas digesters used for oocyst inactivation studies, Effenberger (2008). *: digester was operated at 50°C and 55°C during the evaluations.

Table 2.4 – Characteristics of manure and digested manure from the anaerobic model plant during oocyst inactivation experiments with the RNA based approach (Chapter 6). Details of digester performance are described in Effenberger (2008).

Parameter	Unit	Collection tank	Mesophilic 1	Thermophilic *
Temperature	°C	22	36.2	50
pH	-	7.4	7.9	8.1
TS	%	7.5	6.5	6
VFA	mg/l	6391 ± 1474	362 ± 25	696 ± 253
NH_4-N	mg/l	1402 ± 264	1652 ± 53	1865 ± 191
NH_3-N	mg/l	19.6 ± 8.2	127 ± 21	483 ± 218

*: digester was also operated at 55°C during the inactivation studies; mean values n=4-10.

2.8.2 Experimental design

The following Fig. 2.5 shows the procedures for the sentinel chamber experiments and the accompanying waterbath experiments.

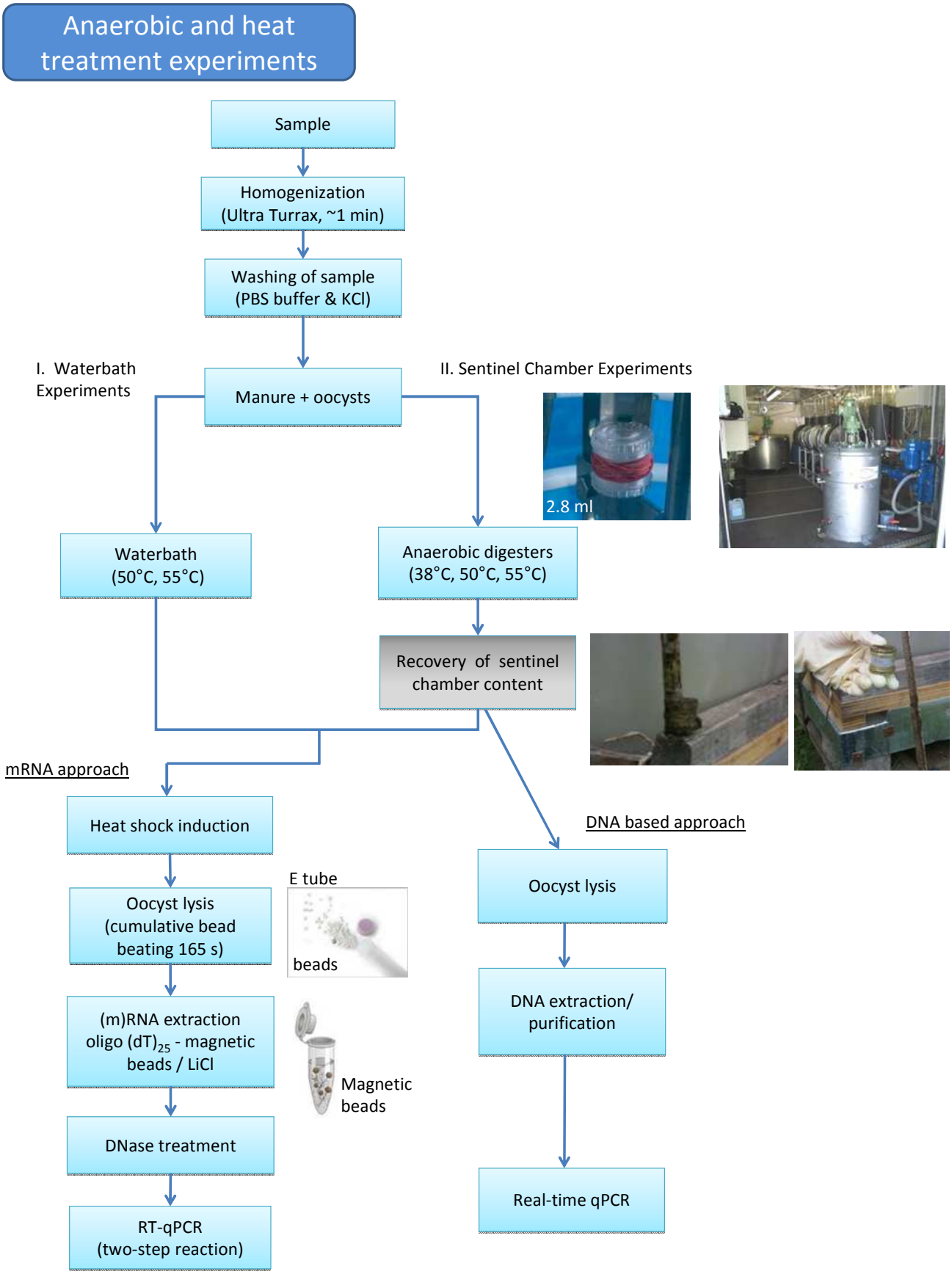


Fig. 2.5 - Experimental procedure for waterbath experiments (i) and sentinel chamber experiments in anaerobic digesters (ii) in evaluations of oocyst inactivation. Evaluations are reported in Chapter 3 (DNA based approach) and Chapter 6 (mRNA based approach).

2.8.3 Experimental procedure

Diffusion sentinel chambers made of polycarbonate (3 ml volume; Labor Dr Rabe) were used for the experiments. The lids of the sentinel chambers were closed with filters of nitrate cellulose (0.4 µm pore size; Millipore HTTP02500). Sentinel chambers were filled with 1.8 ml of manure or digested manure from the respective collection tank or fermenter, respectively, and then seeded with 1 ml of pre-quantified pure oocyst suspension (about 10^7 oocysts / ml). Sentinel chambers were then closed taking care that the filter on the cover lid was correctly placed. Sentinel chambers were attach to a removable tray with a wire and rapidly placed in the digesters (Fig. 2.5, II). Sentinel chambers were maintained inside the reactors for different times (1 h, 4 h, 12 h, and 20 h). The times, 1 and 4 h corresponded to the feeding intervals in the digesters, which were evaluated for process and sanitation performance (Effenberger et al., 2006). 20 h was the retention time for the chain digester (mesophilic-thermophilic-mesophilic). After the respective times, sentinel chambers were removed from the digesters, cleaned with a tissue from the outside and immediately transferred to the lab. Chamber contents were transferred to a 10 ml sterile Falcon tube (Eppendorf) by pipetting and by carefully washing the sentinel chamber with 1x PBS buffer. The sample that remained in the filter membrane and chamber lids was also recovered by washing with 1x PBS buffer. Oocysts in the Falcon tube were concentrated by centrifugation at 2,500 x g for 5 min. The supernatant was discarded leaving in the tube about 2.8 ml of sample, and then the pellet was resuspended in this volume, as initially present in the sentinel chamber. Sample aliquots were used for extraction of DNA (Chapter 3) or mRNA (Chapter 6). Nucleic acids in the extracts were then quantified by (RT)-qPCR.

Additionally, waterbath experiments (Fig. 2.5, I) were performed for the mRNA approach, as described in Chapter 6.

2.9 Preparation of control organism – *Caenhorhabditis elegans*

The nematode *C. elegans* was used as positive control organism for the evaluation of *hsp70* mRNA production in *C. parvum* oocysts (Chapter 7).

2.9.1 Cultivation

Culturing of *C. elegans* was carried out at the Institute of Biotechnology, Technische Universität München. Facility and instrumentation were kindly provided by Dr. Martin Haslbeck, and sample processing followed instructions used in the laboratory of Biotechnology (Sulston and Hodgkin, 1988; Somoza and Buchner, 2006 - lab guidelines). *C. elegans* were grown in NGM agar plates with a lawn of OP50, a leaky uracil-requiring strain of *E. coli*, at 21°C for 2.5 days.

A small piece of the culture was transferred to a new 5 cm NGM agar plate without *E. coli*. Plates were incubated for 2 - 3 days to produce L3 – L4 larvae. *C. elegans* cultures were observed under the microscope to verify their vitality and mobility. Nematodes were transferred to 2 ml Eppendorf tubes by washing with 1x PBS buffer. Samples were then concentrated in 500 µl by a short centrifugation at 800 x g, and immediately placed at 4°C. Aliquots (100 µl) were used for (m)RNA extraction including, or not, a prior heat shock step for *hsp70* mRNA production.

2.9.2 Heat shock induction and RNA extraction

Aliquots of nematode suspension (100 µl) were heated at 35°C for 3 hours to induce the production of *hsp70* mRNA in the organism, according to Snutch and collaborators (1983, 1988). Samples were shortly placed at room temperature, mixed with 60 µl washed manure, placed shortly on ice, and then subjected to cell lysis and RNA extraction, using the same extraction kit as for *C. parvum* oocysts. Other nematode aliquots were mixed with manure, not heat shock induced, and maintained shortly at 21°C (normal temperature growth) before cell lysis. Nematode lysis was done by 30 s bead beating in the lysing matrix E tubes (BIO 101® Systems, Qbiogene) with 600 µl lysing buffer. Samples were placed shortly on ice, centrifuged for 14,000 x g for 4 min, and the supernatant recovered in a new Eppendorf tube. The sediment in the E tubes were again resuspended in 300 µl of lysing buffer, mixed well, vortex for 20 s, centrifuged for 4 min and the supernatant recovered. Supernatants were collected together. Total RNA was extracted with the GITC-PC based method 1 (Fast RNA® Pro Soil-Direct Kit, Qbiogene, MP Biomedicals), following the manufacturer's suggestions and incorporating the optimized steps as indicated in Chapter 4 (section 4.3.2, Chapter 4, text in italic). Extractions from heat induced and no heat induced organisms were done in parallel. Extracts were treated with DNAse to remove co-extracted DNA with the TURBO DNA-*free*™ system following the manufacturer's instructions, except that samples were centrifuged twice at 10,000 x g to allow a better purification of RNA.

CHAPTER 3

Using DNA as target in qPCR to quantify *C. parvum* in environmental samples and anaerobic digesters treating manure

3.1 Introduction

Manure from animal farms is considered the major source of *Cryptosporidium parvum* oocysts, from which they can disseminate to the environment. The reuse of manure in agriculture requires a minimum treatment in order to reduce the number of pathogens in the substrate. *Cryptosporidium* oocysts may not be totally inactivated at mesophilic anaerobic conditions (Burton and Turner, 2003) and may survive suboptimal operating systems. Thus, the control of the presence and the load of *C. parvum* oocysts in environmental samples and effluents from manure treatment systems could inform on the level of contamination of the matrices.

Common detection methods for oocysts, such as IF assay (EPA 2005), are laborious, cannot identify species and are mainly optimized for water samples (see Chapter 1, section 1.10.1).

This work chose to evaluate a qPCR based method to allow a rapid processing and specific pathogen detection. A procedure that extracts DNA directly from the matrix was preferred because techniques used for purification of the pathogen before nucleic acid extraction are time consuming and can yield losses of oocysts (Nieminski et al., 1995; Kuczynska and Shelton, 1999; Ramirez et al., 2006). DNA extraction procedures should be effective to remove potential qPCR inhibitors that otherwise can significantly impede the analysis (Tebbe et al., 1993; Chapter 1, section 1.10.3). The lysis of oocysts within complex samples should also be effective for the extraction of DNA. Limited studies have evaluated the efficiency of the lysis step, and those available were done in pure suspensions or fecal samples (Bialek et al., 2002; Guy et al., 2003), but not in a complex matrix like manure.

This work evaluated and optimized a method based on DNA extraction directly from the matrix and real-time qPCR to specifically quantify *C. parvum* oocysts in manure and soils. The first objective was to evaluate lysis procedures based on bead beating, in combination with a specific DNA extraction / purification system that uses silica matrix in spin columns. The complete assay including lysis, extraction and detection of the *hsp70* gene was evaluated in terms of

specificity and sensitivity. A further objective was to evaluate the applicability of the optimized DNA extraction method and qPCR to indicate the presence and inactivation of *Cryptosporidium* oocysts in manure after exposure to mesophilic and thermophilic anaerobic treatment.

3.2 Materials and methods

3.2.1 Environmental samples and oocyst suspensions

Different *C. parvum* oocysts suspensions were used for seeding manure and soil at defined concentrations. Manure and digested manure samples from an anaerobic model biogas plant were homogenized and washed to reduce the amount of qPCR inhibitory compounds and free nucleic acids present in solution, as described in Chapter 2 (section 2.1). Sample aliquots (40 - 60 µl) were spiked with viable oocysts, mixed with 100 µl 1x PBS buffer and processed for oocysts lysis. Soil samples (50 mg) from tilled and minimum-tilled agricultural land (see soil characteristics in Chapter 2, Table 2.1) were mixed with 100 µl 1x PBS and known amount of oocysts before lysis. The membrane permeability assay (DAPI / PI) and *in vitro* excystation were performed to assess the oocyst viability in manure and pure suspensions, as described in Chapter 2 (section 2.7).

3.2.2 Procedures for oocyst disruption

Lysis involved sample homogenization by bead beating with the lysing matrix E tubes and lysing buffer. Different bead beating processing times were evaluated using fresh oocyst suspensions (1 - 2 months). Manure with oocysts were mixed with SDS-EDTA-sodium phosphate based lysing buffer (FastDNA® SPIN Kit for Soil, Qbiogene) in E tubes (BIO 101®, Qbiogene), mixed by short vortex, and then bead beating was performed in the FastPrep® Instrument at a speed of 5.5, following the protocols described below. After each bead beating time interval, samples were centrifuged at 14,000 x g for 4 min at 4°C, and the supernatant containing the DNA was transferred to a new DNAse free Eppendorf tube (2 ml). The following bead beating protocols were evaluated using lysing buffer contained in the extraction kit:
Protocol 1: 30 s, Protocol 2: 60 s, and Protocol 3: 120 s. Bead beating was done in a single step with 978 µl of the sodium phosphate based buffer (SP) and 122 µl MT buffer from the kit.
The next procedures include the approach of cumulative bead beating. This consisted of bead beating in time intervals with recovery of the lysate fractions (the supernatant) after each centrifugation step (cumulate DNA recovery after bead beating intervals). This approach was evaluated assuming that oocysts, even in the same suspension, may have different wall constitutions (weak or strong walls), and therefore may require different intensities of bead beating to be disrupted. Hence, oocyst lysis by several repetitive single bead beating steps,

rather than one single prolonged treatment, may permit the recovery of nucleic acids from different oocysts in the supernatant.

Protocol 4: 240 s, was performed in the following ways: a) 60 s + 60 s + 60 s + 60 s, b) 30 s + 70 s + 70 s + 70 s, and c) 30 s + 10 s vortex + 90 s (3 x 30 s) + 120 s (4 x 30 s). 978 µl of SP buffer and 122 µl MT buffer was added for the first bead beating interval, and for each of the following intervals 100 µl pure water, 100 µl SP buffer and 12 µl MT buffer was added.

Protocol 5: 165 s, performed as follows: (30+30 s) + (30+30 s) + (20+25 s) with 450 µl SP buffer, 57 µl MT buffer and 50 µl of pure water for the first and second bead beating intervals. For the last time interval, 100 µl of pure water, 200 µl of SP and 25 µl of MT buffer were added. The volume of buffers for extractions with soil was the same as in protocol 5, except that 100 µl of water was added in each bead beating interval to improve the sample homogenization.

If samples warmed up in between bead beating steps, the tubes were placed on ice water for 1 – 3 min to prevent DNA degradation. For cumulative bead beating, the pooled lysate volume (the combined supernatant) was kept on ice water before DNA extraction/purification.

3.2.3 DNA extraction / purification

The DNA extraction/purification chemistry used GITC and silica matrix included in the FastDNA® SPIN Kit for Soil (Qbiogene). The kit was used following the suggestions of the manufacturer and adapted to process more lysate volume. The total lysate from one sample was split in two volumes and processed in parallel in 2 ml Eppendorf tubes by adding half the volume of the ingredients to each fraction. After mixing each fraction with Binding Matrix (500 µl for each tube), the two volumes of a sample were centrifuged consecutively in one SPIN™ filter column (provided in the kit). The final DNA was eluted in 100 µl of DES (DNase/Pyrogen-free ultra-pure water), in order to recover as much DNA from the matrix as possible. If qPCR results were not optimum, DNA extracts were further purified with a filtration system that employs a silica-based membrane, included in the DNeasy® Plant Mini Kit (Qiagen). For this, DNA was mixed with buffer AW3 containing guanidine hydrochloride (provided in the kit) and the procedure was performed following the manufacturer instructions. Buffer volumes were downsized for a 100 µl DNA extract. DNA extraction/purification was also done from pure oocysts in buffer and from manure free of oocysts (negative control).

See a diagram flow for DNA based experiments in Chapter 2 (Fig. 2.2).

3.2.4 Real-time qPCR and quantification of oocysts

Primers and hydrolisis probe specific for the *hsp70* gene in *C. parvum* were used. qPCR reactions were done in 25 µl volume including 2.5 µl of purified DNA and 0.75 units of

Platinum® Taq DNA polymerase (Invitrogen) (see sections 2.5.2 and 2.5.3, Chapter 2, for reaction chemistry and primers/probe sequences). qPCR reactions were done in triplicates. Negative controls contained pure water instead of DNA.

qPCR standard curves for each substrate (manure and soil) were constructed by plotting Ct values from serial dilutions of DNA extracts versus the calculated copy numbers present in each dilution. The DNA extraction and detection efficiency using different oocyst lysis procedures was assessed relative to the procedure that yielded the best result.

3.2.5 Sentinel chamber experiments – oocyst inactivation

The inactivation of *C. parvum* oocysts was evaluated in anaerobic digesters of a model biogas plant treating semi-liquid cattle manure and using diffusion sentinel chambers. The model plant consisted of three digesters connected in series mesophilic-thermophilic-mesophilic (38°C/ 55°C/ 38°C). The experimental procedure and details of the digesters are described in Chapter 2 (section 2.8 and Fig. 2.5). Manure and digested manure were taken from the collection tank and from effluents of the digesters. Sentinel chambers were filled with 1.8 ml of (digested) manure and with 1 ml of oocyst suspension (2×10^7 oocysts). Sentinel chambers were placed in the digesters for different retention times: 4 h in the mesophilic digester, 4 h and 12 h in the thermophilic digester, and a total of 20 h in the digesters connected in series (4 h mesophilic - 12 h thermophilic - 4 h mesophilic). The retention time of 4 h corresponded to the feeding interval evaluated in the model biogas plant (Effenberger et al., 2006). Afterwards, samples were removed from the digesters and immediately thereafter the content was transferred to a clean tube. Oocysts were then concentrated in 2.8 ml volume by centrifugation. The sample was used for DNA extractions (100 µl aliquots), excystation test and infectivity test. Cell culture infectivity test was used to assess oocyst infectivity after exposure to anaerobic treatment. For the infectivity test, oocysts were purified from the sentinel chamber content by percoll gradient centrifugation, and the test was performed as described in Chapter 2 (section 2.7).

3.3 Results

3.3.1 Specificity of primers

The specificity of the *hsp70* primers and probe was evaluated in extracts from manure samples that were free from oocysts but contained metabolically active microorganisms. No qPCR amplification was obtained in these control samples, thus confirming the specificity of the primers/probe to the target organism and the specificity of the assay.

3.3.2 Viability reference measurements

All oocyst suspensions used for DNA extraction were > 80 % viable as verified in excystation test and 80 - 92 % potentially viable in the dye permeability assay (DAPI / PI). Performing the dye permeability test with manure samples was difficult due to particulate material in the matrix, which produced interfering fluorescence background that hindered oocyst detection by microscopy. Due to attachment of oocysts to fibers and particles and difficulties in microscopic analysis, also *in vitro* excystation of oocysts was preferably done in pure suspensions or diluted manure samples.

3.3.3 Comparison of oocyst disruption procedures for DNA extraction

The following Table 3.1 shows the performance of the oocyst lysis procedures based on bead beating. Nucleic acid extraction from oocysts without a lysis step yielded no DNA (no qPCR amplification), indicating that nucleic acids could not be released from the oocysts. Bead beating for 30 s or 60 s resulted in no or very low detection of *hsp70* DNA copies, most likely due to insufficient lysis of oocysts in the manure matrix. Increasing the time of bead beating to 120 s did not yield significantly higher DNA copies from oocysts in manure (Table 3.1). Using 120 s bead beating for lysis of oocysts in pure suspensions yielded a recovery and detection efficiency of DNA of 60 - 70 % for oocysts from one stock suspension, but this was less than 10 % with oocysts from another fresh suspension. That difference suggested that oocysts might have had different physical wall constitutions (strong or weak). Table 3.1 shows that long bead beating steps were needed for sufficient oocyst disruption in manure. The cumulative bead beating strategy in time intervals, which consists of cumulative extraction of nucleic acids after each interval, enhanced total DNA recovery. Cumulative bead beating for 240 s (4x60 s; 30+70+70+70 s; and 30+90+120 s) yielded a DNA recovery and detection efficiency from manure that was in the range of 51 - 71 %. However, despite the initial higher DNA yield obtained with this bead beating procedure, lower DNA detection or no qPCR amplification was obtained in some replicate samples. In those cases, a dense white lysate suspension was observed in the lysing matrix E tube, particularly after the last bead beating step. That probably was the result of abrasion of material from the glass, silica and ceramic beads after prolonged treatment, which made it difficult to recover a clean supernatant containing DNA, without removing the sediment. In addition, excessive foaming was regularly produced in the E tube after the last bead beating step in the 240 s cumulative approach.

In order to improve the recovery of DNA from manure and to resolve problems with foaming and milky lysate, the total bead beating time was reduced to 165 s and performed in time

intervals: (30 + 30 s) + (30 + 30 s) + (20 + 25 s). In order to prevent nucleic acid degradation, a 60 s bead beating interval was divided in two steps (30 + 30 s) to cool down the sample after each 30 s when necessary. This was done because E tubes sometimes warmed up after ~ 60 s, which may risk DNA degradation. Oocyst lysis by 165 s cumulative bead beating yielded efficient recovery of DNA from manure samples (Table 3.1) and high sensitivity, as reported in the next section. This procedure was more rapid and consistently provided a clear lysate, which was easy to recover after the centrifugation step.

Table 3.1 – Efficiency of the DNA extraction and detection of the *hsp70* gene by qPCR from oocysts in manure, using different lysis procedures based on bead beating. Efficiency calculated with reference to standards of the 165 s bead beating procedure. s: seconds.

Oocyst disruption protocol	Extraction / detection efficiency (n=3-4), %
No bead beating	no detection
30 s	no detection
60 s	2.04 ± 1.03
120 s	5.37 ± 6.25
240 s (4x60)	53.9 ± 4.7
240 s (30+70+70+70)	71.1 ± 37
240 s (30+90+120)	51.5 ± 38.7
165 s (60+60+45)	82 ± 6.9

3.3.4 Sensitivity of the extraction and detection method in manure

The sensitivity of the assay including oocyst lysis by cumulative bead beating, DNA extraction, and detection by qPCR was verified in serial dilutions of extracted DNA and with manure spiked with different oocyst concentrations.

The detection limit in the qPCR assay for the procedure including 165 s cumulative bead beating was 1.2 - 5 DNA copies / 25 µl qPCR reaction. This corresponds to detection of qPCR amplification signals for 240 - 830 oocysts / ml manure sample. A real-time qPCR amplification plot depicting the lower detection limit in the qPCR assays for *hsp70* DNA is shown in Fig. 3.1. With the procedure as applied, 1.2 DNA copies / qPCR reaction were measured with a Ct value of 38.93 ± 0.66 (n=3). This is equivalent to detection of 1 oocyst (one organism contains 4 sporozoites, each of them with one target gene; Fig. 3.2). The correlation of Ct values and DNA

copies in regression analysis was high (r^2= 0.98) and the efficiency of the qPCR assay was 97 %, which indicated optimum amplification of DNA in extracts of manure.

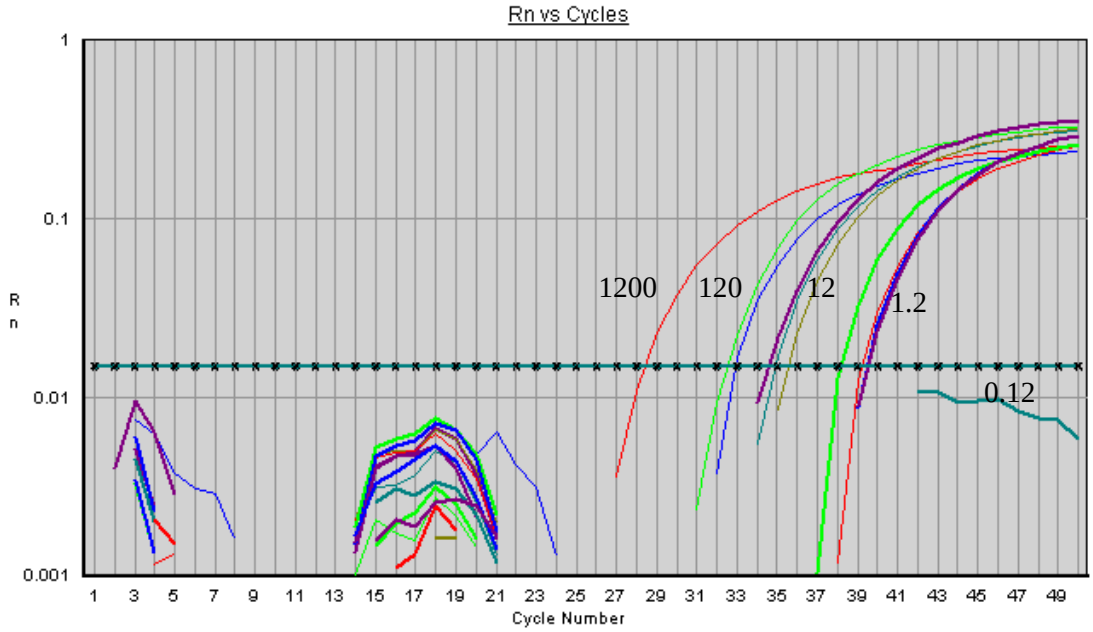


Fig. 3.1 – Real-time qPCR amplification plot showing detection of *hsp70* DNA copies in extracts from oocysts in manure. Lysis of oocysts was done with 165 s cumulative bead beating. The PCR fluorescence signal (Rn) is plotted versus the cycle number of the PCR. Numbers are the DNA copies present in the sample for the corresponding amplification curve. Horizontal line is the threshold that was set above the background fluorescence.

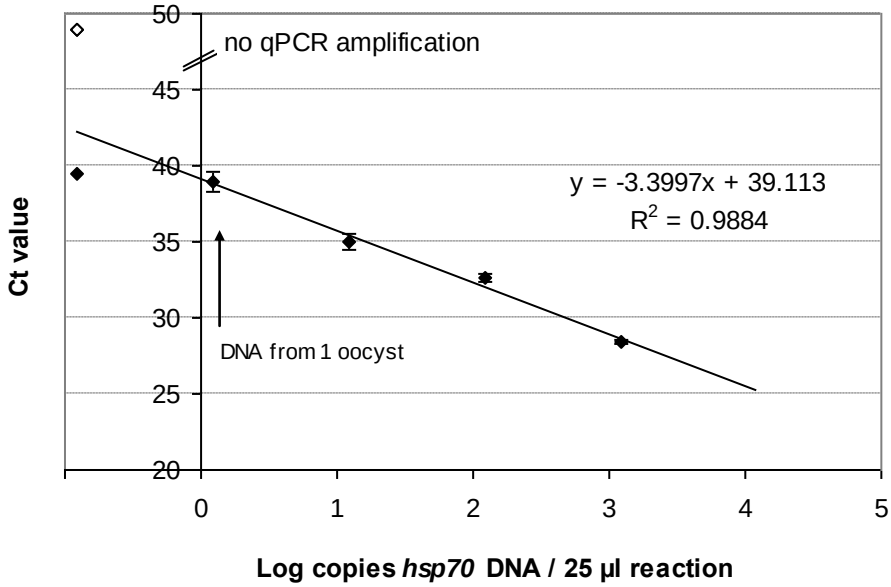


Fig. 3.2 – Linear regression of Ct values versus the copy number of *hsp70* DNA detected in extracts from oocysts in manure. Mean Ct values are shown (n=3-5). It shows detection of DNA equivalent to one oocyst by qPCR. Lysis of oocysts was done with 165 s cumulative bead beating. Open symbols indicate no qPCR amplification.

Table 3.2 – Overview of the method detection limit comprising the extraction and detection assay and employing cumulative bead beating procedures for oocyst lysis in manure.

Protocol	Oocyst disruption	Oocyst suspensions*	Method detection limit (oocysts / ml manure)
4 a	240 s (4 x 60)	M	1×10^3 - 1.2×10^3
4 b	240 s (30+70+70+70)	M1	1×10^3 - 2.7×10^3
		M1	nd
4 c	240 s (30+90+120)	A1	2.7×10^3
		A1	8.9×10^2
		A1	2.7×10^4
		D or A	nd
5	165 s (60+60+45)	different suspensions	2.4×10^2 - 8.3×10^2

*: viable oocysts from different stock suspensions; s: seconds; nd: not detected DNA.

For comparison, the sensitivity of the assay using the 240 s cumulative approach for lysis was down to 4 - 10 DNA copies / 2.5 µl reaction. However, occasionally no qPCR signal was detected using this lysis approach (Table 3.2). For this last procedure, the detection limit of the complete extraction and detection assay using different oocyst suspensions was in the range of 8.9×10^2 - 2.7×10^4 oocysts / ml manure (Table 3.2).

3.3.5 Method performance with soil matrices

The DNA extraction method was evaluated with two types of soils seeded with 5×10^5 oocysts. No qPCR curve was observed using undiluted DNA extracts from both type of soils, but amplification curves were detected in serial dilutions of the samples (Fig. 3.3). By diluting DNA extracts, also other components in the corresponding volume are diluted. Therefore, it is most likely that qPCR inhibition by humic substances occurred for the undiluted samples. In these samples the silica Binding Matrix that collected the DNA in the spin columns turned to a yellowish-brownish color. This generally indicates co-purification of humic material. Further purification of DNA from the soil extracts using another silica-based membrane system, included in the DNeasy® Plant Mini Kit (Qiagen), did not improve qPCR detection for soil samples.

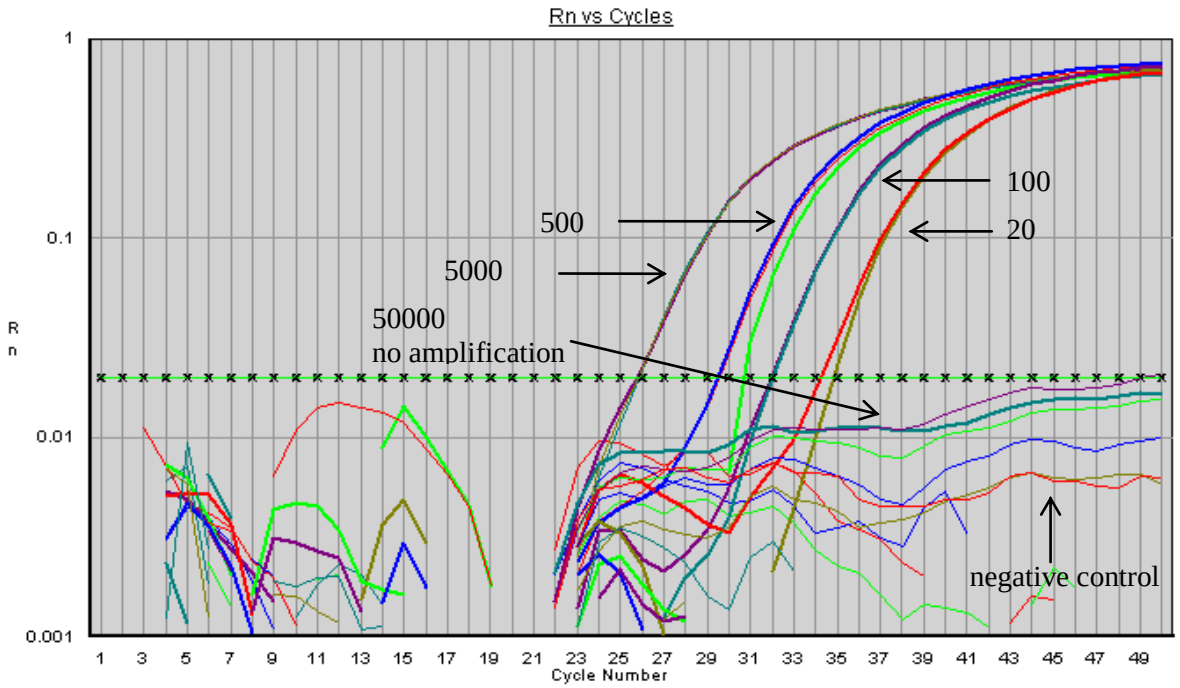


Fig. 3.3 – Real-time qPCR amplification plot showing detection of *hsp70* DNA copies in extracts from oocysts in minimum-tilled soil samples. Oocyst lysis was done with 165 s cumulative bead beating. The PCR fluorescence signal (Rn) is plotted versus the cycle number of the PCR. Numbers indicate the DNA copies spiked to the sample. No qPCR amplification is shown for the non-diluted samples containing high number of DNA copies. Horizontal line is the threshold above the background fluorescence.

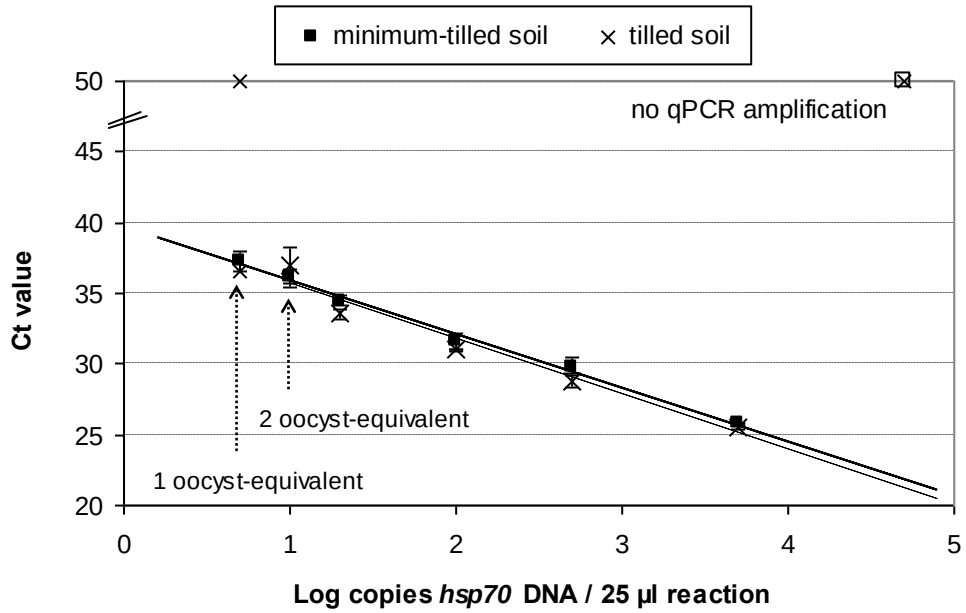


Fig. 3.4 – Linear regression of Ct values versus the copy number of *hsp70* DNA detected in extracts from oocysts in soil samples. It shows the lower detection limit in the qPCR assay (equivalent to 1 and 2 oocysts). Open symbols indicate no qPCR amplification.

For minimum-tilled soils, the lowest qPCR amplification curve was measured for 5 *hsp70* DNA copies, whereas for tilled soil samples, the lowest qPCR amplification was detected for 10 DNA copies. For both type of samples, measured Ct values corresponded to detection of 1 and 2 oocysts / qPCR reaction, respectively (Fig. 3.4). The efficiency of the qPCR assay was 84 % for minimum-tilled soils and 80 % for tilled soils, which were lower than with manure (Table 3.3). The calculated method detection limit for the complete procedure with minimum-tilled soil was 1×10^4 oocysts / g, and 2×10^4 oocysts / g for the tilled soil.

Table 3.3 – Efficiency of the qPCR assay and coefficients of determination (r^2) for linear regressions of Ct values versus *hsp70* DNA copies from extracts of oocysts in manure and soil.

Substrate	**Efficiency of qPCR** (%)	**r^2**
Manure 1	97	0.991
Manure 2	97	0.9884
Minimum-tilled soil	84	0.9941
Tilled soil	80	0.9542

Manure 1 and 2: raw manure samples seeded with different fresh oocyst suspensions.

3.3.6 Screening oocysts in anaerobic digesters by measuring *hsp70* DNA

The optimized DNA extraction method in combination with qPCR was used to evaluate the inactivation of *C. parvum* oocysts in model-scale anaerobic digesters treating manure. Taking as reference the feeding interval in the digesters (4 h), the time the oocysts were maintained in the sentinel chambers was 4 h. This time corresponded to a minimum theoretical retention time of the organisms in the digesters, assuming a continuous operation. Oocysts in the horizontal thermophilic digester were maintained for 4 h and 12 h.

There was no apparent reduction in *hsp70* DNA content from oocysts subjected to a single-stage mesophilic treatment (4 h) or single-stage thermophilic treatment (4 h or 12 h). Also, DNA content was not reduced in oocysts subjected to 20 h digestion in the mesophilic-thermophilic-mesophilic system (38°C/55°C/38°C, 4 h - 12 h - 4 h, respectively) (Fig. 3.5).

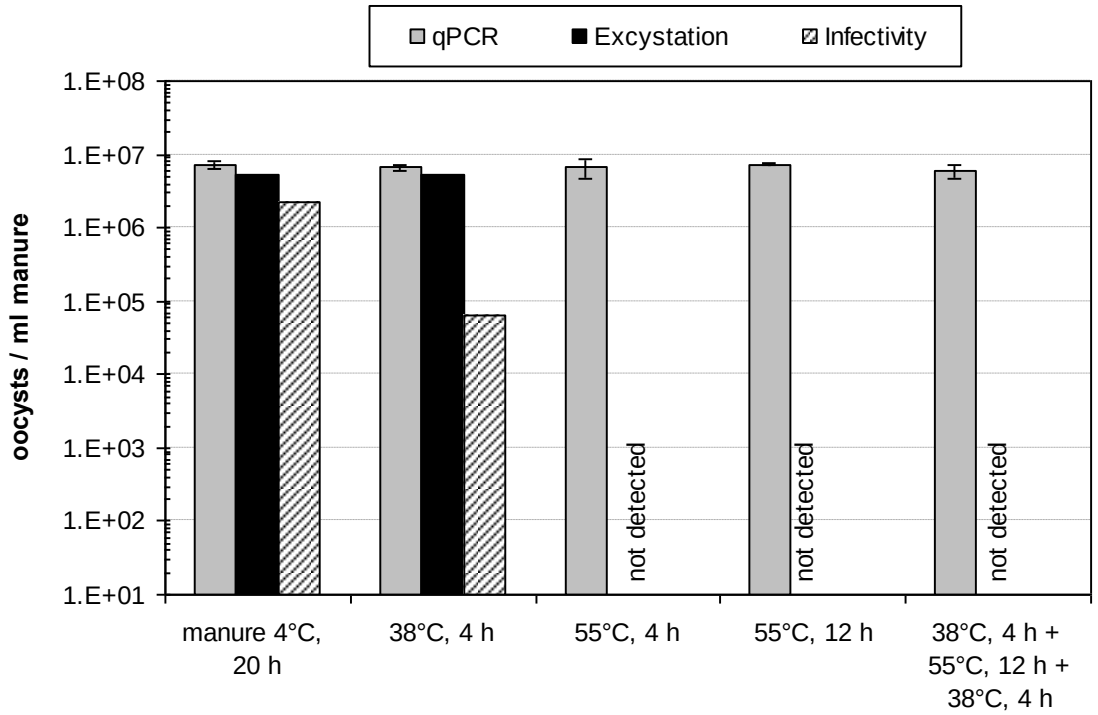


Fig. 3.5 – Number of oocysts in samples from anaerobic digesters measured as *hsp70* DNA. Excystation capability and infectivity of oocysts are shown in parallel.

Results from infectivity test showed that oocysts were reduced by more than 5 log units after 4 h thermophilic treatment in comparison to oocysts in manure control samples. Similarly, the oocyst capability to excyst in the excystation test was reduced to not detectable levels after the thermophilic treatment (Fig. 3.5). There was no correlation between *hsp70* DNA measurements and the viability / infectivity of oocysts for the samples exposed to thermophilic conditions (55°C). The constant DNA content indicated that the treatments did not affect the stability of the strands in inactived oocysts. It also suggested that at the conditions evaluated oocysts were not degraded by the temperature and anaerobic conditions, and that oocysts might have not released their genome to the digester content. Measured DNA by qPCR in samples from the thermophilic digester and the mesophilic-thermophilic-mesophilic system should have corresponded to dead organisms.

The comparison of viability and infectivity tests for the mesophilic anaerobic treatment (Fig. 3.5) showed that oocysts able to excyst (metabolic active) were about 2 log units higher than the number of infectious oocysts, indicating overestimation by the excystation test for this assay. However, 4 h of mesophilic treatment may not always reduce the number of infectious oocysts, as seen in assays with different oocyst suspensions (Lebuhn and Wilderer, 2006).

3.4 Discussion

In this chapter, the aim was to develop a molecular method that permits rapid, sensitive and specific detection of *Cryptosporidium* species that are potential infectious agents to humans and cattle. An optimized DNA extraction and quantification method is presented for *C. parvum* oocysts in manure, digested manure and soil by real-time qPCR targeting the *hsp70* gene. The method was evaluated for its suitability to process complex matrices that contain fibers and particulate material, without the need to purify oocysts from the matrix.

Optimum detection of DNA by qPCR depends on suitable extraction of nucleic acids from the organism and purification from the substrate (Wilson, 1997; Bustin and Nolan, 2004). Although there have been advances in DNA extraction procedures from difficult materials, a number of studies that employed DNA extraction and PCR reported also suboptimal detection due to the presence of PCR inhibitors (Higgins et al., 2001; Bach et al., 2002; Fredricks et al., 2005). For *Cryptosporidium*, extraction methods should also facilitate DNA recovery from oocysts with strong walls and from those with less strong walls (Campbell et al., 1992; Jenkins et al., 2010). Limited studies have evaluated the efficiency of the oocyst disruption step in the extraction procedure, and the available ones were done in pure suspensions or fecal samples (Bialek et al., 2002; Guy et al., 2003). Here a method is presented that employs a lysis step consisting of a fractionated bead beating approach with ceramic and silica beads and lysing buffer containing SDS (for protein solubilization), in combination with a purification procedure based on GITC and silica matrix spin columns, to obtain DNA extracts that are suitable for qPCR from complex environmental samples.

3.4.1 Optimization of DNA extraction

When employing different oocyst disruption protocols, varying DNA yields indicated that an optimum procedure is essential for complete lysis and recovery of nucleic acids from manure and soil (Table 3.1). Higher detection of DNA was obtained when lysis of oocysts in manure was done by cumulative bead beating with fractionated lysate recovery in the lysing matrix E tubes, compared to lower detection with a single bead beating step (Table 3.1). Lysis by mild bead beating (30 s or 60 s), which works good for bacteria (Lebuhn et al., 2003, 2004) is not suitable to extract DNA from fresh oocysts in manure. Clearly, disruption of the oocyst wall in manure required stronger physical force with the bead beating approach. The lower DNA yield for extractions in manure with the 120 s bead beating compared to the yield obtained with pure suspensions indicated that the matrix influenced the lysis performance. Oocysts can be trapped between manure particles and fibers and attached to them (Kuczynska et al., 2005). Particles and

fibers in manure may have sheltered the oocysts during bead beating, and seemed to mitigate the lysis effect.

The bead beating should facilitate the release of oocysts from the matrix and the lysis of oocyst walls. While oocysts with strong walls needed stronger lysis procedures, the high shear force of long bead beating steps can damage DNA by shredding if they are more rapidly released from oocysts with weak walls. *Cryptosporidium* oocysts from different strains or even in the same stock suspension can have different wall constitution. Nichols and Smith (2004) used freezing-heating cycles for oocyst lysis and reported that some isolates were more easily disrupted than others. Moreover, oocyst resistance may vary depending on their previous exposure to (environmental) stresses that may affect their wall structural composition (Fayer, 2004). Thus viable oocysts with weak walls required milder bead beating time for lysis, whereas strong oocysts needed longer processing time and/or stronger shear force. Taken this into consideration the cumulative bead beating strategy was performed. This permits the sequential recovery of DNA in each lysate fraction after each bead beating interval, as oocysts are lysed.

Despite of the good performance of 240 s cumulative bead beating in some replicate experiments (Table 3.1, Table 3.2), there were problems with excessive foaming that impeded the recovery of the supernatant, and with production of glass milk and a milky lysate attributed to abrasion of beads. These factors may have affected the recovery of DNA and their detection by qPCR (Table 3.2). The attachment of DNA to mineral particles has been reported (Schneegurt et al., 2003). Such DNA binding may have occurred also with micro ceramic and glass particles that can result from abrasion of the beads. Thus strong binding of DNA to glass milk may have impeded DNA recovery from the lysate and their subsequent detection.

Oocyst lysis with a cumulative bead beating for 165 s (30+30 s) + (30+30 s) + (20+25 s), was the most suitable procedure and DNA recovery, as determined by qPCR, was 83 %. This shorter protocol reduced foam formation, avoided too intense heating of the beads and yielded consistent qPCR results.

Other procedures have also been applied for the lysis of oocysts (e.g. freezing-thawing cycles or enzymatic treatments). Guy and coworkers (2003) reported adequate DNA recovery from oocysts in wastewater samples with a lysis step that combines 3 freeze-thaw cycles (using liquid nitrogen) and 30 min sonication (in a cup horn). In another study, oocyst lysis by boiling (5 min) and 3 freeze-thaw cycles in ASL lysing buffer yielded higher DNA recovery compared to not lysed oocysts (Bialek et al., 2002). Long freezing-thawing steps (6 and up to 15 times) had also been employed for oocyst lysis in water samples (reviewed in Wiedenmann et al., 1998; Nichols et al., 2006). In contrast to the above mentioned studies, the 165 s cumulative bead beating

approach using an SDS-lysing buffer was faster and did not require hazardous liquid nitrogen which is not available in every laboratory. While lysis by bead beating was previously used for various organisms and substrates, such as bacteria, conidia, yeast, soil and plant litter (Griffiths et al., 2000; Lebuhn et al., 2003; Aneja et al., 2004; Fredricks et al., 2005), a cumulative bead beating lysis with fractionated DNA recovery and DNA purification based on SDS and GITC in manure and soil samples has not been previously reported for *Cryptosporidium* oocysts.

In soil, the lysis and extraction method using bead beating with ceramic and silica beads showed to be effective for DNA extraction from *Cryptosporidium* oocysts. Soil particles were well homogenized after 165 s, as visually observed, and qPCR detection was possible, but only in diluted DNA extracts. It seems that the purification system based on SDS-GITC and silica matrix in spin columns did not completely remove qPCR inhibitory substances from the soils evaluated. The lack of (q)PCR amplification signal can be due to inhibition of the activity of the PCR polymerase enzyme, cross linking of DNA with co-extracted humic substances, or inhibition of the fluorescence signal (Stults et al., 2001; Bustin and Nolan, 2004; Matheson et al., 2010). Here, it appeared that the concentration of inhibitors was reduced in serial dilutions of the extracted DNA, since qPCR signals were measurable in 10-fold dilutions. For the undiluted DNA, inhibition of the DNA polymerase or lower efficiency in primer hybridization may have been caused by relatively high concentration of co-extracted humic acids in the sample.
Inhibition of PCR in extracts from soil has often been reported (e.g. Tebbe et al., 1993; Bach et al., 2002). With the DNA extraction/purification procedure employed here, *hsp70* DNA copies from 1 - 2 oocysts / qPCR reaction could be measured in extracts from soil containing 26 - 28 % clay. This mineral is known to be often co-extracted and to inhibit PCR (Frostegård et al., 1999).

Sensitivity of the method

Sensitive detection of *hsp70* DNA copies was found in the qPCR assay when manure and soil samples containing oocysts were directly processed for DNA extraction without pre-purification steps. Using the procedure as applied, a qPCR amplification signal was detected for as few as 1.2 - 5 DNA copies / qPCR reaction in manure extracts. The detection limit for the complete method as applied was 2.4×10^2 - 8.3×10^2 oocysts / ml manure sample. This is a good value considering the complexity of manure and the strict requirements for sensitive detection by qPCR (i.e. virtually no presence of inhibitory compounds). In comparison, previous works reported inhibition and lower sensitivity in the qPCR assay with extracts from manure (Higgins et al., 2001; Klerk et al., 2006). Higgins and collaborators measured a qPCR signal from 10^4 oocysts / g manure only after nested PCR. They reported inhibition of qPCR when more than 2

µl of extracted DNA was used in a 50 µl qPCR volume (extraction was from 200 - 250 mg manure with a specific extraction kit). Stroup et al. (2006) used bead beating with glass beads (2 - 5 min vortex) and ASL buffer (composition not disclosed, Qiagen) for oocyst lysis and reported a DNA detection limit of 10^2 - 10^3 oocysts / 200 mg stool by qPCR using scorpion probes directed to the 18S rRNA gene. This detection limit (approximately 5 x 10^2 - 5 x 10^3 / g stool) is slightly higher than the one found within the present study using target specific hydrolysis probes. Other studies targeting at the COWP wall protein gene reported a detection limit of 5 x 10^2 oocysts / 200 mg stool using nested PCR (Bialek et al., 2002), and an estimated theoretical detection limit of 10^2 oocysts in the qPCR assay from 500 µl sewage pellet (Guy et al., 2003). The latter corresponds to approximately a detection limit of 800 DNA copies / qPCR reaction, which is higher than the detection limit obtained with the approach in the present study.

The major bottleneck in the use of the present qPCR based detection system is that only small sample volumes can be extracted and only small extracted volumes can be used for qPCR analysis. However, processing small sample volumes reduces analysis costs and helps to avoid inhibition by co-extracted compounds that may remain in the extract. Even purified extract are not 100 % free of inhibitors (Jiang et al., 2005). The above mentioned sensitivity achieved in this work was obtained by performing 45 – 50 qPCR cycles and using 2.5 µl of extracted DNA (DNA present in 50 - 60 µl sample and extracted in 100 µl eluate) in a 25 µl qPCR volume. Under these conditions, no inhibition of qPCR amplification was observed for manure extracts. Small sample volume were also used in previous works, and inhibition was indicated when higher volumes were used (Higgins et al., 2001; Lebuhn et al., 2003; Guy et al., 2003). For wastewater, Guy et al. (2003) suggested a maximum of 2 µl of extracted DNA (DNA from 500 µl sewage pellet extracted in 200 µl eluate) for a 25 µl qPCR volume, but using another extract from wastewater they found that no more than 0.5 µl of extracted DNA could be employed for qPCR. No inhibition of qPCR was observed with manure, provided that samples were washed twice in 1x PBS buffer and once in KCl 0.85 % (Lebuhn et al., 2003). For extractions of manure samples, additional steps to remove PCR inhibitors, such as treatment of the lysate with PVP or Chelex 100, or the addition of bovine serum albumin (BSA), PVP or T4 gene 32 to the qPCR (Morgan et al., 1998; Guy et al., 2003; Jiang et al., 2005), were not found to be helpful, if the sample is clean enough (Lebuhn et al., 2003).

For the soils evaluated, the method detection limit is 1 x 10^4 - 2 x 10^4 oocysts / g of soil considering the inhibition in undiluted DNA extracts. This detection limit is about 2 log units

higher than that with manure; therefore further or modified purification of soil extracts should be considered for practical application with soil samples.

3.4.2 Evaluation of oocyst inactivation in anaerobic digesters using DNA as marker

This work first intended to use a DNA detection approach to indicate the presence and inactivation of oocysts in effluents from anaerobic digesters treating manure. Although DNA molecules can persist in dead cells, the DNA decay rate can vary according to the environment in which the organisms are inactivated (Nielsen et al., 2007). This investigation found undegraded *hsp70* DNA of *C. parvum* oocysts that were inactivated in anaerobic treatments that included a thermophilic stage (55°C). Almost no reduction in the amount of *hsp70* DNA in not viable and not infectious oocysts even after 20 h exposition in the mesophilic-thermophilic-mesophilic system showed persistence of DNA from dead oocysts under the evaluated treatment conditions. Previously, Lebuhn et al. (2004) found some reduction in DNA content from dead bacteria after 20 h of treatment in a comparable three-stage anaerobic system. Here, it seems that *Cryptosporidium* oocysts were more resistant to degradation after inactivation in anaerobic systems, and therefore DNA was not that rapidly exposed to the fermentative anaerobic environment. In a previous study, Widmer et al. (1999) reported decay of DNA from oocysts that became not infectious after 20 weeks storage at room temperature. Longer exposure of oocysts in the anaerobic systems may therefore result in degradation of oocysts, their sporozoites and DNA. The inactivation of oocysts in the anaerobic digesters is mostly attributed to the thermophilic temperature and the chemical composition in the digesters. At the time the experiments were performed, free ammonia content was about 580 mg/l (Effenberger et al., 2008) in the thermophilic digesters. The relatively high NH_3 concentration may have contributed to reduction in oocysts viability (Jenkins et al., 1998).

Microscopic observation of samples from the thermophilic digesters (4 h and 12 h) showed mostly dark and intact oocysts. Analyses confirmed that oocysts lost their viability while their structure was maintained. Both, the capability of oocysts to excyst and their infectivity were decreased after exposure to thermophilic treatments.

It is concluded that measuring DNA can overestimate the fraction of viable oocysts exposed to severe stress such as lethal anaerobic treatments. However, the use of DNA detection by qPCR as employed here is suitable to indicate the presence of and to quantify oocysts in samples from anaerobic digesters and in other environmental matrices, as well as to identify species using target specific probes. Rapid information about the presence of oocysts is needed to identify contaminated matrices that can be a source of infection. The specific and sensitive detection of *C. parvum* in manure and soil indicates that the complete extraction and detection method is

robust, despite the complexity of the matrices and the diverse microbial population present within it.

For practical application, the proposed approach is as follows: i) sample (e.g. manure) homogenization in a sterilized Ultra-Turrax (about 1 min), ii) washing the sample in PBS buffer and KCl, iii) oocyst lysis with cumulative bead beating for 165 s, iv) DNA extraction from 50-60 µl sample volume with buffers containing SDS and GITC (FastDNA® SPIN Kit), v) qPCR with *Taq* DNA polymerase and specific primers/probe, for example, for the *hsp70* gene. Twelve samples can be processed in about 3 h and 3 h more are needed for qPCR.

Although other DNA based methodologies such as nested PCR were found suitable to detect low amount of oocysts in faeces (Bialek et al., 2002; Kato et al., 2003b) nested PCR requires two set of primers, and this assay is highly prone to contamination. In practice, methods should be kept as short as possible to avoid contamination and sample degradation.

Box : key outcomes Chapter 3

- DNA extraction from *Cryptosporidium* oocysts in manure with a lysis step consisting of cumulative 165 s bead beating ((30+30 s) + (30+30 s) + (20 + 25 s)) with glass, ceramic and silica beads and sodium phosphate-SDS lysing buffers yielded 83 % extraction and qPCR detection efficiency. This lysis procedure did not produce glass milk and a milky lysate, and foam formation was reduced compared to prolonged bead beating treatments (240 s).
- DNA extraction employing bead beating, SDS-sodium phosphate based buffer, a GITC based buffer, silica matrix and spin columns, in combination with qPCR and specific primers/probe, showed sensitive detection of genomic DNA without the need for oocyst purification from the environmental matrix prior to extraction. qPCR signals over at least 4 orders of magnitude in extracts from manure and soil were obtained, as well as very good qPCR efficiencies for manure.
- The detection limit of the complete procedure with the given system, including washing manure to remove inhibitors, was 1.2 - 5 *hsp70* DNA copies / qPCR reaction, and the method detection limit for a single extraction was $2.4 \times 10^2 - 8.3 \times 10^2$ oocysts / ml manure.
- There was qPCR inhibition using undiluted DNA extracts from soil samples. A 1:10 dilution of the extracts solved the problem and yielded consistent Ct values in serial dilutions with qPCR efficiencies $\geq$ 80 %. Considering the dilution step, the method detection limit of the overall assay was $1 \times 10^4 - 2 \times 10^4$ oocysts / g of soil. Further optimization is necessary for soil samples.
- Almost unchanged *hsp70* DNA levels were detected in oocysts that were inactivated (not viable and not infectious) by anaerobic treatment with a thermophilic stage (4 h or 12 h retention time). Thus no correlation between *hsp70* DNA measurements and viability was found for thermophilically treated oocysts.
- Oocysts seemed structurally intact but inactivated after 12 h of thermophilic anaerobic treatment and after 20 h in the mesophilic-thermophilic-mesophilic digesters.
- The overall DNA extraction and qPCR quantification procedure can produce results in about 6 h. The approach suits to screen for the presence of *Cryptosporidium* parasites in environmental samples, particularly when first information for the presence of oocysts and their taxonomy is needed.

CHAPTER 4

Development of mRNA and RNA extraction procedures for *C. parvum* and enteroviruses in manure and optimization of RT-qPCR

4.1 Introduction

Pathogenic *C. parvum* oocysts and infectious enteroviruses may be present in manure.

In addition to survey for the presence of *C. parvum* oocysts in environmental matrices, information about the metabolic state of oocysts is needed because only living organisms represent a risk for causing infections to humans and animals. The bottleneck for the routine screening of viable oocysts is still the detection method. Cell culture infectivity test is laborious and requires days to generate results. Also *in vitro* vitality assays cannot process many samples simultaneously, and therefore the analysis can be long (sections 1.10.1 and 1.10.2, Chapter 1).

Real-time RT-qPCR directed to mRNA molecules may provide information about the viability of the pathogens since mRNA strands are produced only in living organisms.

A major challenge in the use of RT-qPCR is still the extraction and purification of (m)RNA from turbid and particulate substrates, such as wastewater and manure. Difficulties are due to the complexity of the matrices and presence of RT and qPCR inhibitors like humic acids, complex polysacharides and polyphenols that can result in suboptimal recovery of (m)RNA during the extraction and low performance of the RT-qPCR (Wilson, 1997; Matheson et al., 2010).

Also enteroviruses, which have RNA as genetic material, may be rapidly identified in complex matrices by RT-qPCR, compared to longer procedures needed for the cell culture test.

This investigation deals with the development and optimization of procedures for (m)RNA extraction and purification from viable *C. parvum* oocysts in manure and for detection by RT-qPCR. The procedures intend to be applicable directly with manure without pre-isolation of oocysts from the matrix. RNA extraction / purification systems were also evaluated to quantify enteroviruses in manure, wastewater and sludge samples by RT-qPCR.

Optimized oocyst lysis is essential for the release of nucleic acids, without damage of mRNA. Intact strands are needed to allow optimum primer/probe hybridization in the RT-qPCR.

Adequate purification procedures are also needed to obtain RNA virtually free from inhibitory substances. Special consideration has to be taken when working with mRNA because they are less stable than DNA and are very prone to degradation by RNAses. In *C. parvum*, mRNA strands represent only about 2 % of the total RNA (Jenkins and Petersen, 1997).

RT-qPCR reactions for *Cryptosporidium* targeted the *hsp70* gene because the production of transcripts is increased after an induction trigger (Lindquist and Craig, 1988). Therefore, *hsp70* mRNA strands may be present in high numbers to allow a sensitive detection by the assay.

The following aspects were evaluated: i) different oocyst lysis approaches, ii) different purification chemistries for (m)RNA extraction from manure, iii) the performance of RT and qPCR was assessed in terms of specificity and sensitivity using one-step and two-step reactions, and iv) the suitability of three RNA extraction procedures for enteroviruses in manure, wastewater and sludge was evaluated for their use with RT-qPCR assays.

See a diagram flow of the RNA base analysis for *C. parvum* in Chapter 2 (Fig. 2.2).

4.2 Materials and methods

4.2.1 Sample preparation

Manure samples were homogenized and washed with 1x PBS buffer and KCl to remove dissolved qPCR inhibitors, as described in Chapter 2 (section 2.1). Extractions from *Cryptosporidium* were done from 50 - 60 µl washed manure spiked with defined concentrations of fresh (up to one month old) viable and infectious oocysts. Samples were mixed with 50 µl 1x PBS buffer in 2 ml Eppendorf tubes, vortex shortly and subjected to heat shock induction to trigger *hsp70* mRNA production, according to Stinear et al. (1996). This protocol consisted of incubation at 45°C for 20 min in a waterbath. Afterwards, samples were placed shortly in ice water to stop the reaction and to prevent mRNA degradation. Lysing buffer was added to the samples, as specified in Table 4.1, and oocyst lysis performed (see next section). Some manure samples were not seeded with oocysts to control for endogenous content.

Two strains of enteroviruses were used: poliovirus strain Sabin 1 and Echovirus 6. Evaluation of RNA extraction procedures for enteroviruses was done with samples that were not washed to remove inhibitors, as done for oocysts. This is because the small viral particles remain in the supernatant after centrifugation. Aliquots of not washed manure (50 or 90 µl), sludge (70 µl) and wastewater pellet (90 µl, see section 2.1, Chapter 2) were seeded with known amount of viruses, mixed with 50 µl of 1x PBS buffer, vortex, and subjected to lysis (see section 4.2.6).

4.2.2 Procedures for lysis of *Cryptosporidium* oocysts

Oocyst lysis was done either by bead beating, by pre-excystation in combination with bead beating or with freeze-thaw cycles, or by only freeze-thaw cycles, following the protocols in Table 4.1. The lysis buffer was chosen according to the employed purification method (method 1 or 2; see sections 4.2.4 and 4.2.5). After oocyst lysis, (m)RNA was extracted.

i) Bead beating. Samples were lysed by bead beating in E tubes containing beads matrix (BIO 101® Systems, Qbiogene) at speed of 5.5 in a FastPrep® instrument. Cumulative bead beating consisted of pooling lysate supernatant fractions after each bead beating interval (Table 4.1). Each interval consisted of two or three steps (e.g. 30 + 30 s for the 60 s interval) with addition of only one buffer volume. Intervals were split to control for heating of the E tubes due to the mechanical friction of beads in the tube, and in such case to place the sample in ice water for at least 1 min for cooling down before bead beating was restarted. Cumulative bead beating may permit progressive extraction of (m)RNA as oocysts in the sample are disrupted, assuming that some may break more easily than others. Cumulative bead beating for 240 s was evaluated because initially it produced high DNA recovery from oocysts (Chapter 3); 120 s and 165 s cumulative bead beating (in time intervals) were used as milder alternatives. In addition to lysing buffer, 100 µl of 1x PBS buffer was added for each bead beating interval in protocols 1a, 1b, 2, 4 and 5, and 50 µl in protocol 3. After each bead beating interval, samples were removed from the FastPrep®, placed shortly in ice water, centrifuged at 14,000 x g for 4 min, and then the supernatant was collected in a new Eppendorf tube (2 ml) and kept on ice. Supernatants (lysates containing nucleic acids) collected from each bead beating interval were collected together in one tube and mixed well.

ii) Treatment with excystation medium (sodium taurocholate, Sigma-Aldrich®, T4009) (SoTa) before bead beating or freezing-thawing cycles. This was done to weaken and disrupt oocyst walls with the first treatment and to facilitate further oocyst and sporozoite lysis with the subsequent step. SoTa is a bile salt solution and anionic detergent used for protein solubilization. Manure with oocysts were incubated with 200 µl of 1x PBS buffer acidified with KCl (pH 2.5) for 1 h at 37°C in shaking conditions. Samples were centrifuged and 100 µl discarded, then 25 µl of SoTa (0.8 %) was added, mixed and incubated at 37°C for 2 h. Samples were then placed on ice.

iii) Freezing-thawing (5 cycles) consisting of 1 min in liquid nitrogen and 1.5 min thaw in a waterbath, derived from Stinear et al. (1996).

Table 4.1 – Procedures evaluated for disruption of *Cryptosporidium* oocysts in manure.

		Oocyst disruption	**Lysing buffer volume** (added to each bead beating interval or vortex step)
Cumulative bead beating (i)**	1a	240 s bb + 2 times vortex: 30 s + 10 s vortex + 90 s (3x30) + 120 s (4x30) + vortex 10 s	(500 + 125 + 125 + 125 + 125) µl
	1b	240 s bb: 30 s + 10 s vortex + 90 s (3x30) + 120 s (4x30)	(500 + 200 + 200 + 200) µl
	2	120 s bb: 30 s + vortex 10 s + 90 s (3x30)	(500 + 125 + 250) µl
	3	165 s bb: (30+30) s + (30+30) s + (20+25) s	(400 + 300 + 300) µl
Treatment with excystation medium (ii)	4	SoTa treatment + 30 s bb [#]	500 µl
	5	SoTa treatment + 60 bb s (bb: 30 s + 10 s vortex + 30 s)	750 + 125 + 125 µl
	6	SoTa treatment + freezing-thawing (5 freeze-thaw cycles: 30 s liquid in nitrogen, 1 min at 65°C)	1 ml
Freeze - thaw (iii)	7	Freezing-thawing, 5 cycles: (1 min in liquid nitrogen, 1.5 min at 65°C)	1 ml

**: indicate the lysis strategy described in the text; bb: bead beating; SoTa: sodium taurocholate; [#]: used only with pure oocyst suspensions.

4.2.3 Oocyst disruption with beads of different material

Oocyst lysis by bead beating using beads of different material were evaluated and compared to lysis with the Lysing Matrix E tube. The homogenization tubes (Precellys®, Peqlab Biotechnology) contained beads of different diameter: glass beads (0.5 mm), ceramic beads (1.4 mm), ceramic beads (2.8 mm), or steel beads (2.8 mm). Lysis of oocysts (5×10^4) seeded to manure was done by 165 s cumulative bead beating, and then total RNA was purified with method 1 using Phase Lock Gel Heavy tubes in the purification (see next section for method description).

4.2.4 Total RNA extraction, method 1

A total RNA extraction with ingredients of the FastRNA® Pro Soil-Direct Kit (Qbiogene, MP Biomedicals) was used to evaluate the oocyst disruption protocols in manure and pure

suspensions. The RNA extraction method included lysing buffer with guanidinium isothiocyanate, purification with phenol-chloroform and isopropanol, and silica-based binding RNAMATRIX® for extracting RNA (GITC-PC method 1). The extraction / purification procedure suggested by the manufacturer was adapted to disrupt *C. parvum* oocysts by adding additional steps (section 4.2.2). For total lysate exceeding 1,100 ml, the volume was split in two parts and processed in parallel, in order to fit the fractions with further addition of 375 µl phenol-chloroform in 2 ml Eppendorf tubes and leaving sufficient space for mixing. After phenol-chloroform purification, the two sample fractions were mixed in one tube. The remaining purification steps were done following the manufacturer's instructions (unless otherwise optimized and stated, see results section 4.3, text in italics). When mentioned, Phase Lock Gel Heavy tubes (2 ml; Eppendorf) were included in the procedure to improve separation of liquid and organic phases during purification with phenol-chloroform and to prevent contamination with traces of organic solvents. Phase lock tubes were shortly centrifuged before used (14,000 x g for 1.5 min). Half volume of the lysate (containing GITC) mixed with 375 µl phenol-chloroform was added to a phase lock tube, incubated for 5 min at room temperature and then centrifuged at 14,000 x g for 5 min at 4°C. The liquid phase containing RNA above the gel in the interface was collected to a new 2 ml clean tube. The entire lysate volume was processed in the same manner, the sample was then collected together and further purification steps performed as indicated in the kit manual.

4.2.5 Direct mRNA extraction, method 2

The second method evaluated consisted of direct extraction of only mRNA strands with oligo $(dT)_{25}$ magnetic beads (Dynabeads® mRNA DIRECT™ Micro Kit, Dynal Biotech). The method is based on binding of the mRNA poly A tail to complementary oligo $(dT)_{25}$-magnetic beads for separation of the strands. Extraction method 2 was evaluated in combination with oocyst lysis by cumulative 165 s bead beating. The extraction and purification method 2 uses lithium chloride (LiCl) and lithium dodecyl sulfate (LiDS) contained in the lysing and binding buffers of the kit. The purification steps suggested by the manufacturer were optimized to suit mRNA purification from manure, taking special considerations to facilitate the binding of mRNA strands to the magnetic beads, since this step may be affected with dense substrates. In addition, the following lysing solutions were evaluated: a) buffer containing GITC (provided in the FastRNA® Pro-Soil Direct Kit), and b) a buffer mixture of GITC and 1x PBS buffer (1:1 v/v). GITC was used as it is known to be a strong denaturant that confers also protection against RNases. The temperature for elution of the final mRNA was verified for enhanced extraction.

Prior to extraction, 20 µl of oligo $(dT)_{25}$-magnetic beads solution was transferred to 1.5 ml tubes, placed on the magnetic particle concentrator (Dynal MPC™-S), the supernatant removed after 30 s, and the beads washed with 100 µl binding buffer (Dynal). For extractions, a 1/10 lysate aliquot (about 100 µl) was added to the 1.5 ml Eppendorf tube containing washed oligo $(dT)_{25}$ beads. Samples were mixed thoroughly with a pipette and then placed on a rotator for 7 min (with occasional gently shake by hand) to allow hybridization of the oligo $(dT)_{25}$ beads to the mRNA strands. Afterwards samples were placed on the magnet to allow separation of the hybrid mRNA-oligo $(dT)_{25}$ from the liquid phase. Samples were then washed basically following the manufacturer's instructions, unless otherwise optimized and stated (see results section 4.3, text in italics). The elution of mRNA was done twice with 25 µl of 10 mM Tris-HCl (pH 7.5) at 45°C, 65°C, 74°C or 84°C, the latter three temperatures in the range suggested by the manufacturer. Low elution temperature was evaluated to prevent mRNA degradation at higher temperatures.

4.2.6 RNA extraction methods for enteroviruses

Three RNA extraction / purification methods were evaluated: a) total RNA extraction with the GITC-PC method 1, b) direct mRNA extraction method 2 with magnetic beads, and c) a extraction system with streptavidin coated plates (Strep-thermo-Fast®, AB gene) for direct capture of RNA with a biotinylated probe, and subsequent attachment of biotin to complementary streptavidin. The first two were the same systems as described for *Cryptosporidium*.

Poliovirus strain Sabin 1 (1.8×10^6 viral units) and Echovirus 6 (3×10^6 viral units) were seeded to the environmental samples. Other samples were not spiked to control for endogenous enterovirus content.

Lysis of the viral coat was done by 30 s bead beating at speed of 5.5 using the lysing matrix in E tubes and with lysing buffer included in the respective extraction kits that were employed.

- For extractions with GITC-PC method 1: spiked samples were mixed with 750 µl of the kit respective lysing buffer and 100 µl 1x PBS buffer, and bead beating performed. Samples were then centrifuged for 5 min at 14,000 x g, the supernatant collected and cooled in ice water. The sample in the E tube was again used for further lysis: 250 µl lysing buffer and 50 µl 1x PBS were added, the sample was vortex for 10 s, centrifuged for 5 min, and the supernatant removed and mixed with the lysate from the first lysis step. The viral lysate was processed for RNA purification with phenol-chloroform and isopropanol following the manufacturer's notes. RNA was eluted in 100 µl volume.

- For extractions with magnetic beads method 2: spiked samples mixed with 600 µl lysing buffer (LiCl-LiDS; Dynal) and 100 µl 1x PBS buffer were lysed by bead beating as described for method 1 (see above). Further RNA purification with the oligo $(dT)_{25}$-magnetic beads was done following the manufacturer's notes and incorporating optimized steps for manure processing (see section 4.3, text in italics).
- For the Streptavidin method: lysis was done with buffer RLT that contains GITC (RNeasy® Mini extraction kit, Qiagen). Previously, RLT buffer was mixed with β-mercaptoethanol (100:1 vol/vol). Spiked samples were lysed as described for method 1 (see above) with 520 µl lysis buffer and 50 µl PBS buffer for the 30 s bead beating step, and with 125 µl lysis buffer plus 50 µl PBS for the vortex step. A 1/10 volume of the lysate was added directly to the streptavidin-coated plates, mixed with 0.25 µl of a biotin labelled capture primer (10 µM, same sequence as the reverse primer, Thermo Hybrid), and hybridization performed at 42°C for 30 min. Afterwards, samples were washed 4 times with cold PCR buffer (4°C, Eppendorf). Reverse transcription was done in the streptavidin plate with the chemistry described in Chapter 2 (section 2.5.1). cDNA was then transferred to a new tube for qPCR.

4.2.7 Second purification of (m)RNA extracts with DNAse

DNAse treatment applied for *Cryptosporidium* mRNA or RNA extracts. This was done to eliminate residual or contaminating genomic DNA from the extracts and to ensure that qPCR signals correspond only to the target mRNA. The TURBO DNA-*free*™ kit (Ambion®, Applied Biosystems) was used with 20 µl of eluted (m)RNA following the manufacturer's instructions (unless otherwise stated). Samples were then placed on ice.

4.2.8 Optimization of reverse transcription and qPCR

Generally, reverse transcription (RT) and qPCR were performed in separate reactions (two-step system) with the ThermoScript™ kit for RT and Platinum® Taq DNA polymerase for qPCR (Invitrogen) following the descriptions in Chapter 2 (section 2.5). RT was done with DNase treated and noDNase treated (m)RNA, including or not the enzyme in each case. To control for the effectiveness of the DNAse treatment, negative controls consisted of qPCR reactions with DNase treated samples and not including the RT enzyme (DNase, -RT). Negative controls with pure water, instead of nucleic acids, were also included in each RT and qPCR assay.

In addition, a one-step RT-qPCR system was evaluated and compared to the two-step RT-qPCR. The one-step assay was done as described in Chapter 2 (section 2.5). The yield of mRNA was also evaluated with (m)RNA extracts that were incubated at 65°C for 7 min in a waterbath and

then placed on ice for 7 - 10 min prior to RT. This was done to unfold mRNA secondary structure and to improve primer hybridization in the RT.

4.3 Results

4.3.1 Evaluation of oocyst disruption protocols

This evaluation was done in combination with total RNA extraction method 1 using oocysts that exhibited 77 - 92 % viability as measured by membrane integrity test (DAPI/PI) and excystation test. First results showed that oocyst disruption by 240 s cumulative bead beating (protocol 1b) produced the highest cDNA yield in manure compared to other protocols evaluated (Fig. 4.1). With this lysis procedure, qPCR signals from 3.4 x 10^3 oocysts / 50 µl manure was detected with a mean Ct of 36.16 (SD 0.36). Lower copy numbers (about 2.5 times less) were obtained for the 240 s cumulative bead beating plus two times vortex that used less volume of lysing buffer (protocol 1a). The use of more lysing buffer (about 1.6 times more) for each bead beating step seemed to improve the lysis and extraction of RNA (Fig. 4.1). Lysis with SoTa followed by a short 60 s bead beating yielded on average 1.8 times less cDNA than 240 s cumulative bead beating, perhaps due to insufficient oocyst and sporozoites lysis to free nucleic acids in the lysate, or the lysis treatment was too strong that resulted in nucleic acid shredding. The use of a similar procedure with an even shorter bead beating step (SoTa treatment + 30 s bead beating) was tested in pure oocyst suspensions, but it yielded no qPCR signal. Extractions in pure suspensions were also performed with lysis by SoTa treatment + freeze-thaw, but no cDNA was detected in those experiments, as opposed to signal detection when manure samples were processed, thus indicating varying results with such lysis procedure (Table 4.2).

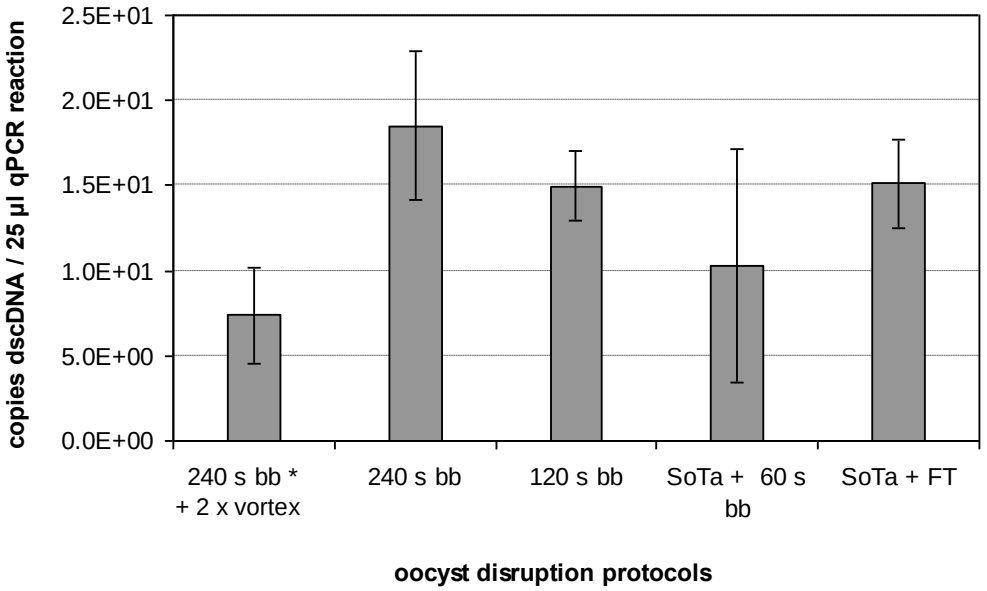


Fig. 4.1 – Performance of different oocyst disruption procedures in manure measured in terms of cDNA. bb: cumulative bead beating; SoTa: treatment with sodium taurocholate; FT: freeze-thaw cycles. Total RNA extracted with method 1 (GITC-PC) from 3.4 x 10^3 oocysts. See description of protocols in Table 4.1. *: reduced lysing buffer volume was used.

The cumulative 240 s bead beating lysis protocol was used with oocysts from different stocks spiked to manure and in pure suspensions (Table 4.2). No qPCR signal was detected for extractions in pure suspensions. It appeared that this lysis procedure in pure oocysts was too strong and may have damaged mRNA strands. In contrast, particulate material and fibers in manure seemed to protect oocysts during bead beating and to mild the lysis step.

Table 4.2 also shows varying mRNA detection with the 240 s cumulative approach using various oocyst stock suspensions. High Ct values (around Ct 38), indicating detection of low amount of cDNA, or no amplification signal was measured in some replicate experiments. The variable qPCR results with this procedure suggested some difference in oocyst wall constitution (weak and strong) although they all were viable and metabolic active. Therefore, long bead beating lysis steps may not suit for oocysts with less strong walls. In addition, this lysis protocol produced a milky suspension most probably due to the prolonged grinding and strong abrasion of the silica and ceramic beads, and of the glass bead. Foam formation was another factor that impeded rapid recovery of the homogenized lysate.

Table 4.2 – Performance of oocyst lysis by the 240 s cumulative bead beating approach and by SoTa treatment with freeze-thaw cycles in pure suspensions and manure. Results given as mean Ct value (n = 3 - 6). Total RNA extraction performed with the GITC-PC method 1.

Oocyst disruption protocol	Oocyst susp.**	Oocysts / sample[$]	Pure susp.	Manure
			mean Ct ± SD	mean Ct ± SD
240 s bb	B	3.4×10^5	no signal	27.72 ± 0.14
	E	3.4×10^5	-	29.9 ± 0.11
	C	3.4×10^5	-	38.71 [#]
	A1	3.4×10^5	-	no signal
SoTa + freeze-thaw	A and B	7.9×10^3	no signal	36.53 ± 0.2

**: oocysts from different fresh suspensions; [$]: oocysts extracted per sample; SoTa: treatment with sodium taurocholate; bb: bead beating; no signal: no detected qPCR amplification from n=6; [#]: one amplification signal detected. Lower Ct values indicate higher cDNA detection.

In order to overcome the difficulties mentioned above, the beating processing time was reduced to 165 s in three intervals: (30+30 s) + (30+30 s) + (20+25 s), with cumulative pooling of the lysate fractions (protocol 3, Table 4.1). The following were considered for choosing this approach:

a) The 165 s protocol excluded long 90 s and 120 s beating intervals to prevent strong abrasion of the ceramic and silica beads, and to prevent the formation of glass milk and excessive foaming.
b) Oocysts with weak and strong walls can be sequentially disrupted and the (m)RNA sequentially collected after each bead beating interval.
c) By shortening the bead beating time, the lysate can be rapidly purified and mRNA strands may be less prone to degradation, e.g. by RNases, that may be still active in the lysate.

Tests with the 165 s protocol showed reduced glass milk formation and produced a more clear suspension. Foam formation was also reduced. Therefore this protocol was applied for further evaluations with *Cryptosporidium*. Fig. 4.2 shows the comparison of the 165 s cumulative bead beating approach and lysis by freezing-thawing cycles, which has been frequently used for oocyst in pure suspensions and water (Stinear et al., 1996; Wiedenmann et al., 1998; Bialek et al., 2002).

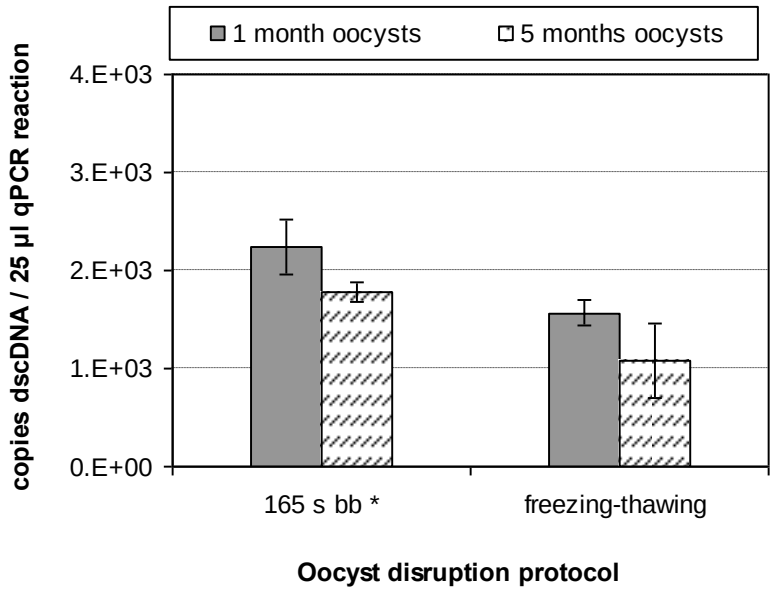


Fig. 4.2 – Comparison of oocyst lysis by 165 cumulative bead beating (proposed protocol) and freezing-thawing cycles using 1 and 5 months old oocysts. Results are shown as detected cDNA copies per qPCR reaction. bb: cumulative bead beating; freezing-thawing cycles (derived from Stinear et al., 1996). Total RNA extraction performed with GITC-PC method 1.*: phase lock gel separator was used during purification.

Fig. 4.2 shows significantly higher detection of mRNA copies ($p \leq 0.05$) from fresh viable oocysts using the 165 s bead beating approach in comparison to freezing-thawing. This was also obtained in tests with oocysts with different wall constitution (5 months aged oocysts). Aged oocysts can exhibit weaker walls, i.e. higher permeability (Campbell et al., 1992). Moreover, the use of 5 freeze-thaw cycles with other fresh viable oocysts in pure samples or manure yielded no cDNA detection in the qPCR presumably due to the presence of oocysts with very strong walls that could not be disrupted.

The following recommendations are derived from the use of 165 s cumulative bead beating:

i) Samples should be controlled for overheating after 30 s intervals because lysate in the E tube can warm up during bead beating despite setting the FastPrep machine at 4°C. In that case, samples should be placed on ice water for 1 - 3 min to cool down in order to avoid mRNA degradation.

ii) After bead beating and centrifugation, small fractions of lysate supernatant should be collected from the E tube using small pipette tips to avoid carry of the sediment containing particles. The lysate should be placed immediately on ice water.

4.3.2 Comparison of different bead materials for oocyst lysis

The evaluation of oocyst lysis in manure using beads of different materials and 165 s cumulative bead beating is shown in the next figure.

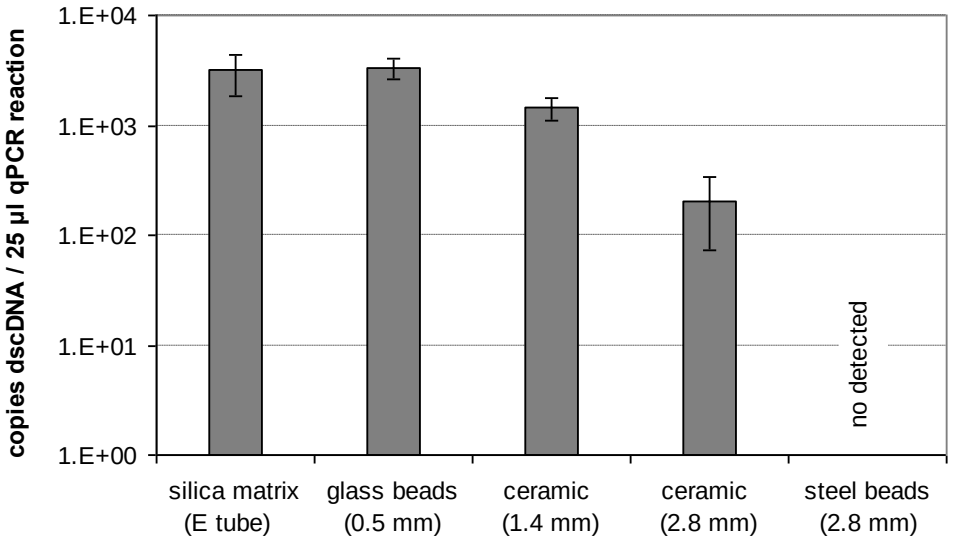


Fig. 4.3 – Comparison of different bead materials for oocyst lysis by 165 s cumulative bead beating in manure. Results are shown as cDNA copies / qPCR reaction. .

Similar amount of *hsp70* cDNA was obtained with the silica matrix in E tubes (a mixture of glass, silica and ceramic beads) and with tubes containing glass beads (size 0.5 mm) ($p > 0.05$, not significant different). Significant lower cDNA was obtained with ceramic beads (size 1.4 mm or 2.8 mm) compared to the silica matrix or the glass beads ($p \leq 0.05$). The use of steel beads with the 165 s bead beating approach did not suit for lysis of oocysts because the mechanical / physical force of bead beating was too strong that resulted in break of the tubes with partial or total loss of the lysate. Subsequent tests were done using the silica lysing matrix E tube.

The following optimized steps were derived from the use of total RNA extraction method 1 based on GITC-PC and isopropanol purification with manure samples:

i) Carefully remove the liquid phase during organic-liquid separation after adding phenol-chloroform, for example, by using 10 µl pipette tips to collect the liquid phase close to the interphase. Phase-lock gel tubes (2 ml) can be used for separation of the organic phase and for easy collection of the liquid phase containing RNA. If part of the gel migrates to the top of the tube after centrifugation, as it was sometimes found, remove the gel with a pipette tip.

ii) After precipitation of RNA in isopropanol, carefully remove the supernatant because the RNA pellet is not visible in the tube. Use for it small pipette tips (10 µl) and if needed leave approximately a small drop of the liquid.

iii) After washing the pellet (containing RNA) with 70 % ethanol, it is recommended to centrifuge the sample again at 14,000 x g for 5 min to sediment the invisible pellet and to avoid losses of RNA before ethanol is discarded.

4.3.3 Performance of DNAse treatment in total RNA extracts from method 1

Ct values for RNA extracts treated or not with DNase are presented in Table 4.3. It shows the specific detection of *hsp70* mRNA (cDNA) in the DNase, +RT samples.

Table 4.3 – Specific detection of *Cryptosporidium hsp70* cDNA in RNA extracts from manure after DNase treatment (DNase, +RT samples), and qPCR results with noDNase treated samples. Results are shown as mean Ct value. Total RNA extraction performed with GITC-PC method 1.

Oocysts / extracted sample *	**cDNA**			
	noDNase, +RT	**noDNase, -RT**	**DNase, +RT**	**DNase, -RT**
	(co-extracted DNA + cDNA)	(co-extracted DNA)	(***hsp70* cDNA**)	negative control
Extraction 1, (3.4 x 10^5)	27.40 ± 0.56	27.84 ± 0.07	29.65 ± 0.21	nd
Extraction 2, (5.0 x 10^4)	29.68 ± 0.38	37.35 ± 1.15	30.66 ± 0.22	nd
Extraction 3, (3.4 x 10^3)	34.87 ± 0.44	35.15 ± 0.08	36.00 ± 0.27	nd

cDNA molecules are produced from mRNA strands by reverse transcription; *: number of oocysts per manure sample; +RT: reverse transcription reactions including the enzyme reverse transcriptase; -RT: reactions without the enzyme; nd: no qPCR amplification signal.

RNA samples that were not treated with DNAse and were used for RT reactions without the enzyme (noDNAse, -RT) yielded qPCR amplification signals, indicating that there was co-extracted genomic DNA in the total RNA extracts from manure. Residual contaminating DNA in total RNA extracts was completely removed by DNases, as indicated by no qPCR signal in negative controls (cDNA samples obtained with DNase treated RNA and without reverse transcriptase in the RT). Thus, DNase treatment was necessary for specific detection of the mRNA target. Note the following step that was added to the description of the manufacturer:

i) Samples were centrifuged twice at 10,000 x g for 1.5 min to elute (m)RNA. This additional step was done to completely eliminate contamination by traces of inactivation reagent that were usually found in the final RNA when samples were spun only once.

4.3.4 Optimization of direct mRNA extraction method 2

The direct mRNA extraction procedure with oligo $(dT)_{25}$-magnetic beads was evaluated with different lysing buffers and mRNA elution temperatures (Table 4.4). A GITC kit-based buffer was used as this compound is known to be effective to remove RNases, which can degrade (m)RNA strands. However, no qPCR amplification signal was obtained when the magnetic Dynabeads system was used with such lysing buffer. A slow separation of the hybrid magnetic bead–mRNA from the lysate in the magnetic field was observed when using GITC based buffers. Dilution of the GITC buffer in 1x PBS (1:1 vol/vol) did not yield better results (Table 4.4). No qPCR amplification was attributed to the GITC chaotrophic solution, which is a strong denaturant that can produce viscous lysates. This might have also affected the hybridization of the poly A tail of the mRNA to complementary oligo $(dT)_{25}$–magnetic beads, with the subsequent loss of transcripts during the washing steps. Better results were obtained with the LiCl based buffer that included Tris-HCl, EDTA, LiDS and 5mM DTT.

Table 4.4 – Evaluation of lysing buffers and elution temperature employed with the oligo $(dT)_{25}$-coated magnetic beads system, method 2, for direct mRNA extraction from manure. qPCR results are given as mean Ct value.

Lysing buffer	**Temperature mRNA elution** (°C)	**Oocysts / extracted sample**	**Calculated cDNA copies / 25 µl qPCR**	**Mean Ct**	**SD**	**Relative detection factor** [§]
GITC*	45	3.15×10^4	1.1×10^2	nd	-	-
GITC	65	3.15×10^4	1.1×10^2	nd	-	-
GITC : 1x PBS (1:1)	45	3.15×10^4	1.1×10^2	nd	-	-
GITC : 1x PBS (1:1)	65	3.15×10^4	1.1×10^2	nd	-	-
LiCl **	45	3.15×10^4	1.1×10^2	34.46	0.48	0.25
LiCl	65	3.15×10^4	1.1×10^2	32.47	0.36	1
LiCl [§]	65	4.90×10^5	1.5×10^3	27.89	0.32	1
LiCl	74	4.90×10^5	1.5×10^3	27.81	0.26	1.06
LiCl	84	4.90×10^5	1.5×10^3	28.20	0.41	0.8

[§]: result from extraction with LiCl and mRNA elution at 65°C was used as reference sample for relative comparison; *: contains guanidinium isothiocyanate; **: contains lithium chloride, lithium dodecyl sulfate, EDTA and DTT; nd: no detected qPCR amplification signal; SD: standard deviation (n=5). Lower Ct values indicate higher cDNA detection.

The final mRNA elution step in the mRNA purification procedure was done at 45°C to reduce the risk of mRNA degradation that may have occurred at a higher temperature. However, elution at 45°C yielded lower *hsp70* cDNA compared to elution at 65°C probably due to insufficient detachment of mRNA strands from the complementary oligonucleotide-tagged magnetic beads at lower temperature. A double elution of mRNA at 65°C or 74°C in 10 mM Tris-HCl (pH 7.5) produced comparable results (Table 4.4). Elution at 84°C showed slightly higher Ct values (lower cDNA numbers) but results were not significantly different than with the former temperatures ($p > 0.05$).

The following optimized steps and recommendations were derived from the use of the direct mRNA extraction system with oligo $(dT)_{25}$-magnetic Dynabeads (method 2) with manure samples:

i) For enhanced performance with viscous lysates (e.g. from manure), hybridization of the poly A tail of the mRNA to the complementary oligo$(dT)_{25}$-coated magnetic beads should last at least 7 min.

ii) To fully concentrate the magnetic beads–mRNA hybrid to the site of the magnet during the washing steps, it was necessary to perform semi-circular movements of the reaction tubes when placed on the magnet. By doing this, it was possible to easily remove the washing buffers from the tubes by pipetting without the risk of carrying mRNA. Failing to include this recommendation can yield suboptimal results, as seen in some replicate experiments in this work.

iii) Beads washing with buffer A after mRNA binding to the magnetic beads in the manure lysate needs at least 2 minutes for the attachment of the magnetic beads to the magnet.

iv) Total separation of the magnetic beads–mRNA hybrid from the lysate with the magnet during the washing step with buffer B required a longer time than the recommended by the manufacturer (about 10 min is here recommended). The reason for the slow separation of the magnetic beads is most probably due to the viscous sample and buffer B composition (containing 0.15 M LiCl and 1 mM EDTA).

v) Elution of mRNA can be done at 65°C or 74°C, 2 times each with 25 µl of elution buffer.

4.3.5 Quality of mRNA extracts purified with method 2

Low amount or no co-extracted DNA was measured in mRNA extracts that were not treated with DNase (noDNase,-RT) when extraction / purification was done with the magnetic beads system, method 2 (Table 4.5), in contrast to the higher co-extracted DNA obtained in extracts from the total RNA extraction method 1 (Table 4.3). Although the oligo $(dT)_{25}$-magnetic beads

system would theoretically extract only mRNA strands, some residual DNA was carried in some replicate assays. Therefore DNAse treatment is still recommended to ensure that qPCR signals correspond only to *hsp70* mRNA. Specific detection of *hsp70* cDNA (in DNAse,+RT samples) was verified by no signals in negative controls.

Table 4.5 – Specific detection of *Cryptosporidium hsp70* cDNA in mRNA extracts from manure after DNase treatment (DNase, +RT samples), and qPCR values from noDNase treated samples. Results are shown as mean Ct value. Direct mRNA extraction performed with the oligo $(dT)_{25}$-magnetic beads system, method 2.

Oocysts / extracted sample *	**cDNA**			
	noDNase, +RT	**noDNase, -RT**	**DNase, +RT**	**DNase, -RT**
	(co-extracted DNA + cDNA)	(co-extracted DNA)	(***hsp70* cDNA**)	negative control
Extraction 1, (4.9×10^5)	27.00 ± 0.16	39.44 ± 0.82 / nd[§]	27.89 ± 0.32	nd
Extraction 2, (5.0×10^5)	27.08 ± 0.59	35.97 ± 0.15 / nd[§]	28.8 ± 0.42	nd
Extraction 3, (3.1×10^4)	32.15 ± 0.1	nd	32.68 ± 0.28	nd

cDNA molecules are produced from mRNA strands by reverse transcription; *: number of oocysts per manure sample; +RT: reverse transcription reactions including the enzyme reverse transcriptase; -RT: reactions without the enzyme; nd: no qPCR amplification signal; [§]: or no signal in replicate samples.

4.3.6 Evaluation of RT-qPCR

The RT-qPCR detection assay is affected by the efficiency and performance of the RT and qPCR reactions. The performance of a one-step RT-qPCR (one tube reaction) and a two-step RT-qPCR (separate reactions for RT and qPCR) were evaluated in mRNA extracts from manure that were purified with the oligo $(dT)_{25}$-magnetic beads method 2. qPCR amplification curves for both systems are shown in Fig. 4.4. No real amplification curve was obtained for samples containing 78 or less dscDNA copies with the one-step system (A) in contrast to the two-step system (B) that allowed detection of lower amount of copies. The RT in the one step-system was done at 42°C in comparison to 57°C for the two-step system, and both systems (from different manufacturers) also employed different reaction chemistry. The two-step RT-qPCR allowed higher reaction efficiency and apparently higher primer hybridization to the nucleic acids. The detection efficiency for the one-step system, calculated at the linear region on the plot Ct vs cDNA copies was 41.7 % with respect to the two-step system (see arrow in Fig. 4.5). The detection limit for the two-step RT-qPCR was 1.5 *hsp70* dscDNA copies / qPCR reaction and

occasionally qPCR signals for the lowest dilution theoretically containing 0.15 copy were detected. In contrast, for the one-step system the lowest analytical detection limit in the RT-qPCR assay was obtained for samples with 780 dscDNA copies.

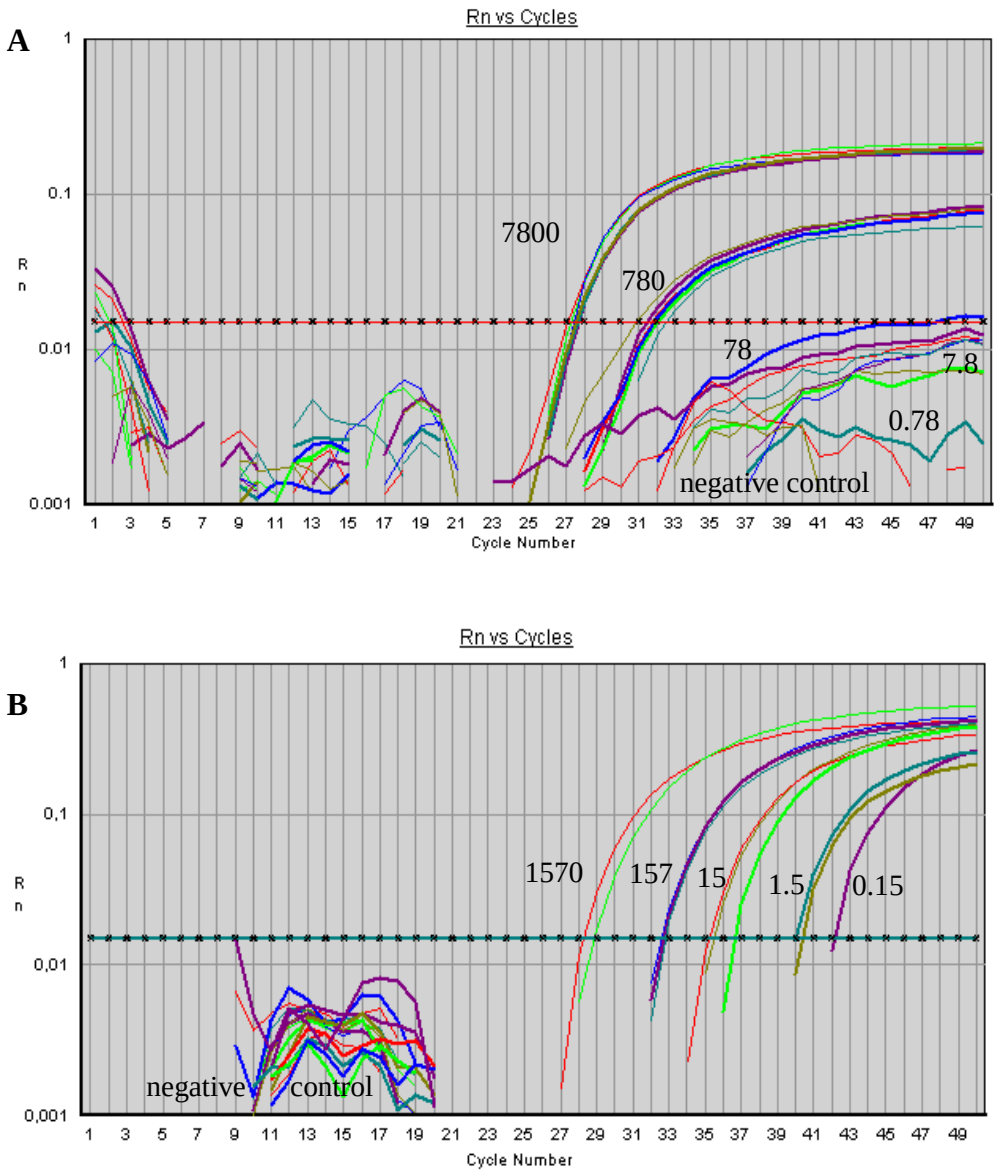


Fig. 4.4 – Real-time qPCR plots depicting amplification curves for *hsp70* cDNA. **A:** one-step RT-qPCR and **B**: two-step RT-qPCR. Amplification curves above the threshold (horizontal line at 0.015 Rn, above the background fluorescence) correspond to samples containing the indicated number of dscDNA copies, which is equivalent to DNA copies present in the sample.

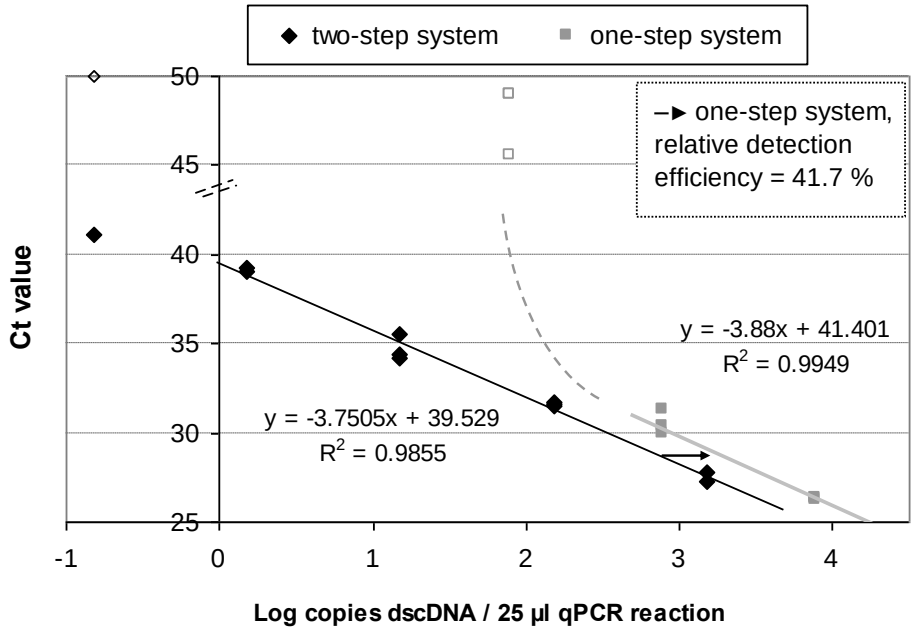


Fig. 4.5 – Linear regression of measured Ct values versus the copy number of *hsp70* dscDNA in mRNA extracts processed with one-step and two-step RT-qPCR. It depicts the higher sensitivity for the two-step system and suboptimal cDNA detection for the one-step system in manure extracts. Open symbols: no qPCR amplification.

The secondary structure of the (m)RNA, which is the folding of the strand and the formation of internal hydrogen bonds between complementary bases, can affect the performance of the reverse transcription and the subsequent detection by RT-qPCR. Heating the extracted mRNA at 65°C for 7 min to dissolved any secondary structure before the two step RT-qPCR yielded lower cDNA content compared to not-heat treated extracts (Fig. 4.6). Apparently, the mRNA content was slightly degraded by the treatment. It is possible that this additional sample manipulation may have reduced the stability of the mRNA strands. Results with mRNA that was not pre-heated showed on average 1.9 fold more copies. The RT reaction at a high temperature, i.e. 57°C, seemed sufficient to allow a correct reaction.

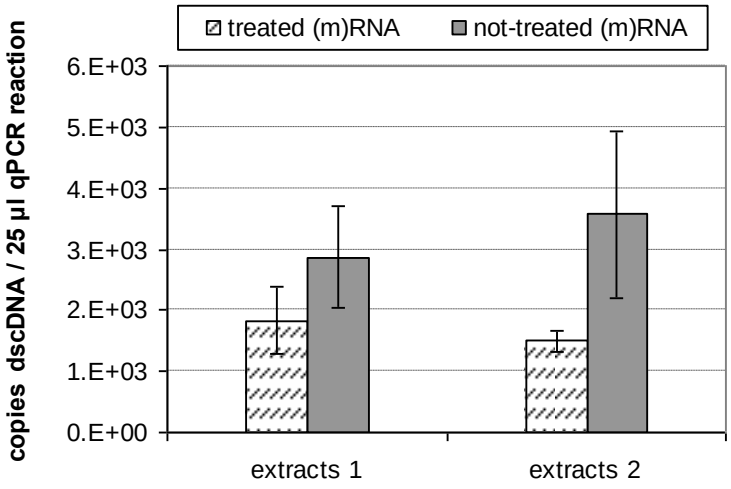
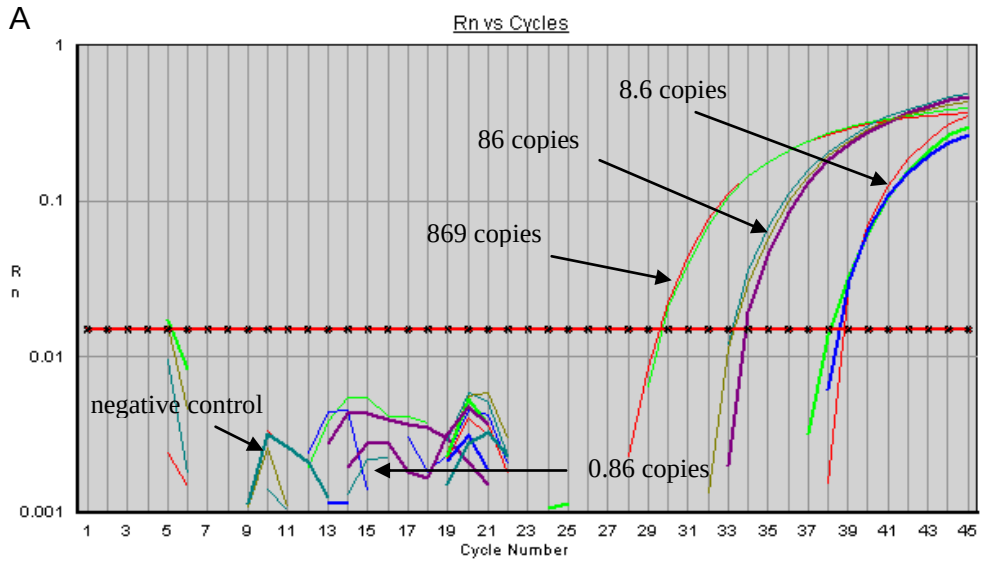


Fig. 4.6 – Yield of *hsp70* cDNA in mRNA extracts subjected or not to 65°C for 7 min to unfold secondary structure before reverse transcription.

4.3.7 Method sensitivity for *Cryptosporidium* oocysts

The sensitivity of the complete method comprising: washing manure, lysis by 165 s cumulative bead beating, mRNA extraction / purification, DNase treatment of the extracts, and RT and qPCR in separate reactions (two-step reaction) is shown in Fig. 4.7 and Fig. 4.8. Real-time qPCR amplification plots depicting curves for *hsp70* cDNA in extracts from manure are shown in Fig. 4.7.



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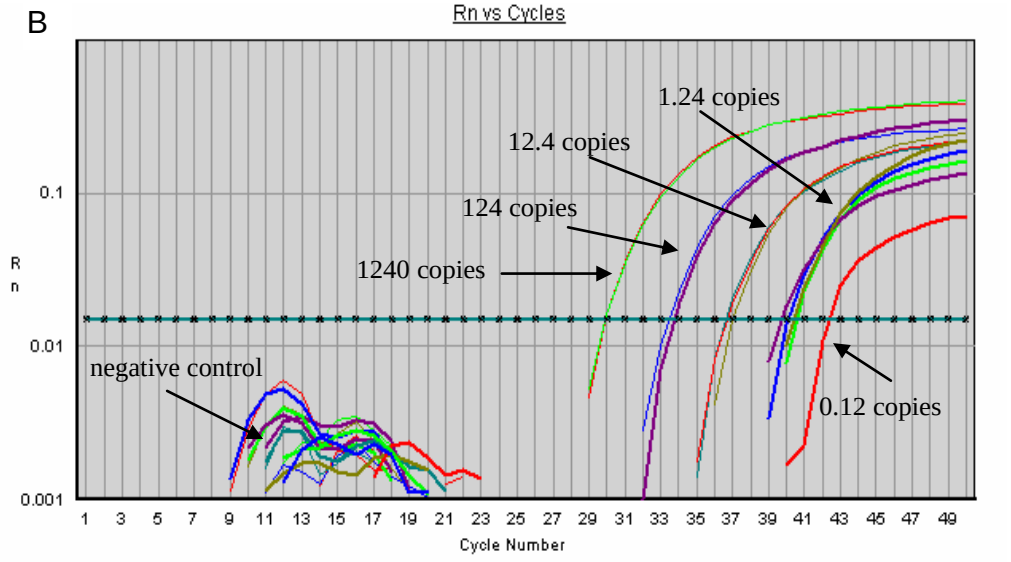

Fig. 4.7 – Real-time qPCR plots showing amplification curves for *hsp70* cDNA in extracts from oocysts in manure. **A**: total RNA extraction performed with GITC-PC method 1; **B**: direct mRNA extraction performed with oligo $(dT)_{25}$-magnetic beads method 2. Threshold (horizontal line) was set at 0.015 Rn.

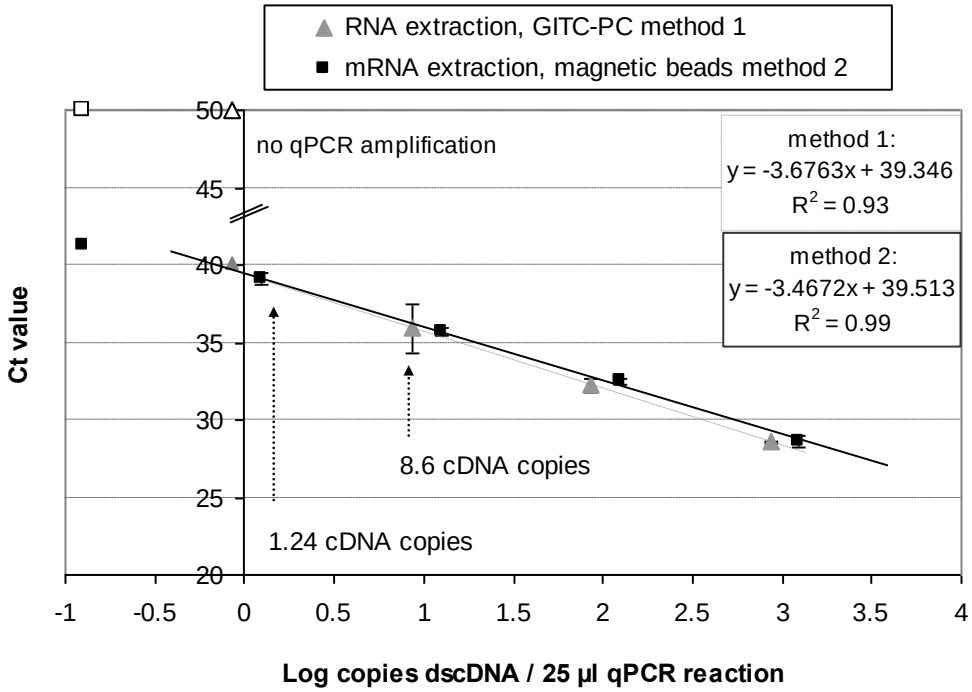

Fig. 4.8 – Linear regression of measured Ct values versus the copy number of *hsp70* dscDNA in (m)RNA extracts from oocysts in manure using total RNA extraction method 1 and direct mRNA extraction method 2. It shows the lower analytical detection limit in the qPCR. Open symbols: no qPCR amplification.

qPCR signals were measured from as low as 1.24 dscDNA copies when direct mRNA extraction was done with the oligo $(dT)_{25}$-magnetic beads, method 2 (Fig. 4.7, B). These amplification

curves were measured from 390 oocysts / 60 µl of manure extracted (Fig. 4.8). An amplification signal for a calculated 0.12 cDNA copy was also measurable, however not in all qPCR replicates. This corresponded to signal from about 39 oocysts per extracted sample. For the total RNA extraction method 1, the lowest analytical detection limit in the RT-qPCR was obtained for 8.6 dscDNA copies, which was measured with high Ct values (Fig. 4.7, Fig. 4.8). For the highest dilution that contains cDNA from an equivalent of 50 oocysts extracted / 60 µl manure, one signal (Ct = 40.18) was obtained, but detection was not consistent at this level.

The linear correlation of Ct values and dscDNA copies was $r^2 = 0.99$ for method 2, and $r^2 = 0.93$ for method 1 (Fig. 4.8). The efficiency of the qPCR was 94 % using extracts from method 2 and 87 % for extracts from method 1.

Table 4.6 shows the measured Ct values for *hsp70* mRNA for the corresponding number of double strand cDNA spiked copies and oocyst numbers in the samples. The variation of Ct values, measured as the standard deviation, was typically high for samples containing low amount of copies. For method 1, Ct variation for the sample dilution containing 8.6 dscDNA copies was higher than that found for 1.24 copies in extracts from method 2.

Table 4.6 – Ct qPCR values for *hsp70* dscDNA in mRNA extracts from manure using two extraction methods.

A: Total RNA extraction, GITC-PC method 1			
Oocysts / sample[$]	**Copies dscDNA / qPCR reaction**	**Mean Ct**	**SD**
5.0E+04	869.0	28.56	0.07
5.0E+03	86.9	32.22	0.39
5.0E+02	8.6	35.92	1.58
5.0E+01	0.86	40.18* / nd	-
B: Direct mRNA extraction, magnetic beads and LiCl method 2			
Oocysts / sample[$]	**Copies dscDNA / qPCR reaction**	**Mean Ct**	**SD**
3.9E+05	1240	28.60	0.38
3.9E+04	124	32.51	0.19
3.9E+03	12.4	35.73	0.24
3.9E+02	1.24	39.08	0.41
3.9E+01	0.12	41.29* / nd	-
no oocysts [c]	0	nd	-

[$]; calculated number of oocysts per 60 µl manure sample; SD: standard deviation (n = 3 - 6); *: one signal from n=6; nd: not detected qPCR amplification in replicates; [c]: manure without oocysts (negative control) evaluated with extraction method 1 and method 2.

4.3.8 Comparison of RNA extraction methods for enteroviruses

No endogenous enteroviruses were measured in not spiked wastewater, sludge or manure samples (no qPCR signal was detected).

The evaluation of RNA extraction methods for enteroviruses yielded lower extraction efficiency for the streptavidin-biotin system in sludge and manure, as seen by the higher Ct values in comparison to the GITC-PC method 1 (Table 4.7). This difference was 32.5 times for extractions in sludge and it was much higher for manure samples (300 fold difference). Similar results were obtained in other replicate experiments (not shown), indicating the lower performance of the streptavidin based method with complex environmental matrices most likely due to interferences during the hybridization step. Another lysing buffer containing only guanidinium thiocyanate as denaturant and excluding β-mercaptoethanol (RNaid® Plus Kit, MP Biomedicals) was also evaluated for manure extractions with this system, but it did not yield results.

Table 4.7 – Comparison of RNA extraction methods for enteroviruses in wastewater, sludge and manure. Ct values and fold difference in cDNA detection between the methods are shown.

Matrix / enterovirus	**Vol. extracted**	**Streptavidin-biotin (Sb)**		**GITC-PC method 1 (m1)**		**Detection ratio**
Sabin 1	(µl)	mean Ct	SD	mean Ct	SD	(m1 / Sb)
sludge	70	35.61	0.31	29.91	0.54	32.5
manure	50	38.94	2.16	29.80	0.24	300
	Vol. extracted	**Oligo $(dT)_{25}$ method 2**		**GITC-PC, method 1**		**Detection ratio**
Echovirus 6	(µl)	Mean Ct	SD	mean Ct	SD	(m1 / m2)
wastewater	90	27.97	0.07	27.07	0.16	1.8
manure	90	29.34	0.37	27.81	0.51	2.7
pure suspension	90	29.01	0.4	28.12	0.08	1.8

Extractions from strain Sabin 1 (1.8×10^6 virions / sample) and Echovirus 6 (3×10^6 virions / sample); n=3 - 5.

The GITC-PC method 1 yielded slightly higher detection of cDNA copies than the oligo $(dT)_{25}$-magnetic beads method 2 (Table 4.7). It is possible that the slightly higher Ct values with method 2 were due to some fragmentation of viral RNA during sample processing, which may have subsequently result in reduced primer hybridization in the RT-qPCR. Intact mRNA strands spanning from the poly A tail until the primer binding sites is needed for the oligo $(dT)_{25}$ system. The slightly higher Ct values (lower detection) for extractions in pure suspensions

suggest that some degradation of RNA may have occurred most likely during the lysis step as typically this matrix is a simpler substrate to extract and purify RNA.

Extractions from 90 µl manure or wastewater with total RNA extraction method 1, showed no sign of inhibition of the qPCR assay as verified by the linear plot in serial dilutions of extracts (Fig. 4.9). The amplification efficiency, which is dependent on the slope, was 95 %. The figure also shows that RT and qPCR amplification was possible using enteroviruses in pure clean suspensions that were not extracted. In that case the lysis of the viral capsid to release the genomic RNA was most probably achieved by the high temperature during the RT reaction. The efficiency of the qPCR was 90 % and the assay could detect as low as 1.5 viral particle / reaction.

The efficiency of the total RNA extraction method 1, including a 30 s bead beating step to release the viruses from particulate material and for lysis of the capsid, was 37 % calculated at the linear region on the plot Ct vs cDNA copies (see arrow in Fig. 4.9). The lowest detectable qPCR signal with manure extracts was obtained for 15 viral particles (15 RNA copies detected), and a detection limit of 5.7 viral particles can be calculated after regression analysis.

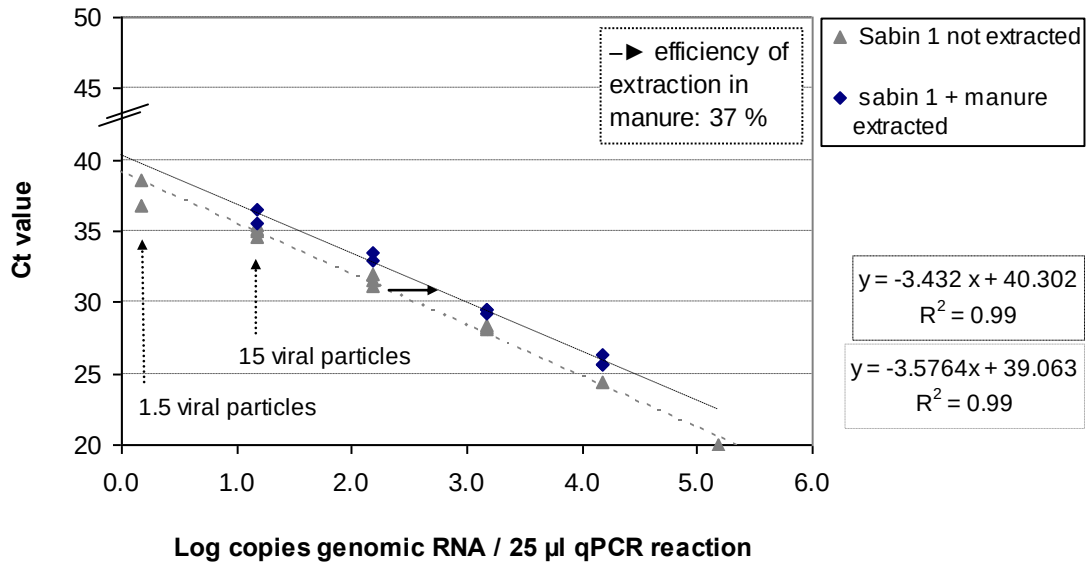

Fig. 4.9 - Linear regression of measured Ct values and the copy number of enterovirus genomes from manure extracts and from pure viral suspensions that were not extracted. Arrow shows the efficiency of extraction and detection from 90 µl manure. Total RNA extraction was performed with GITC-PC method 1. The number of viral particles indicates the lower detection limit of the qPCR.

4.4 Discussion

Procedures for RNA and mRNA extraction and for detection by RT-qPCR were developed and optimized for direct application with complex environmental substrates. Here are presented

optimized steps for the specific and sensitive detection of *hsp70* mRNA from viable (metabolic active) *Cryptosporidium* oocysts in manure and for RNA enteroviruses in manure, sludge and wastewater. Suitable molecular detection assays that provide information about the metabolic state of pathogenic *Cryptosporidium* oocysts are needed because only living organisms represent a risk of infection to human and animals. For hygiene control of potentially contaminated matrices, rapid, specific and sensitive detection methods are required. The accuracy of RT-qPCR results strongly depends on effective (m)RNA extraction procedures to produce extracts of good quality and purity, and on optimum RT-qPCR performance.

4.4.1 Oocyst lysis and (m)RNA recovery

Oocyst lysis is the first important step. The differently strong wall of oocysts must be disrupted, while avoiding mRNA degradation. The differences found in mRNA yield with the evaluated lysis procedures underline that the lysis step to recover mRNA strands from oocysts in manure must be suitably designed (Fig. 4.1, Fig. 4.2, Table 4.2). In initial experiments, the extended 240 s cumulative bead beating approach with pooled fraction recovery performed well for oocyst lysis in manure and yielded more cDNA in comparison to other milder bead beating steps or lysis procedures. However, 240 s did not perform well with pure oocyst samples and yielded also inconsistent results when oocysts from different stock suspensions were used with manure (Table 4.2). The variable cDNA yield in these samples was not due to reduced oocyst viability because the oocysts exhibited ≥ 77 % of excystation capability and were infectious in the cell culture test. This indicated that the wall of oocysts from different suspensions may have had different resistance, and required therefore different lysis intensities. The suboptimal extraction with the 240 s bead beating protocol was likely due to shredding of mRNA strands liberated from oocysts during the long bead beating intervals (i.e. after 90 or 120 s) or to formation of nucleic acid binding glass milk. Quick release of mRNA that is not readily collected from the lysate can be a factor of pronounced mRNA degradation by RNases or increased shredding by the bead beating force. The milky suspension (the lysate) sometimes obtained with this procedure was most likely the result of significant abrasion of beads after prolonged bead beating. Glass milk formation may have been a factor for the reduced mRNA recovery after extraction / purification, since transcripts can strongly bind to glass and ceramic particles that originated from beads abrasion. Ogram et al. (1988) reported on the ionic binding of nucleic acids to minerals or particulate material.

A shorter alternative for oocyst lysis, i.e. 165 s cumulative bead beating in intervals with pooling of fractions: (30+30 s) + (30+30 s) + (20 + 25 s) (protocol 3, Table 4.1), showed to be suitable for extractions from manure. The milky suspension and foam formation was reduced

without the need to supply of extra chemical additives. Controlling these factors was important for the practical performance of the procedure and the resulting cDNA yield. The protocol was kept relatively long to allow for lysis of oocysts with weak and strong walls. This particularly suits for environmental samples, which may contain oocysts from different sources and variable strength. Oocysts with relatively weak and more permeable walls (Campbell et al., 1992) can still be viable and occur in the environment. The cumulative 165 s bead beating approach did not include long bead beating intervals (90 s or 120 s) in order to reduce the risk of overheating and mRNA shredding by the bead beating force, or rapid mRNA degradation by residual RNAses that remained active.

Lysis by 165 s cumulative bead beating showed higher yield of mRNA from oocysts in manure than lysis by five freeze-thaw cycles (Fig. 4.2). Moreover, using different fresh oocysts suspensions, lysis by freeze-thaw yielded insufficient extraction without positive qPCR results, probably due to the presence of oocysts with very strong walls. The effectiveness of oocyst lysis by freeze-thaw cycles was previously reported to vary with the oocyst isolate (Nichols and Smith, 2004), probably due to different wall constitution of the oocysts. Thus, 5 freeze-thaw cycles may not always be suitable for lysis of oocysts with strong walls in manure, as found in this study.

Previous studies employed relatively harsh treatments for lysis of oocysts (Deng et al., 2000; Bialek et al., 2002; and Guy et al., 2003). Freezing-thawing has frequently been employed for lysis in water or buffer, e.g. 5 cycles (Stinear et al., 1996; Connelly et al., 2008), 8 – 15 cycles (DiGiovanni et al., 1999; Nichols and Smith., 2004), and enzymatic digestion has also been employed for lysis (Wiedenmann et al., 1998).

Oocyst lysis by bead beating is rapid and can easily be applied in practice. For lysis by cumulative bead beating, an appropriate volume of lysing buffer was necessary to allow complete mixing of the sample and lysis. Less than 200 µl buffer for each bead beating step is not recommended. GITC and LiCl-LiDS based lysing buffers employed for extractions with method 1 and method 2, respectively, showed to be suitable for mRNA extraction from manure.

4.4.2 Method specificity and sensitivity

Previous studies have employed different mRNA / rRNA targets for RT-PCR, and all reported specific detection to *Cryptosporidium* species (Rochelle et al., 1997; Widmer et al., 1999; Jenkins et al., 2000; Fontaine and Guillot, 2003). In this investigation, *hsp70* mRNA was chosen because it can be induced by a heat trigger and thus may be present in high numbers in the organism (Lindquist and Craig, 1988), increasing the sensitivity of the RT-qPCR assay.

Specific detection of *hsp70* mRNA by RT-qPCR was achieved when (m)RNA extracts from manure were treated with DNase with the TURBO DNA-*free* system (Table 4.3 and Table 4.5). Co-extracted DNA can be present in (m)RNA extracts from environmental samples (Bach et al., 1999; Bürgmann et al., 2003). Co-extracted DNA that is not removed can produce false positive signals if DNA is used as template in (q)PCR, or false negative results for instance if DNA–RNA interactions occur (Alm et al., 2000). The present work found that even if the direct mRNA extraction procedure with oligo $(dT)_{25}$-magnetic beads (method 2) was used, which theoretically would extract only mRNA strands, traces of genomic DNA were occasionally detected despite maintaining manufacturer-suggested conditions during sample preparation. It can be that DNA was partially denatured during the lysis step, then attached to mRNA and was co-extracted. Hence, DNase treatment of sample extracts is essential. As a measure of control, it is generally recommended to include a DNase,–RT (negative) control in each qPCR assay to prove specificity of the RT-qPCR amplification of the target of interest (*hsp70* mRNA).
The specificity and suitability of the developed primers for analysis of environmental matrices, typically containing a very diverse microbial population, was verified by the absence of signals obtained with mRNA extracts from manure free of oocysts (negative controls, Table 4.6). This verified the primer specificity analysis performed *in silico* with the bioinformatics tools FASTA and BLAST N2. The specific primers for the *hsp70* gene or transcripts generated short amplicons (144 bp) which also supports high (RT)-qPCR amplification efficiency.

Manure is considered a difficult matrix to extract mRNA because it contains particulate material, various phenolic and humic compounds. Long and harsh extraction and purification procedures for environmental samples can reduce the yield of mRNA because the transcripts are very labile and prone to degradation. In addition, humic and phenolic compounds co-extracted with (m)RNA can also inhibit the reverse transcription and qPCR reactions (e.g. Tebbe et al., 1993; Matheson et al., 2010). In this work, no inhibition of qPCR was found with manure samples and the purification procedures employed, as seen by the good exponential qPCR amplification (Fig. 4.7), the log-linear fit obtained with Ct values and serial sample dilutions, the y-intercept closely to the Ct value 38 and the slope of the dilution plot closely to -3.3 (Fig. 4.8). This was possible provided that manure samples were washed prior to extraction to reduce inhibitors (Lebuhn et al., 2003).
Successful extraction / purification of (m)RNA from *Cryptosporidium* oocysts was achieved with either of the purification chemistries: GITC-phenol-chloroform with isopropanol precipitation in the total RNA extraction method 1, and LiCl-LiDS purification with oligo $(dT)_{25}$ coated magnetic beads in the mRNA extraction method 2. Extraction with method 1 required

extra care during the organic phase separation step because carry-over of proteins and traces of phenol into the extract can compromise transcript detection (Wilson, 1997). Including the system "Phase Lock" during the organic separation facilitated the recovery of the supernatant (see section 4.3.2, text in italics).

Both purification methods allowed sensitive detection of *hsp70* mRNA from manure by RT-qPCR, but higher variability of Ct values was obtained at low template concentration with extracts from total RNA extraction method 1. The methods produced amplification signals for 1.24 and 8.6 *hsp70* dscDNA copies / qPCR reaction, corresponding to a signal detection limit of 390 and 500 viable oocysts in 60 µl manure extracted by method 2 and method 1, respectively. This assay sensitivity was achieved with the specific two-step RT-qPCR system (in separate reactions; Fig. 4.5, Fig. 4.8) and induction of *hsp70* mRNA production in the oocysts by heat shock before the extraction. It was even possible to detect occasionally qPCR signals for 39 - 50 oocysts in 60 µl extracted sample, most likely due to random variation that can occur at low cDNA concentration (Wilson, 1997). The lower variation of Ct values for replicates with low template concentration for extracts of the oligo $(dT)_{25}$-magnetic beads method 2 and the good qPCR efficiency indicates a slightly better RT-qPCR performance and quality of the extracts. The analytical detection limit of the qPCR assay was defined here as the number of cDNA copies that produced amplification signals in all qPCR repetitions for the same run.

Using more simple substrates, such as water samples, Stinear et al. (1996) reported detection of *hsp70* RT-PCR signals from a single oocyst using a magnetic bead system. For extractions in pure and clean oocyst suspensions, Baeumner et al. (2001) reported assay sensitivity of 5 - 10 oocysts (with a detection limit of 10 *hsp70* cDNA copies / reaction), and sensitivity of 50 oocysts for clean or dirty water samples using a GITC-based purification method, NASBA amplification and electrochemiluminescence detection. Another study that employed purified oocysts from environmental water samples reported detection of 100 oocysts using oligo (dT) magnetic beads for mRNA extraction, NASBA and detection by a nucleic acid hybridization lateral flow assay (Connelly et al., 2008). In the last two studies, oocysts were isolated and concentrated from water by IMS, which does not fully suit to process particulate material, such as manure (Davies et al., 2003). In general, RT-qPCR allows higher analytical throughput than other amplification assays such as NASBA or nested PCR.

The application of RT-qPCR with complex environmental matrices is still a new field of research, with limited reports for mRNA detection from viable organisms (e.g. Bürgmann et al., 2003; Smith et al., 2007; Bui et al., 2011). In the present study, the optimized (m)RNA extraction procedures for *Cryptosporidium* oocysts in manure and RT-qPCR targeting *hsp70*

mRNA yielded highly sensitive and specific detection results. The method detection limit per ml manure for the complete procedure was about 8.3 x 10^3 viable oocysts with method 1, occasionally, transcript signals could be detected from 8.3 x 10^2 oocysts. For direct mRNA extraction with method 2, *hsp70* mRNA was measurable typically from about 6.5 x 10^3 viable oocysts / ml manure, and occasionally in some replicates qPCR results could be obtained for 6.5 x 10^2 oocysts / ml manure.

The higher sensitivity and larger dynamic range (range of cDNA copies that qPCR can detect) obtained with the two-step RT-qPCR system indicated a better reaction performance compared to the one-step RT-qPCR (Fig. 4.4, Fig. 4.5). A one-step system allows more rapid processing and avoids risk of contamination between the RT and qPCR steps. However, the lower temperature of the RT reaction with the one-step system probably decreased the priming efficiency and thus the cDNA yield. In contrast, the RT in the two-step system could be performed at a higher temperature (57°C), using a cloned avian thermal stable reverse transcriptase (ThermoScript kit), for enhanced specificity of the enzyme, and for improved and stringent primer hybridization. In addition to an adequate RT biochemistry, the elevated RT temperature probably had dissolved higher order RNA structures, which may have improved the RT reaction (Fig. 4.6) (Bustin and Nolan, 2004). A high efficiency and sensitivity in the RT-qPCR assay is also essential for the construction of standard curves using extracts from standard-spiked environmental matrices (Lebuhn et al., 2003)

4.4.3 Detection of enteroviruses

The detection and quantification of human enteroviruses in wastewater and sludge is of interest for health authorities because of the epidemiological significance of these viruses and their relatively long persistence in the environment (EPA, 2004; WHO, 2006). Also the reuse of animal manure containing viruses can lead to contamination of water resources, soil and crops.

Molecular detection methods for enteroviruses have been mainly described for water samples and more recently for wastewater samples (e.g. Fong and Lipp, 2005; Fuhrman et al., 2005; La Rosa et al., 2011). The present study evaluated the suitability of three RNA extraction procedures for manure, wastewater and sludge samples. The streptavidin system and the oligo $(dT)_{25}$-magnetic beads method 2 are based on hybridization of the RNA to complementary sequences, and the hybridization topography will affect the overall RNA recovery rate.

The streptavidin-biotin system was fast, but lower cDNA yield was obtained with the tested sludge samples and particularly with manure, in comparison to the total RNA extraction method 1. The lower yield could have been due to reduced attachment efficiency of the hybrid biotin-

RNA to the complementary streptavidin in the manure lysate. The streptavidin protein may also have been affected by proteases that could have remained active in the lysate.

With the oligo $(dT)_{25}$ extraction method 2, however, it was possible to detect viral RNA from manure extracts that approximated the yield of the total RNA extraction method 1 (Table 4.7). Differences in the RNA hybridization site between the extraction method 2 and the streptavidin-biotin system may have influenced the results. In extraction method 2, complementary base sequences hybridized to the poly A tail of the RNA, and the strand is captured by its 3' end. Previously, Pusch et al. (2005) reported a more efficient recovery by hybridization of base sequences to the poly A tail of viral RNA than hybridizations that targeted other sequences of the strand.

The GITC-PC based extraction method 1 and RT-qPCR (without sampling washing) showed to be applicable to detect and quantify RNA viruses in complex environmental matrices such as manure and wastewater. It includes a 30 s bead beating lysis step that serves to release viruses from matrix clusters or particles and for lysis of the viral capsid. The extraction method 1 is a rapid assay that can be useful for viral RNA extraction from samples with particles. No sign of qPCR inhibition was observed in this evaluation (Fig. 4.9).

The direct extraction method with oligo $(dT)_{25}$-magnetic beads can also be used for viral extraction in manure, but with a slightly lower efficiency than method 1. Shredded RNA strands will not give an RT-qPCR signal if the cut lies between the primer annealing sites and the poly A tail at the 3' end. Based on this evaluation, preference is given to extraction method 1 for application with viral RNA.

Previously, Ley et al. (2002) reported an RT-PCR detection limit of about 5 - 10 viral genomes using a conventional RT-PCR based method. Simmons et al. (2011) reported a detection limit of 10 cDNA copies per qPCR reaction using purified plasmids as template, which is similar to the 15 viral particles / RT-qPCR that were quantified in the present study from manure extracts using the total RNA extraction method 1 and the two-step RT-qPCR system.

Box : key outcomes Chapter 4

- Sensitive and specific detection of *hsp70* mRNA by RT-qPCR from *C. parvum* oocysts in manure was possible without the need to pre-isolate oocysts from the matrix.
- An optimized oocyst lysis step is essential for mRNA extraction from manure. Cumulative bead beating for 165 s (in time intervals with pooling of lysate fractions) yielded consistent and higher cDNA yield than five freeze-thaw cycles. It allows

extraction of mRNA from oocysts with different wall constitution. Long bead beating times (e.g. 240 s) can shred mRNA and / or produce loss of mRNA (binding to formed glass milk), and is therefore not suited for mRNA extraction.

- Oocyst lysis by bead beating with the beads in matrix E tubes (mixture of a glass, ceramic and silica beads) or with glass beads (0.5 mm) were comparable and could be both applied. Other beads yielded lower nucleic acid recovery rates.
- For *Cryptosporidium* in manure, extraction and purification of total RNA with the GITC-phenol-chloroform (method 1) or the direct mRNA extraction with oligo $(dT)_{25}$-magnetic beads and LiCl-LiDS purification (method 2), following the optimizations described here, was successful to efficiently extract and transcribe (m)RNA and to detect *hsp70* cDNA by RT-qPCR, without apparent qPCR inhibition by inhibitors like humic acids. With method 2, at least 7 min of hybridization of the oligo $(dT)_{25}$ to the mRNA were sufficient for manure lysates, and 10 min separation time of the magnetic beads-mRNA hybrid was needed for washing with buffer B. A GITC-based lysing buffer was found unsuitable together with the oligo $(dT)_{25}$ extraction system.
- For manure samples, the oligo $(dT)_{25}$-magnetic beads extraction method showed a slightly higher sensitivity in the qPCR assay (1.24 dscDNA copies), compared to the total RNA extraction method 1 (8.6 dscDNA copies). For the two methods, the qPCR detection limit typically was $6.5 \times 10^3 – 8.3 \times 10^3$ viable oocysts / ml manure.
- Co-extraction of residual DNA in extracts from manure, even in small amounts as found with the direct mRNA extraction system 2, indicated that DNAse treatment of the (m)RNA extracts is essential.
- Two-step RT-qPCR in separate reactions using a heat resistant reverse transcriptase yielded a higher efficiency with manure extracts than the one-step reaction.
- The extraction and detection procedures for *Cryptosporidium* are fast, easily applicable and produce results in one day. About 8 h (including qPCR) are needed for assays with the oligo $(dT)_{25}$ beads method 2, and about 10 h (including qPCR) for assays with the total RNA extraction method 1.
- Quantification of enteroviruses in manure, wastewater and sludge was possible with both extraction / purification systems. However, a slightly higher yield was obtained with the total RNA extraction method 1. Using this method with a 30 s bead beating lysis step, 15 viral particles / qPCR reaction were measurable, and theoretically the system should allow the detection of as few as 5.7 viral particles.

CHAPTER 5

Evaluation of two methods for mRNA quantification from oocysts with environmental samples

5.1 Introduction

Methods based on (m)RNA extraction and detection by RT-qPCR are promising molecular tools for the screening of viable pathogenic microorganisms such as *Cryptosporidium* in environmental matrices.

Viable oocysts present in animal manure that is spread or reused in agricultural land can disseminate in the environment with the runoff and leachates. This can result in contamination of water sources and the land. Control for pathogens including *C. parvum* in environmental matrices, may be advised in cases when health risks are identified and / or as part of hygiene control of slurry or treated manure from animal farming (EPA, 2005; EC, 2009).

In the previous Chapter 4, a direct mRNA and a total RNA extraction procedure for *Cryptosporidium* oocysts were developed and optimized for its use with RT-qPCR assays.

This chapter applies those procedures to quantify *hsp70* mRNA from heat induced fresh and aged oocysts in manure samples. The amount of manure processed and the age of oocysts on the extraction and detection efficiency was assessed. These factors have an impact on the sensitivity of the assay and their suitability for environmental analysis. The detection of *hsp70* mRNA in oocysts was evaluated with respect to their viability.

The direct mRNA extraction method with the oligo $(dT)_{25}$-magnetic beads (method 2) was evaluated in samples of manure, fermented manure and in pure oocyst suspensions, and the performance of the complete extraction and detection assay was assessed using viable oocysts. Since *C. parvum* oocysts can survive also in pasture land and soil (Kato et al., 2004; WHO 2006), it was also foreseen to evaluate the applicability of the (m)RNA extraction and detection methods for oocysts in soil samples from an agricultural land.

5.2 Materials and methods

5.2.1 Sample preparation

Soil samples

Soil samples were from integrated, conventional farming (minimum tillage and conventional tillage) taken at depths 0 - 8 cm and 8 - 22 cm. Soil texture was silty-loam (26 - 28 % clay), which has been characterized and described previously (see Table 2.1, Chapter 2; Fuß et al., 2011). Oocysts (1 - 5 x 10^5) in 200 µl of 1x PBS buffer were seeded to soil samples (50 mg), mixed by vortex and immediately thereafter heat shock induced at 45°C for 20 min for *hsp70* mRNA production. Samples were then lysed and (m)RNA extracted.

Manure samples

Fresh manure and fermented material were collected from an anaerobic digestion plant. Samples were screened for *C. parvum* DNA to verify the presence of endogenous contents, and it was confirmed the absence of oocysts in the tested matrices. Fresh viable oocysts from different suspensions and oocysts aged for 5, 9 and 12 months at 4°C were used for (m)RNA extraction and RT-qPCR. The viability of oocysts was verified by excystation test on pure suspensions following the description in Chapter 2 (section 2.7). Aliquots of washed manure (90 µl or 60 µl) were spiked with defined concentrations of oocysts: 5 x 10^4 or 10^5 for RNA extractions with GITC-PC method 1 (as indicated) and 5 x 10^5 for mRNA extraction with magnetic beads method 2 (unless otherwise stated). Different oocyst numbers were spiked, as one tenth of the total lysate volume was used for purification with method 2. Samples were heat shock induced for *hsp70* production at 45°C for 20 min, placed shortly in ice water, and then mRNA extraction was immediately started. Negative controls consisted of mRNA extraction from manure with oocysts that were inactivated at 95°C for 20 min in a waterbath. These samples were then cooled down at room temperature for 5 min, placed shortly on ice, and then heat shock induced for *hsp70* mRNA production.

The efficiency of mRNA extraction with the oligo $(dT)_{25}$-magnetic beads method 2 was further evaluated with samples of oocysts in pure double distillate water, manure, fermented manure (content of the thermophilic digester), and liquid manure supernatant. The latter had no particulate matter and it was the supernatant after centrifugation of fermented manure at 2,500 x g for 5 min. Like this, the sample still maintained the osmotic pressure and (potentially toxic) composition of the fermented material, which allowed evaluating the performance of the extraction and detection method. Samples (60 µl) were spiked with 5 x 10^5 oocysts, mixed with 50 µl 1x PBS buffer in 2 ml Eppendorf tubes, and heat shock induced for mRNA production. Immediately thereafter, mRNA extraction was performed.

5.2.2 Nucleic acid extraction and RT-qPCR

Oocyst lysis was done by 165 s cumulative bead beating as described in Chapter 4 (section 4.2.2), except that for soil samples 100 µl of 1x PBS buffer was added to each bead beating interval. Extraction and purification of mRNA or total RNA was done following the procedures in Chapter 4 (sections 4.2.4, 4.2.5): total RNA extraction based on guanidinium isothiocyanate and phenol-chloroform (GITC-PC, method 1) and the direct mRNA extraction with oligo $(dT)_{25}$-coated magnetic beads and LiCl-based buffer (method 2). Total RNA with method 1 was eluted in 50 µl or 100 µl DEPC water. Extracts were treated with DNAse to remove co-extracted DNA. A two-step RT-qPCR was performed as described in Chapter 2 (section 2.5).

5.2.3 Analysis

The performance of the extraction/detection assay was assessed with Ct values, whereby lower Ct indicates higher efficiency. Evaluations were done by comparison of the detected copy numbers and Ct values using equation 2 (Chapter 2, section 2.6). For comparative analysis, Ct values were always obtained by setting the same threshold in the exponential phase of the amplification curves (usually at 0.015 or 0.020 Rn) above the fluorescence background. The precision of the RT-qPCR and replicate variations were measured by the standard deviation and the coefficient of variation of Ct values.

5.3 Results

5.3.1 Effect of the manure volume in the detection efficiency

Manure seeded with fresh viable oocysts was used for total RNA (method 1) and direct mRNA (method 2) extractions (Table 5.1). Extractions from 90 µl manure produced higher Ct values (lower *hsp70* cDNA yield) than with 60 µl manure. The higher manure volume may contain a higher load of RT-qPCR inhibitors, which can explain the lower cDNA yield with these extracts. In all cases with 90 µl manure, the oligo $(dT)_{25}$-magnetic beads system (method 2) yielded higher cDNA yields (lower Ct values), indicating a more efficient purification of mRNA (Table 5.1). For extractions with 60 µl manure, increased cDNA yields were obtained with both extraction methods. Elution of RNA in 50 µl pure water was tested in method 1 in order to concentrate the extracted nucleic acids in a small volume, but the cDNA yield was lower than with elution in 100 µl. This was most likely due to insufficient recovery of strands from the silica binding RNAMATRIX® during RNA elution with method 1.

Table 5.1 - Detection of *hsp70* cDNA from fresh oocysts in 90 µl or 60 µl manure using two extraction / purification methods. Ct qPCR values are shown. Underlined values correspond to high yields of cDNA for extractions from 60 µl manure.

Experiment	**Manure sample** (µl)	Elution vol. of RNA for method 1 (µl)	**GITC-PC, method 1**		**Magnetic beads – LiCl, method 2**[$]	
			mean Ct	SD	mean Ct	SD
1	90	50	30.64	0.18	28.81	0.38
2	90	50	31.25	0.27	29.74	0.10
3	90	100	29.60	0.31	28.81	0.55
4	60	100	<u>28.07</u>	0.06	<u>27.58</u>	0.54

Experiment 1 (n=5), experiment 2 (n=4), experiment 3 (n=3), experiment 4 (n=6). Total RNA extraction with GITC-PC method 1, and direct mRNA extraction with oligo $(dT)_{25}$-magnetic beads method 2. [$]: elution of mRNA was done in 50 µl Tris-HCl. cDNA is transcribed from mRNA by RT.

The qPCR efficiency and assay sensitivity for extractions with 90 µl manure is shown in Fig. 5.1. Higher Ct values for *hsp70* cDNA were obtained for method 1, indicating reduced copy detection than with method 2. With total RNA extraction method 1, at least 1 Ct value from the dilution with (calculated) 1.7 dscDNA copies was higher than expected from the regression curve, whereas all Ct values for the magnetic beads method 2 matched the corresponding curve at this point (Fig. 5.1). No qPCR amplification signal was obtained with either extraction method for the highest dilution theoretically containing 0.17 dscDNA copies. For extractions with 90 µl manure still the analytical detection limit was low: 1.7 *hsp70* dscDNA copies / qPCR reaction for the magnetic beads method 2. The efficiency of the qPCR amplification was 89 % for the extraction method 1 and 88 % for method 2, indicating that the performance of the qPCR reactions was similar with both extracts.

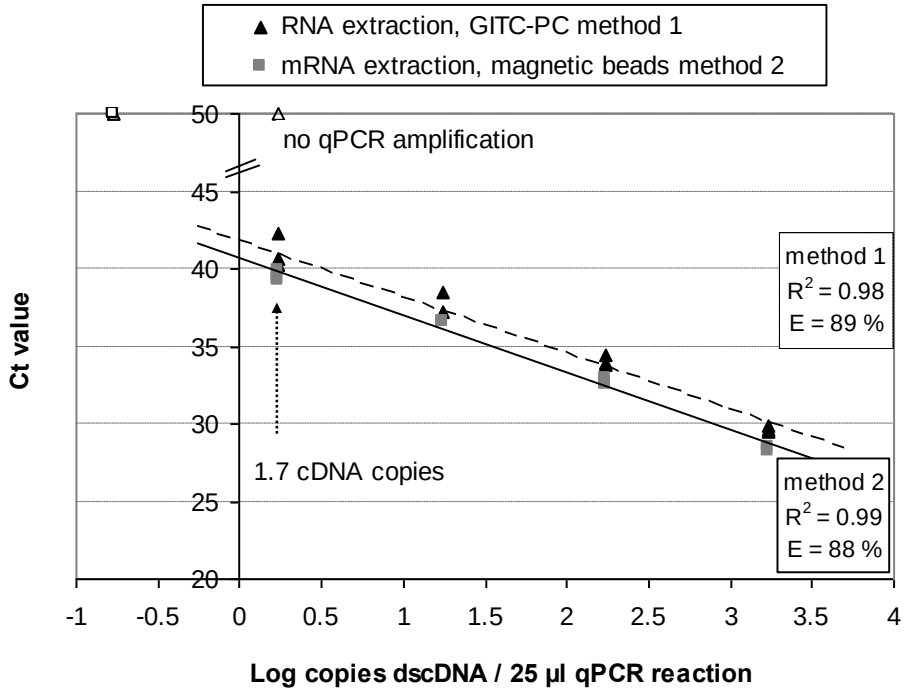

Fig. 5.1 - Linear regression of measured Ct values versus the copy number of *hsp70* dscDNA in serial dilutions of extracts from 90 µl manure. It shows the sensitivity of the assays and the higher efficiency of the extraction method 2 (lower Ct values) than the total RNA extraction method 1. Open symbols: no qPCR amplification; E: qPCR efficiency.

Figure 5.2 shows the comparison of extraction and detection efficiency using 60 and 90 µl manure with the direct mRNA extraction method 2. The extraction / detection efficiency of assays with 90 µl manure, determined by comparison of the linear regression curves, was 43 % with respect to extractions with 60 µl sample. A slightly higher efficiency of the qPCR reactions was obtained when using extracts from 60 µl manure than with 90 µl sample (91 % versus 88 %). Nevertheless, high sensitivity in the qPCR assay was obtained using both manure volumes with the mRNA extraction method 2: qPCR amplification signals for 1.5 - 1.7 calculated dscDNA copies / qPCR reaction were generated.

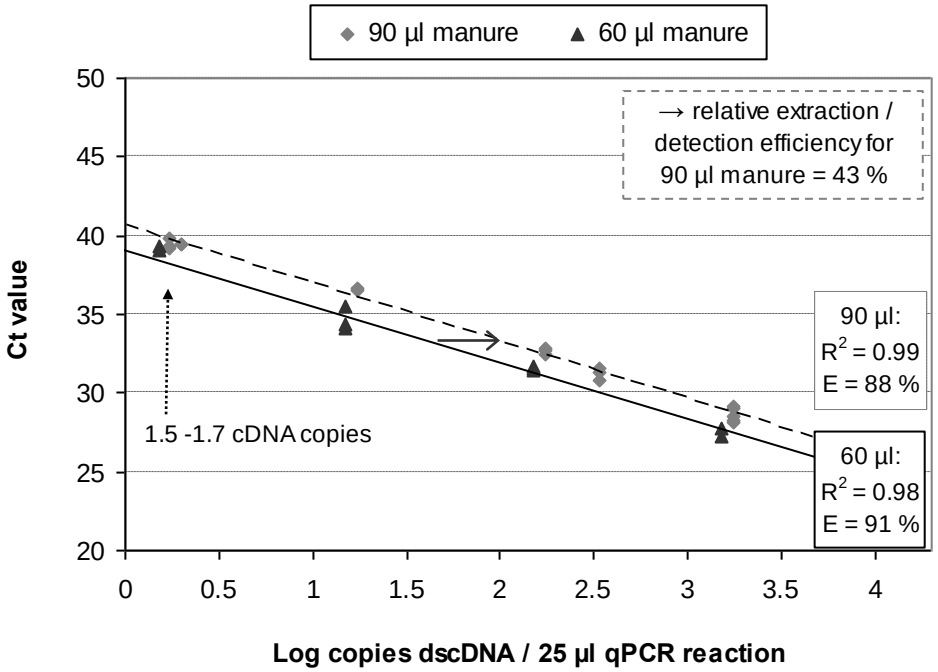

Fig. 5.2 - Linear regression of measured Ct values versus the copy number of *hsp70* dscDNA in serial dilutions of extracts from 60 µl and 90 µl manure using the direct mRNA extraction method 2 with oligo $(dT)_{25}$-magnetic beads. It shows a higher efficiency of extraction and detection with 60 µl manure sample. E: qPCR efficiency.

5.3.2 Yield of mRNA in aged oocysts using two extraction methods

Fresh (1 month) and aged oocysts showed different viability, as measured by their ability to excyst in the excystation test (Fig. 5.3). Both (m)RNA extraction methods yielded reduced *hsp70* mRNA content with aged oocysts in manure, in comparison to samples with fresh organisms. Copy numbers in 5 and 9 months old oocysts were on average 0.7 log units lower than in fresher oocysts when extractions were done with method 1, and this reduction was about 1.1 log units when extractions were done with the oligo $(dT)_{25}$-magnetic beads method 2 (Fig. 5.3). Accordingly, results from excystation test showed that 74 % of oocysts were able to excyst in samples aged for 5 months, and about 10 % were viable in samples with 9 months old oocysts.

Samples with 12 months old oocysts did not show excysted organisms after excystation trigger (oocysts were not viable). Very few *hsp70* mRNA copies were measured in these samples and were only detectable when the total RNA extraction GITC-PC method 1 was used. In these samples, the detected copy numbers were 1.8 log units lower than in fresh oocysts that were fully viable. No *hsp70* mRNA copies was found in samples with inactivated oocysts (after

exposure to 90°C / 20 min) using both extraction methods. Similarly, the excystation capability of oocysts was totally reduced (oocyst were not metabolic active).

Fig. 5.3 also shows that the decline of mRNA content in 5 months old oocysts was more pronounced than for their ability to excyst, indicating that excystation test can overestimate the fraction of viable oocysts in samples with aged organisms. Production of *hsp70* mRNA in oocysts aged for 5 months may be reduced as a result of metabolic decline in aged oocysts.

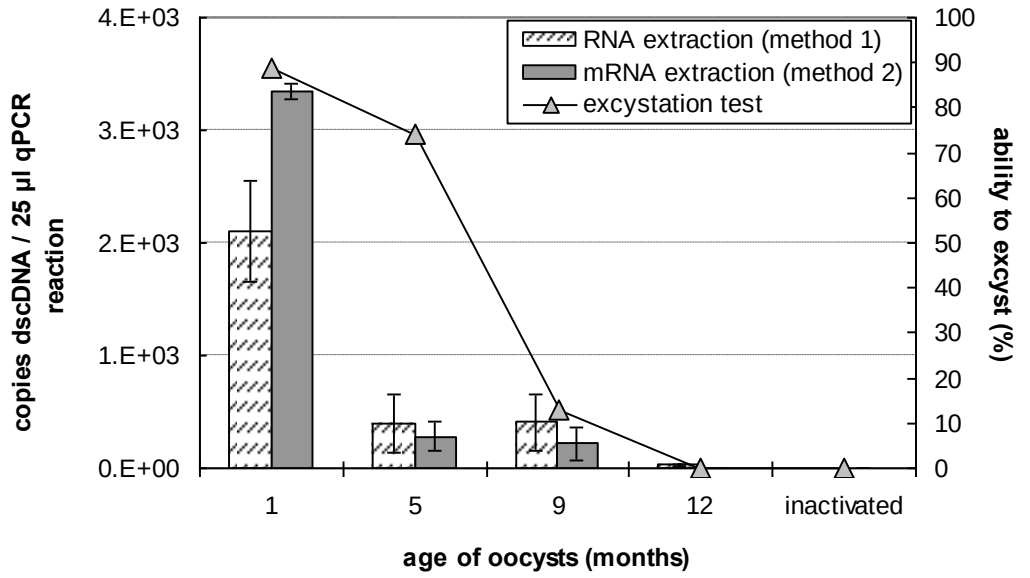

Fig. 5.3 – Detection of *hsp70* cDNA in fresh, aged and inactivated oocysts in manure evaluated with the total RNA extraction GITC-PC method 1 and the direct mRNA extraction with oligo $(dT)_{25}$-coated magnetic beads, method 2. Secondary y-axis: viability of oocysts measured by their ability to excyst in excystation test.

5.3.3 Application of the direct mRNA extraction method with different matrices

The applicability of the optimized mRNA extraction method 2 and RT-qPCR was assessed with different matrices and oocyst suspensions. Table 5.2 shows mean Ct values for *hsp70* cDNA measured in each matrix. Ct values correspond to detection of about 1,700 analytical dscDNA copies / qPCR reaction (or detection of 5 x 10^5 oocysts / extracted sample).

All mean Ct values irrespective of the matrix and oocyst suspension were in the range of 26.3 – 28.6, indicating no major variation due to the matrix, as it was the case for soil samples (see next sub-section), and a good performance of the extraction and detection method. Pure water and liquid manure supernatant had no fibers and particulate material, as opposed to fermented manure and manure samples that contained 6 % and 7.5 % of dry matter, respectively.

Table 5.2 - Precision of the extraction and detection method in terms of variation of Ct values (standard deviation and coefficient of variation) for oocysts in different matrices. Mean Ct of *hsp70* cDNA are shown. Intra-assay variation for qPCR replicates in the same qPCR run (left side) and sample variation for replicates in the same matrix (right side). mRNA extraction was done with the oligo $(dT)_{25}$-coated magnetic beads, method 2.

Substrate	Oocyst * suspension	Intra-assay variation			Sample variation		
		Mean Ct , same qPCR run (n=3-4)	SD	CV (%)	Mean Ct$^{\$}$ (n=7-11)	SD	CV (%)
Manure	S1	28.67	0.25	0.86	28.24	0.43	1.52
	S2	27.78	0.25	0.91			
	S4	28.27	0.16	0.55			
Liquid manure supernatant	S3	27.66	0.07	0.26	27.65	0.42	1.51
	S4	27.98	0.14	0.51			
	S5	27.00	0.08	0.28			
Fermented manure	S2	26.34	0.18	0.68	26.92	0.76	2.81
	S4	27.71	0.26	0.94			
Pure water	S2	26.84	0.28	1.05	27.10	0.85	3.15
	S2	26.49	0.49	1.85			
	S3	28.30	0.36	1.25			

*: oocyst stock suspensions seeded to substrates; $^{\$}$: mean Ct of all qPCR reactions with extracts of the same matrix.

The intra-assay variation for samples in the same qPCR run and for the same matrix was evaluated in terms of variation of Ct values (left side, Table 5.2). Low standard deviations of < 0.49 and CV < 1.85 % indicated a good assay performance. The variation of the complete extraction / detection assay, measured for replicate samples of the same matrix and from different qPCR runs (performed in different days), showed a CV < 3.15, indicating the reproducibility of the assay. This sample variation includes the inherent variance due to qPCR reactions performed in different runs.

The performance of the mRNA extraction and detection method with different matrices was evaluated in parallel experiments by comparison of Ct values and the detected copy numbers (Table 5.3). The yield of cDNA obtained in the matrices was similar. Extractions in pure water and (fermented) manure yielded similar results in parallel experiments, which indicated an adequate purification and detection of *hsp70* mRNA from manure containing samples, and no RT-qPCR interference due to inhibitory substances present in these samples. The slightly lower cDNA measured in water with suspension S3 could have been due to a slight variation in

mRNA extraction efficiency, but this was most likely due to inherent variation in qPCR reactions. The latter is because individual Ct values obtained for these samples were within a relatively small range, i.e. 27.6 – 28.6 with a 0.42 SD.

Table 5.3 – Comparative detection of *hsp70* cDNA in different matrices seeded with 5 x 10^5 viable oocysts.

Oocyst * suspension	Substrate	Mean Ct	Delta Ct	Measured cDNA copies / qPCR	Relative detection factor
S2	Pure water *vs*	26.65		4.0 x 10^3	
	Fermented manure	26.34	0.31	4.8 x 10^3	1.19
S3	Pure water *vs*	28.30		3.2 x 10^3	
	Liquid manure supernatant	27.66	0.64	4.8 x 10^3	1.50
S4	Pure water *vs*	29.72		8.1 x 10^2	
	Manure	29.23	0.49	1.1 x 10^3	1.34
S4	Liquid manure supernatant *vs*	27.98		2.5 x 10^3	
	Fermented manure	27.71	0.27	3.0 x 10^3	1.21
S4	Manure *vs*	28.27		2.0 x 10^3	
	Liquid manure supernatant	27.98	0.29	2.5 x 10^3	1.25

*: oocyst stock suspension seeded to substrate; *vs*: versus.

Real-time qPCR plots in Fig. 5.4 shows amplification curves for *hsp70* cDNA in extracts from pure water, manure, and liquid manure supernatant spiked with viable oocysts from stock suspension S4. For pure water, an amplification signal was detected for the lowest dilution containing 1.7 dscDNA copies. For manure and liquid manure supernatant, qPCR amplification signals were measurable in all replicates containing 1.7 dscDNA copies. With these samples, even an amplification curve was occasionally detected for samples containing < 1.7 calculated dscDNA copies (e.g. Fig. 5.4-B). The detection limit of the extraction and detection assay was 490 oocysts per 60 µl extracted manure or liquid manure supernatant, using the oligo $(dT)_{25}$ based method 2 for direct mRNA extraction.

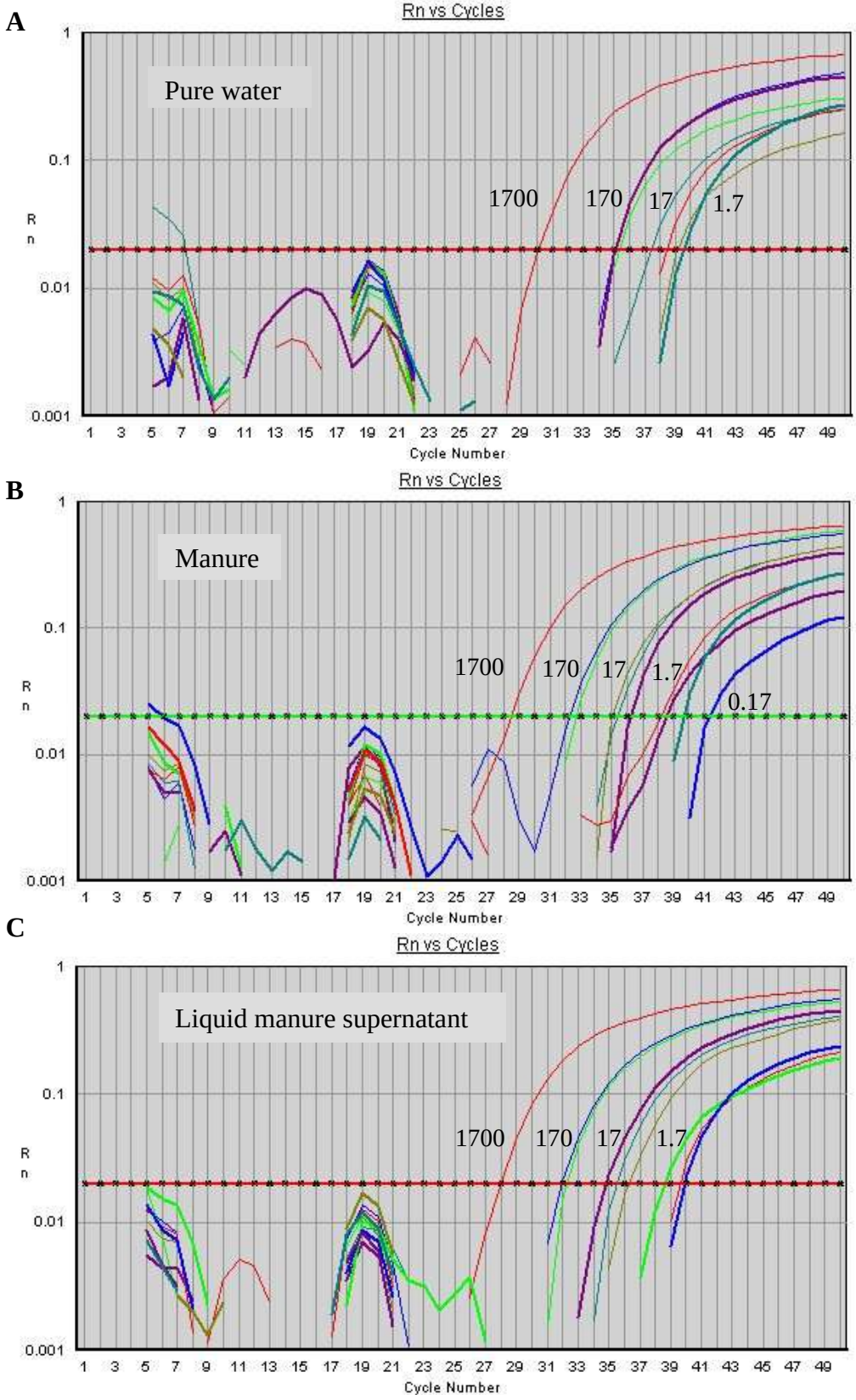

Fig. 5.4 – Real-time qPCR amplification plots: cycle number (X-axis) versus cumulated fluorescence (Y-axis), depicting amplification curves for *hsp70* cDNA in pure water (A), manure (B) and liquid manure supernatant (C). Numbers represent the calculated dscDNA copies equivalent to DNA present in the sample. Threshold (horizontal line) was set at 0.020 Rn.

Soil matrices

Processing soil samples with the optimized total RNA and mRNA extraction methods yielded practically no *hsp70* cDNA copies by RT-qPCR. Only low amplification curves were obtained in two sample replicates with minimum-tilled and tilled soils seeded with 5 x 10^5 oocysts: Ct = 40.35 and Ct = 38.17, respectively (Table 5.5). The cDNA detected in the soil samples was 2 – 2.6 log units lower than that measured in manure using the same oocyst stock suspension. The following was observed during sample processing, which may have influenced the results: a) extractions from soil samples produced a dense lysate after bead beating, most likely due to soil components, and b) there was a slow migration of the hybrid mRNA-magnetic beads towards the magnet during mRNA extraction with the magnetic beads system (method 2), perhaps due to the viscous suspension. Increasing the time of hybridization of the mRNA to complementary magnetic beads from 7 min (as usually done for manure) to 15 min, to ensure an adequate attachment of the strands, did not yield better results.

To verify possible inhibition of RT-qPCR by inhibitory substances in the soil samples, qPCR reactions were done with serial dilutions of RT product, as diluting cDNA would also reduce the concentration of potential inhibitory compounds. This has worked for the detection of DNA by qPCR in the soil matrices, as reported in Chapter 3. With mRNA extracts, however, no cDNA was measured in the diluted samples (Table 5.4, Table 5.5). Reverse transcription reactions were also performed with serial dilutions of extracted mRNA to verify if inhibition was occurring at the RT level. No qPCR amplification (Ct=50) was detected using these samples.

Table 5.4 - Mean Ct values for *hsp70* cDNA in extracts from oocysts in soil (upper part) and manure. Total RNA extraction with the GITC-PC based system, method 1.

Oocysts / sample*	**Copies *hsp70* dscDNA / qPCR**	**Tilled soil** (0 - 8 cm)	**Minimum-tilled soil** (0 - 8 cm)	**Manure**
		mean Ct (n=6)	mean Ct (n=6)	mean Ct (n=4)
1 x 10^5	1730	nd	nd	30.76 ± 0.16
1 x 10^4	173	nd	nd	-
1 x 10^3	17	nd	-	-

*: calculated number of oocysts per 50 mg soil and 60 µl manure; nd: no qPCR amplification detected.

Table 5.5 - Mean Ct values for *hsp70* cDNA in extracts from soil (2 depths) and manure. Direct mRNA extraction with oligo $(dT)_{25}$-magnetic beads, method 2.

Oocysts / sample*	Copies *hsp70* dscDNA / qPCR	Minimum-tilled soil		Tilled soil		Manure
		(0 - 8 cm)	(8 - 22 cm)	(0 - 8 cm)	(8 - 22 cm)	
		mean Ct (n=6)	Mean Ct (n=6)	mean Ct (n=6)	mean Ct (n=6)	mean Ct (n=3)
5×10^5	1730	40.35**	nd	38.17**	nd	30.75 ± 0.3
5×10^4	173	nd	-	nd	-	34.58 ± 0.5
5×10^3	17	nd	-	nd	-	36.95 ± 0.8

*: calculated number of oocysts in each sample (50 mg soil or 60 µl manure); a: soil 8 cm; b: soil 22 cm;
**: one qPCR amplification signal; nd: no qPCR amplification detected.

5.4 Discussion

A total RNA extraction method that uses GITC-PC (method 1) and a direct mRNA extraction method with oligo $(dT)_{25}$-magnetic beads (method 2) were evaluated to quantify *hsp70* mRNA from heat induced viable and from injured oocysts. Evaluations were done with respect to factors that can affect the extraction efficiency and the overall assay sensitivity. These are the amount of substrate processed, the age/constitution of oocysts, and the sample matrix.

5.4.1 Total RNA versus direct mRNA extraction for manure

Manure contains humic substances that can affect RT-qPCR reactions (Wilson, 1997). Optimized mRNA purification procedures should therefore produce extracts of good quality that can be used for the enzymatic reactions. Here it was found that even small differences in the manure volume extracted (90 µl versus 60 µl) had an effect on the overall efficiency of the assay. This was most likely due to a different load of RT-qPCR-inhibitory substances. The performance of the extraction / detection assay was slightly lower when processing 90 µl of manure compared to 60 µl sample with both, the total RNA and the mRNA extraction method (Table 5.1 and Fig. 5.2). The higher cDNA yield with 90 µl manure obtained in extracts of the magnetic beads method 2 than in extracts of the total RNA extraction method 1 (about 2 fold difference, Fig. 5.1) indicated that method 2 was more effective to recover clean mRNA from the sample. It is possible that with a higher concentration of interfering compounds in the 90 µl sample, the total RNA was less efficiently recovered from the silica RNAMATRIX® slurry during the final RNA elution step in method 1. Interfering substances like humic compounds can also bind to the silica matrix, trap RNA strands (as well as DNA), and decrease the capacity of the silica material in the slurry to bind RNA (Tian et al., 2000).

The direct mRNA extraction with magnetic beads (method 2) does not use a silica based purification system. The principle of extraction / purification is based on hybridization of a specific mRNA segment (the poly A tail) to complementary bases of the coated magnetic beads. Using 60 µl manure for extraction, both extraction / purification procedures (method 1 and method 2) yielded comparable results. The negative effect of processing an increased volume of complex material on the efficiency of the mRNA extraction and RT-qPCR detection points out that the performance of an optimized adjusted method must be checked if the amount of sample used for analysis is increased.

Nevertheless, using 90 µl manure still allowed sensitive detection of cDNA amplification signals by qPCR. Thus processing of no more than 90 µl, preferably 60 µl, of manure per extraction is recommended with the tested systems in order to minimize counteracting effects of humic acids or other inhibitors during (m)RNA purification or RT-qPCR.

In the selection of extraction methods for routine application, also other factors such as the time required for sample processing, the practicability of the methods, equipment needed, and costs must be considered. The oligo $(dT)_{25}$ based method 2 provides results more quickly in about 5 h (8 h when including qPCR) compared to 7 - 8 h (10 h with qPCR) with the total RNA extraction GITC-PC method 1. The total RNA extraction method 1 needs extra care due to the use of phenol-chloroform, and this step impedes the processing of many samples, whereas extraction method 2 avoids these toxic substances. This is considered a valid point for lab work.

5.4.2 Yield of mRNA in aged oocysts

Aged oocysts were used to evaluate the two optimized (m)RNA extraction methods and to assess the detection of *hsp70* mRNA in viable and aged organisms. RT-qPCR assays that target mRNA molecules can be useful to identify viable pathogens (Coutard et al., 2005; Smith et al., 2007; Bui et al., 2011) because mRNAs are transcribed from DNA only in living organisms. mRNA has a much shorter-half life than DNA and is therefore a better parameter to assess viable organisms. The absence of an *hsp70* mRNA signal in control measurements with inactive oocysts is in accordance with the capability of only viable organisms to produce mRNA. Results showed a time-dependent decline in *hsp70* mRNA content in aging oocysts measured with both extraction / purification methods (Fig. 5.3), which correlated positively with the viability of oocysts. The reduced mRNA content in aged oocysts is attributed to the smaller fraction of viable organisms present in the sample, and this was accompanied by lower production of *hsp70* mRNA after heat shock induction. *Hsp70* mRNA molecules were still detected with both extraction methods in samples with 9 months old oocysts that were still 10 % viable (Fig. 5.3). These results also indicate the high resistance of oocysts at cool temperatures (4°C), suggesting

that in natural environments at similar conditions, oocysts may be potentially infectious agents for a long time. Robertson et al. (1992) assessed the viability of oocysts after 5.8 months at 4°C and found viable organisms.

The loss of viability in 12 months aged oocysts indicated that the low RT-qPCR amplification signals measured only in extracts from total RNA extraction method 1 corresponded to residual mRNA from inactive oocysts. It seems that *hsp70* transcripts can, to a limited extent, be stable and detectable in injured organisms, if the surrounding environment has low biological (degradation) activity (such as at low temperatures, 4°C). At low temperatures, the action of endogenous RNAses that can persist in inactive organisms is limited, and the metabolic activity of the surrounding microbial community, which can metabolize components of dead organisms, is also reduced.

The slightly lower *hsp70* mRNA content measured in aged oocysts with the direct mRNA extraction method 2 in comparison to total RNA extraction method 1 (Fig. 5.3) may have been due to changes in the structure of mRNA strands in aged oocysts and to differences in primer / probe hybridization during RT-qPCR. One of the mechanisms of mRNA decay in eukaryotes is initiated by shortening of the poly A tail (Beelman et al., 1995; Körner et al., 1998). Therefore it is possible that less intact transcripts were available for extraction by the direct extraction method 2, which is based on binding the poly A tail of the mRNA to oligo $(dT)_{25}$-coated magnetic beads. This can explain the detection of weak *hsp70* signal from 12 months old inactive oocysts in total RNA extracts from method 1, but not in mRNA extracts from extraction method 2.

Even though the *hsp70* mRNA content in oocysts aged for 5 months was reduced to a level that was similar than after 9 months, the excystation capability after 5 months aging was still relatively high, suggesting that excystation can overestimate the fraction of viable and potentially infectious oocysts in aged and injured organisms at 4°C. This explanation is in accordance with findings, that the excystation capability of oocysts is dependent on the content of carbohydrate energy reserves in the form of amylopectin (Jenkins et al., 2003; Fayer, 2008). Thus it can be that oocysts after 5 months at 4°C are able to respond to excystation triggers but display reduced metabolic activity with a concomitantly reduced transcription rate.

5.4.3 Analysis with soil

Soil was used for analysis because it is a very complex substrate, and it is still a challenge to obtain clean mRNA extracts that can be used for RT-qPCR, as described by Saleh-Lakha et al. (2005). This work showed that the procedures optimized for manure were suboptimal for soil samples from an agricultural field. Suboptimal detection of *hsp70* cDNA in the evaluated soils

indicated strong interference with soil components. Soil contains high amounts of humic substances as a result of organic fertilization and organic matter degradation, a great diversity of soil microbes, and minerals, that makes soil a difficult matrix to extract nucleic acids, particularly to recover mRNA strands with short-half life. No qPCR amplification signal in reactions with cDNA dilutions, with reduced concentration of inhibitors (but also of cDNA), indicated that inhibition of the DNA-polymerase may not have been the reason for the lack of qPCR signal, provided that cDNA was not diluted out. Since also no qPCR amplification was detected when diluted mRNA extracts were used for the RT reaction, strong inhibition of the reverse transcription or an insufficient amount of mRNA copies in the extracted sample may have been reasons for the amplification failure. For the latter case, the cause may have been an inadequate extraction / purification. The tested soil samples contain 26 - 28 % of clay particles. Clay colloids can adsorb nucleic acids through ionic binding, and this can hinder their extraction (Ogram et al., 1988; Frostegård et al., 1999; Cai et al., 2006). Thus, it is possible that mRNA molecules interacted with minerals or humic compounds in the soil lysate resulting in strongly reduced extraction efficiency. Even if mRNA is extracted, interaction of co-extracted RT-qPCR inhibitors or minerals with mRNA may also prevent mRNA reverse transcription and consequently qPCR amplification (Alm et al., 2000).

Co-extracted impurities can sometimes be observed visually during purification. With the GITC-PC method 1, a dark yellow-brownish silica pellet was sometimes seen before the final RNA elution step. Using Quick-Clean spin filter membranes (BIO 101® Systems, Qbiogene) as a second purification of total RNA extracts did not yield better results with the tested soils. It is possible that complexes of humic compounds and clay particles bind to silica matrix or filter membranes, resulting in saturation or clogging of ionic adsorption sites that should serve for the binding of mRNA strands (Alm et al., 2000). Reduced nucleic acid recovery has been reported using silica membranes with samples containing humic acids (Hata et al., 2011).

Problems with the extraction method 2 may have been due to suboptimal attachment of mRNA to oligo $(dT)_{25}$–coated beads in the dense soil lysate and the presence of complex mineral compounds, resulting in reduced mRNA attachment to the beads, even with the extended hybridization time.

Furthermore, soils can contain high amount of exogenous nucleases which can degrade mRNA during extraction / purification, if they are not completely inactivated. mRNA strands are more prone to degradation by nucleases than DNA (Bach et al., 1999). This can also explain why DNA copies were very well detected in the soils (Chapter 4), but almost no mRNA strands in the same matrices (this chapter).

In contrast to studies that employed total RNA extraction methods to detect ribosomal rRNA from soil samples (e.g. Griffiths et al., 2000; Sessitsch et al., 2002), mRNA molecules were the target in this study, which are present in much lower numbers than total RNA (about 1 – 5 % of total RNA). This makes extraction from complex matrices much more difficult.

Overall, the performance of molecular methods can vary according to the type of substrate, and therefore, it must be verified if the applied procedures are suitable for the particular complex substrate (e.g. soils) that is analyzed.

5.4.4 Assay performance with the direct mRNA extraction method 2

The optimized extraction and detection procedures developed for manure are also applicable for more simple matrices like pure water. Obtaining precision and reproducible results is essential for the establishment of RT-qPCR based methods for the analysis of complex environmental matrices. Consistent detection of *hsp70* mRNA, measured as Ct values, were obtained in RT-qPCR replicates in the same run, and the variation of Ct values from assays performed in different days was also low (Table 5.2). These variations were in the range of realistic measurements for RT-qPCR (Pfaffl, 2004). For qPCR replicates in the same run, the low SD of Ct values, with the majority lower than 0.28 SD, indicates a good assay performance. Results showed the good performance of the developed procedures (comprising the extraction, purification and detection system) using different oocysts suspensions and different matrices.

In practice, sample to sample variations can be due to small variation in sample pipetting, the use of different reaction tubes, the preparation of reactants and buffers, the use of different real-time thermocyclers and variations in the qPCR reaction itself (Bustin, 2000). Therefore, the control for Ct variation is essential to ensure the quality of results. In any case, it is recommended the use of same equipment and reaction tubes, and when possible to perform parallel reactions to avoid inter-assay variation between different qPCR runs (Larionov et al., 2005). Ct variation measured from replicate extractions using the same matrix (Table 5.2) includes differences that may exist due to the oocyst source, as *Cryptosporidium* oocysts from different suspensions can differ in wall strength or physiological constitution (Campbell et al., 1992; Xiao et al., 2004) with concomitantly varying content or productivity of mRNA. For this study, all fresh oocyst sources were field isolates of *C. parvum* from cattle infections in Germany (Najdrowski et al., 2007b).

The parallel comparison of different matrices yielded similar extraction and detection efficiencies, as verified by the Ct values (Table 5.3). Good performance of the developed assay was achieved particularly with manure that contains high amounts of qPCR inhibitory substances. The slightly lower cDNA yield for oocysts in water in comparison to oocysts in

liquid manure supernatant can be due to intra-assay variation because the variation of the Ct values was small (SD = 0.42), and not due to differences in oocysts because the comparisons were done with the same oocyst suspension. Amplification signals were detectable for 1.7 dscDNA copies / qPCR reaction in these matrices (Fig. 5.4). Generally, higher variation of Ct values is expected for samples containing low concentrations of the target nucleic acid. For samples with low template concentration, as shown in Fig. 5.1, higher Ct variation was obtained with the total RNA extraction method 1 compared to the magnetic beads mRNA extraction method 2, thus suggesting a slightly better performance of extraction method 2 with the given samples.

In summary, the evaluations in this chapter confirm the good performance of the optimized (m)RNA extraction procedures for processing manure and water-based matrices. *Hsp70* mRNA was consistently detected by RT-qPCR. The analytical detection limit using the system oligo $(dT)_{25}$-magnetic beads method 2 was 1.5 - 1.7 dscDNA copies / qPCR reaction. With the given extraction procedure including extraction from 90 µl (preferably 60 µL) manure, the method detection limit for cDNA from one extraction was in the range of $5.5 \times 10^3 - 8.1 \times 10^3$ / ml manure containing fresh viable oocysts. qPCR amplification signals were also occasionally measured from 550 - 810 oocysts / ml manure, but with these concentrations not all of the qPCR repetitions gave a positive signal. At least two positive signals from three qPCR repetitions should be obtained to conclude that the reactions are not caused by a qPCR artefact. It is to note that no amplification signals were produced in no-template controls. This ensured that measuring high Ct values at low template concentration (close to the detection limit of the assay) still corresponded to *hsp70* mRNA copies, and were not the product of enzymatic artefacts (D'haene et al., 2010), which may occur with some other qPCR chemistry.

Typically infected cattle shed oocysts in the range of 10^6 - 10^7 oocysts / g of faeces (WHO, 2006), and this can reach 10,000 millions / day (WHO, 2011). Asymptomatic cattle, registered as healthy animals, can also shed oocysts, but in a lower fraction. The level of contamination with oocysts in the environment will vary according to the load of contaminated manure and the dynamics of oocyst dissemination in the environment (Kuczynska et al., 2005).

In order to decrease the detection limit of the complete assay, the direct extraction of mRNA with the magnetic beads system can be scaled up to process more lysate volume, or the extracted mRNA can be concentrated. The following can be performed:

i) 500 µl to 1 ml lysate can be purified, taking into account that an additional reagent volume has to be added to the reaction. Processing 500 µl lysate (with 50 µl magnetic beads suspension) can reduce the detection limit of the complete assay by 5 times.

ii) the bound mRNA can be eluted in a single step in 25 µl Tris-HCl. While a second elution could elute a few more mRNA copies (about 0.15 %, not shown), mRNA elution in one volume is feasible.

With these two modifications the method detection limit (including the extraction and detection) can be reduced to 5.5 x 10^2 or 8.1 x 10^2 oocysts / ml manure (for 90 or 60 µl manure extracted, respectively).

In conclusion, both the total RNA and the direct mRNA extraction method are suitable in combination with RT-qPCR for the detection of heat induced *hsp70* mRNA production, with the latter method yielding a slightly higher efficiency. The direct mRNA extraction is a shorter procedure and therefore can reduce chances of mRNA loss by degradation. Extraction method 2 can be used with RT-qPCR for the screening of *C. parvum* oocysts in environmental samples to provide fast information on the presence and viability of this pathogen.

Box : key outcomes Chapter 5

- Using 60 µl manure, comparable extraction and detection of *hsp70* mRNA by qPCR was obtained with both extraction methods: total RNA extraction with GITC-PC and isopropanol purification, and direct mRNA extraction with magnetic beads and LiCl-LiDS purification. Using 90 µl manure, direct extraction method 2 produced extracts of better quality, as verified by slightly lower Ct values compared to the total RNA extraction method 1.
- The volume of extracted material affected the extraction and detection efficiency, most likely due to a higher load of inhibitory substances in 90 µl manure than in 60 µl (43 % extraction and detection efficiency with 90 µl manure using the magnetic beads method). Despite this, extractions from 90 µl manure still yielded sensitive detection (1.7 dscDNA copies / qPCR reaction), but no signal was measured for 0.17 calculated copies in the reaction volume, as opposed to signals occasionally detected when 60 µl manure was used.
- The performance of the extraction and detection method 2 was similar with and applicable for (digested) manure and water samples. Low variation of Ct values (SD < 0.49) for reactions in the same qPCR run indicated good assay performance and qPCR precision in extracts from complex material such as manure.
- The method detection limit (comprising the extraction and detection system) for extractions

from 60 - 90 µl manure with the oligo $(dT)_{25}$-magnetic beads method 2 was obtained for samples containing 5.5 x 10^3 – 8.1 x 10^3 viable oocysts / ml sample. Occasionally, amplification signals were measured for 5.5 x 10^2 – 8.1 x 10^2 oocysts / ml, but consistent detection could not be confirmed for all qPCR replicates.

- The optimized procedures for manure were suboptimal for the tested agricultural soils, most probably due to interference with humic compounds and minerals such as clay that impeded mRNA extraction and purification and/or produced strong inhibition of the RT.
- There was a positive relation between the reduction of the excystation capability and the measured *hsp70* mRNA content in oocysts aged at 4°C, and no transcripts were detected in heat inactivated oocysts.
- Occasionally, a low amount of residual mRNA was measured for inactive oocysts aged for 12 months only in samples extracted with total RNA extraction method 1, suggesting some stability of the transcripts in environments with low biological degradation, as is the case at 4°C. With the direct mRNA extraction method 2, mRNA was not detected in aged inactivated oocysts, most likely due to mRNA fracture and thus, lower recovery of mRNA (containing the PCR target region) by its poly A tail. Only intact transcripts were recovered from the lysate with this system.

CHAPTER 6

Measuring *C. parvum* inactivation in anaerobic digesters treating cattle manure by mRNA detection

6.1 Introduction

The uncontrolled discharge of manure containing *C. parvum* oocysts can lead to dissemination of this pathogen in the environment and to contamination of surface water, groundwater and land (Fayer, 2004; Kato et al., 2004). Oocysts are very resistant to different environmental stresses and apparently survive under a wide range of temperatures and stress conditions (Fayer et al., 2000; Chapter 1, section 1.7). The World Health Organization refers to *Cryptosporidium* as a health relevant pathogen (WHO, 2009), and therefore this organism is of interest in hygiene control of potentially contaminating organic sources (EPA, 2005).

Manure should be treated before it is reused or applied to agricultural land. Anaerobic digestion of manure is effective for reducing pathogens at high temperatures (thermophilic conditions) provided the substrate is retained in the digesters for sufficient time to ensure pathogen reduction (Oropeza et al., 2001; see Chapter 1, section 1.8). *C. parvum* oocysts, however, may not be significantly affected at mesophilic anaerobic conditions (Kato et al., 2003; see Chapter 1, section 1.8). Moreover, variations in process parameters or substrate composition, or suboptimal operation of the system can lead to insufficient sanitation of the treated substrate (Burton and Turner, 2003). It is because of this that monitoring for the presence of viable oocysts in digested manure can help to ensure the sanitizing performance of treatment processes.

The objective of this study was to evaluate the use of a molecular tool consisting of direct extraction of mRNA from the sample with magnetic beads and real-time RT-qPCR to indicate the inactivation of *C. parvum* oocysts after mesophilic and thermophilic anaerobic digestion of manure or after heat treatment only. This was done by quantification of the *hsp70* mRNA.

6.2 Materials and Methods

6.2.1 Anaerobic treatment of oocysts in manure

The evaluation of oocyst inactivation in anaerobic systems was done in sentinel chamber experiments (see a description of the experimental procedure in section 2.8, Chapter 2). Oocysts were subjected to anaerobic treatment at mesophilic (36.2°C) and thermophilic (50°C, 55°C) conditions, and were retained in the digesters for 1 h or 4 h. The time intervals corresponded to the feeding intervals for the digesters (Effenberger et al., 2006). Diffusion sentinel chambers were filled with manure or digester content (1.8 ml) and seeded with 1.1 x 10^7 and 1.2 x 10^7 oocysts / ml of fresh suspensions for experiments with suspension C and B, respectively. For experiments with suspension C, sentinel chambers were filled with the liquid phase after centrifugation of manure at 2,500 x g for 5 min in order to reduce particles in the substrate and to improve the performance of the subsequent infectivity test. After treatment at the respective retention time, samples were removed from the digesters and the contents of the sentinel chamber were carefully recovered by washing with 1x PBS buffer. Samples were concentrated by centrifugation and resuspended in a 2.8 ml volume.

Aliquots (100 µl) were subjected to heat shock treatment at 45°C for 20 min, according to Stinear et al. (1996), to induced the production of *hsp70* mRNA in the oocysts. Samples were placed shortly on ice and then mRNA extraction was started. For comparison, other samples were not subjected to heat shock induction. About 1.8 ml of the sample volume was used for the infectivity test in cell culture. Positive controls consisted of manure spiked with known amount of oocysts and not subjected to anaerobic treatment. Controls were heat shock induced, or not, for *hsp70* mRNA production.

6.2.2 Heat treatment of oocysts in waterbath

C. parvum oocysts were subjected to thermophilic conditions (50°C, 55°C) in a waterbath to determine only the effect of temperature on their metabolic activity. Experiments were performed with the same oocyst suspensions used for sentinel chamber experiments. Manure samples in 2 ml Eppendorf tubes were seeded with defined concentrations of oocysts (5 x 10^5 and 4 x 10^5 oocysts for tests with suspension C and B, respectively). Samples were heated in a waterbath for 1 h or 4 h and then placed shortly at room temperature. Samples were then induced for *hsp70* mRNA production at 45°C / 20 min. Other replicate samples were not heat shock induced. mRNA extraction was then performed.

6.2.3 Nucleic acid extraction and RT-qPCR

Oocyst lysis and mRNA extraction and purification were done according to the optimized procedures in Chapter 4. This included oocyst lysis with 165 s cumulative bead beating and extraction of mRNA with oligo $(dT)_{25}$-magnetic beads and LiCl based buffer (method 2). Extracted mRNA was treated with DNAse to remove co-extracted DNA. Two-step RT-qPCR was done with a specific chemistry composition as described in Chapter 2 (section 2.5).

6.2.4 Viability and infectivity tests

In vitro excystation was done to measure the oocyst viability in purified suspensions or in samples with digested manure. The dye permeability test (DAPI/PI) was also used to assess oocyst viability in pure suspensions. The infectivity of oocysts after anaerobic and heat treatment was verified in cell culture tests. The tests were done following the descriptions in Chapter 2 (section 2.7).

6.3 Results

6.3.1 Inactivation of *C. parvum* after anaerobic digestion and heat treatment

Oocysts from a stock suspension designated as B (Fig. 6.1, Fig. 6.2) were 85.5 % viable after 2 h incubation in the excystation test and 90 - 92 % viable after 24 h incubation. Sentinel chamber experiments (Fig. 6.1) showed a noticeable effect of the anaerobic thermophilic treatments on oocyst measurements, indicated by the lower cDNA content in comparison to controls not exposed to the digesters. There were detectable reductions of *hsp70* cDNA after 1 h or 4 h in the anaerobic digesters at 50°C or 55°C. cDNA copy numbers in samples exposed to 50°C for 1 h and 50°C for 4 h in the digesters were 15.8 % and 3.4 %, respectively, relative to that in untreated manure. Further reduction in copy numbers was obtained after digestion at 55°C for 1 h (1.4 – 1.8 % relative to controls).

Observations of sentinel chamber contents under the microscope showed intact oocysts, not bright, apparently functionally inactive after thermophilic anaerobic digestion in manure. Oocysts purified from digester content after treatment were unable to excyst in the excystation test, indicating no viable organism after 50°C or 55°C. Also, they were not infectious in cell culture after thermophilic anaerobic treatment at either substrate retention time.

In terms of *hsp70* mRNA content, the comparison of anaerobic treatment for 1 h at 50°C versus 55°C yielded about 10 fold less cDNA for oocysts at 55°C (significantly different, $p \leq 0.05$).

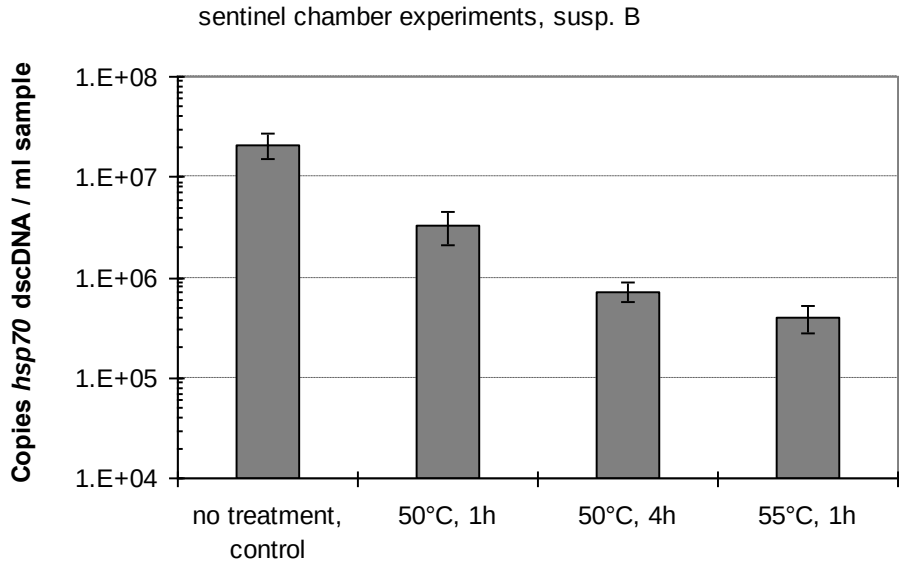

Fig. 6.1 – Reduction in *hsp70* cDNA content in oocysts (suspension B) subjected to thermophilic anaerobic treatment at different substrate retention times. cDNA content is significantly different than in controls ($p \leq 0.05$).

Oocysts exposed to heat treatment in waterbath experiments (Fig. 6.2) were not viable in the excystation test after the thermophilic treatments. Similarly as in the digesters, significantly reduced *hsp70* cDNA content ($p \leq 0.05$) was measured after 50°C and 55°C for 1 h, with respect to the non-treated samples. It was noted that this reduction was more pronounced than that measured after exposure to anaerobic digestion at the same temperature and time conditions: cDNA copy numbers were 2.4 % after 50°C for 1 h, and 0.3 – 0.6 % after 55°C for 1 h, relative to controls. The comparison of treatments at 50°C versus 55°C for 1 h yielded a significant lower content of *hsp70* cDNA for oocysts exposed to 55°C (14 – 24 %, Fig. 6.2).

The inactivation of oocysts by heat treatment was also verified by their ability to respond to heat shock induction under sub-lethal conditions (at 45°C; Lindquist and Craig, 1988). Oocysts were heat shock induced for *hsp70* mRNA production immediately after the thermophilic treatment, or not heat shock induced. Results showed comparable cDNA content in both type of samples, suggesting inability of oocysts for transcription, whereas in controls there was a slight difference, though not significant, in cDNA production (Fig. 6.2).

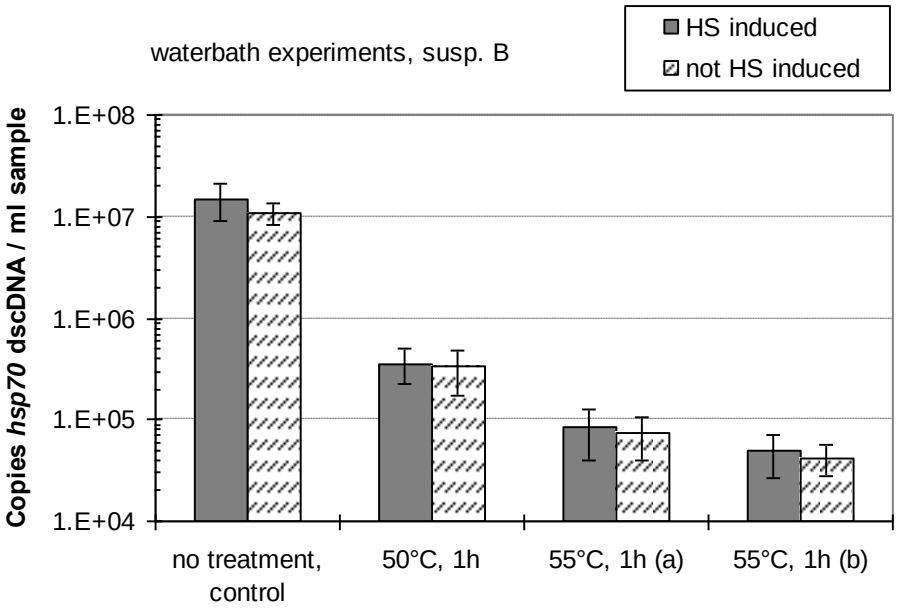

Fig. 6.2 – Reduction in *hsp70* cDNA content in oocysts (suspension B) subjected only to heat treatment in waterbath experiments. cDNA content is significantly different ($p \leq 0.05$) than in controls. Samples were heat shock induced (HS) or not (no HS) for mRNA production after heat treatment. a and b are replicate experiments performed with different stock samples of the same suspension.

The significantly lower *hsp7*0 cDNA content ($p \leq 0.05$) in the 50°C-waterbath samples compared to copies in samples from the anaerobic digesters at the same temperature suggested that oocysts in the anaerobic milieu were more resistant before their inactivation. For thermophilic anaerobic digestion or heat treatment only, the best agreement and the best results for cDNA reduction was found after 55°C for 1 h (0.3 – 1.8 % copy numbers relative to non-treated controls), indicating that temperature was the primary factor influencing mRNA degradation in the inactivated oocysts.

In general, detection of *hsp70* cDNA from oocyst that appeared inactive at 55°C (not viable and not infectious), indicated that oocysts were not completely degraded, neither by the heat treatment nor by combined heat and anaerobic conditions.

The qPCR signals were from residual mRNA from oocysts inactived at the conditions evaluated. Table 6.1 shows Ct values measured for residual mRNA in oocysts inactivated at thermophilic conditions. High Ct values (close to the detection limit of the qPCR assay) indicating low amount of cDNA copies are shown for 4.5×10^4 - 4.5×10^5 oocysts / ml sample after exposure to 55°C for 1 h or 50°C for 4 h.

Table 6.1 – Ct qPCR values measured for *hsp70* mRNA in samples exposed to thermophilic anaerobic treatment.

Oocysts / ml *	**Copies *hsp70* dscDNA / qPCR ****	**Control (no treatment)**	**50°C / 1 h**	**50°C / 4 h**	**55°C / 1 h**
		mean Ct (n=3-4)	mean Ct (n=3-4)	mean Ct (n=3-5)	mean Ct (n=3-4)
4.5×10^6	1650	28.19 ± 0.25	30.75 ± 0.64	32.9 ± 0.29	33.87 ± 0.53
4.5×10^5	165	30.76 ± 0.47	36.27 [#]	37.48 ± 0.44	36.83 ± 0.3
4.5×10^4	16.5	35.76 ± 0.16	38.61 ± 1.0	39.4 ± 0.54	41.4 ± 0.73
4.5×10^3	1.65	38.68 ± 0.3	40.89 ± 1.23 [§]	nd	nd
4.5×10^2	0.65	42.36 ± 0.23	nd	nd	nd

*: Oocysts suspension B calculated number per ml manure before treatment; **: calculated copy numbers in 25 µl qPCR volume for samples not treated; nd: no qPCR amplification signal detected; [#]: one signal detected from n=3; [§]: amplification signal in n=2 from total n=6.

Similar experiments were done with another fresh oocyst stock (suspension C) to verify the stability of *hsp70* mRNA strands in heat treated and anaerobic treated samples (Fig. 6.3, Fig. 6.4). Oocysts before treatment were 85 % viable in the excystation test (after 2 h incubation), and 88 % were potentially viable in the DAPI / PI test.

Anaerobic treatment at 50°C for 4 h or at 55°C for 1 or 4 h rendered oocysts inactive in all replicates as verified by excystation and infectivity tests. In these samples, the amount of *hsp70* cDNA (including or not the heat shock protocol) was lower compared to copy numbers found after treatment at 50°C for 1 h.

Digestion at 50°C for 1 h yielded only slightly lower cDNA copies compared to non-treated controls (63.1 % of cDNA). In these samples, 6 % of oocysts were viable in the excystation test (4 positive measurements from n=10) but oocysts were not infectious in cell culture infectivity test. The limited reduction of *hsp70* mRNA content suggested a slow degradation of mRNA from the inactive fraction of oocysts at 50°C for 1 h. However, longer retention of oocysts in the digesters, i.e. 4 h at 50°C, yielded significantly lower cDNA copies ($p \leq 0.05$): 10 % of copies relative to the amount detected after 1 h digestion. Thus, it appeared that the substrate retention time in the digesters played a role in the reduction of residual mRNA content from the inactivated oocysts (Fig. 6.1, Fig. 6.3).

Also an increase of temperature exposure from 50°C to 55°C for 1 h significantly reduced the number of *hsp70* mRNA copies to 4 % (Fig. 6.3). After treatment at 55°C for 1 or 4 h, the number of cDNA copies with respect to untreated samples was less than 2.7 %.

The temperature in the collection tank and the high content of volatile fatty acids (6391 mg/l) in the substrate (see Table 2.4, Chapter 2 for chemical composition) neither seemed to affect the oocyst viability nor their *hsp70* mRNA content (Fig. 6.3). Oocysts in the mesophilic digester (36.2°C, 4 h) were 76 % viable in the excystation test, and yielded 32 % of copy numbers compared to control samples. This slight reduction in copy numbers at around 36°C might have been the result of rupture (natural excystation) of a small fraction of oocysts in the digesters. That may be because 36°C approximates the human host inner temperature.

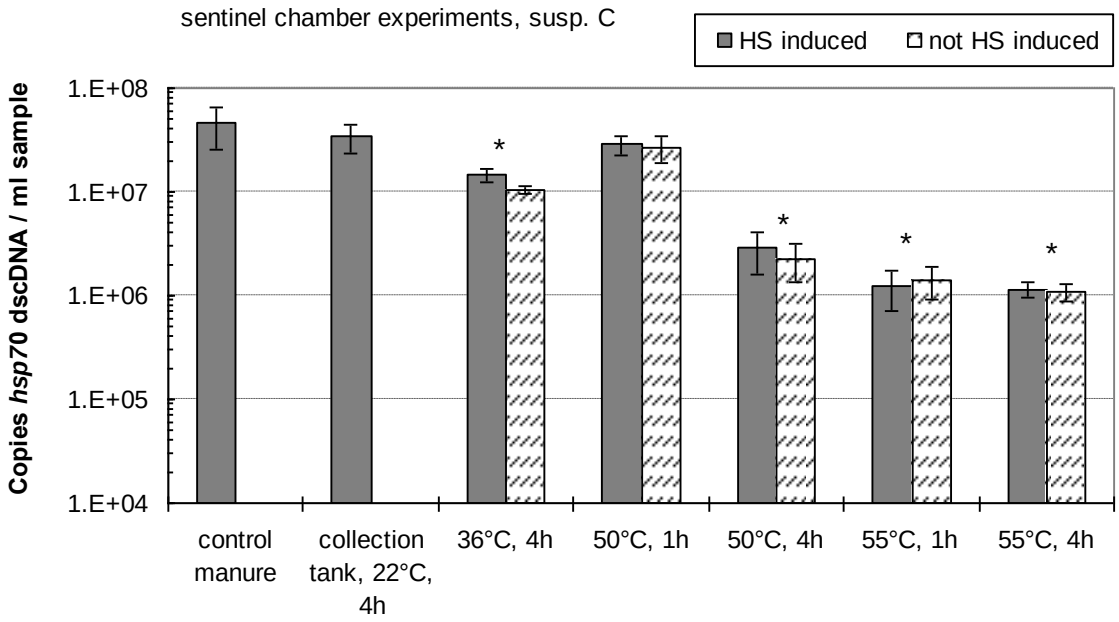

Fig. 6.3 – Reduction in *hsp70* cDNA content in oocysts (suspension C) subjected to anaerobic treatment at different temperatures and substrate retention times. Samples were heat shock induced (HS) or not (no HS) for mRNA production after anaerobic treatment. *: cDNA content is significantly different than in controls ($p \leq 0.05$).

The following Fig. 6.4 shows the yield of cDNA in heat treated oocysts (suspension C) in a waterbath. Copy numbers approximate to results from the anaerobic digesters in Fig. 6.3, indicating the effect of temperature (55°C) and retention time (4 h) in oocysts inactivation and mRNA degradation.

Inducing oocysts to produce *hsp70* mRNA by heat shock (45°C / 20 min) after being exposed to thermophilic anaerobic treatment or only heat treatment (Fig. 6.3, Fig. 6.4) yielded a content of *hsp70* cDNA similar to that found in not heat shock induced samples (induction factor < 1.1), but for samples exposed to 50°C for 4 h this factor was, with 1.29, exceptionally high and seems to span the level of sample variability (Fig. 6.3). Oocysts in controls and the mesophilic anaerobic digester (Fig. 6.3, Fig 6.4) that were heat shock induced showed > 1.35 fold of cDNA than not heat shock induced samples.

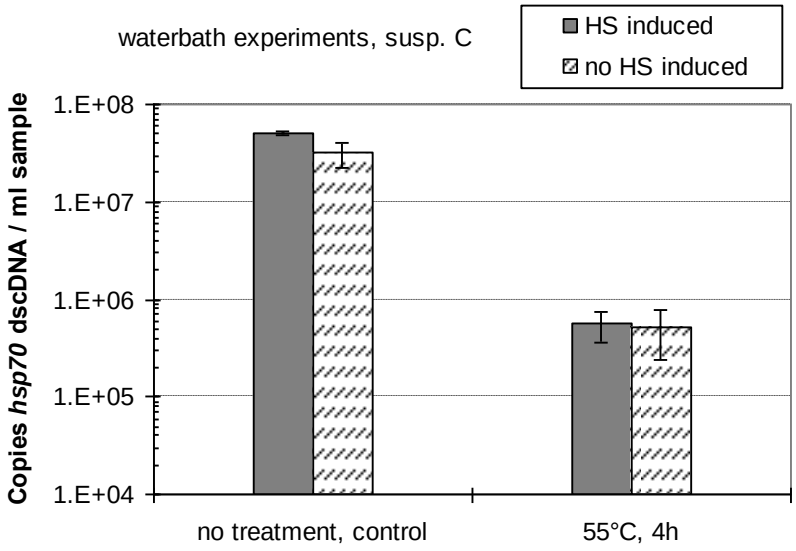

Fig. 6.4 – Reduction in *hsp70* cDNA content in oocysts (suspension C) subjected to heat treatment only. cDNA content is significantly different than in the control ($p \leq 0.05$). Samples were heat shock induced (HS) or not (no HS) for mRNA production after heat treatment.

Correlation between *hsp70* cDNA measurements and oocyst infectivity

Hsp70 cDNA measurements in oocysts exposed to thermophilic treatment in anaerobic digesters did not fully correlate with the infectivity of oocysts. However, there was a positive relation between the two test measurements. There was a clear reduction in *hsp70* cDNA content in oocysts that lost their infectivity. Oocysts infectivity was reduced by 5 log units after exposure to either thermophilic treatment evaluated. This reduction was the maximum that could be obtained with the number of oocysts spiked to the sample. Fig. 6.5 shows over 1 log unit reduction in cDNA for oocysts exposed to 50°C for 4 h. The highest reduction in *hsp70* cDNA content was ≥ 1.5 log units for exposures to 55°C for 1 or 4 h.

Results show some stability of *hsp70* mRNA molecules after oocysts inactivation, which seemed to depend on the intensity of the heat treatment and the time the organisms were retained in the digesters or subjected to heat.

For mesophilic anaerobically treated samples, *hsp70* mRNA copies were reduced by only 0.44 log units (Fig. 6.5) and oocysts were fully infectious in cell culture test.

The major variability in mRNA content was measured from oocysts subjected to 50°C for 1 h (a range from 0.2 – 1.6 logs units reduction in mRNA copies). Here it is to note that infectivity results for purified oocysts that were exposed to 50°C for 1 h in a waterbath experiment, occasionally produced infection in cell culture; however reduced infectivity compared to controls (Najdrowski and Wackwitz, project communications). This is displayed in Fig. 6.5 as reference. Hence, oocysts may not be totally inactivated at this condition.

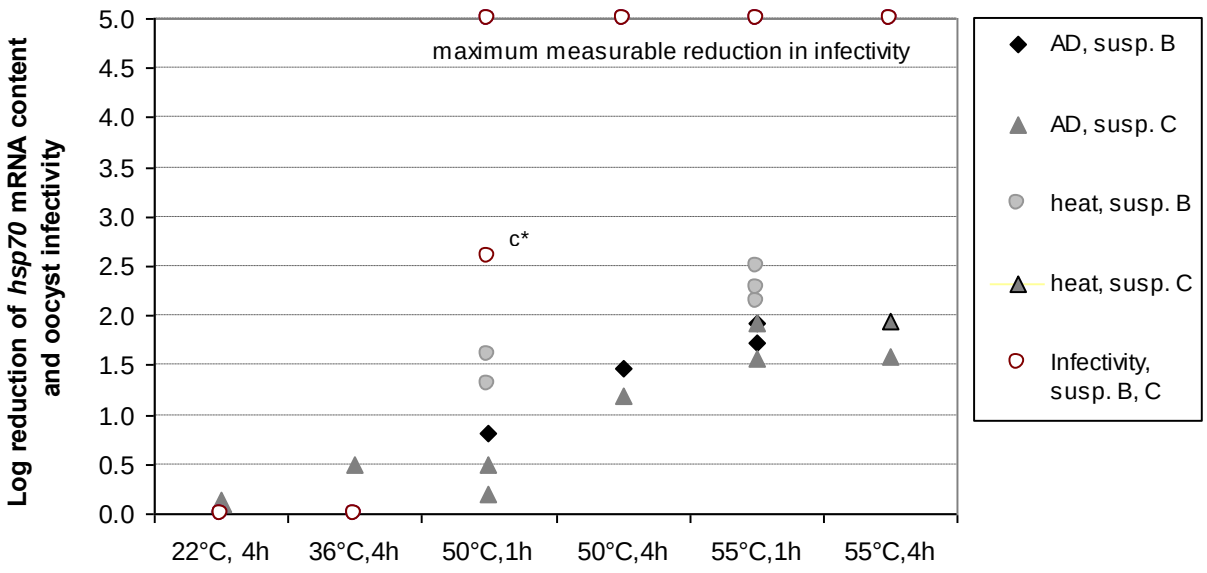

Fig. 6.5 – Logarithmic reduction of oocysts subjected to anaerobic treatment (AD) and heat treatment in waterbath measured in terms of infectivity and the log reduction in *hsp70* cDNA copies. c: infectivity of oocysts from another fresh suspension, displayed as reference.

6.4 Discussion

6.4.1 Oocyst inactivation

This evaluation intended to determine *C. parvum* oocyst inactivation in anaerobic digesters by means of measuring *hsp70* mRNA by RT-qPCR.

The effect of thermophilic conditions and anaerobic digestion on the oocyst metabolic activity after the respective retention times evaluated (1 and 4 h) was apparent by measuring the

infectivity or excystation capability (viability), and by measuring the *hsp70* mRNA content. Unlike *hsp70* DNA strands that remain constant after the death of organisms in thermophilic digestion, as reported in Chapter 4, *hsp70* mRNA quantities decreased.

Results showed a reduction in mRNA content from 0.23 to 2.5 log units in oocysts exposed to thermophilic treatment, with respect to control samples. The degree of mRNA reduction was driven by increasing temperature and by the time oocysts were retained in the digesters. At the retention times evaluated, *hsp70* mRNA strands were still measured in oocysts inactivated at thermophilic anaerobic conditions or heat treatment, and corresponded to residual mRNA. The time the inactivated oocysts were retained under thermophilic treatment contributed to degradation of the mRNA strands that remained inside sporozoites and relatively intact oocysts (Fig. 6.1, Fig. 6.3). It was found that oocysts in thermophilic anaerobic digesters were inactivated but not totally degraded after 1 h or 4 h exposure to 50°C or 55°C. A microscopic evaluation of oocysts after heat treatment in a waterbath test showed that 3.4 – 8.3 % of inactive oocysts were ruptured (excysted naturally) by the effect of such temperatures after 1 - 4 h. It is expected that at increasing retention times of the pathogen in the digesters (longer substrate retention time) the conformational structure of dead oocysts and of sporozoites inside of them will be further degraded, and thus less mRNA would be detected. This is expected as temperature would further affect the permeability of the oocyst wall (Jenkins et al., 1997). Also the degradation of decayed microbial material by the active microbial biomass depends on the accessibility to the decayed organic material and is a function of time. The degradation of mRNA in dead organisms is affected by internal RNases, but also by external metabolites, external RNases and other compounds from the external milieu. For instance, the time dependence of nucleic acid decay in dead organisms was observed in the level of bacterial DNA (Lebuhn et al., 2004) and in DNA from *Cryptosporidium* after approximately 5 months of storage at room temperature (Widmer et al., 1999).

The exposure of oocysts to 1 h or 4 h at thermophilic conditions was investigated because these time intervals were chosen as feeding intervals in the anaerobic digestion system that was evaluated for manure digestion and sanitation performance (Effenberger, 2008). The time between the feeding of manure to the digester represents the minimum guaranteed retention time of pathogens in the system, assuming some of them would be withdrawn from the digester with the effluent during the subsequent feeding step. This residence time (or treatment time) plays a role in the inactivation of pathogens during manure treatment (Burton and Turner, 2003; Popat et al., 2010).

From the positive relation between inactivation of oocysts and the log reduction in *hsp70* mRNA content after treatment, it is derived that the reduction in *hsp70* mRNA copies can serve to indicate a decrease in the number of vital oocysts. According to Fig. 6.5, there was more than 1.5 log units reduction in mRNA content for fully vital oocysts that were inactivated after exposure to 55°C for at least 1 h. Using RT-qPCR, a previous work found that the levels of 18S rRNA and 18S rDNA in purified oocysts were constant within 1 h of inactivation at 65°C (Fontaine and Guillot, 2003), and the same was for oocysts maintained for 1 h at 95°C. Therefore, these molecules appear to be more heat stable than *hsp70* mRNA.

The slightly increased *hsp70* content from oocysts in the thermophilic digesters in comparison to amounts in heated oocysts in a waterbath suggests that oocysts resisted in the digesters at an initial phase of the treatment before metabolic halt. This may have been possibly influenced by the time to reach temperature inside the sentinel chamber, anaerobic conditions and / or chemical composition of the substrate.

Besides temperature as the main factor for oocyst inactivation in the digesters, the chemical composition in digested manure can also affect oocyst metabolic activity. Contribution of ammonia content in the digesters to oocyst inactivation may have played a role. Free ammonia in the digesters was in the range of 120 – 500 mg/l (pH ~8, NH_4-N: 1600 – 1900 mg/l; see Table 2.4, Chapter 2). In a laboratory study, Jenkins et al. (1998) reported a reduction of about 10 – 30 % in excystation capability for oocysts exposed to ammonia concentrations in the range of 119 - 660 mg/l for 1 h at 24 °C. These ammonia values are similar to those values measured in the digesters.

Digestion at 50°C for 1 h can render oocyst not infectious, as cell culture infectivity test showed, but exposure to this temperature may also result in reduced infectivity and not in total oocyst inactivation (Fig. 6.5). Thus, 50°C for 1 h cannot guarantee total inactivation. Also in terms of *hsp70* mRNA, the effect of 50°C for 1 h in the metabolic activity of oocysts was seen with variable results. Because mRNA levels were occasionally similar to those after mesophilic or no treatment, *hsp70* mRNA measurements cannot indicate reduction in oocyst viability for such short retention time (Fig. 6.3, 6.5).

Mesophilic digestion cannot guarantee oocysts inactivation. Oocysts from the mesophilic digesters were infectious and able to excyst (viable), so only marginal reduction in the level of *hsp70* mRNA was obtained after 4 h. This slight reduction in mRNA copies compared to controls is likely due to break of a small fraction of oocysts, as the temperature approximates inner human / animal host temperature. Sporozoites from broken oocysts are more prone to

degradation during digestion than inactive oocysts that maintain their structure and sporozoite cells inside.

At mesophilic conditions, oocyst inactivation in manure is time dependent and can also be influenced by the chemical composition of the substrate (Burton and Turner, 2003). However for complete oocyst inactivation at this temperature, prolonged retention of the substrate, i.e. long feeding intervals of more than 24 h, may be needed, which is not realistic for manure digestion operations. The limited effect of mesophilic conditions on oocyst inactivation was shown by Whitmore and Robertson (1995). The authors reported about 18 days of hydraulic retention time to achieve 1 log reduction during sludge digestion, and Kato et al. (2003a) reported 10 days to reach 2 log reduction.

In conclusion, the inactivation of fully viable *Cryptosporidium* oocysts in manure after anaerobic / heat treatment can be measured by the RT-qPCR approach (including the heat shock protocol) as follows: reduction of *hsp70* mRNA content by > 1 log unit with respect to controls as indication of reduction in oocyst viability in anaerobic systems. However, a reduction of ≥ 1.5 log units relative to controls ensures to conclude the inactivation of oocysts with a higher margin of safety (Fig. 6.5). As stated before, a substrate retention time of > 4 h at 55°C may yield a higher mRNA reduction with respect to untreated samples.

The detection system RT-qPCR directed to *hsp70* mRNA is useful to indicate the change in oocyst viability and can provide information on the effectiveness of sanitation performance of anaerobic systems. Furthermore, for new anaerobic treatment systems that have to be checked for sanitizing performance, a verification of inactivation of *Cryptosporidium* oocysts can be performed using sentinel chambers and the molecular approach.

Quantification of the number of oocysts that remain after manure treatment cannot be done by measuring *hsp70* mRNA because the exact number of transcripts per sporozoite cell and per oocyst is unknown. However, the measurement of the reduction in *hsp70* mRNA content from samples after treatment can indicate the reduction in the fraction of living oocysts.

The control for the presence of living pathogens in digested manure can better ensure its safe use in agriculture. In comparison to other viability measurements, such as excystation test, *hsp70* mRNA quantification by RT-qPCR does not need the high expertise required for the identification of oocysts under the microscope. In addition, qPCR allows for processing multiple samples during one qPCR run.

Box : Key outcomes Chapter 6

- There was a positive relation between measured *hsp70* mRNA in viable oocysts that were inactivated in thermophilic anaerobic digesters or in heat treatments and their infectivity measured in cell culture test. In all cases a reduction in mRNA content by ≥ 1.5 log units was found for fully viable oocysts that were rendered not infectious after 1 or 4 h retention in the digesters at 55°C. Mesophilically (36°C) treated oocysts remained viable and *hsp70* mRNA was only reduced by less than 0.5 log units.
- Detection of residual *hsp70* mRNA copies in oocysts inactivated in the themophilic digesters showed some stability of the transcripts at the experimental conditions (1 or 4 h retention). This indicated that oocysts can remain intact after heat inactivation (50 or 55 °C) and short retention times in the anaerobic digesters. Microscopic observations from heated oocysts in a waterbath agreed with this statement.
- The inactivation of *C. parvum* at 50°C for 1 h in the digesters, however, cannot be guaranteed. At this condition, the inactivation may be in progress or advanced. For such samples, *hsp70* mRNA was reduced by less than 1 log unit and occasionally it was similar than in untreated controls. Therefore, also in terms of mRNA, no conclusion could be made.
- Manure treatment at 55°C for 1 h rendered pathogenic oocysts not viable (not able to excyst) and not infectious. Therefore this inactivation temperature for hygienization may be used to have a margin of safety,
- Measuring the reduction of *hsp70* mRNA content in living oocysts exposed to anaerobic treatments can be used as rapid indication of the reduction in oocyst viability, whereby complete inactivation of oocysts is associated with reduction of > 1 log unit relative to untreated controls, and of ≥ 1.5 log units to ensure a higher margin of safety. With less than 1 log reduction in mRNA copies, no conclusion could be made. No reduction in *hsp70* mRNA content after ≥ 4 h of heat treatment can serve to indicate that viable oocysts are present and were not affected within the system.

CHAPTER 7

Assessment of *hsp70* mRNA induction in *C. parvum* oocysts for viability determination

7.1 Introduction

The application of RT-qPCR based methods targeting mRNA molecules can provide information about viability, since transcription of mRNA occurs only in living organisms. In Chapter 6, it was shown that some residual amount of mRNA remained in oocysts that were inactivated at thermophilic conditions (50 - 55°C), apparently for some hours after the loss of their viability. Determination of the *hsp70* mRNA production rate in oocysts in environmental matrices can further be used to indicate their metabolic activity.

The production of HSP70 proteins and their coding *hsp70* mRNA can be induce by external stress factors in living organisms. This is known as the heat shock response (Lindquist and Craig, 1988) that confers the organism protection against environmental stress (Chapter 1, section 1.10.5). The degree of expression of the *hsp70* gene due to heat shock can vary in different eukaryotic organisms (e.g. Lindquist and Craig, 1988).

This chapter assessed the inducibility of *hsp70* mRNA production by heat shock in fresh, injured (aged) and inactivated oocysts, and examined the suitability of transcript production as a marker of oocyst viability. A *C. parvum* heat shock stimulus at 45°C for 20 min, in line with Stinear et al. (1996), was evaluated for a set of fresh oocyst suspensions. It was also foreseen to evaluate the inducibility of *hsp70* mRNA at or below 45°C for different exposure times and by other heat shock treatments, in order to better understand the response of oocysts to different triggers and trigger intensities.

The yield of *hsp70* in viable organisms was compared to contents of another transcript, β-tubulin, which is constitutively produced in an organism and that was reported to correlate with the viability of oocysts (Widmer et al., 1999). The nematode *Caenorhabditis elegans* (a eukaryote) was used as control organism for the production of *hsp70* mRNA (Snutch et al.,1988).

7.2 Materials and Methods

7.2.1 Samples and procedures for induction of *hsp70* mRNA production

C. parvum oocysts from pure suspension (1 - 2 months old) and oocysts aged for 9 and 12 months at 4°C were used to evaluate the inducibility of *hsp70* mRNA by heat shock. The viability of oocysts was determined by excystation test and their infectivity verified in cell culture test. Manure samples were free of *C. parvum* DNA, as verified by no signal in qPCR. The supernatant after centrifugation of manure at 2,500 x *g* for 5 min (liquid manure supernatant) was also used as matrix. Defined amounts of oocysts were seeded to 60 µl washed manure, liquid manure supernatant and pure double distilled water, mixed by vortex with 50 µl 1x PBS buffer, and immediately subjected to heat shock for *hsp70* mRNA production.

Heat shock for *hsp70* mRNA production was first induced at 45°C for 20 min, according to Stinear et al. (1996), for a set of oocyst suspensions (4×10^5 - 5×10^5 seeded oocysts). This procedure was employed unless otherwise stated. After heat shock in a waterbath, reactions were interrupted by immersion in ice water. Samples were immediately mixed with lysing buffer and mRNA extraction was started using the direct mRNA extraction method 2. Respective controls were directly transferred from 4°C (storage conditions), not heat shock induced, kept shortly in ice water (no longer than 5 min), and then transferred to the E-tubes with lysing buffer for oocyst lysis and mRNA extraction.

Eight heat procedures were evaluated for inducing *hsp70* mRNA production. Washed manure spiked with 5×10^4 oocysts from one stock suspension were exposed to one of the following treatments: i) 45°C for 20 min, ii) 45°C for 30 min, iii) 45°C for 1 h, iv) 45°C for 2 h, v) 41.5°C for 10 min, vi) 41.5°C for 1 h, vii) 35°C for 1 h + 41.5°C for 30 min, and viii) freeze at -20° C for 20 min, thaw for 5 min at 4°C and then heat to 41.5°C for 30 min. 41.5°C was selected on the basis of previous reports which suggested that mRNA production would be enhanced at about this temperature (42°C; Baeumner et al., 2001; Hallier-Soulier and Guillot, 2003). Samples were processed in parallel for RNA extraction with the GITC-PC method 1 and RT-qPCR. For negative controls, oocysts were inactivated at 95°C for 20 min in a waterbath, cooled down at room temperature for 5 min, and placed shortly on ice (< 5 min).

Aged and inactivated oocysts seeded to 60 µl manure were heat shock induced for mRNA production, placed shortly in ice water, and then immediately processed for oocyst lysis and (m)RNA extraction. For comparison, separate sample aliquots spiked with aged oocysts were not heat shock induced. In addition, other manure aliquots were spiked with oocysts that were exposed to 75°C for 30 min. These samples were placed shortly on ice, and then subjected to

heat shock induction at 45°C for 20 min. Heating oocyst at 75°C was done to evaluate the *hsp70* mRNA content after oocyst inactivation at this temperature.

Suspension aliquots (100 µl) of the nematode *Caenorhabditis elegans* (used as positive control organism) were subjected to heat shock induction at 35°C for 3 h for *hsp70* mRNA production, according to Snutch et al. (1983, 1988). Cultivation of *C. elegans*, sample preparation and RNA extraction were done following the descriptions in Chapter 2 (section 2.9).

7.2.2 Extraction of (m)RNA and RT-qPCR

Extraction and purification of (m)RNA was done with the oligo $(dT)_{25}$-magnetic beads system (method 2) and with the total RNA extraction system (GITC-PC, method 1), following the optimized procedures described in Chapter 4. Two-step RT-qPCR was done as described in Chapter 2 (section 2.5). The specific detection of β-tubulin mRNA was done using primers/probe system 3PS that were designed to match the respective gene sequence in *C. parvum.* RNA extraction for the nematode *C. elegans* was done with the same kit-based purification chemistry as for *C. parvum* (Chapter 2, section 2.9). Primers/probe specific for *C. elegans* targeted the *hsp4* gene, member of the *hsp70* family. Refer to Chapter 2, section 2.5 for the oligonucleotide sequences and the RT-qPCR chemistry.

7.3 Results

7.3.1 Inducibility of *hsp70* mRNA production in fresh oocyst suspensions

Oocysts from fresh suspensions that were heat shock induced at 45°C for 20 min for *hsp70* mRNA production yielded slightly higher mRNA copies than the no heat shock induced samples, and this was significantly higher for some of the replicate samples (Fig. 7.1). The induction ratio (HS / not HS) was in the range of 1.27 – 2.6. Oocysts were > 80 % viable as measured in the excystation test. Detection of *hsp70* mRNA copies in oocysts that were not heat shock induced indicated that oocysts transcribed *hsp70* mRNA in a constitutive way as part of their metabolism.

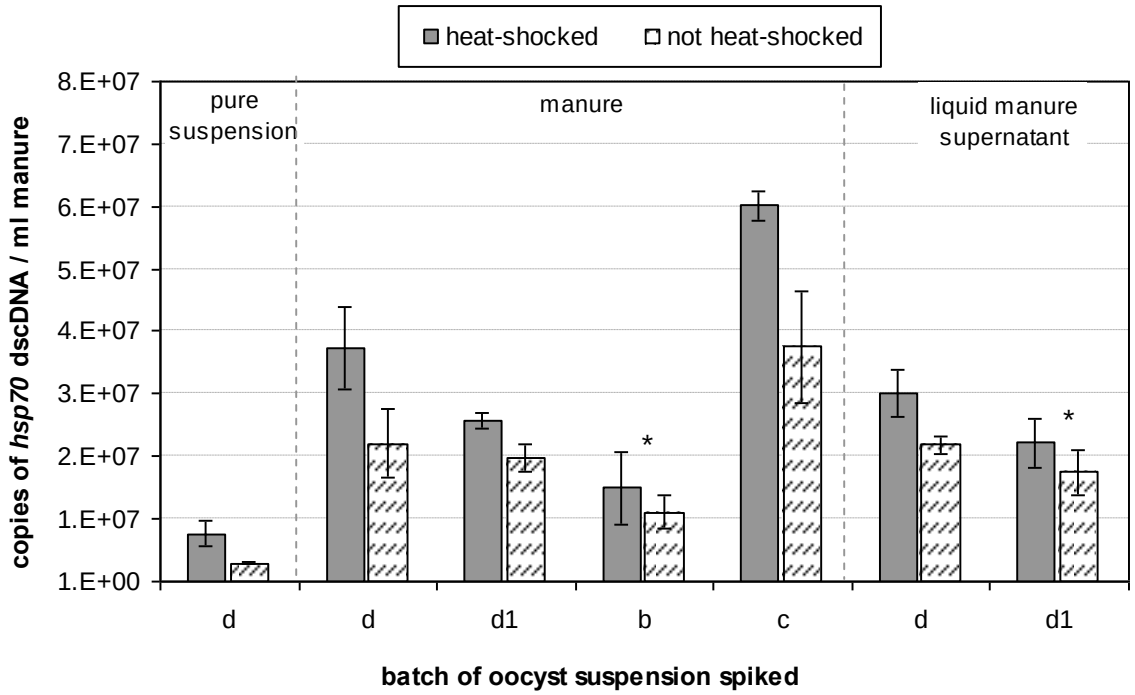

Fig. 7.1 - Yield of *hsp70* mRNA production in heat shock induced oocysts compared to not heat induced samples for a set of fresh oocyst suspensions in different matrices. mRNA content in heat shocked oocysts was significantly higher ($p \leq 0.05$) than without heat shock, except for replicates samples marked with (*).

7.3.2 Comparison of different mRNA induction procedures

The comparison of various procedures to trigger *hsp70* mRNA transcription is presented in Fig. 7.2. Two of these protocols could be performed in less than an hour and produced high mRNA yields in comparison to the other protocols evaluated. These procedures were: 45°C for 20 min, and a freezing-heating procedure consisting of -20°C for 20 min, gently thawing at 4°C for 5 min, and then heating at 41.5°C for 30 min. Both yielded no significantly different *hsp70* mRNA copy numbers ($p > 0.05$) in samples with oocysts that were 88.3 % viable. Experiments using these protocols were reproducible. The inter-assay variation measured as the coefficient of variation of the detected cDNA copies for replicate experiments of different days was CV = 0.27 for the heat shock at 45°C for 20 min, and CV = 0.29 for the freezing-heating protocol (-20°C for 20 min + 41.5°C for 30 min). These are realistic variations for qPCR results (Pfaffl, 2004).

The induction of *hsp70* mRNA production at 45°C for 20 min and at 41.5°C for 1 h was comparable, but the former one is shorter. Oocysts that were exposed to heat shock treatments that combined two stimuli: 35°C for 1 h + 45°C for 30 min, or freezing + 41.5°C for 30 min, yielded slightly higher *hsp70* mRNA copies compared to one trigger. This may have been due to the different and consecutive stimuli applied to the oocysts, but the cDNA yield was not

significantly higher than the copy numbers obtained with the heat shock at 45°C for 20 min ($p > 0.05$).

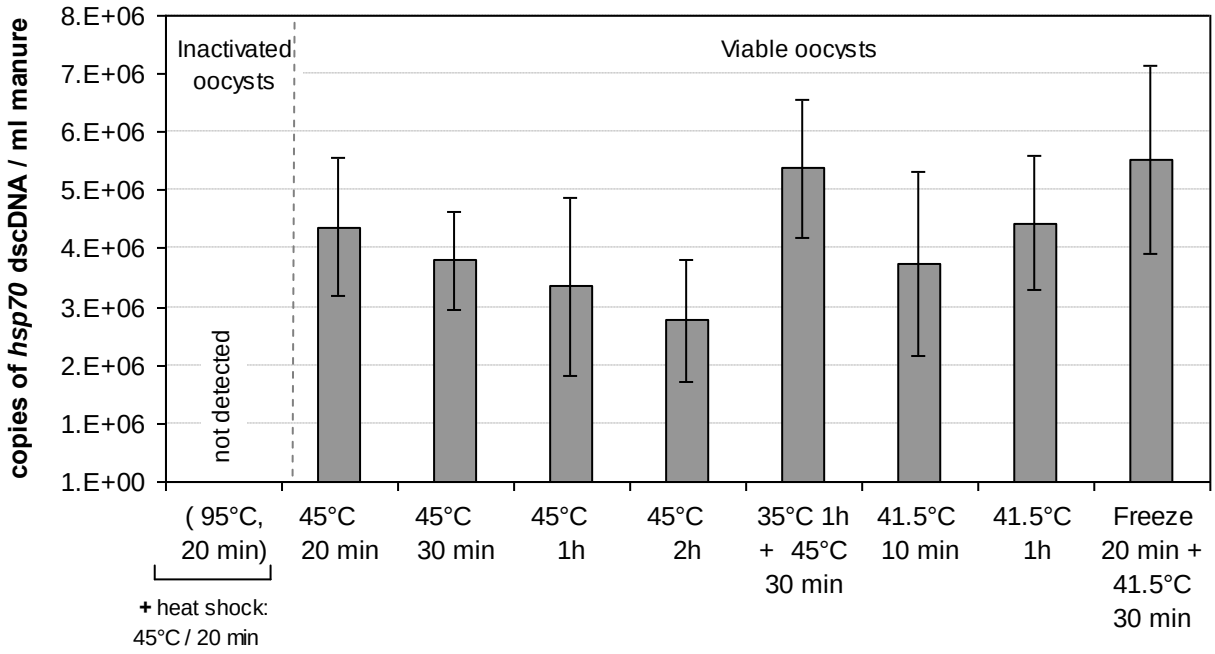

Fig. 7.2 - Induction of *hsp70* mRNA production using different heat shock procedures in viable and inactivated *C. parvum* oocysts in manure. Samples contained the same oocyst suspension.

Samples exposed to 45°C for 20 min yielded more *hsp70* mRNA than samples maintained for longer time at the same temperature. Analysis of variance for all heat shock treatments showed significant difference in cDNA yield only for the incubation at 45°C for 2 h ($p \leq 0.05$).

The lower analytical detection limit in the qPCR assay when oocysts in manure were heat shock induced by freezing + 41.5°C for 30 min was 6.9 dscDNA (with Ct = 38.12 ± 1.2). This was similar to the 8.6 dscDNA copies (with Ct = 37.34 ± 0.85) obtained with the 45°C for 20 min protocol.

Oocysts that were inactivated at 95°C for 20 min, and subsequently heat shock induced for mRNA production, yielded no RT-qPCR amplification.

7.3.3 Comparison of two mRNA as target in RT-qPCR

The yield of *hsp70* mRNA, which is an inducible transcript, was compared to the amount of β-tubulin mRNA (a constitutive transcript, not inducible) in 2 months old viable oocysts in manure. Oocyst numbers, 5×10^4 and 5×10^5 were spiked for extractions with method 1 and method 2, respectively. β-tubulin cDNA was only measurable in some replicate experiments,

whereas *hsp70* mRNA was detected in all analyzed samples (Fig. 7.3). Copies of β-tubulin mRNA in the oocysts seem to be present and / or transcribed at a much lower rate than *hsp70* mRNA. The yield of *hsp70* mRNA was on average 3.2 log units higher than β-tubulin in experiments using the same (m)RNA pool. As a measure of control, DNA extraction from about 1.2 x 10^4 viable oocysts showed detectable qPCR amplification signals for the β-tubulin gene, which verified the specificity and good performance of β-tubulin primers and probe. No qPCR signal for β-tubulin was detected in extracts from manure that were not seeded with oocysts.

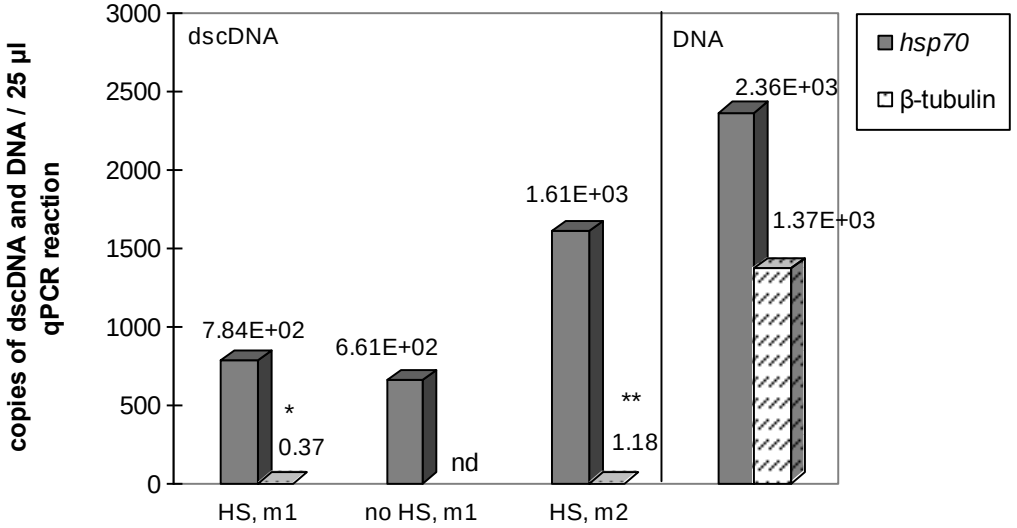

Fig. 7.3 – Copies of *hsp70* and β-tubulin dscDNA measured in viable oocysts in manure. Heat shock induced oocysts (HS) or not heat shock induced (no HS); m1 and m2: (m)RNA extractions performed with the GITC-PC method 1 and the oligo $(dT)_{25}$-magnetic beads method 2. Mean values are shown; *: qPCR amplification in n=4 from n=8; **: qPCR amplification in n=1 from n=6; nd: no qPCR signal. The detection of both genes in DNA extracts is shown (n=4).

The specific detection of β-tubulin and *hsp70* mRNA in manure extracts is shown in "DNase, +RT" samples (extracts purified from co-extracted DNA) in Table 7.1. Ct values obtained for β-tubulin cDNA were close to the detection limit of the qPCR. No amplification signal in negative controls verified that these low signals corresponded to β-tubulin transcripts. Signal detection for co-extracted DNA in the noDNAse, -RT samples showed that primer hybridization to the respective DNA sequence was correct in the qPCR. The detection limit for β-tubulin was approximately 860 dscDNA copies / qPCR reaction after ca. 40 qPCR cycles. This is a method detection limit of 4.9 x 10^4 oocysts / 60 µL manure extracted).

Table 7.1 – Control for the specific detection of *hsp70* and β-tubulin cDNA in RNA extracts from manure after DNAse treatment (DNase, +RT samples), and qPCR results with no DNAse treated samples. Mean Ct values are shown. RNA was extracted from 5 x 10^4 oocysts in manure using the total RNA extraction method 1.

Transcript		cDNA			
		noDNAse, +RT	**noDNase, -RT**	**DNase, +RT**	**DNase, -RT**
		(co-extracted DNA + cDNA)	(co-extracted DNA)	(cDNA)	negative control
β-tubulin,	Exp.1	34.08 ± 1.23	34.79 ± 1.81	42.35*	nd
	Exp. 2	37.03 ± 4.8	37.83 ± 1.4	40.68 ± 0.87	nd
hsp70,	Exp.1	27.57 ± 0.16	30.21 ± 0.31	28.64 ± 0.29	nd
	Exp. 2	27.78 ± 0.96	29.82 ± 0.58	28.5 ± 0.07	nd

cDNA molecules are produced from mRNA strands by reverse transcription. +RT: reverse transcription reactions including the enzyme reverse transcriptase; -RT: reactions without the enzyme; nd: no qPCR amplification signal; *: one qPCR signal.

7.3.4 Induction of *hsp70* mRNA production in fresh, aged and inactive organisms

The content of *hsp70* mRNA was measured before and after heat shock treatment to induce mRNA production (Fig. 7.4). For 1 - 2 months old oocysts, cDNA copies in heat shock induced organisms were higher than in not heat induced samples. The induction factor was 1.4 and 1.6 with respect to the background level for extracts from method 1 (A) and method 2 (B), respectively (Fig. 7.4). Oocysts in these suspensions were 88 - 91 % viable, as determined by their ability to excyst in the excystation test and were fully infectious in cell culture infectivity test.

Lower amount of *hsp70* cDNA was obtained in samples with aged oocysts than with fresher ones. Oocysts in samples stored for 9 and 12 months showed low or completely reduced viability. Nine months old oocysts that were heat shock induced for *hsp70* mRNA production yielded similar copy numbers than not heat induced oocysts. For these samples, the induction ratio of *hsp70* mRNA production was 1.18 and 0.97 with respect to the background level (A and B, respectively; Fig. 7.4), indicating that oocysts in these samples had a reduced capability to produce *hsp70* mRNA. In samples aged for 9 months, an average of 13.6 % (11 – 15.5 % range) of oocysts were able to excyst (viable), and on average the amount of *hsp70* mRNA was reduced by 0.7 log units with respect to measurements with fresh organisms.

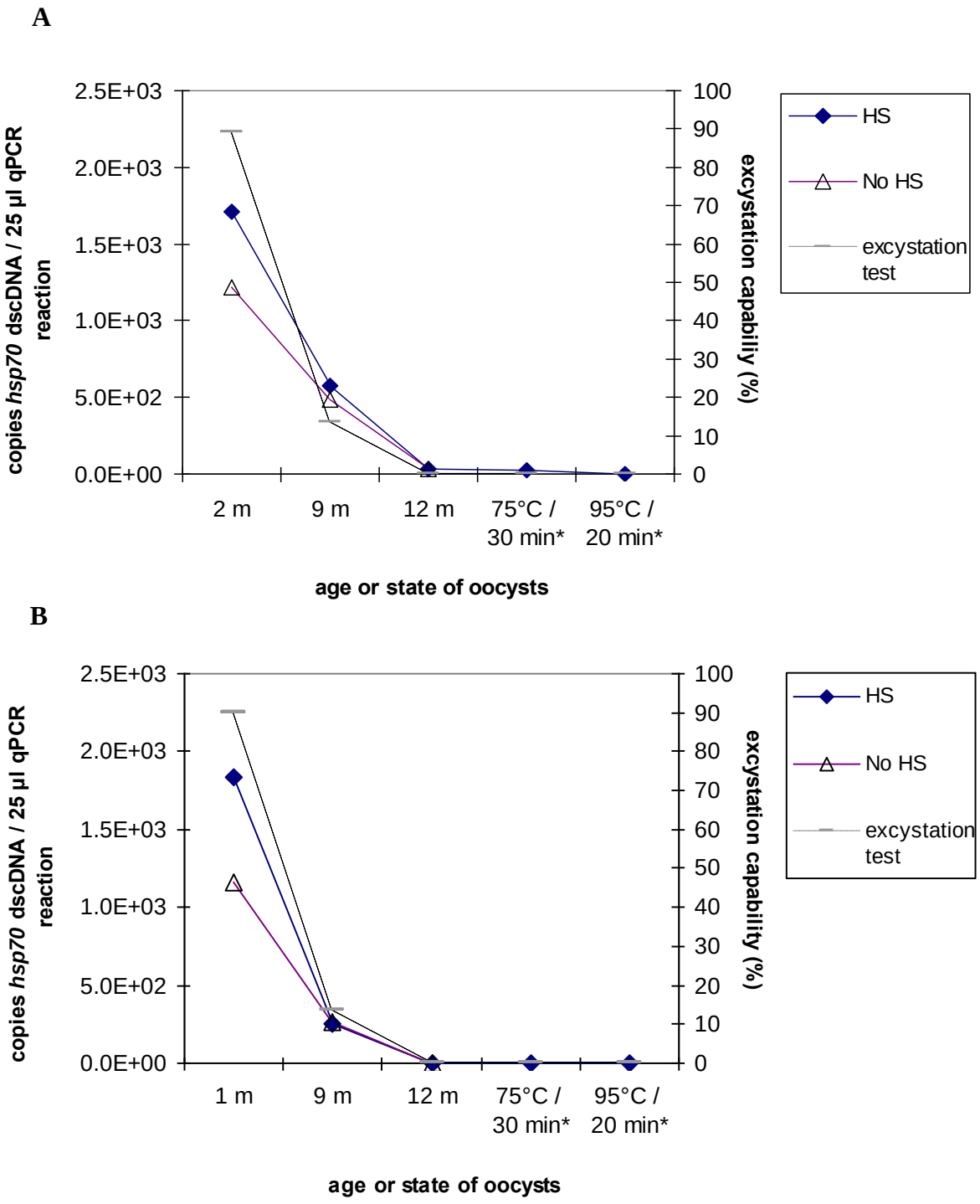

Fig. 7.4 - Induction of *hsp70* mRNA production by heat shock at 45°C for 20 min in fresh, aged and inactivated oocysts in manure. HS: heat shock induced oocysts; no HS: not heat shock induced. m: months of storage of oocysts; *: oocysts inactivated at 75°C and 95°C were subsequently induced for mRNA production by the heat shock treatment. The mean of the detected dscDNA copy numbers are shown (n=3-8). **A**: Total RNA extraction was done with the GITC-PC method 1; **B**: mRNA extraction was done with the oligo $(dT)_{25}$-magnetic beads method 2. Secondary y-axis: viability of oocysts measured by excystation test.

In samples with 12 months old oocysts, that were not viable, no induced levels of *hsp70* mRNA were measured. *Hsp70* cDNA was only occasionally detected with high Ct values (34.6 ± 0.4, n=8 from n=17) in RNA extracts from the GITC-PC method 1 (detection in 47 % of samples). In mRNA extracts from the oligo $(dT)_{25}$-magnetic beads method 2, *hsp70* cDNA was measured, exceptionally, only in one qPCR repetition from n=15, with a Ct of 38.15 that was at the detection limit of the assay. These high qPCR Ct values corresponded to very low amount of transcripts that were sometimes still measurable in the oocysts (Fig. 7.4). Oocysts aged for 12 months were not able to excyst in the excystation test.

No mRNA copies were measurable in oocysts that were inactivated at 95°C for 20 min. Oocysts inactivated at 75°C for 30 min were not viable in the excystation test. Exposure of oocysts to the latter temperature rendered oocysts non-infective in the cell culture infectivity test. Only in some replicate experiments with oocysts inactivated at 75°C, *hsp70* mRNA could be measured in small amounts when extractions were done with the GITC-PC method 1, but no qPCR results were obtained using mRNA extracts from the magnetic beads method 2 (Fig. 7.4). For extracts of method 1, the low amplification curves were measured with a mean Ct of 36.48 (1.08 SD) in 33 % of replicates, and with a high variation in terms of copy numbers (CV = 60.6 %). The cDNA copy numbers were about 2 log units less than that measured in fully viable oocysts.

Induction ratio of *hsp70* mRNA and oocyst viability

Fig. 7.5 shows the relation between the induction ratio of *hsp70* mRNA production and the oocyst viability measured by their ability to excyst. Oocysts samples from different suspensions that were > 80 % viable yielded induction ratios of ≥ 1.27. The average induction ratio for viable and fully infectious oocysts was 1.64 ± 0.3 (n=14). Lower induction ratios (factor < 1.2) were typically obtained for samples with oocysts that exhibited reduced excystation capability. Since the induction ratio of *hsp70* mRNA for samples that contained about 13 % of viable organisms ranged from 0.97 to 1.18, induction measurements in that range or close to zero could not indicate that the sample was free of viable oocysts. Such low induction ratios corresponded to samples that contained oocysts with reduced metabolic activity.

For qualitative analysis, the measured data was fitted to a logistic curve using program Curve Expert 1.4. The regression curve shows that for induction factors above 1.2, theoretically more than 50 % of oocysts in a tested sample would be expected to be metabolically active and capable to excyst.

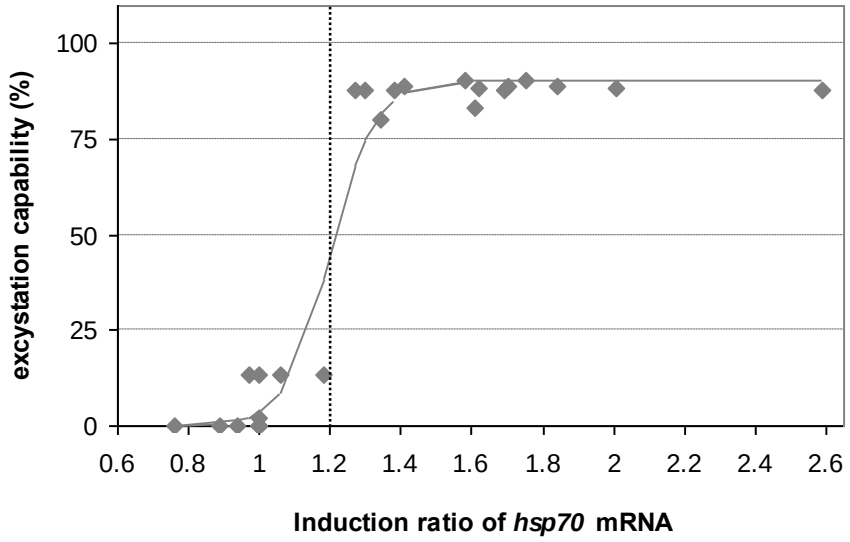

Fig. 7.5 - Induction ratio of *hsp70* mRNA production after heat shock treatment (HS / not HS) versus the percent of excystation capability in *Cryptosporidium parvum* oocysts.

Table 7.2 - Average induction factor of *hsp70* mRNA by a heat shock treatment in different eukaryotic organisms, including the findings in this work.

Organism	***hsp70* induction protocol**	**Induction ratio (HS / noHS)* (mean ± SD)**	**Reference**
Drosophila melanogaster	37°C / 30 min	1000	Velasquez et al. (1983); Petersen and Lindquist, (1989)
Trypanosoma cruzi	37°C / 2 h 42°C / 3 h	4 2.5*	Requena et al. (1992); Olson et al. (1994)
Caenorhaditis elegans	35°C / 3 h or 4 h	8 (50)[$]	Snutch et al. (1983, 1988)
	35°C / 3 h	5.4 ± 1.2	this work
Cryptosporidium parvum	45°C / 20 min	no induction [#]	Stinear et al. (1996)
	42°C / 30 min	6.9	Javier et al. (2009)
	45°C / 20 min	1.6 ± 0.4	this work
	-20°C / 20 min + 41.5°C / 30 min	1.8 ± 0.2	this work

*: measured in terms of HSP70 protein; [$]: maximum measurement displayed in brackets; [#]: assessed by conventional qualitative RT-PCR.

Table 7.2 displays the rate at which *hsp70* mRNA is induced by a heat shock treatment in different eukaryotic organisms and includes the findings in this work. For the nematode

Caenorhabditis elegans that was used as positive control organism, an induction factor of 5.4 fold (1.4 SD; n=12) for the *hsp-4* mRNA (*hsp70* family member) was found with respect to not heat shock induced samples. This value is close to data reported in the literature. It verified the inducibility of this transcript, as well as the good performance of the RNA extraction procedure and RT-qPCR detection system, as applied in this investigation.

7.4 Discussion

7.4.1 Induction of *hsp70* mRNA production

Before using *hsp70* mRNA as target molecule in qPCR to indicate viable oocysts in manure, it was necessary to verify the capability of oocysts to respond to a heat shock trigger and to determine the rate at which the molecule is induced after such trigger. This chapter evaluated the inducibility of *hsp70* mRNA production by heat shock in viable, partially inactivated, and inactivated oocysts, and the suitability of the induced mRNA production to indicate metabolic activity. Only metabolically active oocysts are capable to respond to an induction trigger by producing *hsp70* mRNA strands and translating them into proteins, as a response to adverse environments or triggers to protect themselves against cellular damage.

First measurements showed the presence of a background level of *hsp70* mRNA in viable oocysts from different stock suspensions that were not heat shock induced (Fig. 7.1). In these cases, mRNA production induced by heat shock was 1.27 – 2.6 fold the background level. It was evident that a certain level of transcription occurred without heat stimuli, indicating that *Cryptosporidium* oocysts constitutively produce *hsp70* mRNA. *Cryptosporidium* oocysts, which are typically resistant to several stress factors, may express *hsp70* gene constitutively to protect the organism from environmental stresses and to increase, for example, their thermotolerance. In eukaryotes, the constitutive production of some HSP70 proteins at normal growth temperature provides thermotolerance and serves to support vital functions (Feder and Hofmann, 1999). Moreover, the expression of the *hsp70* gene was reported to be influenced by the development stage in some parasitic organisms (Maresca and Kobayashi, 1994; Newton-Howes et al., 2006). Also in the dauer larvae of the nematode *C. elegans*, which is the form capable to resist environmental stress, *hsp70* mRNA is transcribed constitutively, the molecules are stable, and transcription is also heat induced (Snutch et al., 1988; Dalley and Golomb, 1992). Resistant development stages of organisms, such as viable *C. parvum* oocysts, might produce *hsp70* mRNA at higher rate than other developmental stages and/or increase transcript stability in order to be ready to resist external stress factors. This does not seem to be the case for other transcripts

that are constitutively produced. The respective findings for β-tubulin mRNA in oocysts support this view. The constitutive and inducible character of *hsp70* mRNA makes it suitable as target in RT-qPCR, as it can allow sensitive detection of transcripts, and therefore, qPCR signals may be detected even for samples containing a low number of oocysts.

The evaluation of other heat shock triggers to induce *hsp70* mRNA production showed no major differences to the induction at 45°C for 20 min. This heat shock procedure induced mRNA production by 1.6 ± 0.4 fold in 1 and 2 months old viable oocysts. A comparable induction ratio of 1.8 ± 0.2 was obtained for a temperature shock with several steps, consisting of freezing at -20°C for 20 min, gently thawing at 4°C for 5 min, and heating at 41.5°C for 30 min (Fig. 7.2). A previous study reported that freezing oocysts at -80°C for 5 min followed by incubation at 42°C for 30 min was a suitable procedure for *hsp70* mRNA induction, but no comparison was shown with other heat shock procedures (Hallier-Soulier and Guillot, 2003). In the present work, freezing oocysts at -20°C rather than at -80°C was preferred because strong freezing can affect the viability of oocysts (Fayer et al., 1996). Instead, oocysts can survive moderate freezing temperatures (Fayer et al., 1996). The freezing-heating procedure, as employed here, was used assuming that each of the stimuli (performed consecutively) would trigger mRNA production. Consequently more *hsp70* mRNA might be recovered. Organisms that were exposed to two heat shock stimuli could survive longer than those exposed to only one trigger, and HSP70 most likely played a role in such response (Lindquist, 1986). For oocysts, however, the consecutive steps freezing and then heating did not increase *hsp70* mRNA recovery to a significantly higher level than after a single trigger at 45°C for 20 min. This was also true for exposure to two temperatures, i.e. 35°C for 1 h, cooling to room temperature, and subsequent 45°C for 30 min. Since the induced level of *hsp70* mRNA was not that different, and freezing-heating as well as 45°C for 20 min yielded sensitive detection of cDNA copies in the qPCR assay, this work chose the more rapid procedure.

Longer heat shock treatments (45°C for 2 h) did not enhance *hsp70* mRNA production, suggesting a shorter reaction to the stimulus and subsequent degradation of mRNA. For *C. elegans*, Satyal et al. (1998) reported that the attenuation of the heat shock response starts during the heat shock treatment. The reduced mRNA content after exposure to 45°C for 2 h suggests that the exposure of *C. parvum* oocysts to prolonged heating at about 45°C may not substantially trigger more transcription and may not result in mRNA accumulation.

Relatively low induction ratios after a heat shock trigger have also been reported for other eukaryotes such as *Trypanosoma cruzi* or for the *hsp-4* in *Caenorhabditis elegans* (the latter reported here, and in accordance to previous results). This is in contrast to the much higher yield

as reported for *Drosophila melanogaster* (Table 7.2). A recent work reported induction ratios of 6.9 times for *hsp70* mRNA in *C. parvum* oocysts that were stored at 22°C (Javier et al., 2009) (Table 7.2), but no further specification was given about sample preparation and processing, which could permit to determine the reasons of the difference. In the present work, low induction ratios were found for samples that were stored at 4°C in PBS buffer and that were rapidly subjected to heat shock induction. Results were verified in parallel qPCR reactions. Despite the differences, both works found a response to a heat shock trigger in viable oocysts but none in not viable ones.

Measuring the heat shock response in terms of *hsp70* mRNA production can be useful to differentiate between living and dead oocysts. Verification with inactivated oocysts (95°C for 20 min) showed no detectable *hsp70* mRNA signal and no induction of *hsp70* mRNA production after heat shock, indicating loss of metabolic activity in the oocysts and rapid degradation of mRNA. For oocysts that were inactivated by pasteurization, i.e. 75°C for 30 min (Fayer et al., 1994), the low level of cDNA measured only in some qPCR replicates with extracts of extraction method 1, corresponded to mRNA that were not totally degraded (Fig. 7.4). A possible explanation for the detected signals is that the time between inactivation and extraction was relatively short (< 30 min), thus some mRNA fractions may have remained intact. After inactivation of oocysts at these conditions, a progressive degradation of *hsp70* mRNA strands is expected. Using the extraction method 2 based on oligo $(dT)_{25}$-magnetic beads, it was possible to consistently demonstrate that *hsp70* mRNA was not detected in inactivated oocysts (Fig. 7.4). Extraction of mRNA with the oligo $(dT)_{25}$ system requires that the complete mRNA strand between the primer regions and the poly A tail is present. Thus, it is likely that *hsp70* mRNA strands in inactivated oocysts were not intact. This was probably the reason for the small amount of *hsp70* mRNA found with method 1 but not with method 2.

Previous studies evaluated ribosomal 18S RNA as a marker of oocyst viability because high numbers of copies are present in living cells. However, considerable amounts of this target were found in oocysts inactivated at 95°C, even after 4 hours (Fontaine and Guillot, 2003). Also, other transcripts can persist in *C. parvum* oocysts for many weeks after oocyst death (Widmer et al., 1999).

The similar reduction in *hsp70* mRNA content and the reduction until the loss of viability in oocysts stored for up to 12 months underlines that mRNA is produced only in living oocysts. The very low induction ratio of *hsp70* mRNA in aged oocysts after the heat shock induction seems to be the result of changes in metabolic activity, until no transcription occurs in dead organisms (Fig 7.4). Probably residual *hsp70* mRNA was measured only occasionally when

total RNA extraction method 1 was used with 12 months aged (not viable) oocysts, indicating some stability of fractions of strands after the death of oocysts at constant 4°C. This evaluation confirms that using the direct mRNA extraction system based on oligo $(dT)_{25}$-magnetic beads yields results that correlate better with the viability of oocysts. It is most likely that *hsp70* mRNA from decayed oocysts will degrade more rapidly in active environments at a temperature higher than 4°C. This is because dead cells and nucleic acids are usually metabolized by the active microbial population, and mRNA will be degraded by the action of RNases or other external factors.

This work verified that only viable organisms were capable to produce *hsp70* mRNA after heat shock. No production of *hsp70* mRNA was obtained in samples with inactivated oocysts that were not able to excyst, whereas induction ratios ≥ 1.27 (on average 1.6) were consistently found in viable oocysts from different stock suspensions that were > 80 % able to excyst in the excystation test and infectious in the cell culture test (Fig. 7.5). For practical applications, measuring the *hsp70* mRNA content in a sample can indicate the presence of viable, potentially infectious oocysts. No residual mRNA from inactivated oocysts would be detected when using extraction method 2. The induction ratio of *hsp70* mRNA in the analyzed samples can verify that fully viable oocysts are present. Thus, the induction ratio is useful as indicator of the metabolic fitness.

It has to be considered that at low concentration of oocysts in a sample, low qPCR signals that are inconsistently detected may occur just below or at the detection limit of the assay. In such a case, measuring the induction ratio cannot provide information about the viability of oocysts in the sample. This is because the variation of Ct values at that level is high, and can result in inconsistent determination of the induction ratio. Therefore, the use of the induction ratio for verification of the metabolic fitness is limited by the detection limit of the assay. The verification of the capability of living oocysts to respond to the heat shock stimulus and the positive relation between the induced *hsp70* mRNA content and oocyst viability confirms that the chosen transcript is suitable as indicator of viability.

The results for viability of *C. parvum* oocysts are in accordance to previous studies that reported an excystation capability of 29 % in oocysts after 6 months storage in reverse osmosis water (Robertson et al., 1992). About 10 % of *C. andersoni* oocysts remained viable after 9 months at 4°C (Kváč et al., 2007). Oocysts were also reported to remain infectious during storage for up to 7 – 9 months at 4°C (Widmer et al., 1999; Jenkins et al., 2000), and up to 7 months at 15°C (Jenkins et al., 2003).

7.4.2 Comparison of *hsp70* and β-tubulin mRNA

The lower yield of β-tubulin mRNA found in fresh viable oocysts in comparison to *hsp70* mRNA indicates that using β-tubulin as target in RT-qPCR can underestimate the presence of *Cryptosporidium* oocysts in a sample. β-tubulin is a constitutive transcript present in a living cell and is translated to tubulin, a polymer and major component of microtubules in cells. *Hsp70* mRNA encodes for a major chaperone protein that functions in folding and translocation of polypeptides, in protein formation and during or without stress events, and maintains the structure of proteins in a cell (Beckmann et al., 1990). *Hsp70* mRNA appears to be present already in relatively high numbers in the oocyst stage of *C. parvum*. In the resistant developmental form of another human parasite, the bradyzoite cyst of *Toxoplasma gondii*, β-tubulin transcripts were also reported to be present in lower amounts than *hsp70* mRNA (Radke et al., 2005). The selection of suitable transcripts for the determination of viability is essential. Other RNA and mRNA molecules such as those encoding amyloglucosidase, β-tubulin, or ribosomal RNA, have been evaluated as target molecules in RT-PCR to indicate oocyst viability in pure water or pure suspensions (Widmer et al., 1999; Jenkins et al., 2000). While β-tubulin mRNA from oocysts stored at room temperature was reported to correlate with the infectivity of oocysts (Widmer et al., 1999), transcripts for amyloglucosidase seemed to underestimate infectious oocysts (Jenkins et al., 2003). *Hsp70* mRNA was used previously as target in conventional RT-PCR in water samples (Stinear et al., 1996; Hallier-Soulier and Guillot, 2003). In conclusion, using real-time RT-qPCR directed to *hsp70* mRNA and the optimized extraction / purification procedures allow the sensitive detection of cDNA and the determination of the presence of viable (metabolically active) oocysts in environmental samples.

Box : Key outcomes Chapter 7

- There was a background level of *hsp70* mRNA measured in oocysts that were not exposed to a heat shock induction, suggesting a constitutive production in the oocyst developmental stage.
- The induction ratio of *hsp70* mRNA in 1 - 2 months old viable and infectious *C. parvum* oocysts was 1.6 ± 0.4 after a heat shock stimulus at 45°C for 20 min, and 1.8 ± 0.2 with a freezing-heating heat shock procedure (-20°C for 20 min, thawing at 4°C, and 41.5°C for 30 min).

- The induced mRNA content after exposure of oocysts to consecutive heat shock triggers such as freezing-heating (as applied) or 35°C for 1 h + 45°C for 30 min was similar to the mRNA produced after a short treatment (45°C for 20 min).
- Prolonging the heat shock induction at 45°C from 20 min to 2 h did not result in more *hsp70* mRNA production; mRNA levels seemed rather to return to initial levels. No accumulation of transcripts was observed after the prolonged heat shock exposure.
- Transcripts of β-tubulin were present in much lower amounts than *hsp70* mRNA in viable oocysts, indicating that the latter transcript is more suitable as target in RT-qPCR to detect cDNA in samples containing low number of oocysts.
- Very low *hsp70* mRNA induction ratios or no induction after a heat shock trigger were obtained in oocyst samples that were stored for 9 months at 4°C and that exhibited reduced viability. No production of *hsp70* mRNA was measured for oocysts that lost viability after long-term storage.
- *Hsp70* mRNA detection in environmental samples can indicate the presence of viable oocysts. A reduced mRNA content was present in oocysts with reduced viability after long-term storage. There were no transcripts in oocysts that lost viability after 12 months or after heat inactivation (> 75°C) when the oligo $(dT)_{25}$ mRNA extraction method 2 was used. With extraction method 1, low amounts of (residual) mRNA, or fractions of it, could be occasionally detected at these conditions. Hence, using the oligo $(dT)_{25}$ based method yield results that better correlate with viability.
- Measuring the capability of oocysts to respond to a heat shock is useful to indicate the metabolic fitness: Induction ratios ≥ 1.27 (on average 1.6 fold induction) were found for oocysts that were > 80 % capable to excyst and fully infectious. Lower induction ratios (less than 1.2), as found in samples with 9 months old oocysts, indicates the presence of oocysts with reduced metabolic activity. However, due to variation of qPCR results at low template concentration at the assay detection limit or just below it, an assessment of induction ratios lower than 1.2 cannot fully verify that samples contain oocysts with reduced metabolic activity.

General discussion

Why are detection methods needed for *C. parvum* and enteroviruses?

Manure, slurry, wastewater and sludge can contain *Cryptosporidium* parasites and enteroviruses (Gerba and Smith, 2005; FAO, 2006). Controlling for the presence of these pathogens in environmental matrices is intended to evaluate possible sources of contamination of water resources and food, and to assess the potential risk of infection to humans and animals.

In order to prevent chemical and microbial contamination, measures for manure management and control points can be integrated within the complete reuse cycle (WHO, 2006), from the source, transport and treatment systems to the final application on land, including hygiene verification and monitoring. Hygiene control for *C. parvum* and enteroviruses using surrogate organisms may not always suit to indicate the reduction of these pathogens (Chapter 1, section 1.8), because they are more resistant to environmental stresses, inactivation and to disinfection procedures than conventional fecal indicator organisms (Rose et al., 2004; Skraber et al., 2004).

The screening of *C. parvum* in environmental matrices has been hindered by the lack of suitable methods that provide fast and reliable results. Techniques for *C. parvum* and enteroviruses in water samples (i.e. EPA, 2005b, 2010) do not necessarily fit for manure or even more particulate matrices. Recognizing that controlling possible sources of contamination is important rather than only controlling the contamination level of water resources is driving the development of suitable techniques for pathogens in more complex environmental substrates, production residues and crops. An EPA led expert group emphasized the need of suitable techniques to detect pathogens in sediments, manure and soil, and to develop microbial criteria for recreational waters (Dorevitch et al., 2010); and the WHO (2009) has underlined the need for sensitive detection methods for viable *Cryptosporidium* oocysts.

We aimed to develop suitable nucleic acid extraction and real-time qPCR and RT-qPCR-based procedures targeting DNA and mRNA to detect and quantify *C. parvum* and enteroviruses in complex environmental matrices.

This study intended to answer the following initial questions: (i) Can a DNA based qPCR approach be useful to quantify *C. parvum* oocysts in environmental samples / effluents from manure biogas digesters, and to indicate their inactivation after anaerobic digestion?; (ii) How specific and sensitive is an optimized direct extraction procedure for mRNA or total RNA from *C. parvum* and enteroviruses in combination with specific RT-qPCR using manure and soils?; (iii) Is *hsp70* mRNA suitable as target molecule in RT-qPCR to indicate viable, potentially infectious oocysts in complex matrices?; (iv) Is an optimized mRNA extraction and RT-qPCR

based method applicable to control the inactivation and degradation of *C. parvum* oocysts in thermophilic anaerobic digesters treating manure?.

Determining the presence of pathogens by a DNA based approach and qPCR

The use of qPCR for detection of pathogens in the environment has been advancing in recent years, mainly driven because qPCR allows specific detection, quantification, and automation for rapid sample processing. For example, pathogenic bacteria, have been identified in wastewater (Shannon et al., 2007); manure and digested manure (Lebuhn et al., 2003; Lebuhn et al., 2004; Klerk et al., 2006), aquatic sediments (Lloyd et al., 2010), and soil (Bach et al., 2002).

This work started with the optimization of a procedure for direct DNA extraction from *C. parvum* oocysts in manure. Specific detection of *hsp70* DNA by qPCR in manure extracts was obtained, with no interferences during the detection system (Chapter 3). The procedure includes a rapid and optimized washing and lysis protocol consisting of a cumulative bead beating for 165 s, and a kit-based extraction/purification with SDS, GITC and silica matrix. Following this procedure, the mean extraction and detection efficiency was 83 %. For nucleic acid extraction, the release of organisms from the matrix and their lysis is considered the first critical step in the analysis of environmental samples. Bead beating (based on physical and chemical lysis) was optimized for oocysts with typically thick walls, which are not disrupted with procedures usually employed for bacteria. Chapter 3 showed that while mild bead beating steps could not completely disrupt oocysts, too strong procedures can produce DNA losses.

DNA measurements are useful to identify organisms in a sample, but not always their viability, as DNA can be stable in dead cells. However, dead microbial biomass is reused by active organisms, and the rate of DNA decay in dead cells depends on the environment to which they are exposed (Nielsen et al., 2007). Targeting the *hsp70* gene, here it was found that DNA copies persisted in oocysts that were inactivated in anaerobic thermophilic digesters and that DNA was almost not reduced even in an environment with a high turnover rate, as it is the active biomass in manure during fermentation. Unlike DNA from dead pathogenic bacteria that seem to decay more rapidly after anaerobic thermophilic treatment (about 51°C, Lebuhn et al., 2004), dead oocysts remained apparently almost intact after thermophilic digestion and seemed more resistant to disintegration, and therefore their DNA less prone to degradation. DNA measurements were not useful to indicate oocysts viability.

The DNA based optimized method using the specifically designed primer-probe system can be applied to quantify oocysts in environmental matrices and to identify target species that are a threat to humans and cattle. The lower detection limit of the complete DNA based assay was 2.4 x 10^2 – 8.3 x 10^2 oocysts / ml manure, for a single extraction from 50 - 60 µl manure. This is

similar to the limit for fecal samples reported previously by Bialek et al. (2002), who employed a more laborious technique, nested PCR, which is used to improve the sensitivity of PCR. However nested PCR, which consists of 2 rounds of PCR, is susceptible to contamination, and does not allow automation and rapid processing of many samples, as qPCR. The detection limit achieved in the present study is on average similar or lower than that found for water, sewage and stool samples, using other extraction procedures and qPCR directed to other genes (COWP wall protein, 18S rRNA; Guy et al., 2003; Stroup et al., 2006). In a recent work that used LNA probes specific for the COWP gene in order to increase the specificity of the assay, a limit of 1.65 oocysts / qPCR in purified oocysts from wastewater was reported (Alonso et al., 2011). This was similar as in the present work (1 oocyst / qPCR) using DNA extracts from manure and specific hydrolysis probes.

Oocysts may survive in stored manure, manure amended soil or attached to crops and pasture plants for a relatively long time. Therefore, rapid assessment of the degree of contamination of environmental sites can give rapid information on the contamination risk of water resources.

The method proved also to be applicable for soil samples from agricultural fields. Including a 10 fold dilution step, it allowed to overcome qPCR inhibition due to the chemical composition of soils, e.g, with high clay content (26 - 28 %). In another work where soils with clay content of about 6 - 15 % were used, also inhibition in undiluted samples was reported (Töwe et al., 2010). Yankson et al. (2009) reported loss of DNA due to their strong adsorption to clay particles and also qPCR inhibition by humic compounds. It remains to further reduce extraction and qPCR interferences by clay minerals and humic compounds. It is to note, however, that in the present work DNA was detected without further addition of chemical facilitators to remove humic acids. Only a phosphate based lysing buffer and a SDS-EDTA based buffer are used. This suggests to keep this chemistry. In the praxis, the use of only one extraction procedure for different substrates is convenient, as it reduces costs and time for analysis.

The direct DNA extraction procedure and qPCR can produce results in 6 h. Other traditional techniques that are still in use for the screening of oocysts in environmental samples, such as IF based assays (e.g. Castro-Hermida et al., 2008; Chapter 1, section 1.10.1) require purified oocysts and microscopy, thus analysis is limited to a few samples at a time and they cannot determine species, in contrast to multiple processing by a qPCR set up.

Advances in RT-qPCR methods for environmental analysis

Assays based on RT-qPCR have been employed for the specific detection of viable organisms. The presence of mRNA is generally correlated with activity of organisms, since transcription of

mRNA from DNA occurs only in living organisms. Particularly the extraction and purification of mRNA from complex matrices to obtain intact strands virtually free of inhibitors of RT and qPCR is still a challenge because mRNA is very labile and present in low concentration compared to total RNA (Saleh-Lakha et al., 2005; Matheson et al., 2010). Because of these difficulties, RT-PCR directed to mRNA to detect active organisms in the environment or to identify specific microbial functions has been used only in limited studies, compared to ribosomal RNA based studies. These include the use of water samples (Fey et al., 2004; Gonzalez-Escalona et al., 2006), estuarine sediments (Smith et al., 2007), soils (Tebbe et al., 1995; Bürgmann et al., 2003), and manure for bacterial pathogens (Jacobsen et al., 2007).

This work presents two optimized procedures for extraction and detection of *hsp70* mRNA from viable *C. parvum* oocysts in manure, which are suitable for the direct processing of the matrix (no need to pre-purify oocysts). Method 1 uses chemistry based on GITC (for lysis and purification), phenol/chloroform purification, isopropanol precipitation, and silica matrix. Method 2 consists of extraction with oligo $(dT)_{25}$-magnetic beads and purification with buffer containing LiDS as anionic detergent and LiCl salt. The methods are applied with two-step RT-qPCR (separate reactions), a thermal stable reverse transcriptase and a high-performance Taq DNA polymerase. With these rapid methods, the isolation last about 5 h for method 2 and 7 - 8 h for method 1 (without the qPCR step).

An advantage of these specialized extraction procedures is the integration of an optimized lysis step based on 165 s fractionated cumulative bead beating with ceramic and silica beads. The approach overcomes long protocols frequently used for lysis, i.e. many freeze-thaw cycles (up to 15, Nichols et al., 2006; Alonso et al., 2011), and the need of liquid nitrogen. A lysis step should be sufficient to liberate oocysts from particles in the matrix and to disrupt oocysts without damaging mRNA strands. It was reported that the strength of the oocyst wall can differ in different strains (Nichols and Smith, 2004) and even in oocysts from the same isolate (Campbell et al., 1992). For the lysis of oocysts in wastewater and feces, Guy et al. (2003) and Bialek et al., (2005) used freeze-thaw cycles in combination with a second lysis procedure. Varying yield of nucleic acids with freeze-thaw cycles using different oocyst isolates was reported (Nichols and Smith, 2004).

Lysis by cumulative bead beating for 165 s is fast and suitable for oocysts in manure, and it also worked for water samples. Since 165 s cumulative bead beating is done in time intervals with fractionated harvest of nucleic acids, it permits DNA and (m)RNA recovery from oocysts that disrupt more easily as well as from oocysts with strong walls, thus reducing risks of nucleic acid shredding by bead beating and underestimation of results. It can be used for extractions of

manure samples from leachates or land spreading, or in storage tanks, which may contain oocysts with different conformational resistance and age. This is in contrast to clinical samples, which are mainly very fresh and may contain only strong oocysts.

The optimized extraction/purification procedures and RT-qPCR for manure were not reproducible with the tested agricultural soils, most probably due to high concentration of humic acids and mineral content (particularly clay) that can affect RT and qPCR. These compounds can adsorb nucleic acids (Cai et al., 2006) and prevent their purification (Frostegård et al., 1999). While using other soils and different purification chemistry RNA extraction is in principle possible (Griffiths et al., 2000; Sessitsch et al., 2002), inhibition and variable detection can still occur to some extent, depending also on the type of soil (Töwe et al., 2011). Soil remains a difficult matrix to extract mRNA. For specific soils, method optimizations may be needed to overcome the effect of specific soil components. In microbial ecology, the evaluation of diversity and functioning ecosystems has usually targeted rRNA molecules in RT-(q)PCR (Griffiths et al., 2000). Ribosomal RNA is present in high numbers in diverse organisms, and is therefore an easier target for extraction, but it does not fit to assess viable organisms (Widmer et al., 1999; Smith et al., 2004).

Sensitive and specific detection of *hsp70* mRNA by RT-qPCR from *C. parvum* oocysts in manure was achieved with the two optimized extraction methods. Direct extraction of mRNA by hybridization to oligo $(dT)_{25}$ capture probes (method 2), as described in Chapter 4, yielded slightly better sensitivity than the total RNA extraction method 1, with 1.2 - 1.7 dscDNA copies / qPCR reaction. This level of detection was achieved without the use of additional chemical facilitators to remove humic compounds or to inactivate RNases, such as Chelex 100 or PVP, which have been employed in PCR based assays with water and wastewater samples (Guy et al., 2003, Jiang et al., 2005). In contrast to inhibition of qPCR as previously reported with DNA and RNA extracts from manure and marine sediments (Higgins et al., 2001; Klerk et al., 2006; Lloyd et al., 2010), no signs of inhibition were found in the present study, even in undiluted (m)RNA extracts from manure. The assays here included rapid and cost effective washing steps of manure prior to (m)RNA extraction to reduce the concentration of dissolved inhibitory substances in the sample, which appeared to be sufficient. The buffer chemistry used in the extraction method 1 (GITC-PC) and method 2 (LiDS-LiDS and DTT) provided sufficient protection against RNases and PCR-inhibitors like humic acids and heavy metals.

For *C. parvum*, the method detection limit achieved for the complete assay (from sample washing, the extraction step until RT-qPCR) using either total RNA extraction (method 1) or direct mRNA extraction (method 2) was in the range of $5.5 \times 10^3 - 8.3 \times 10^3$ fresh infectious,

heat shocked oocysts / ml manure (Chapters 4 and 5). Because, method 2 could occasionally allow detection of mRNA from 5 x 10^2 – 8.1 x 10^2 oocysts / ml, it is a more sensitive option. Scaling up the purification procedure to process all lysate volume with extraction method 2 could yield a detection limit of 5 x 10^2 – 8.1 x 10^2 oocysts / ml manure. This is similar to the detection limit of 100 oocysts detected in environmental water concentrates (Connelly et al., 2008). Water can be better concentrated by filtration or IMS than manure, and therefore a higher sensitivity can be possible (e.g. Hallier-Soulier and Guillot, 2003). Also, water is a simpler matrix to extract mRNA and may contain fewer amounts of inhibitory substances for RT-PCR (Stinear et al., 1996) than manure or other more complex matrices. Other evaluations of (m)RNA extraction and RT-qPCR based methods for oocysts in manure were not found.

For enteroviruses in manure and wastewater samples, this study proposes the use of a 30 s bead beating lysis step, extraction method 1 based on GITC-PC and silica matrix, and a two-step RT-qPCR. Following this procedure, it was possible to detect 15 viral particles by RT-qPCR with an extraction efficiency of 37 % and no sign of inhibition with manure. Recent applications of qPCR and RT-qPCR for DNA and RNA viruses have been performed mainly in environmental water samples. This includes the recent EPA procedure (EPA, 2010). Advances have started to optimize methods for wastewater samples (e.g. Katayama et al., 2008; Aw and Gin, 2010). RT-qPCR applications in more complex matrices, such as sludge or manure, have been more limited (e.g. Monpoeho et al., 2001; Costantini et al., 2007). A previous study performed a survey of enteroviruses in water and bovine fecal samples using a one-step RT-qPCR system (Jimenez et al., 2005). However, an evaluation of the efficiency of their method and the assay sensitivity was not reported using fecal extracts, which have the potential to cause inhibition of the reactions by co-extracted substances and to reduce the sensitivity of the assay (Jimenez et al., 2005). Using concentrated viruses from pure suspensions, the authors reported a detection limit of 11.5 RNA copies / RT-qPCR reaction. Simmons et al. (2011) also reported a detection limit of 10 cDNA copies / RT-qPCR. These values are similar to that found in the present work with another extraction system and manure samples.

Given the importance to prevent viral contamination of water, crops and forage, and the related risk of human and animal infections (EPA, 2005), this rapid and specific procedure for RNA viruses can be useful to quantify the presence of these agents in wastewater, sludge and manure as a hygiene control measure. Enteroviruses can remain infective even for months in wastewater, soil and crops at 20 - 30°C (WHO, 2006; Chapter 1, section 1.7) and can be easily mobilized with the runoff after manure fertilization of soils with little vegetation (Ferguson et al., 2007). This runoff is a potential contaminator of water resources.

In comparison to *in vitro* cultivation techniques that are still used as standard to determine infectious viral particles, an RT-qPCR approach can provide results more rapidly (in one day). Moreover, potential (partial) inhibition of infection of the cell culture or death of the cultures can occur with virus extracts from complex samples, such as sludge and shellfish, due to the toxic and inhibitory compounds that can remain after virus purification from such matrices (Greening et al., 2002). Detection of genomic RNA is, in a first instance, indicative of the presence of viruses but not necessarily of their infectivity. Nevertheless, with some overestimated infective viral particles, one is on the safe side. In addition, there have been indications that positive qPCR results for enteroviruses have related with community health effects (EPA, 2010).

Since, cell culture infectivity tests for viruses are demanding and particularly for noroviruses still very difficult, employing molecular methods can present major advantages. Rapid data collection from RT-qPCR can be used for hygiene quality controls of various substrates.

RT-qPCR based methods versus other methods for viability assessment

In contrast to traditional methods used to determine viability and infectivity of *Cryptosporidium* oocysts, such as those that use fluorescence microscopy (i.e. dye permeability assay and *in vitro* cell culture infectivity), the optimized methods based on RT-qPCR can deliver results more rapidly, as purification of oocysts from the matrix is not needed, and the time required for skilled microscopic examination of oocysts or infected cells is spared. Microscopic identification of excysted (viable) oocysts in *in vitro* excystation tests can be difficult due to the turbidity of the sample. Even if dilutions are made, this may not be suitable since the number of organisms will also be diluted and may not be detected.

Routine screenings for viable and infectious oocysts are not performed mainly because of high costs of analysis and the long procedures of the traditional methods. The advances made in recent years with RT-qPCR, including those in this work, can lead to the uniformization of monitoring tests and the establishment of standard hygiene controls for manure containing substrates and wastewater. RT-qPCR based assays for oocysts in manure showed sensitivity in the order of about 10^3 oocysts / ml manure. This detection limit was also reported for the detection of infectious oocysts in the cell culture tests (Najdrowski et al., 2007).

For the screening of viable *Cryptosporidium* oocysts in environmental samples, also other molecular methods, like fluorescence *in situ* hybridization (FISH) were described (Vesey et al., 1998; Davies et al., 2005). However, FISH epifluorescence microscopy assays need purified oocysts and the specificity of the molecular probes in the assay may be compromised by

particles that may remain even after oocyst purification. The recovery efficiency of oocysts in water by IMS (EPA, 2005b) can vary, and IMS seems not to suit for more dense substrates containing (sand) particles and fibers. For example, Ramadan et al. (2010) reported about 36 – 38 % recovery efficiency of oocysts in secondary effluents from water reclamation plants by IMS. Another problem is that FISH assays targeting 18S rRNA in eukaryotes do not fully suit to assess the viability and infectivity of oocysts, as rRNA can persist for days after inactivation (Widmer et al., 1999; Smith et al., 2004).

Screening for viable pathogens by measuring mRNA molecules

In contrast to DNA that can persist longer in dead cells, mRNAs are degraded rapidly by exogenous and endogenous RNAses. Ribosomal 18S RNA is also more resistant to degradation after death cell in comparison to mRNA molecules. rRNA can persist in heat inactivated oocysts (65°C or 95°C) even after hours and days (Widmer et al., 1999; Fontaine and Guillot, 2003) and can be unaffected in non-infectious oocysts after long-term storage (Widmer et al., 1999).

The present work showed that quantification of *hsp70* mRNA content correlates positively with the viability (excystation capability) of the oocysts, as verified in samples that were stored for different time periods (Chapters 5 and 7). *Hsp70* mRNA was not detected in not viable inactivated oocysts at 95°C. The long-term storage experiments demonstrated that *hsp70* mRNA has a limited stability in inactive oocysts. Low amounts of residual mRNA, strand fractions, remained in oocysts that lost their viability after storage for months at low temperatures, and were occasionally detected with the total RNA extraction method 1. The presence of partially degraded mRNA in such oocysts was confirmed by not detecting *hsp70* mRNA with the direct method 2, which requires the intact mRNA strand for extraction, including its poly A tail at its 3' end. A degradation mechanism of mRNA can begin from the deadenylation of the poly A tail (Beelman et al., 1995). This difference in the detection of residual mRNA with the two extraction systems indicates that the choice of the extraction procedure is important for the results. For viability determination, preference is given to the direct extraction method 2, as screening procedures that detect less residual mRNA from inactive organisms are most convenient.

The degradation rate of mRNA molecules is most likely faster in inactive oocysts in the environment than in stored pure suspensions, as the structure of inactive oocysts will be more rapidly affected by external environmental factors. Survival of oocysts in the environment at temperate conditions may be shorter than 9 months, as laboratory studies from Jenkins et al. (2003) suggest. Environmental factors (e.g. predators, humidity and temperature) can affect the

survival of oocysts, and accelerate the degradation of dead oocysts (starting from the glycocalyx lipo-proteinic wall). More permeable oocyst walls and broken oocysts would allow external RNAses and other metabolites to increase mRNA degradation in the sporozoites that are in the oocysts.

Appropriate RNA targets for RT-qPCR should be chosen as marker of viability, and the selection will also influence the sensitivity of the assay. Previous studies have shown overestimation or underestimation of viability when other than *hsp70* mRNA strands were evaluated as viability indicator in pure oocyst suspensions (Widmer et al., 1999; Jenkins et al., 2003). β-tubulin mRNA production was reported to correlate with oocyst infectivity after long-term storage (Widmer et al., 1999), but the transcript is present in lower numbers than *hsp70* mRNA, as found in the present study (Chapter 7). This means that a higher number of oocysts in the manure sample is needed to generate a qPCR signal using β-tubulin. It can be derived that β-tubulin mRNA has a lower production rate and / or a lower stability than *hsp70* mRNA in the oocyst stage. The production rate of different mRNAs can vary, for example, according to the physiological state of the organism, and in parasites, it may be influenced by the development stage in which the organism is present (Maresca et al., 1999). In studies with other parasitic organism also higher contents of *hsp70* mRNA than other transcripts were found (Radke et al., 2005).

Hsp70 mRNA was previously used in RT-(q)PCR for extracts from water or pure oocyst suspensions (Stinear et al., 1996; Hallier-Soulier and Guillot, 2003; Lee et al., 2008). The transcript was also used to assess the inactivation of oocysts after treatment with disinfectants and ozone, commonly employed for drinking water treatment (Hallier-Soulier and Guillot, 2003). In contrast, the present study evaluated the application of RT-qPCR assays in more complex matrices and the suitability of *hsp70* as a marker of viability in long-term assays. Aging is a process that oocysts undergo in the environment, and it was intended to evaluate the transcript content at different oocyst constitution over a longer period, at mesophilic and thermophilic conditions in anaerobic digesters, and after heating at pasteurization temperatures (≥ 70°C) as required for hygienization of biowaste and animal residues (Annon., 1998; EC, 2011). No *hsp70* mRNA was measured by RT-qPCR using the direct extraction method 2 after oocyst treatment at 75°C for 30 min in manure, which fully agreed with the absence of viable oocysts in the samples.

For application in hygiene controls of environmental samples (i.e. slurry, manure, runoff), this study suggests to use the oligo $(dT)_{25}$-magnetic beads extraction procedure and RT-qPCR directed to *hsp70* mRNA to indicate the presence of viable oocysts. It can also be employed for

validation tests, for example, of heat treated manure with biowaste at high temperatures, when requested by competent authorities.

Hsp70 mRNA production and viability

The production of *hsp70* mRNA in eukaryotes and its translation to proteins can be induced by an induction trigger only in viable organisms as a response that provides protection and resistance against stress factors and degradation (Lindquist and Craig, 1988). Initially, it was assumed that by inducing *hsp70* mRNA production and correspondingly increasing the amount of the target, RT-qPCR signals would be detected from samples with low number of oocysts. This study found that *hsp70* production is up-regulated in oocysts by environmental induction. It was also found that *hsp70* mRNA is produced constitutively in the oocyst metabolic stage without a heat shock trigger. Oocysts are the resistant form and the free living metabolic stage of *Cryptosporidium* species. Considering that *hsp70* mRNA products (proteins) are needed to confer resistance against stress in the environment and initially in the host after infection, and also for oocyst metabolism during stable conditions, it is possible that the transcript has a constitutive rate of production besides stress induction.

Hsp70 mRNA production in 1 - 2 months old oocysts in manure was induced on average only 1.6 times after a heat shock trigger at 45°C for 20 min. In another study, it was reported that mRNA production of oocysts held at 22°C was induced 6.9 fold after a heat shock at 42°C for 30 min (Javier et al., 2009), which it is still considered low.

Although the induction ratio was not as high as in other eukaryotes, like 1,000 fold in *Drosophila melanogaster* (Petersen and Lindquist, 1989), the response to a stimulus was present and reproducible in living oocysts. This response was shown to be correlated to the viability and infectivity of oocysts. Induction ratios ≥ 1.27 were found for oocysts that were > 80 % viable (able to excyst).

The capacity to respond to heat shock stimuli is also dependent on the metabolic fitness of the oocysts. In evaluations with samples aged for 9 months, where a fraction of oocysts was still viable, as determined by excystation tests and as verified by detecting *hsp70* mRNA, oocysts responded less to heat shock stimuli than fresh oocysts. Aging produces structural conformation changes in oocysts (Nichols and Smith, 2004), which is accompanied by changes in metabolic activity. The findings indicate that aged oocysts may still be able to transcribe *hsp70* mRNA, but they seem to lose their capacity to respond to a heat shock trigger. Other studies with *D. melanogaster* found reduced expression of *hsp70* mRNA in aged organisms as well as reduced resistance to stress (Sorensen et al., 2002).

In practice, the induction ratio could be used to assess the vitality of living oocysts. Accordingly, an induction of ≥ 1.27 fold would indicate the presence of fully vital oocysts in the samples. Low induction ratios < 1.2 fold, however, do not fully guarantee that pathogens are dead. The analysis of *Cryptosporidium* oocysts in environmental samples should therefore comprise the verification of the capability to respond to heat shock induction (45°C for 20 min). With positive results, this information can also indicate that the contamination was recent. However, at low template concentration, close to or just below the detection limit of the qPCR assay (e.g. Ct values in the range of ~39 – 42), an induction may not be clearly detectable, due to the random distribution of cDNA copies in the sample and variation of Ct values. This needs to be considered when using the induction ratio of *hsp70* mRNA to verify if oocysts are fully vital.

The information obtained from *hsp70* mRNA induction after different triggers (which included a heating step) improved the understanding on the response of oocysts. Exposure to 45°C for 2 h did not result in higher *hsp70* mRNA production than after 20 min. Hereby it can be derived that heating environments below inactivation levels, for example 45°C in manure treatment systems, should not end in accumulation of *hsp70* mRNA. It seems that a regulatory mechanism for *hsp70* transcription exists that is independent of the duration of the exposure to the increased temperature. A transcriptional regulation of *hsp70* mRNA production was reported for other eukaryotes (Petersen and Lindquist, 1989) and the degradation of induced *hsp70* mRNA can start even before the induction trigger is eliminated (Satyal et al., 1998). After exposure of oocysts to environmental stresses, it is expected that any induced level of *hsp70* mRNA will return to background level, and allow to measure a further heat shock response during sample analysis. For example, chemical oxidants used for water treatment can also induce *hsp70* production, but the accumulated transcripts decrease to basal levels, allowing a further response to a heat shock (Bajszár and Dekonenko, 2010).

There was only a low variation in *hsp70* mRNA content after the different induction triggers evaluated. This suggests that the heat shock response is reproducible in oocysts of a similar physiological state, and therefore the transcript suits as a viability marker.

Screening for *Cryptosporidium* pathogens in anaerobic and other treatment systems

Manure is increasingly used in anaerobic biological systems for the generation of renewable energy (biogas), and anaerobic digestion can also serve for hygienization purposes. In Germany, most biogas systems that co-digest manure with energy crops are operated at mesophilic

conditions (Weiland, 2010). Systems that use biowaste or category 3 animal by-products as co-substrate are operated at thermophilic conditions, or mesophilic with a hygienization (70°C) step (Paavola et al., 2006; EC, 2009). While pathogen reduction may be achieved at thermophilic temperatures, mesophilic conditions cannot guarantee the reduction / inactivation of pathogens, including *Cryptosporidium* oocysts, to levels that are risk-free for humans and animals (Whitmore et al., 1995; Chapter 1, sections 1.7, 1.8). Even operation at thermophilic conditions may suffer from process variations and operational failures (e.g. temperature, mixing) that can result in insufficient reduction of pathogenic microorganisms (Godfree and Farrel, 2005; Lebuhn and Wilderer, 2006; Effenberger et al., 2008).

The optimized methods based on quantification of DNA and mRNA can be used to control the reduction of *Cryptosporidium* oocysts during treatment processes. Although DNA was still measurable in dead organisms after the evaluated retention times (up to 20 h - comprising mesophilic and thermophilic digestion) in very active environments, the DNA based qPCR method allows the quantification of total oocysts (alive and dead) in the samples. The DNA based approach is useful to screen for the presence of oocysts, and only positive samples need further analysis for the presence of viable organisms. High numbers of oocysts are shed by infected animals (up to 10^{10} oocysts / day; WHO 2011), and high loads of oocysts represent a potential source of living organisms.

By measuring *hsp70* mRNA with the RT-qPCR based approach (with and without heat shock induction) it was possible to verify a reduction in the fraction of viable oocysts after anaerobic and heat treatment (Chapter 6). Although some residual *hsp70* transcripts were still detectable after inactivation of oocysts at thermophilic conditions (after 4 h), the reduction in *hsp70* content was clear in comparison to the content measured for fresh oocysts in untreated manure. There was consistently ≥ 1.5 log units less *hsp70* mRNA in oocysts that were totally inactivated at 55°C for at least 1 h.

In practice, the RT-qPCR based approach with heat shock induction and the log reduction in *hsp70* mRNA content can be used as marker to assess the decay of viable oocysts, for example, in treated manure after anaerobic digestion, heat treatment processes, composting, and also in storage piles. Less than 1 log reduction in *hsp70* mRNA content cannot ensure reduction in the number of viable oocysts, as found in this work, whereas more than 1 log reduction, and more than 1.5 log for a higher margin of safety, with respect to not treated manure is associated with inactivation of oocysts. Higher reduction in *hsp70* mRNA is expected from oocysts that are heat inactivated and maintained longer than 4 h under treatment.

A further way to assess the change in the fraction of viable oocysts in a sample can be the

measurement of the cDNA/DNA ratio in treated samples as compared to controls. This approach is currently used to evaluate the activity of methanogenic bacteria in biogas systems (Munk et al., 2012).

These hygiene controls are useful to rapidly verify the hygiene efficacy of treatment processes, particularly when suboptimal operating conditions or fluctuations are observed. Treatments at temperatures below 55°C may not guarantee reduction of pathogenic oocysts. While in the present study non-infectious oocysts were found after 4 h digestion at 50°C, Meyer (2002) reported that about 15 % of oocysts remained viable (on an excystation test) after 24 h at 51 - 52°C in biogas systems. Differences in oocysts strength can exist and strong oocysts may be more resistant to inactivation at 50°C. It seems that a treatment at about 50°C, particularly with relatively short retention time, may not always ensure total oocyst inactivation. The RNA based approach targeting the *hsp70* mRNA can be used to verify the inactivation capacity of such systems. For example, the co-digestion of manure with biowaste can be done at 50°C and a defined retention time, according to the German waste legislation (Annon., 1998). Hygiene controls have to be performed for salmonellae. However, with such a temperature and a short retention time, the inactivation of *Cryptosporidium* spp. may not be fulfilled.

The inactivation of oocysts in anaerobic digesters and the concomitant reduction in *hsp70* mRNA production is mainly the result of temperature and the exposition time, but also of the chemical composition of the substrate, including ammonia (Jenkins et al., 1998).

In this study, the retention times used for *C. parvum* inactivation were the same as the feeding intervals evaluated in a model digester system. They represent the minimum guaranteed time the pathogens can be retained in the digesters. Based on results for intestinal enterococci and of tracer experiments, Lebuhn and Wilderer (2006) and Effenberger (2008) suggested a minimum guaranteed substrate retention time of 8 h for a stirred thermophilic (55°C) anaerobic digester treating manure, in order to obtain sufficient sanitation. Further mRNA decay in dead oocysts is expected at longer retention times. The time of exposure to high temperatures is most likely to further affect the structure of oocyst walls and sporozoite membranes, while mRNA in dead organisms would be further denatured by the increased temperature, the action of RNAses, and chemical metabolites in the biological matrices.

Studies have shown that the use of standard indicator organisms for hygiene controls do not always suit to indicate the decay of other pathogens (Olsen and Larsen, 1987; Skraber et al., 2004). Spores of the bacterium *Clostridium perfringens* have been suggested as surrogate to control for the presence of *Cryptosporidium* oocysts, and in other cases helminth eggs have been used for hygiene tests (Medema et al., 1997; Bowman, 2008; WHO, 2009). However, those

organisms may not ensure reduction of *C. parvum* and *C. hominis* oocysts. Rose et al. (2004) found that oocysts were less affected during the biological treatment of wastewater than *C. perfringens* or other indicators. *C. perfringens* spores can be removed differently than oocysts maybe due to their smaller size. Spores are also less suitable as indicator organisms due to their strong resistance to heat treatment. Intestinal enterococci were found to be good hygiene indicator also for the protozoa (Lebuhn and Wilderer, 2006), but Rose et al. (2004) reported no direct correlation for oocysts numbers and indicator organisms, including enterococci, in final effluents of wastewater treatment, and a higher persistence of the indicators. Therefore, verification by analysis for reduction of oocysts is recommended (WHO, 2009). This is currently implemented in state regulations for reuse of reclaimed water in the US (FAC, 2007). In any case, the rapid identification of the target pathogen itself during hygiene control provides a more accurate control and it is always better than using surrogates.

Further considerations for practical applications

Given the current increase in the reuse of wastewater for agriculture, and the increasing impact of livestock farms as source of pathogen contamination of water resources and land (EPA, 2005; FAO, 2006), new regulations and more strict requirements for manure management and treatment are likely to come. For manure treatment systems, the establishment of quality assurance and hygiene control of effluents before being disposed on the land is recommended (Böhm, 2004), preferably within an holistic approach for manure management.

For the purpose of monitoring the hygiene efficiency of (anaerobic) treatment systems, including effluents of concentrated animal feeding operations (CAFO), and also for the screening of environmental matrices (e.g. slurry, manure in soil, runoff), the following approaches, as developed in this study, can be performed:

- Screening for *hsp70* DNA from *C. parvum* oocysts → indicates the presence and number of this pathogen. ⎪rapid assay of about 6 h⎪.
- Determining the presence of viable, potentially infectious oocysts by measuring the *hsp70* mRNA content upon heat shock → For treatment systems: indicates the reduction in the fraction of living oocysts relative to controls by measuring the reduction in mRNA content. For surveys in the environment: measuring the *hsp70* mRNA content can indicate the presence of viable potentially infectious oocysts in the samples.

  Measuring the induction ratio of *hsp70* mRNA production upon heat shock verifies the presence of fully vital oocysts. ⎪duration of the assay: 1 day; relatively rapid decisions can be made⎪.

– Optional infectivity tests of samples that were positive in the RT-qPCR mRNA approach or the qPCR DNA approach → verification of the presence of infectious oocysts.

Quantification of *hsp70* mRNA alone cannot provide an accurate estimate of the number of viable pathogens present in a sample because the level of mRNA production per oocyst would be unknown.

With respect to method sensitivity, the optimized methods yielded detection limits per ml manure of 2.4 x 10^2 – 8.3 x 10^2 oocysts for the DNA based approach, and cDNA from 5.5 x 10^3 – 8.1 x 10^3 oocysts for the mRNA based approach with the direct mRNA extraction method 2 (magnetic beads system). Such numbers are found in the surroundings of animal farms and in wastewater sludge. Typical numbers of *C. parvum* oocysts excreted by infected calves are 10^6 - 10^7 / g of faeces, and a range from 25 to 10^4 oocysts / g can be shed by asymptomatic adult animals. The concentration of oocysts in slurry from calve housing was reported to be about 7.5 x 10^3 / g (Medema et al., 2001; WHO, 2009). Surveys for cryptosporidia in dewatered sludge reported concentrations of around 3.8 x 10^2 oocysts / g (Guzman et al., 2007). The possible routes of transmission of this pathogen to humans and animals are multiple (Fig. 1.1, Chapter 1). Inherent of (RT)-qPCR based assays for environmental analysis is the processing of small sample volumes. This also helps to reduce potential inhibition of the reactions by co-extracted inhibitors. For analysis of environmental samples that may contain low amount of pathogenic oocysts, the following steps, as specified in Chapter 5 for the mRNA based approach, can be done to lower the detection limit of the assay: For example, the processing of 1 ml of the lysate volume with the direct mRNA extraction method 2, could reduce the detection limit of the complete mRNA assay to detect 5.5 x 10^2 – 8.1 x 10^2 oocysts / ml sample. In addition, effluents of manure anaerobic digestion systems can be concentrated by centrifugation (i.e. 10 ml aliquot can be concentrated in about 1 – 1.5 ml sample).

For DNA, PCR repetitions of the entire DNA extract (100 µl) is feasible in one time, as nowadays qPCR instrumentation allows the processing of more than 350 samples at a time. The sum of the detected gene signal in all repetitions would reduce the detection limit of the assay to about 6 - 20 oocysts / ml manure. A qPCR detection limit of 10 oocysts in faeces and soil samples was reported for other procedures that include a pre-purification step of oocysts from the matrix (Ramirez and Sreevatsan, 2006).

– Screening for viral RNA from enteroviruses by RT-qPCR →indicates the number of viral particles in the sample

Quantification of enteroviruses using the proposed extraction method can measure as few as 15

viral particles / qPCR reaction and 1.2 x 10^4 viral particles / ml manure. This corresponds to the number of viruses that can produce an infection. High numbers of zoonotic viruses can be shed by animals in farms (Ley et al., 2002). High numbers of enteric viruses can also be found in effluents from wastewater treatment plants (i.e. 10^5 - 10^8 / l; Aw and Gin, 2010; La Rosa et al., 2010). For (waste)water samples, viral particles can be concentrated by an ultracentrifugation step. For manure samples, particulate material can be removed by centrifugation (Costantini et al., 2007), and viruses contained in the supernatant can be concentrated by ultracentrifugation.

The rapid evolution of viral mechanisms to survive and resist treatments, as well as the high genetic variability, opens the possibility of cross infections and the appearance of new hosts for new viral strains. This underlines the need for controlling the presence of viral particles in a more rapid way in environmental matrices, preferentially at the source, e.g. at animal farms, and wastewater treatment facilities. Since enteroviruses are rapidly degraded by thermophilic treatments (Lund et al., 1996; Böhm, 2004; Popat et al., 2010) and RNA is a labile molecule, any measurement of viral particles would indicate that a thermophilic treatment system did not perform in an efficient way. A guaranteed retention time of 6 h at 55°C in anaerobic digesters is sufficient to reduce bovine enteroviruses by 4 log units. In contrast, enteroviruses can survive longer at mesophilic treatments (Lund et al., 1996; Böhm, 2004). Bovine enteroviruses have been suggested as indicator organisms for animal fecal pollution (Lund et al., 1996). The screening for viruses using a suitable rapid molecular tool such as the proposed one can be applied to obtain further knowledge on the survival of viruses after different manure or wastewater treatment systems, and for the screening of other environmental matrices.

Conclusions and outlook

This study deals with the pathogenic microorganisms *Cryptosporidium parvum* and enteroviruses, their relevance for public health and their importance when manure and wastewater are reused in agricultural land.
The rapid and specific detection of these microorganisms in environmental matrices, such as manure, slurry, wastewater and soil, has been identified as one of the major challenges for the development of measures to control the dissemination of these pathogens in the environment. Real-time qPCR based techniques have the potential to provide a valuable contribution in this field, given that they can be made applicable to such complex matrices.

Targeting the *hsp70* DNA, this work has developed and optimized oocyst lysis and extraction procedures for the detection of genomic DNA by real-time qPCR in manure. The lysis via cumulative bead beating allows a rapid and efficient recovery of DNA from oocysts with varying wall constitutions. The developed specific primers allow the selective detection of the target species. The complete method has shown to provide results of high sensitivity (2.4×10^2 – 8.3×10^2 oocysts / ml manure) in short time (6 h).
The DNA based method allowed also to quantify oocysts in soil from agricultural fields. By integrating into the procedure a sample dilution step that mitigated inhibitory effects, concentrations as low as 1×10^4 oocysts / g of soil could be detected from soils containing relatively high amounts of clay. The optimized qPCR based detection method has shown to be appropriate to determine the presence and to quantify the number of oocysts in complex environmental matrices.

For *hsp70* mRNA based RT-qPCR detection of oocysts, two extraction methods with different purification chemistry have been shown to provide good results for manure. They include lysis by cumulative bead beating (165 s), treatment with DNase, and a two-step RT-qPCR system. With these methods, a sensitive and specific detection of *hsp70* mRNA from *C. parvum* oocysts in manure is possible without pre-isolation of oocysts from the substrate. Detection of cDNA from 1 - 2 oocysts / qPCR assay was achieved. The lower method detection limit for *hsp70* cDNA was equivalent to 5.5×10^3 - 8.1×10^3 oocysts / ml manure for the direct mRNA extraction method based on oligo $(dT)_{25}$–magnetic beads, and signals could occasionally be obtained from 5.5×10^2 - 8.1×10^2 oocysts / ml. Including the qPCR step, the procedures take between 8 h for the oligo $(dT)_{25}$ system (method 2) and 10 h for the total RNA GITC-PC-silica matrix system (method 1), and can be performed in one day.

For mRNA detection in soil from agricultural fields with high clay content, the procedures have been found to provide suboptimal performance. This is attributed to interference with clay particles, humic compounds and potentially other minerals, and could not be overcome by sample dilution. Further methodological improvement is thus needed for soils with high clay content.

Quantification of enteroviruses in manure, wastewater and sludge was possible with both extraction systems (method 1 and method 2). Using the total RNA extraction method 1 with a 30 s bead beating lysis step, 15 viral particles / RT-qPCR assay were measured.

With respect to viability assessment, oocyst *hsp70* DNA content was shown to not be representative for the actual viability of the pathogen. More than with bacteria, the DNA can persist in inactivated oocysts even in environments with high metabolic turn-over rate, such as in anaerobic digestion.
Hsp70 mRNA content, however, correlated positively with viability. This was found for defined oocyst batches treated by pasteurization temperatures, in anaerobic digesters as well as for oocysts aged over a period of 12 months in a controlled environment (at 4°C). Particularly with the direct extraction method 2 based on oligo $(dT)_{25}$–magnetic beads together with the proposed RT-qPCR assay, the *hsp70* mRNA approach with heat shock induction can be used as a marker to indicate the presence of viable protozoa in environmental matrices.

Measuring the reduction of *hsp70* mRNA content in fully infective oocysts exposed to anaerobic treatments, for example in sentinel chamber tests, can be a rapid indicator of reduction in oocyst viability. Here, complete inactivation of oocysts is associated with reduction of at least 1 log unit of mRNA, and preferably ≥ 1.5 log units.
In addition to the decrease of the mRNA amount during treatments, the measurement of the *hsp70* induction response to a heat shock trigger gives an indication of the vitality of oocysts eventually contained in an environmental sample. An induction of ≥ 1.27 fold mRNA production has been shown to correspond to oocysts that are fully infectious and capable to excyst (excystation capability > 80 %). *Hsp70* mRNA was found to be constitutively produced in viable *Cryptosporidium* oocysts. That is probably the reason why the mean induction ratio was only 1.6 fold.

The developed RT-qPCR based detection methods have been shown to be applicable to manure and effluents from anaerobic digesters operating at mesophilic and thermophilic conditions. Mesophilic operating regimes, commonly used in Germany, do not provide sufficient sanitation

to ensure complete inactivation of recalcitrant pathogens. Even at 50°C, oocysts may not be totally inactivated if the retention time is not long enough. Applying the optimized molecular methods, it was seen that for the operation at 50°C and short retention times, no reduction in oocyst viability could be derived by measuring the mRNA content because *hsp70* mRNA copies were not consistently reduced, while 55°C led to reduction of ≥ 1.5 log units in *hsp70* mRNA and a corresponding oocyst inactivation. Process parameter variations can influence on the pathogen reduction. It is therefore important the availability of appropriate detection methods to screen for such changes.

This research advanced the application of molecular tools based on qPCR and RT-qPCR for environmental screening of pathogenic microorganisms. They can be useful to identify possible microbial contamination of water resources and crops, for example when manure is applied to land. The rapid, specific and sensitive detection methods employed are applicable to detect *Cryptosporidium* oocysts and enteroviruses in complex environmental matrices.

In order to develop and improve manure management, further knowledge is needed about the fate of pathogens in the environment and the related application of molecular methods. In this regard, the optimized (RT)-qPCR procedures can be applied to investigate the transport of oocysts and enteroviruses in the environment once manure or slurry is spread to the land either under controlled operations or when spills occur, particularly from large scale animal husbandry.

References

Alm E.W., Zheng D., Raskin L. (2000) The Presence of Humic Substances and DNA in RNA Extracts Affects Hybridization Results. Appl. Environ. Microbiol. 66, 4547–4554.

Alonso J.L., Amorós I., Cañigral I. (2011) Development and evaluation of a real-time PCR assay for quantification of *Giardia* and *Cryptosporidium* in sewage samples. Appl. Microbiol. Biotechnol. 89, 1203–1211.

Aneja M.K., Sharma S., Munch J.C., Schloter M. (2004) RNA fingerprinting - a new method to screen for differences in plant litter degrading microbial communities. J. Microbiol. Methods 59, 223-231.

Anonymous (1998) Verordnung über die Verwertung von Bioabfällen auf landwirtschaftlich, forstwirtschaftlich und gärtnerisch genutzten Böden (Bioabfallverordnung - BioAbfV) / Federal Regulation on the Utilization of Bio-Wastes in Agriculture von 21.09.1998 (BGBl. I S. 2955).

Anonymous (2004) Tierische Nebenprodukte-Beseitigungsgesetz (TierNebG) / Federal Animal By-Product Disposal Act (BGBl. I S. 82).

Anonymous (2006) Verordnung über die Anwendung von Düngemitteln, Bodenhilfsstoffen, Kultursubstraten und Pflanzenhilfsmitteln nach den Grundsätzen der guten fachlichen Praxis beim Düngen (Düngeverordnung - DüV) / Federal Regulation on the Application of Fertilizers (BGBl. I S. 221).

Anonymous (2008) Verordnung über das Inverkehrbringen von Düngemitteln, Bodenhilfsstoffen, Kultursubstraten und Pflanzenhilfsmitteln (Düngemittelverordnung - DüMV) / Federal Regulation on the Circulation of Fertilizers (BGBl. I S. 2524).

Aw T.G., Gin K.Y.-H (2010) Environmental surveillance and molecular characterization of human enteric viruses in tropical urban wastewaters. J. of Applied Microbiol. 109, 716–730.

Bach H.J., Hartmann A., Trevors J. T., Munch J. C. (1999) Magnetic capture–hybridization method for purification and probing of mRNA for neutral protease of Bacillus cereus. J. Microbiol. Methods 37, 187–192.

Bach H.J., Tomanova J., Schloter M., Munch J.C. (2002) Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. J. Microbiol. Methods 49, 235– 245.

Baeumner A.J., Humiston M.C., Montagna R.A., Durst R.A. (2001) Detection of Viable Oocysts of *Cryptosporidium parvum* Following Nucleic Acid Sequence Based Amplification. Anal. Chem. 73, 1176-1180.

Bajszár G., Dekonenko A. (2010) Stress-Induced *Hsp70* Gene Expression and Inactivation of *Cryptosporidium parvum* Oocysts by Chlorine-Based Oxidants. Appl. Environ. Microbiol. 76, 1732–1739.

Baque R.H., Gilliam A.O., Robles L.D., Jakubowski W., Slifko T.R. (2011) A real-time RT-PCR method to detect viable *Giardia lamblia* cyst in environmental waters. Water Research 45, 3175-3184.

Beach M.J. (2008) Waterborne: Recreational Water. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. and Xiao L. (eds.). CRC Press, ISBN 978-1-4200-5226-8.

Beckmann R.P., Mizzen L.A., Welch, M.J. (1990) Interaction of *Hsp70* with newly synthesized proteins: Implications for protein folding and assembly. Science 248, 850-854.

Beelman C.A., Parker R. (1995) Degradation of mRNA in Eukaryotes, Cell 81, 179-183.

Bernard P.S., Wittwer C.T. (2002) Real-Time PCR Technology for Cancer Diagnostics. Clinical Chemistry 48, 1178–1185.

Bialek R., Binder N., Dietz K., Joachim A., Knobloch A., Zelck U.E. (2002) Comparison of fluorescence, antigen and PCR assays to detect *Cryptosporidium parvum* in fecal specimens. Diagnostic Microbiology and Infectious Disease 43, 283–288.

Bicudo J.R., Goyal S.M., Zhu J. (2000) Animal Production, Manure Management and Pathogens: A Review. Proceedings of the Eighth International Symposium on Animal, Agricultural and Food Processing Wastes, J.A. Moore ed., St. Joseph, Mich., ASAE, 507-521.

Blackburn B.G., Mazurek J.M., Hlavsa M., Park J., Tillapaw M., Parrish M., Salehi E., Franks W., Koch E., Smith F., Xiao L., Arrowood M., Hill V., da Silva A., Johnston S., Jones, J.L. (2006) Cryptosporidiosis associated with ozonated apple cider. Emerg. Infect. Dis. 12, 684–686.

Blumenthal U., Mara D., Peasey A., Ruiz-Palacios G., Stott R. (2000) Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines. Bulletin WHO, 78, 1104-1116, World Health Organization, Geneva.

Böhm R. (2004) Investigations on microbial indicators and/or test organisms in supervision of hygienic safety in co-digestion of animal slury, biowaste and/or animal byproducts. International Society for Animal Hygiene, Saint-Malo, 283-284.

Borchardt M.A., Bradbury K.R., Gotkowitz M.B., Cherry J.A., Parker B.L. (2007) Human Enteric Viruses in Groundwater from a Confined Bedrock Aquifer. Environ. Sci. Technol. 41, 6606–6612.

Bowman D.D. (2008) Waste Management. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. and Xiao L. (eds.). CRC Press, ISBN 978-1-4200-5226-8.

Boyer D.G., Kuczynska E., Fayer R. (2009) Transport, fate, and infectivity of *Cryptosporidium parvum* oocysts released from manure and leached through macroporous soil. Environmental Geology, 58, 1011-1019.

Buckley B.A., Owen M.E., Hofmann G.E. (2001) Adjusting the thermostat: the threshold induction temperature for the heat shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. The Journal of Experimental Biology 204, 3571–3579.

Bui X.T., Wolff A., Madsen M., Bang D.D. (2012) Reverse transcriptase real-time PCR for detection and quantification of viable *Campylobacter jejuni* directly from poultry faecal samples. Research in Microbiology 163, 64-72.

Bukhari Z., Smith H.V. (1995) Effect of three concentration techniques on viability of *Cryptosporidium* oocysts recovered from bovine feces. J. Clinical Microbiol. 33, 2592-2595.

Bürgmann H., Widmer F., Sigler W.V., Zeyer J. (2003) mRNA Extraction and Reverse Transcription-PCR Protocol for Detection of nifH Gene Expression by *Azotobacter vinelandii* in Soil. Appl. Environ. Microbiol. 69, 1928–1935.

Burton C. H., Turner C. (2003) Manure Management - Treatment strategies for sustainable agriculture. Silsoe Research Institute, ISBN 0 9531282-6-1.

Bustin S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25, 169-193.

Bustin S.A., Nolan T. (2004) Analysis of mRNA expression by real-time PCR. In: Real time PCR – An essential guide, Edwards K., et al., (ed.). Horizon Bioscience, ISBN 0-9545232-7-X.

Cai, P., Huang Q., Zhang X., Chen H. (2006) Adsorption of DNA on clay minerals and various colloidal particles from an Alfisol. Soil Biology & Biochemistry 38, 471–476.

Campbell A.T., Robertson L.J., Smith H.V. (1992) Viability of *Cryptosporidium parvum* Oocysts: Correlation of In Vitro Excystation with Inclusion or Exclusion of Fluorogenic Vital Dyes. Appl. Environ. Microbiol. 58, 3488-3493.

Castro-Hermida J.A., García-Presedo I., Almeida A., Gonzalez-Warleta M., Correia Da Costa J.M., Mezo M. (2008) Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium spp*. and *Giardia duodenalis*. Water Research 42, 3528-3538.

Chalmers R.M. (2009) Advances in Diagnosis: is Microscopy Still the Benchmark? In: *Giardia* and *Cryptosporidium* From Molecules to Disease, Ortega-Pierres G. et al., (ed.). CAB International, ISBN 978-1-84593-391-3.

Chappell C.L., Okhuysen P.C., Sterling C.R., Wang C., Jakubowski W., Dupont H.L. (1999) Infectivity of *Cryptosporidium parvum* in healthy Adults with pre-existing Anti-C. parvum Serum Immunoglobulin G. Am. J. Trop. Med. Hyg. 60, 157–164.

Chapron D.C., Ballester A.N., Fontaine H.J., Frades N.C, Margolin B.A. (2000) Detection of Astroviruses, Enteroviruses, and Adenovirus Types 40 and 41 in Surface Waters Collected an Evaluated by the Information Collection Rule and an Integrated Cell Culture-Nested PCR Procedure. Appl. Environ. Microbiol. 66, 2520–2525.

Clancy J.L., Hargy M. (2008) Waterborne: Drinking Water. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. and Xiao L. (eds.). CRC Press, ISBN 978-1-4200-5226-8.

Connelly J.T., Nugen S.R., Borejsza-Wysocki W., Durst R.A., Montagna R.A., Baeumner A.J. (2008) Human pathogenic *Cryptosporidium* species bioanalytical detection method with single oocyst detection capability. Anal. Bioanal. Chem. 391, 487–495.

Costantini V.P., Azevedo A.C., Li X., Williams M.C., Michel F.C., Saif L.J. (2007) Effects of Different Animal Waste Treatment Technologies on Detection and Viability of Porcine Enteric Viruses. Appl. Environ. Microbiol. 73, 5284–5291.

Coutard F., Pommepuy M., Loaec S., Hervio-Heath D. (2005) mRNA detection by reverse transcription-PCR for monitoring viability and potential virulence in a pathogenic strain of *Vibrio parahaemolyticus* in viable but nonculturable state. J. of Applied Microbiol. 98, 951–961.

Dalley B.K., Golomb M. (1992) Gene expression in the *Caenorhabditis elegans* dauer larva: developmental regulation of Hsp90 and other genes. Dev. Biol. 151, 80–90.

Davies C.M., Kaucner C., Deere D.A, Ashbolt N.J. (2003) Recovery and enumeration of *Cryptosporidium parvum* from animal fecal matrices. Appl. Environ. Microbiol. 69, 2842–2847.

Davies C.M., Ferguson C.M., Kaucner C., Krogh M., Altavilla N., Deere D.A., Ashbolt N.J. (2004) Dispersion and Transport of *Cryptosporidium* Oocysts from Fecal Pats under Simulated Rainfall Events. Appl. Environ. Microbiol. 70, 1151–1159.

Davies C.M., Altavilla N., Krogh M., Ferguson C.M., Deere D.A., Ashbolt N.J. (2005) Environmental inactivation of *Cryptosporidium* oocysts in catchment soils. J. of Applied Microbiol. 98, 308–317.

Deng M.Q., Lam K.M., Cliver D.O. (2000) Immunomagnetic separation of *Cryptosporidium parvum* oocysts using MACS MicroBeads and high gradient separation columns. J. Microbiol. Methods 40, 11–17.

Desmarais T.R., Solo-Gabriele H.M., Palmer C.J. (2002) Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment. Appl. Environ. Microbiol. 68, 1165-1172.

D'haene B., Vandesompele J., Hellemans J. (2010) Accurate and objective copy number profiling using real-time quantitative PCR. Methods 50, 262–270.

Di Giovanni G.D., Hashemi F.H., Shaw N. J., Abrams F.A., LeChevallier M.W., Abbaszadegan M. (1999) Detection of infectious *Cryptosporidium parvum* oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. Appl. Environ. Microbiol. 65, 3427-3432.

Dixon B.R. (2009) The Role of Livestock in the Foodborne Transmission of *Giardia duodenalis* and *Cryptosporidium spp*. to Humans. In *Giardia* and *Cryptosporidium* From Molecules to Disease. Ed. Ortega-Pierres et al., CAB International, ISBN 978-1-84593-391-3.

Duffy G., Moriarty E. M. (2003) *Cryptosporidium* and its potential as a food-borne pathogen. Anim. Health Res. Rev. 4, 95–107.

Dorevitch S., Ashbolt N.J., Ferguson C.M., Fujioka R., McGee C.D., Soller J.A., Whitman R.L. (2010) Meeting Report: Knowledge and Gaps in Developing Microbial Criteria for Inland Recreational Waters. Environ. Health Perspect. 118, 871–876.

DuPont H. L., Chappell C.L., Sterling C. R., Okhuysen P. C., Rose J.B., Jakubowski W. (1995) The Infectivity of *Cryptosporidium parvum* in healthy volunteers. The New England Journal of Medicine 332, 855 859.

DVGW (1995) Richtlinien fur Trinkwasserschutzgebiete, I. Teil: Schutzgebiete fur Grundwasser. Arbeitsblatt W 101, Deutscher Verein des Gas- und Wasserfaches.

Edwards K.J. (2004) Performing Real-Time PCR. In: Real time PCR – An essential guide, Edwards K., et al. (ed.). Horizon Bioscience, ISBN 0-9545232-7-X.

Effenberger M., Bachmaier J., Garcés G., Gronauer A., Wilderer P.A., Lebuhn M. (2006). Mesophilic-thermophilic-mesophilic anaerobic digestion of semi-liquid dairy cattle manure. Water Sci. Technol. 53, 253-261.

Effenberger M. (2008) Influence of temperature and feeding mode on digestion and sanitation efficiency during multiple-stage anaerobic treatment of liquid dairy cattle manure. PhD. Thesis, Technische Universitat München. Cuvillier Verlag, ISBN 978-3-86727652-8.

EPA (U.S. Environmental Protection Agency). (1993) Standards for the use or disposal of Sewage Sludge. United States Environmental Protection Agency, 40 CFR Part 503.

EPA (2004) Guidelines for Water Reuse. US EPA/625/R-04/108.

EPA (2005) Detecting and Mitigating the Environmental Impact of Fecal Pathogens Originating from Confined Animal Feeding Operations: Review. U.S. EPA/600/R-06/021.

EPA (2005b) Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA. U.S. EPA 815-R-05-001.

EPA (2009) Review of published studies to characterize relative risks from different sources of fecal contamination in recreational waters. U.S. EPA 822-R-09-001.

EPA (2010) 1615 Measurement of Enterovirus and Norovirus, Occurrence in Water by Culture and RT-qPCR. Office of Research and Development, EPA/600/R-10/181, December 2010.

European Commission (1986) Council Directive of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture, 86/278/EEC. Official Journal of the European Communities L 181, 6-12.

European Commission (1991) Council Directive of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural Sources, 91/676/EEC. Official Journal of the European Communities L 375, 1-8.

European Commission (2003) Draft Discussion Document for the Ad Hoc Meeting on Biowastes and Sludges, Brussels. European Commission Directorate-General Environment, Directorate A - Communications, Governance, Production, Consumption and Waste, ENV.A2.

European Commission (2009) Regulation (EC) No 1069/2009 of the European Parliament and Council of the European Union of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). Official Journal of the European Union L 300, 1-33.

European Commission (2010) Directive 2010/75/EU of the European Parliament and Council of the European Union of 24 November 2010 on industrial emissions (integrated pollution prevention and control). Official Journal of the European Union L 334, 17-119.

European Commission (2011) Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive. Official Journal of the European Union L 54, 1-254.

FAC - Florida Administrative Code (2007) Rule Chapter 62-610: Reuse of reclaimed water and land application. Florida Department of State, United States of America. http://www.dep.state.fl.us/legal/rules/wastewater/62-610.pdf, accessed October, 2012.

FAO - Food and Agricultural Organization of the United Nations (2006) Livestock's long shadow – environmental issues and options. ISBN-13: 978-925105571-7.

Fayer R. (1994) Effect of High Temperature on Infectivity of *Cryptosporidium parvum* Oocysts in Water. Appl. Environ. Microbiol. 60, 2732-2735.

Fayer R., Nerad T. (1996) Effects of low temperatures on viability of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 62, 1431-1433.

Fayer R., Graczyk T.K., Lewis E.J., Trout J.M., Farley C.A. (1998) Survival of Infectious *Cryptosporidium parvum* Oocysts in Seawater and Eastern Oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl. Environ. Microbiol. 64, 1070–1074.

Fayer R., Morgan U., Upton S. J. (2000) Epidemiology of *Cryptosporidium*: transmission, detection and identification. International Journal for Parasitology 30, 1305-1322.

Fayer R. (2004) *Cryptosporidium*: a water-borne zoonotic parasite. Vet. Parasitol. 126, 37-56.

Fayer R. (2008) General Biology. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. and Xiao L. (eds.). CRC Press, ISBN 978-1-4200-5226-8.

Feder M.E., Hofmann G.E. (1999) Heat-shock Proteins, Molecular Chaperones and the Stress Response. Annu. Rev. Physiol. 61, 243–282.

Ferguson C.M., Davies C.M., Kaucner C., Krogh M., Rodehutskors J., Deere D.A., Ashbolt N.J. (2007) Field scale quantification of microbial transport from bovine faeces under simulated rainfall events. Journal of Water and Health 5, 83-95.

Fey A., Eichler S., Flavier S., Christen R., Höfle M.G., Guzmán C.A. (2004) Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using Salmonella as a model organism. Appl. Environ. Microbiol. 70, 3618-23.

Fong T.T., Lipp K.E. (2005) Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools. Microbiology and Molecular Biology Reviews 69, 357-371.

Fontaine M., Guillot E. (2003) Study of 18S rRNA and rDNA stability by real-time RT-PCR in heat-inactivated *Cryptosporidium parvum* oocysts. FEMS Microbiology Letters 226, 237-243.

Fredslund L., Ekelund F., Jacobsen C.S., Johnsen K. (2001) Development and application of a most probable number-PCR assay to quantify flagellate populations in soil samples. Appl. Environ. Microbiol. 67, 1613–1618.

Fredricks D.N., Smith C., Meier A. (2005) Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol. 43, 5122-5128.

Frostegård A., Courtois S., Ramisse V., Clerc S., Bernillon D., Le Gall F., Jeannin P., Nesme X., Simonet P. (1999) Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol. 65, 5409–5420.

Fuhrman A.J., Liang X., Noble T.R. (2005) Rapid Detection of Enteroviruses in Small Volumes of Natural Waters by Real-Time Quantitative Reverse Transcriptase PCR Appl. Environ. Microbiol. 71, 4523–4530.

Fuß R., Ruth B., Schilling R., Scherb H., Munch J.C. (2011) Pulse emissions of N_2O and CO_2 from an arable field depending on fertilization and tillage practice. Agric. Ecosyst. Environ. 144, 61-68.

Garcés G., Effenberger M., Najdrowski M., Wackwitz C., Gronauer A., Wilderer P.A., Lebuhn M. (2006) Quantification of *Cryptosporidium parvum* in anaerobic digesters treating manure by (reverse-transcription) quantitative real-time PCR, infectivity and excystation tests. Water Sci. Technol. 53, 195-202.

Garcés-Sanchez G., Wilderer P.A., Munch J.C., Horn H., Lebuhn M. (2009) Evaluation of two methods for quantification of *hsp70* mRNA from the waterborne pathogen *Cryptosporidium parvum* by reverse transcription real-time PCR in environmental samples. Water Research 43, 2669-2678.

Georgiev V.S. (2009) National Institute of Allergy and Infectious Diseases Volume 2 Impact on Global Health. Humana Press, ISBN 978-1-60327-296-4.

Gerba C.P., Smith J.E.Jr. (2005) Sources of Pathogenic Microorganisms and Their Fate during Land Application of Wastes. J. Environ. Qual. 34, 42-48.

Godfree A. (2003) Health constraints on the agricultural recycling of wastewater sludges. In: Handbook of Water and Wastewater Microbiology, Mara D. and Horan N. (eds.). Academic Press, ISBN 012-470-1000.

Godfree A., Farrell J. (2005) Processes for Managing Pathogens. J. Environ. Qual. 34, 105–113.

Goldstein S.T., Juranek D.D., Ravenholt O., Hightower A.W., Martin D.G., Mesnik J.L., Griffiths S.D., Bryant A.J., Reich R.R., Herwaldt B.A. (1996) Cryptosporidiosis: An Outbreak Associated with Drinking Water Despite State-of-the-Art Water Treatment. Annals of Internal Medicine 124, 459-468.

González-Escalona N., Fey A., Höfle M.G., Espejo R.T., Guzmán C.A. (2006) Quantitative reverse transcription polymerase chain reaction analysis of Vibrio cholerae cells entering the viable but non-culturable state and starvation in response to cold shock. Environ. Microbiol. 8, 658–666.

Greening G.E., Hewitt J., Lewis G.D. (2002) Evaluation of integrated cell culture-PCR (C-PCR) for virological analysis of environmental samples. J. of Applied Microbiol. 93, 745–750.

Griffiths R.I., Whiteley A.S., O'Donnell A.G., Bailey M.J. (2000) Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNAand rRNA-Based Microbial Community Composition. Appl. Environ. Microbiol. 66, 5488-5491.

Guy R.A., Payment P., Krull U.J., Horgen P.A. (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. 69, 5178–5185.

Guzmán C., Jofre J., Montemayor M., Lucena F. (2007) Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. J. of Applied Microbiol. 103, 2420–2429.

Hallier-Soulier, Guillot E. (2003) An Immunomagnetic separation-reverse transcription polymerase chain reaction (IMS-RT-PCR) test for sensitive and rapid detection of viable waterborne *Cryptosporidium parvum*. Environ. Microbiol. 5, 592-598.

Hancock C.M., Rose J.B., Callahan M. (1998) Crypto and *Giardia* in US groundwater. J. Am.Wat.Works Assoc. 90, 58–61.

Hata A., Katayama H., Kitajima M., Visvanathan Ch., Nol Ch., Furumai H. (2011) Validation of Internal Controls for Extraction and Amplification of Nucleic Acids from Enteric Viruses in Water Samples. Appl. Environ. Microbiol. 77, 4336–4343.

Higgins J.A., Fayer R., Trout J.M., Xiao L., Lal A.A., Kerby S., Jenkins M.C. (2001) Real-time PCR for the detection of *Cryptosporidium parvum*. J. Microbiol. Methods 47, 323–337.

Holland P.M., Abramson R.D., Watson R., Gelfand D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5 '- 3 ' exonucleaseactivity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. 88, 7276-7280.

Jacobsen C.S., Holben W.E. (2007) Quantification of mRNA in *Salmonella* sp. seeded soil and chicken manure using magnetic capture hybridization RT-PCR. J. Microbiol. Methods 69, 315-321.

Javier D., Castellanos-Gonzalez A., Weigum E. S., White A.C., Richards-Kortum R. (2009) Oligonucleotide-Gold Nanoparticle Networks for Detection of *Cryptosporidium parvum* Heat Shock Protein 70 mRNA. J Clin Microbiol. 47, 4060–4066.

Jean J.-S., Guo H.-R., Chen S.-H., Liu, C.-C., Chang W.-T, Yang, Y.-J., Huang M.C. (2006) The association between rainfall rate and occurrence of an enterovirus epidemic due to a contaminated well. J. of Applied Microbiol. 101, 1224–1231.

Jenkins M.C., Petersen C. (1997) Molecular Biology of *Cryptosporidium*. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. (ed.). CRC Press, Boca Raton. ISBN 0-8493-7695-5.

Jenkins B.M., Anguish J.L., Bowman D.D., Walker J.M., Ghiorse C.W. (1997) Assessment of a Dye Permeability Assay for Determination of Inactivation Rates of *Cryptosporidium parvum* Oocysts. Appl. Environ. Microbiol. 63, 3844-3850.

Jenkins M.B., Bowman D.D., Ghiorse W.C. (1998) Inactivation of *Cryptosporidium parvum* Oocysts by Ammonia. Appl. Environ. Microbiol. 64, 784-788.

Jenkins M.B., Walker M.J., Bowman D.D., Anthony L.C., Ghiorse W.C. (1999) Use of a sentinel system for field measurements of *Cryptosporidium parvum* oocyst inactivation in soil and animal waste. Appl. Environ. Microbiol. 65, 1998–2005.

Jenkins M., Trout J.M, Abrahamsen M.S., Lancton C.A., Higgins J., Fayer R. (2000) Estimating viability of *Cryptosporidium parvum* oocysts using reverse transcriptase-polymerase chain reaction (RT-PCR) directed at mRNA encoding amyloglucosidase. J. Microbiol. Methods 43, 97-106.

Jenkins M., Trout J.M., Higgins J., Dorsch M., Veal D., Fayer R. (2003). Comparison of tests for viable and infectious *Cryptosporidium parvum* oocysts. Parasitol. Res. 89, 1-5.

Jenkins M.B., Liotta J.L., Lucio-Forster A., Bowman D.D. (2010) Concentrations, Viability, and Distribution of *Cryptosporidium* Genotypes in Lagoons of Swine Facilities in the Southern Piedmont and in Coastal Plain Watersheds of Georgia. Appl. Environ. Microbiol. 76, 5757-5763.

Jenkins M.B., Eaglesham B.S., Anthony L.C., Kachlany S.C., Bowman D.D., Ghiorse W.C. (2010) Significance of Wall Structure, Macromolecular Composition, and Surface Polymers to the Survival and Transport of *Cryptosporidium parvum* Oocysts. Appl. Environ. Microbiol. 76, 1926–1934

Jiang J., Alderisio K.A., Singh A., Xiao L. (2005) Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. Appl. Environ. Microbiol. 71, 1135-1141.

Jiménez Clavero M.A., Escribano-Romero E., Mansilla C., Gómez N., Córdoba L., Roblas N., Ponz F., Ley V., Sáiz J.C. (2005) Survey of Bovine Enterovirus in Biological and Environmental Samples by a Highly Sensitive Real-Time Reverse Transcription-PCR. Appl. Environ. Microbiol. 71, 3536–3543.

Jiménez B., Asano T. (2008) Water reclamation and reuse around the world (Chapter 1). In: Water Reuse: An International Survey Common Practice and Current Needs in the World, Jiménez B. and Asano T. (eds.). IWA Publishing, Inc. London, ISBN 978- 1843390893.

Johnson D.W., Pieniazek N.J., Griffin W., Misener L., Rose J.B. (1995) Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl. Environ. Microbiol. 61, 3849-3855.

Johnston S.P., Ballard M.M., Beach M.J., Causer L., Wilkins P. (2003) Evaluation of Three Commercial Assays for Detection of *Giardia* and *Cryptosporidium* Organisms in Fecal Specimens. Journal of Clinical Microbiology 41, 623-626.

Jothikumar N., Lowther J.A., Henshilwood K, Lees D.N., Hill V.R., Vinjé J. (2005) Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. Appl. Environ. Microbiol. 71, 1870-75.

Karpiscak M.M., Sanchez L.R., Freitas R.J., Gerba C.P. (2001) Removal of bacterial indicators and pathogens from dairy wastewater by a multi-component treatment system. Water Sci Technol. 44, 183-190.

Kato S., Fogarty E., Bowman D.D. (2003a) Effect of aerobic and anaerobic digestion on the viability of *Cryptosporidium parvum* oocysts and *Ascaris suum* eggs. Int. J. Environ. Health. Res. 13, 169–179.

Kato S., Lindergard G., Mohammed H.O. (2003b) Utility of the *Cryptosporidium* oocyst wall protein (COWP) gene in a nested PCR approach for detection infection in cattle. Vet. Parasitol. 11, 153–159

Kato S., Jenkins M., Fogarty E., Bowman D. (2004) *Cryptosporidium parvum* oocyst inactivation in field soil and its relation to soil characteristics: analysis using geographic information systems. Sci. Tot. Environ. 321, 47-58.

Katayama H., Haramoto E., Oguma K., Yamashita H., Tajima A., Nakajima H., Ohgakia S. (2008) One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water Research 42, 1441–1448.

Kirchmayr R., Scherzer R., D.L. Baggesen, Braun R., Wellinger A. (2003) Animal by-products and anaerobic digestion. IEA Bioenergy Task 37.

Körner C.G., Wormington M., Muckenthaler M., Schneider S., Dehlin E., Wahle E. (1998) The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of Xenopus oocytes. EMBO Journal 17, 5427–5437.

Klerks M.M., van Bruggen A.H.C, Zijlstra C., Donnikov M. (2006) Comparison of Methods of Extracting *Salmonella enterica* Serovar Enteritidis DNA from Environmental Substrates and Quantification of Organisms by Using a General Internal Procedural Control. Appl. Environ. Microbiol. 72, 3879–3886.

Kreader C.A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62, 1102–1106.

Kuczynska E., Shelton D.R. (1999) Method for detection and enumeration of *C. parvum* oocysts in feces, manures and soils. Appl. Environ. Microbiol. 65, 2820-2826.

Kuczynska E., Shelton D.R., Pachepsky Y. (2005) Effect of Bovine Manure on *Cryptosporidium parvum* Oocyst Attachment to Soil. Appl. Environ. Microbiol. 71, 6394–6397.

Kváč M., Květoňová D., Salát J. (2007) Viability staining and animal infectivity of *Cryptosporidium andersoni* oocysts after long term storage. Parasitol. Res. 100, 213-217.

La Rosa G., Pourshaban M., Iaconelli M., Muscillo M. (2010) Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. Ann. Ist. Super. Sanità. 46, 266-273.

Larionov A., Krause A., Miller W. (2005) A standard curve based method for relative real time PCR data processing. BMC Bioinformatics, 6:62, doi: 10.1186/1471-2105-6-62.

Lebuhn M., Effenberger M., Gronauer A., Wilderer P.A., Wuertz S. (2003) Using quantitative real-time PCR to determine the hygienic status of cattle manure. Water Sci. Tech. 48, 97-103.

Lebuhn M., Effenberger M., Garces G., Gronauer A.,Wilderer P. A. (2004) Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples. Water Sci. Tech. 50, 263-270.

Lebuhn M., Effenberger M., Garcés G., Gronauer A. and Wilderer P.A. (2005). Hygienization by Anaerobic Digestion: Comparison between Evaluation by Cultivation and Quantitative Real-Time PCR. Water Sci. Tech. 52, 93-99.

Lebuhn M., Wilderer P.A. (2006). Biogastechnologie zur umweltvertraglichen Flussigmistverwertung und Energiegewinnung in Wasserschutzgebieten: wasserwirtschaftliche und hygienische Begleituntersuchung. In: LfL Schriftenreihe Nr. 23/2006, Bavarian State Research Center for Agriculture (LfL), Freising, Germany, ISSN 1611-4159, 149-239.

Lee S., Joung M., Ahn M., Huh S., Song H., Park W., Yu J. (2008) CP2 gene as a useful viability marker for *Cryptosporidium parvum*. Parasitol. Res. 102, 381–387.

Lemarchand K., Berthiaume F., Maynard B., Harel J., Payment P., Bayardelle P., Masson L., Brousseau R. (2005) Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J. Microbiol. Methods 63, 115-126.

Lemos V., Graczyk T.K., Alves M., Lobo M.L., Sousa M.C., Antunes F., Matos O. (2005) Identification and determination of the viability of *Giardia lamblia* cysts and *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts in human fecal and water supply samples by fluorescent in situ hybridization (FISH) and monoclonal antibodies. Parasitol. Res. 98, 48–53.

Leoni F., Amar C., Nichols G., Pedraza-Díaz S., McLauchlin J. (2006) Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. Journal of Medical Microbiology 55, 703–707.

Ley V., Higgins J., Fayer R. (2002) Bovine Enteroviruses as Indicators of Fecal Contamination. Appl. Environ. Microbiol. 68, 3455-3461.

Lie Y.S, Petropoulos C.J. (1998) Advances in quantitative PCR technology: 5' nuclease assays. Curr. Opin. Biotechnol. 9, 43-8.

Lindquist S., Craig E. A. (1988) The Heat-Shock Protein. Annu. Rev. Genet. 22, 631-677.

Lipson S.M., Walderman R., Costello P., Szabo K. (1998) Sensitivity of rhabdomyosarcoma and guinea pig embryo cell cultures to field isolates of difficult-to-cultivate group A coxsackieviruses. J Clin. Microbiol. 26, 1298-1303.

Lloyd K.G., Macgregor B.J., Teske A. (2010) Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. FEMS Microbiol. Ecol. 72, 143-151.

Lobo M.L., Xiao L., Antunes F., Matos O. (2009) Occurrence of *Cryptosporidium* and *Giardia* genotypes and subtypes in raw and treated water in Portugal. Letters in Applied Microbiology 48, 732-737.

Lund B., Frøkjaer Jensen V., Have P., Ahring B. (1996) Inactivation of virus during anaerobic digestion of manure in laboratory scale biogas reactors. Antonie van Leeuwenhoek 69, 25-31.

Mara D., Horan N. (2003) Handbook of Water and Wastewater Microbiology. Academic Press, ISBN 012-470-1000.

Maresca B., Kobayashi G.S. (1994) *Hsp70* in parasites: as an inducible protective protein and as an antigen. Experientia 50, 1067-1074.

Matheson C.D., Gurney C., Esau N., Lehto R. (2010) Assessing PCR Inhibition from Humic Substances. The Open Enzyme Inhibition Journal 3, 38-45.

Meyer J. (2002) Hygienisch-mikrobiologische Untersuchung an drei Güllebehandlungsanlagen unter Berücksischtigung von *Cryptosporidium spp.* Klaus Bielefeld Verlag Friedland, ISBN-3-898333-056-7.

Mead P. S., Slutsker L., Dietz V., McCaig L. F., Bresee J. S., Shapiro C., Griffin P. M., Tauxe R.V. (1999) Food-Related Illness and Death in the United States. Emerging Infectious Diseases 5, 607-625.

Medema G.J., Bahar M., Schets F.M. (1997). Survival of *Cryptosporidium parvum, Escherichia coli*, faecal enterococci and *Clostridium perfringens* in river water: Influence of temperature and autochthonous microorganisms. Water Sci. Technol. 35, 249-252.

Medema G.J., Ketelaars H.A.M., Hoogenboezem W. (2001) *Cryptosporidium* and *Giardia*: occurrence in sewage, manure and surface water. In: WHO (2009) Risk Assessment of *Cryptosporidium* in Drinking-water. World Health Organization, WHO/HSE/WSH/09.04.

Monpoeho S., Dehée A., Mignotte B., Schwartzbrod L., Marechal V., Nicolas J.-C., Billaudel S., Férré V. (2000) Quantification of Enterovirus RNA in Sludge Samples Using Single Tube Real-Time RT-PCR. BioTechniques 29, 88-9.

Monpoeho S., Maul A., Mignotte-Cadiergues B., Schwartzbrod L., Billaudel S., Ferre V. (2001) Best Viral Elution Method Available for Quantification of Enteroviruses in Sludge by Both Cell Culture and Reverse Transcription-PCR. Appl. Environ. Microbiol. 67, 2484–2488.

Monteith H.D., Shannon E.E., Derbyshire J.B. (1986) The inactivation of a bovine enterovirus and a bovine parvovirus in cattle manure by anaerobic digestion, heat treatment, gamma irradiation, ensilage and composting. J. Hygiene Camb. 97, 175-184.

Morens D.M., Pallansch M.A. (1995) Epidemiology. In: Human Enterovirus Infections, Rotbart H.A. (ed.). American Society for Microbiology Press, 3-23. ISBN 155-581-0926.

Morgan U.M., Pallant L., Dwyer B.W., Forbes D.A., Rich G., Thompson R.C.A. (1998) Comparison of PCR and Microscopy for Detection of *Cryptosporidium parvum* in Human Fecal Specimens: Clinical Trial. Journal of Clinical Microbiology 34, 995–998.

Najdrowski M., Heckeroth A.R., Wackwitz C., Gawlowska S., Mackenstedt U., Kliemt D., Daugschies A. (2007a) Development and validation of a cell culture based assay for in vitro assessment of anticryptosporidial compounds. Parasitol. Res. 101, 161–167.

Najdrowski M., Joachim A., Daugschies A. (2007b) An improved in vitro infection model for viability testing of *Cryptosporidium parvum* oocysts. Vet. Parasitol. 150, 150–154.

Neumann N.F., Gyürek L.L., Gammie L., Finch G.R., Belosevic M. (2000) Comparison of Animal Infectivity and Nucleic Acid Staining for Assessment of *Cryptosporidium parvum* Viability in Water. Appl. Environ. Microbiol. 66, 406-412.

Newton-Howes J., Heath D.D., Shoemaker C.B., Grant W.N. (2006) Characterisation and expression of an *Hsp70* gene from *Parastrongyloides trichosuri*. International Journal for Parasitology xx, 1–8.

Nichols R.A.B., Smith H.V. (2004) Optimisation of DNA extraction and molecular detection of *Cryptosporidium* oocysts in natural mineral water sources. J. Food Prot. 67, 524–532.

Nichols R.A.B., Campbell B.M., Smith H.W. (2006) Molecular Fingerprinting of *Cryptosporidium* Oocysts Isolated during Water Monitoring. Appl. Environ. Microbiol. 72, 5428-5435.

Nichols G., Lake I.R., Chalmers R. M., Bentham G., Harrison F.C.D., Hunter P.R., Kovats S., Grundy C., Anthony S., Lyons H., Agnew M., Proctor C. (2009) Intervention in Waterborne Disease. In: *Giardia* and *Cryptosporidium* From Molecules to Disease, Ortega-Pierres G. et al. (ed.). CAB International, ISBN 978-1-84593-391-3.

Nielsen K.M, Johnsen P., Bensasson D., Daffonchio D. (2007) Release and persistence of extracellular DNA in the environment. Environ. Biosafety Res. 6, 37-53.

Nieminski E.C., Schaefer III F.W., Ongerth J.E. (1995) Comparison of two methods for detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl. Environ. Microbiol. 61, 1714-1719.

Ogram A., Sayler G.S., Gustin D., Lewis R.J. (1988) DNA adsorption to soils and sediments. Environ. Sci. Technol. 22, 982–984.

Olsen J.E., Larsen, H.E. (1987). Bacterial decimation times in anaerobic digestion of animal slurries. Biological Wastes 21, 153-168.

Olson C.L., Nadeau K.C., Sullivan M.A., Winquist A.G., Donelson J. E., Walsh C.T., Engman D.M. (1994) Molecular and biochemical comparison of the 70-kDa heat shock proteins of *Trypanosoma cruzi*. J. Biol. Chem. 269, 3868–3874.

Oropeza M.R., Cabirol N., Ortega S., Ortiz L.P.C., Noyola A. (2001) Removal of fecal indicator organisms and parasites (fecal coliforms and helminth eggs) from municipal biologic sludge by anaerobic mesophilic and thermophilic digestion. Wat. Sci. Technol. 44, 97–101.

Ortega Y.R., Cama V.A. (2008) Foodborne Transmission. In: *Cryptosporidium* and Cryptosporidiosis, Fayer R. and Xiao L. (eds.). CRC Press, ISBN 978-1-4200-5226-8.

Ottoson J., Hansen A., Björlenius B., Norder H., Stenström T.A. (2006) Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Research 40, 1449-1457.

Paavola T., Syvåsalo E., Rintala J. (2006) Co-digestion of manure and biowaste according to the EC Animal By-Products Regulation and Finnish national regulations. Wat. Sci. Technol. 53, 223–231.

Payment P., Tremblay M., Trudel M. (1985) Relative Resistance to Chlorine of Poliovirus and Coxsackievirus Isolates from Environmental Sources and Drinking Water. Appl. Environ. Microbiol. 49, 981-983.

Peeters J. E., Villacorta I. (1995) *Cryptosporidium*. In: Biotechnology - Guidelines on techniques in coccidiosis research, Eckert, J., et al. (ed.). Office for official Publications of the European Communities, Luxemburg, pp. 202-240.

Petersen R.B., Lindquist S. (1989) Regulation of *hsp70* synthesis by messenger RNA degradation. Cell Regulation 1, 135-149.

Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids Research 29, 2002-2007.

Pfaffl W.M. (2004) Quantification strategies in real-time PCR. In: A-Z of quantitative PCR, Bustin S.A (ed). International University Line, ISBN: 0-9636817-8-8.

Popat S.C., Yates M.V., Deshusses M.A. (2010) Kinetics of inactivation of indicator pathogens during thermophilic anaerobic digestion. Water Research 44, 5965- 5972.

Pusch D., Ihle S., Lebuhn M., Graeber I., López-Pila J.M. (2005) Quantitative detection of enteroviruses in activated sludge by cell culture and real-time RT-PCR using paramagnetic capturing. J. Water Health 3, 313-324.

Quintero-Betancourt W.,Gennaccaro A.L., Scott T.M., Rose J.B. (2003) Assessment of Methods for Detection of Infectious *Cryptosporidium* Oocysts and Giardia Cysts in Reclaimed Effluents. Appl. Environ. Microbiol. 69, 5380-5388.

Radke J.R., Behnke M.S., Mackey A.J., Radke J.B., Roos D.S., White M.W. (2005) The transcriptome of *Toxoplasma gondii* BMC Biology 3:26, doi: 10.1186/1741-7007-3-26.

Rahman S., Rockeman K., Erb K. (2011). Manure Spills: What You Need to Know and Environmental Consequences. NDSU - North Dakota State University, extension service, NM-1555, (http://www.ag.ndsu.edu/manure/documents/nm1555.pdf, accessed October, 2012.

Ramadan Q., Christophe L., Teo W., Li S., Feng H.H. (2010) Flow-through immunomagnetic separation system for waterborne pathogen isolation and detection: application to *Giardia* and *Cryptosporidium* cell isolation. Analytica Chimica Acta 673, 101-108.

Ramirez N.E., Sreevatsan S. (2006) Development of a sensitive detection system for *Cryptosporidium* in environmental samples. Vet. Parasitol. 136, 201–213.

Requena J. M., Jimenez-Ruiz A., Soto M., Assiego R., Santaren J. F., Lopez M. C., Patarroyo M. E., Alonso C. (1992) Regulation of *hsp70* expression in *Trypanosoma cruzi* by temperature and growth phase. Molec. Biochem. Parasit. 53, 201-211.

RKI (2010) Epidemiologisches Bulletin. Robert Koch Institut 37/2010, 369-278.

RKI (2011) Epidemiologisches Bulletin. Robert Koch Institut 3/2011, 17-22.

Robertson L.J., Campbell A.T., Smith H.V. (1992) Survival of *Cryptosporidium parvum* under various environmental pressures. Appl. Environ. Microbiol. 58, 3494-3500.

Robertson L.J., Paton C.A., Campbell A.T., Smith P.G., Jackson M.H., Gilmour R.A., Black S.E., Stevenson D.A., Smith H.V. (2000) *Giardia* Cysts and *Cryptosporidium* Oocysts at Sewage Treatment Works in Scotland, UK. Water Research 34, 2310-2322.

Robertson L.J., Hermansen L., Gjerde B.K. (2006) Occurrence of *Cryptosporidium* Oocysts and *Giardia* Cysts in Sewage in Norway. Appl. Environ. Microbiol. 72, 5297-5303.

Rochelle P.A., Ferguson D.M., Handojo T.J., De Leon R., Steward M.H., Wolfe R.I. (1997) An Assay Combining Cell Culture with Reverse Transcriptase PCR To Detect and Determine the Infectivity of Waterborne *Cryptosporidium parvum*. Appl. Environ. Microbiol. 63, 2029–2037.

Rochelle P.A., Marshall M.M., Mead J.R., Johnson A.M., Korich D.G., Rosen J.S., De Leon R. (2002) Comparison of In Vitro Cell Culture and a Mouse Assay for Measuring Infectivity of *Cryptosporidium parvum.* Appl. Environ. Microbiol. 68, 3809-3817.

Rose J.B., Farrah S.R., Harwood V.J., Levine A.D., Lukasik J., Menendez P., Scott T.M. (2004) Reduction of Pathogens, Indicator Bacteria, and Alternative Indicators by Wastewater Treatment and Reclamation Processes. IWA Publishing, ISBN 1-84339-730-7.

Saleh-Lakha S., Miller M., Campbell R.G., Schneider K., Elahimanesh P., Hart M.M., Trevors J.T. (2005) Microbial gene expression in soil: methods, applications and challenges. J. Microbiol. Methods 63, 1-19.

Satyal S.H., Chen D., Fox S.G., Kramer J.M., Morimoto R.I. (1998) Negative regulation of the heat shock transcriptional response by HSBP1. Genes & Development 12, 1962–1974.

Scheierling S.M., Bartone C.R., Mara D.D., Drechsel P. (2011) Towards an agenda for improving wastewater use in agriculture. Water International 36, 420–440.

Schneegurt M.A., Dore S.Y., Kulpa C.F.Jr. (2003) Direct Extraction of DNA from Soils for Studies in Microbial Ecology. Curr. Issues Mol. Biol. 5, 1-8.

Sedmak G., Bina D., MacDonald J., Couillard L. (2005) Nine-Year Study of the Occurrence of Culturable Viruses in Source Water for Two Drinking Water Treatment Plants and the Influent and Effluent of a Wastewater Treatment Plant in Milwaukee, Wisconsin (2005). Appl. Environ. Microbiol. 71, 1042–1050.

Semenza J., Nichols G. (2007) Cryptosporidiosis Surveillance and Water-borne Outbreaks in Europe. Euro Surveillance 12, 120-123.

Sessitsch A., Gyamfi S., Stralis-Pavese N., Weilharter A., Pfeifer U. (2002) RNA isolation from soil for bacterial community and functional analysis: evaluation of different extraction and soil conservation protocols. J. Microbiol. Methods 51, 171– 179.

Shannon K.E., Lee D.Y., Trevors J.T., Beaudette L.A. (2007) Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Science of the Total Environment 382, 121-129.

Shets M.F., Engels G.B., During M., de Roda Husman A.M. (2005) Detection of Infectious *Cryptosporidium* Oocysts by Cell Culture Immunofluorescence Assay: Applicability to Environmental Samples. Appl. Environ. Microbiol. 71, 6793-6798.

Shuval H., Fattal B. (2003) Control of pathogenic microorganisms in wastewater recycling and reuse in agriculture. In: Handbook of Water and Wastewater Microbiology, Mara D. and Horan N. (eds.). Academic Press, ISBN 0-12-470100-0.

Sinclair R.G., Jones E.L., Gerba C.P. (2009) Viruses in recreational water-borne disease outbreaks: a review. J. of Applied Microbiol. 107, 1769–1780.

Simmons F.J., Xagoraraki I. (2011) Release of infectious human enteric viruses by full-scale wastewater utilities. Water Research, 45, 3590-3598.

Skraber S., Gassilloud B., Schwartzbrod L., Gantzer C. (2004) Survival of infectious Poliovirus-1 in river water compared to the persistence of somatic coliphages, thermotolerant coliforms and Poliovirus-1 genome. Water Research 38, 2927–2933.

Slifko T.R., Friedman D., Rose J.B., Jakubowski W. (1997) An In Vitro Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture. Appl. Environ. Microbiol. 63, 3669-3675.

Smith H.V., Rose J.B. (1998) Waterborne cryptosporidiosis: current status. Parasitol. Today 14, 14-22.

Smith J.J., Gunasekera T.S. Barardi C.R.M, Veal D.,Vesey G. (2004) Determination of *Cryptosporidium parvum* oocyst viability by fluorescence in situ hybridization using a ribosomal RNA-directed probe. J. of Applied Microbiol. 96, 409–417.

Smith C.J., Nedwell D.B., Dong L.F., Osborn A.M. (2007) Diversity and Abundance of Nitrate Reductase Genes (narG and napA), Nitrite Reductase Genes (nirS and nrfA), and Their Transcripts in Estuarine Sediments. Appl. Environ. Microbiol. 73, 3612-3622.

Snelling W.J., Xiao L., Ortega-Pierres G., Lowery C.J., Moore J.E., Rao J.R., Smyth S., Millar B.C., Rooney P.J., Matsuda M., Kenny F., Xu J., Dooley J.S.G. (2007) Cryptosporidiosis in developing countries. Journal of Infection in Developing Countries 1, 242-256.

Snutch P.T., Baillie L.D. (1983) Alterations in the pattern of gene expression following heat shock in the nematode *Caenorhabditis elegans*. Can. J. Biochem. Cell Biol. 61, 480-487.

Snutch P.T., Heschl F.P.M., Baillie L.D. (1988) The *Caenorhabditis elegans hsp70* gene family: a molecular genetic characterization. Gene 64, 241-255.

Sørensen J.G., Loeschcke V. (2002) Decreased heat-shock resistance and down-regulation of Hsp70 expression with increasing age in adult *Drosophila melanogaster*. Functional Ecology 16, 379–384.

Stadterman K.L., Sninsky A.M., Sykora J.L., Jakubowski W. (1995) Removal and Inactivation of *Cryptosporidium* Oocyst by Activated Sludge Treatment and Anaerobic Digestion. Wat. Sci. Technol. 31, 97-104.

Stinear T., Matusan A., Hines K., Sandery M. (1996) Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by Reverse Transcription-PCR. Appl. Environ. Microbiol. 62, 3385-3390.

Stroup S.E., Roy S., Mchele J., Maro V., Ntabaguzi S., Siddique A., Kang G., Guerrant R.L., Kirkpatrick B.D., Fayer R., Herbein J., Ward H., Haque R., Houpt E. R. (2006) Real-time PCR detection and speciation of *Cryptosporidium* infection using Scorpion probes. Journal of Medical Microbiology 55, 1217–1222.

Stults J.R., Snoeyenbos-West O., Methe B., Lovley D.R., Chandler D.P. (2001) Application of the 59 Fluorogenic Exonuclease Assay (TaqMan) for Quantitative Ribosomal DNA and rRNA Analysis in Sediments. Appl. Environ. Microbiol. 67, 2781–2789.

Sturbaum G.D., Klonicki P.T., Marshall M.M., Jost B.H., Clay B.L., Sterling R. (2002) Immunomagnetic Separation (IMS)-Fluorescent Antibody Detection and IMS-PCR Detection of Seeded *Cryptosporidium parvum* Oocysts in Natural Waters and Their Limitations. Appl. Environ. Microbiol. 68, 2991-2996.

Sulston J., Hodgkin J. (1988) Methods. In: The nematode *Caenorhabditis elegans*, Wood W.B. (ed.). Cold Spring Harbor, New York. ISBN 0-87969-307.

Svoboda I.F., Read I.A., Kemp J.S, Wright S.E., Coop R.L., Mawdsley J.L.I., Bargett R.D., Merry R.J., Pain B.F., Theodorou M.K., Bukhari Z., Smith H.V. (1997) *Cryptosporidium* on cattle farms. In: *Cryptosporidium* in Water - The Challenge to Policy Makes and Water Managers. Symposium by Chartered Institute of Water an Environmental Management, Glasgow, 3-20.

Tamburini A., Pozio E. (1999) Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels *(Mytilus galloprovincialis*). Int. J. Parasitol. 29, 711–715.

Tebbe C.C., Vahjen W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol., 59, 2657-2665.

Tebbe C.C, Wenderoth D.F., Vahjen W., Lübke K., Munch J.C. (1995) Direct Detection of Recombinant Gene Expression by Two Genetically Engineered Yeasts in Soil on the Transcriptional and Translational Levels. Appl. Environ. Microbiol. 61, 4296-4303.

Tian H., Hühmer A.F.R., Landers J.P. (2000) Evaluation of Silica Resins for Direct and Efficient Extraction of DNA from Complex Biological Matrices in a Miniaturized Format. Analytical Biochemistry 283, 175-191.

Töwe S., Kleinedam K., Schloter M. (2010) Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples. J. Microbiol. Methods 82, 338–341.

Töwe S., Wallisch S., Bannert A., Fischer D., Hai B., Haesler F., Kleineidam K., Schloter M. (2011) Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. J. Microbiol. Methods 84, 406–412.

Tsai Y.-L., Park M.J., Olson B.H. (1991) Rapid Method for Direct Extraction of mRNA from Seeded Soils. Appl. Environ. Microbiol. 57, 765-768.

Velasquez J., Sonoda S., Bugaisky G.E., Lindquist, S. (1983) Is the major *Drosophila* heat shock protein present in cells that have not been heat shocked? J. Cell Biol. 96, 286-290.

Vesey G., Ashbolt N., Fricker E.J., Deere D.K., William K.L., Veal D.A., Dorsch M. (1998) The use of ribosomal RNA targeted oligonucleotide probe for fluorescent labeling of viable *Cryptosporidium parvum* oocyst. J. Appl. Microbiol. 85, 429-440.

Walters S.P., Yamahara K.M., Boehm A.B. (2009) Persistence of nucleic acid markers of health-relevant organisms in seawater microcosms: implication for their use in assessing risk in recreational waters. Water Res. 43, 4929–4939.

Wagner-Wiening, C., Kimmig P. (1995). Detection of viable *Cryptosporidium parvum* oocysts by PCR. Appl. Environ. Microbiol. 61, 4514–4516.

Wei J., Jin Y., Sims T., Kniel K.E. (2010) Survival of Murine Norovirus and Hepatitis A Virus in Different Types of Manure and Biosolids. Foodborne Pathogens and Disease, 7, 901-906.

Weiland P. (2010) Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85, 849-860.

Whitmore T.N, Robertson L.J. (1995). The effect of sewage sludge treatment processes on oocysts of *Cryptosporidium parvum*. Journal of Applied Bacteriology 78, 34–38.

WHO (2003) Guidelines for Safe Recreational Water Environments, Volume 1 Coastal and Fresh Waters. World Health Organization, ISBN 924-154-5801.

WHO (2006) WHO Guidelines for the Safe Use of Wastewater, Excreta and Greywater. World Health Organization, ISBN 924-154-6867.

WHO (2009) Risk Assessment of *Cryptosporidium* in Drinking-water. World Health Organization, WHO/HSE/WSH/09.04.

WHO (2011) Guidelines for Drinking-water Quality Fourth Edition. World Health Organization, ISBN 978-924-154-8151.

Widmer G., Orbacz E., Tzipori S. (1999) β-Tubulin mRNA as a marker of *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. 65, 1584-1588.

Wiedenmann A., Krüger P., Botzenhart K. (1998) PCR detection of *Cryptosporidium parvum* in environmental samples—a review of published protocols and current developments. Journal of Industrial Microbiology & Biotechnology 21, 150–166.

Wilson I.G. (1997) Inhibition and Facilitation of Nucleic Acid Amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Xiao L., Fayer R., Ryan U., Upton S. J. (2004) *Cryptosporidium* Taxonomy: Recent Advances and Implications for Public Health. Clinical Microbiology Reviews 17, 72–97.

Yankson K.K., Steck T.R. (2009) Strategy for Extracting DNA from Clay Soil and Detecting a Specific Target Sequence via Selective Enrichment and Real-Time (Quantitative) PCR Amplification. Appl. Environ. Microbiol. 75, 6017-6021.