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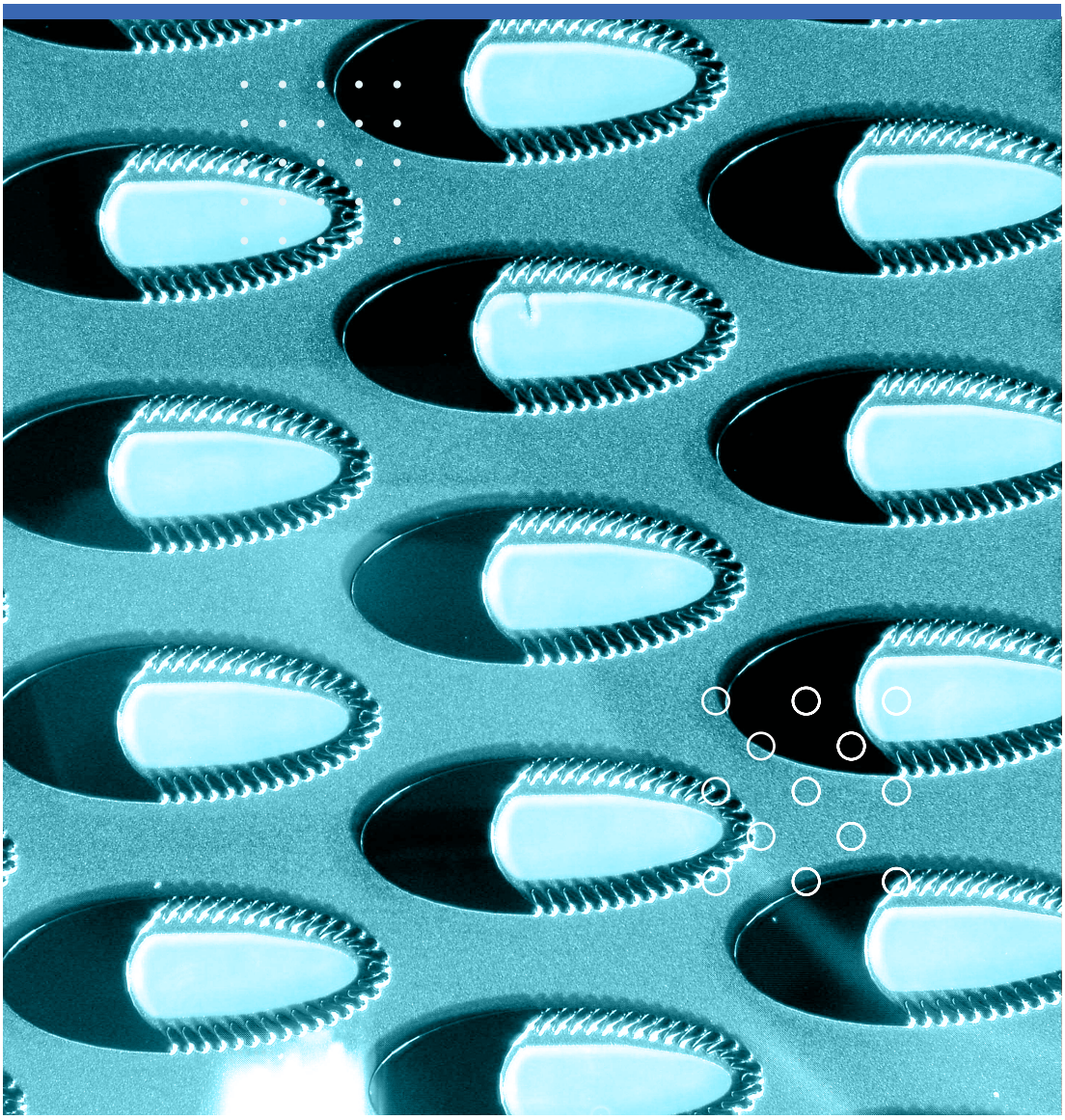
Birgitte Kasin Hønsvall

Environmental microorganisms - microsystem approaches to separation and analysis



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Environmental microorganisms – microsystem approaches to separation and analysis

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Forord

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Takk til Eirik for innspill, tålmodighet og optimisme!
Så vil jeg takke Dag for kaffe, Skype og gode ideer. Og for mikroalgene. Og mer kaffe.
Takk til mamma og pappa, for alle måter dere hjelper meg gjennom utfordrende perioder i livet.
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Sammendrag

I dette PhD-prosjektet ble tre typer teknologi testet, som alle har potensiale for bruk innen mikrosystemer for miljøanalyser: Trilobite®-teknologien for partikkelseparering og rensing, analysemetoden NASBA (nucleic acids sequence-based amplification), og POCNAD, som er et såkalt lab-on-a-chip (lab-i-en-brikke)-system for automatisk prøveanalyse.

Den mikrofluidiske Trilobite®-brikken, som er utviklet for å oppkonsentrere og separere partikler i væske, ble testet for bruk på biologiske partikler. Tre arter mikroalger ble brukt for å undersøke om denne brikken kan passe som en alternativ metode i avvanningsprosessen når algene høstes. Trilobite®-brikken viste potensiale for oppkonsentrering av rigide kiselalger, og for separering der celler kunne skilles basert på størrelsene sine. Men det viste seg også at de ulike egenskapene partiklene har, slik som størrelse, fleksibilitet og produksjon av ekstracellulære polymerer, har stor innvirkning på hvor effektiv chipen er.

Brikken ble også testet for bruk på miljøprøver med vannbårne protozo-parasitter. Disse eksperimentene antydet at Trilobite®-brikken, på nåværende tidspunkt ikke er optimal for å konsentrere opp cyster og oocyster i vannprøver der det er viktig å få hentet inn alle partiklene for å før analyse. Disse partiklene er ofte i lavt antall i prøvene, og det skal ofte få cyster eller oocyster til for å forårsake infeksjon hvis mennesker og dyr får dem i seg.

Real-time NASBA-analyser ble utvikler for sensitiv deteksjon av et konsortium med oljenedbrytende bakterier, samt for protozo-parasitter.

Cryptosporidium parvum og *Cryptosporidium hominis* er de to *Cryptosporidium*-artene som oftest forbindes med cryptosporidiose i mennesker. Ved å utvikle en real-time NASBA-analyse rettet mot MIC1-transcript hos *C. parvum* og *C. hominis*, ble oocyster fra disse artene påvist ned til 5 oocyster. mRNA er målmolekylet for NASBA, og har vært foreslått som markør for levedyktighet, siden mRNA brytes raskt ned etter celledød. Men analysen som ble utviklet her detekterte mRNA i inaktiverte oocyster også, og analysen ser derfor ikke ut til å være passende for å måle levedyktighet.

Primersett for real-time NASBA ble også designet for flagellaten *Giardia duodenalis*, for Assemblage (type) A, B og E. Dessverre ble ikke disse primersettene fullstendig testet på

grunn av begrenset tid og mangel på *Giardia* cyster fra passende Assemblage. Men dette kan være et grunnlag for fremtidig testing.

NASBA-primersett ble også utviklet for et konsortium med fire stammer oljenedbrytende bakterier. Ved å bruke disse primersettene ble alle stammene detektert med NASBA. Men ikke alle stammene kunne skilles fra hverandre ved hjelp av primersettene. Det var også ønskelig å utvikle primersett som kunne gi informasjon om metabolismen hos disse bakteriestammene, men dette ble ikke oppnådd. Til og med når det kun er få transkript tilgjengelig fra et nedregulert gen, kan disse være nok for den sensitive amplifiseringen NASBA gir. Men primersettene var gode nok til å bli inkludert i NASBA-delen av POCNAD-systemet, som er et lab-on-a-chip-system, og NASBA ble gjennomført her. NASBA-reaksjonen i seg selv virket ikke å ha noen problemer i den mikrofluidiske POCNAD-brikken, men blant annet pumping av reagenser i brikken og sanntidsdeteksjon (real-time) viste seg å være vanskelig i systemet. Alle de nødvendige komponentene, samt et kontrollinstrument som ikke var ferdig utviklet, gjorde systemet veldig komplisert. Fullstendig håndfri og automatisk analyse kunne ikke gjennomføres i POCNAD systemet slik det var. Disse eksperimentene viste tydelig hvor vanskelig det kan være å utvikle et brukbart system av denne typen.

Abstract

Three technologies were tested in this PhD project, all of which have potential for use in micro systems for environmental analyses: The Trilobite® technology for particle separation and purification, the analysis technique nucleic acids sequence-based amplification (NASBA), and POCNAD, a lab-on-a-chip system for automatic sample analysis.

The Trilobite® microfluidic chip for concentration and separation of particles in fluids was tested for its use in applications for biological particles. Three species of microalgae were used to investigate the chip's potential as an alternative dewatering technique in microalgae harvesting. The Trilobite® chip was promising for concentration of rigid diatoms, and for separation of cells based on their sizes. However, the various properties of the particles, such as size, flexibility and production of extracellular polymers, have great impact on efficiency of the chip.

The chip was also tested for its application in analysis of environmental samples for waterborne protozoan parasites. These experiments suggested that the Trilobite® chip might not currently be optimal for concentration of cysts and oocysts for water samples, where efficient particle recovery is crucial for detection. The numbers of these particles in water are generally low, and few cysts or oocyst may be enough to cause infection if they are ingested by humans or animals.

NASBA assays were developed for sensitive detection of a consortium of oil-utilizing bacteria and also for protozoan parasites.

Cryptosporidium parvum and *Cryptosporidium hominis* are the two *Cryptosporidium* species most often associated with human cryptosporidiosis. By developing a NASBA assay targeting the MIC1 transcript of *C. parvum* and *C. hominis*, oocysts of these two species were successfully detected down to 5 oocysts. The assay was also able to distinguish *C. parvum* oocysts from *C. hominis*. mRNA, which is the target molecule for NASBA, has been suggested to be a suitable marker for viability, as mRNA is degenerated quickly after cell death. However, the assay did not seem to be suitable for using as a viability assay, as transcripts from inactivated oocysts could also be detected.

NASBA primer sets were designed for the binucleated flagellate *Giardia duodenalis*, Assemblages A, B, and E. Unfortunately, due to time constraints and the lack of *Giardia*

cysts of the appropriate Assemblages, these primer sets were not fully tested. This remains a subject for further studies.

NASBA primer sets were also developed for a consortium of four strains of oil-utilizing bacteria. Using these, all strains were detected by NASBA. However, not all strains could be distinguished from each other using the primer sets. An additional intention was to develop primer sets that could provide information on the metabolism of these strains, but this was not achieved. Even when there are very few transcripts available from a down-regulated gene, these may still be sufficient for the sensitive amplification NASBA provides. However, the primer could be used in the NASBA assay component of the POCNAD lab-on-a-chip system. The NASBA reaction was carried out in the POCNAD system. The NASBA reaction itself did not seem to have any difficulties in the microfluidic POCNAD chip, but factors regarding pumping of reagents in the chip, and real-time detection proved to be challenging in the system. All the required components of the system, and a control instrument that was not sufficiently developed, made this system very complicated. Complete hands-off automatic analysis could not be conducted in the POCNAD system as it stood. These experiments provided a clear demonstration of the difficulty in developing usable systems of this type.

Table of contents

Papers omitted from online edition due to publisher's restrictions

1. Introduction

The original objective of my PhD research project was to develop an existing total analysis microsystem (POCNAD – point-of-care nucleic-acid diagnosis) for the analysis and monitoring of oil-utilizing bacteria. In order to concentrate the bacteria from the samples prior to analysis, the intention was to integrate a microfluidic chip, provided by Trilobite Microsystems AS, into the analysis system. For this project, I was employed as an industry PhD student, with salary and employment also provided by Trilobite Microsystems AS.

The intention of POCNAD analyses was to obtain information on the presence of four particular strains of bacteria, and, if possible, their level of viability. For this purpose, using a sensitive analysis method that was based on RNA was proposed to be incorporated into the system.

However, due to factors beyond my control, this original project was terminated prior to completion, and therefore the objectives of my research were altered accordingly.

After discussion with other collaborators, the focus of my project turned towards parasites, with the idea that some of the technologies and techniques from the POCNAD project could be of relevance for environmental analysis. Thus, the objectives became to develop a method for analysis of the waterborne parasites *Cryptosporidium* and *Giardia duodenalis*, in their transmission stages as oocysts (*Cryptosporidium*) or cysts (*Giardia*). As only viable parasites are infectious, information on the viability of the parasites is also important. This aspect also tied in with original work on the POCNAD system. As not all species of *Cryptosporidium* or genotypes of *Giardia duodenalis* are of public health significance, the intention was also to develop an analysis procedure that could distinguish between species/genotypes; the current standard detection methods (with identification based on microscopy of stained samples), is unable to distinguish between many of the *Cryptosporidium* species or the different genotypes of *Giardia*.

As these waterborne parasites normally occur in low numbers in water samples, a concentration and purification step is required before the samples are analysed by standard methods. Currently, the standard method for concentration is filtration, and the (oo)cysts are recovered from the filter by simple elution. Filtration is carried out by dead-end filtration, and new methods for recovery of (oo)cysts are necessary, with the objective of enabling filtration of large volumes without clogging. Thus, one objective of

the research was to explore whether the microfluidic technology of Trilobite Microsystems AS could be used for this purpose: to concentrate (oo)cysts from water samples through a non-clogging microfluidic chip. Again, this research provided an overlap with the original POCNAD research.

As the distribution of (oo)cysts in water samples can be very low, I decided to begin the research by examining the microfluidic chip's abilities at concentrating and purifying more densely distributed microorganisms. Microalgae are used in numerous applications, such as food and animal feed, nutritional supplements, and in cosmetics, among others. In microalgae cultivation, the biggest challenge and the most expensive step, is separating them from the water phase during harvesting. There is therefore a need for new and cheaper methods of water removal. My objective was to examine the efficacy of the Trilobite® microfluidic chip as a method for removing water from algal cultures. This research would both provide information regarding whether we could provide an alternative water removal step for microalgae harvesting, but also would provide preliminary information on the chip's ability to handle biological micro-particles, before we progressed the research to exploring the use of the chip on the less densely distributed biological particles, parasite (oo)cysts.

Thus, the research described in this PhD project falls into the area of exploring the use of microsystem approaches for the separation and analysis of environmental microorganisms, and the research work described in this thesis is divided into three main topics, the Trilobite® concentration and separation chip; the analysis method, NASBA; and finally, the POCNAD analysis technology, into which NASBA is integrated. All these topics are considered for their application in environmental-type samples, from dewatering of micro-algae cultures to analysis of environmental samples for specific parasites.

The various microorganisms that I used in my project are first introduced in Chapter 2. These were bacteria, protozoan parasites, and microalgae that were used for testing of the Trilobite® chip and/or were analysed by NASBA.

The Trilobite® technology consists of a microfluidic chip that can be used for purification of fluids, and for separation and concentration of particles in fluids. The chip has many

potential uses, and in my work I explored some of its potential applications for biological particles. Other technologies that may have been alternative approaches for achieving the same aim are described in Chapter 3, but, although of potential relevance, were not investigated in the research described here because the focus was on the specific technology of my employer. Samples with a high density of particles were tested with microalgae, and for low particle density, cysts and oocyst of protozoan parasites were used. The Trilobite® chip and its potential for biological applications are described is described in Chapter 4.1.

The molecular method that I used for analysis, nucleic acid-sequence-based amplification (NASBA) is described in Chapter 4.2. This method was explored for its potential use for identifying protozoan parasites, and for use in monitoring of a consortium of oil-utilizing bacteria.

The NASBA method also has some advantages that made it the analysis tool of choice for the lab-on-a-chip (LOC) system that was used in the initial project of my research. This system was called POCNAD (point-of-care nucleic acid detection), and my role in its development and associated investigations is described in Chapter 4.3.1.

Main objective:

Exploration of microsystem approaches for the separation and analysis of environmental microorganisms.

Sub-objectives:

1) The use of the Trilobite® chip for concentration of microalgae.
2) The use of the Trilobite® chip for concentration of waterborne protozoan parasites of public health significance (*Cryptosporidium* spp. oocysts, *Giardia duodenalis* cysts, *Toxoplasma gondii* oocysts)
3) The use of NASBA for identifying oil-utilizing bacteria, with particular emphasis on distinguishing between strains and growth conditions.
4) The use of NASBA for identifying *Cryptosporidium* oocysts, with particular emphasis on species identification and assessment of oocyst viability.

5) The use of NASBA for identifying *Giardia* cysts, with particular emphasis on genotype identification.
6) Development of the use of the POCNAD system, integrating both the Trilobite® chip and NASBA for environmental analysis, with particular emphasis on oil-utilizing bacteria.

The publications resulting from my work describe different parts of the technologies I worked with during the PhD project.

The Trilobite® system is presented in Paper 1: Continuous harvesting of microalgae by new microfluidic technology for particle separation, published in Bioresearch Technology. This paper describes the experimental work in using the Trilobite® chip for dewatering of microalgae.
The NASBA method is the focus in Paper 2: Real-time Nucleic Acid Sequence-based Amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts, published in Experimental Parasitology, and Paper 3: From research lab to standard environmental analysis tool: will NASBA make the leap?, published in Water Research. In Paper 2, the development and testing of a NASBA assay for distinguishing *C. parvum* from *C. hominis* is described, while Paper 3 is a review on the potential for NASBA to be used for routine environmental monitoring.

During my experimental work on NASBA, I encountered some issues with loss of material during the isolation of nucleic acids. These issues and the solving of them are described in Paper 4: Washed away; minimising RNA losses during isolation, submitted to Journal of Biomolecular Techniques.

The POCNAD system is described in Paper 5: Detection of oil-utilizing microorganisms by nucleic acid sequence-based amplification in a total analysis lab-on-a-chip device, published and presented at the microTAS conference. This conference paper describes preliminary extraction results, and describes the POCNAD system. Unfortunately, the project was terminated before results for further publications were achieved.

2. Biological particles

2.1. A small world

Living organisms come in a wide spectrum of sizes (Figure 1), from the largest animal, the blue whale at 30 metres, and the honey mushroom (*Armillaria gallica*) spreading across the largest area (15 hectares), to the many smallest organisms that are not even visible with the naked eye. The greatest diversity is found at the micro-end of the size-scale of life. Life sprang from the small-scale, and the highest metabolic variance exists in micro-scale environments. There are microbes "breathing" sulphur or nitrite instead of oxygen; single-celled organisms living in extreme conditions like hot springs, or thousands of metres below the sea surface; organisms capable of including oil, heavy metals, and iron in their diets, to name a few. The 0.5 mm large water-bear (tardigrade) has even survived the harsh conditions of space (Jönsson et al., 2008)!

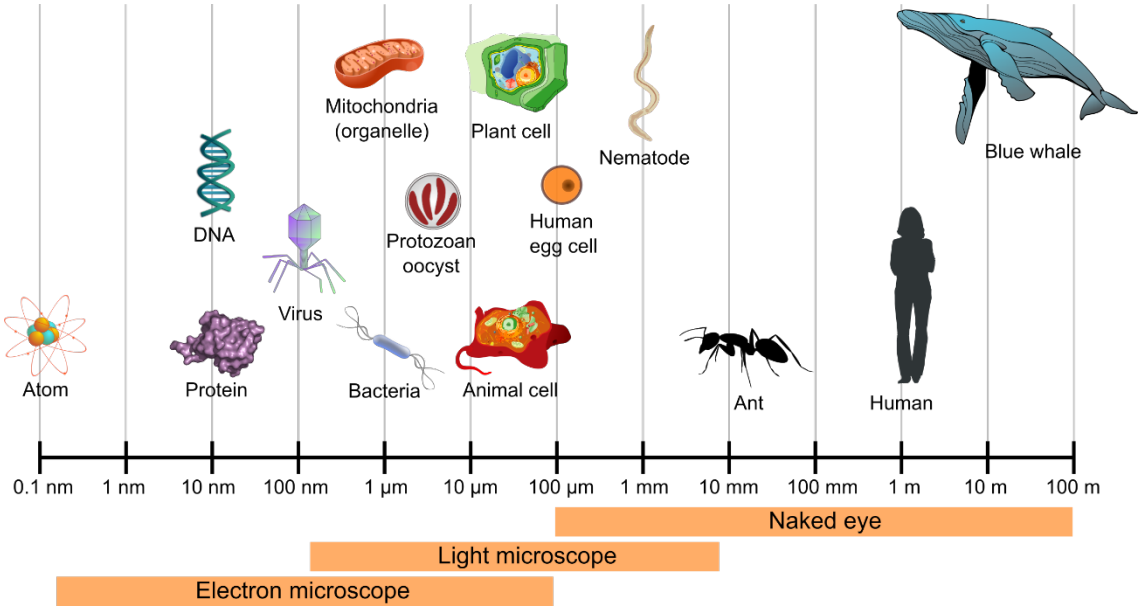

Figure 1 Sizes of life: relative sizes of organisms, cells, and cell components

Many of these small-scale organisms are also useful; by providing microbes with favourable living conditions, they can repay us in food, animal feed, medicine, antibiotics, and other useful molecules. Microalgae, for instance, are readily grown in controllable bioreactors and can be utilized for food and feed, amongst other uses. Microorganisms are also useful for breaking down pollution both in the environment (bioremediation) and

in bioreactors. Microbiological communities in biofilm and sludge are, for example, used to break down organic compounds, and compounds containing phosphorus and nitrogen that occur in wastewater; some species are even able to clean up heavy metals. Heavy metal ions can be taken up actively or passively and stored in the microbes biomass, or the bioavailability of heavy metal compounds is reduced through metabolic biotransformation (Marques, 2016).

However, not all microbes are so friendly, and, even if they cannot be seen, some are capable of causing harm and diseases; monitoring waters and environments is essential as part of the strategy in reducing the risk of them infecting or harming us. The smallest of viruses are capable of inflicting great harm to individuals, by causing severe, sometimes deadly, diseases. In addition, they are difficult to evade, as they are everywhere, and small enough to escape through water filters (sizes vary from some ten to several hundred nanometres).

Bacteria and archaea are prokaryotic cells, and their sizes range from 0.3 µm (*Mycoplasma genitalium* (Tully et al., 1983)) to 300 µm (*Thiomargarita namibiensis* (Schulz et al., 1999)). Even with relatively simple cells (or perhaps due to this), it is among the prokaryotes that the highest metabolic variations are found.

Eukaryotic cells are typically larger and have more complex structures than prokaryotic cells. Eukaryotes vary in size from 0.8 µm of the phytoplankton *Ostreococcus tauri* (Courties et al., 1994) to the *Caulerpa,* whose cell length can exceed one metre (Marshall et al., 2012). Eukaryotic microorganisms include algae, fungi, protozoa, and slime moulds.

Monitoring water-borne microorganisms and pathogens, as part of the measures to ensure that our water is safe to drink or bathe in, often requires reduction of large volumes of water down to more manageable volumes for analysis. Sensitive methods of analysis are then needed, since there are often few microorganisms present in the samples. However, even low numbers of organisms are important, as ingestion of just a few microbes may be enough to cause infection (low infectious dose). In my work, a microfluidic chip technology for continuous particle concentration was explored for its ability to concentrate microorganisms from water samples. A sensitive molecular technique for detection was also investigated, and assays for this technique were developed for detection of some of these microbes.

2.2. My biological particles

Various different microorganisms were used in my research. These microorganisms were selected for analysis (NASBA) and/or as biological test-particles for the Trilobite® separation and concentration technology (The Trilobite® technology).

The initial work focused on oil-utilizing bacteria, and an analysis method for their monitoring. Protozoan parasites that may be transmitted via water, became organisms of interest in my further work.

These latter organisms may cause diseases, and some are of particular risk to immunocompromised people. These parasites were subject to both concentration experiments in the chip, and to experiments concerned with their detection during the analysis of water concentrates.

Microalgae are more useful to us than parasites. They can be easily cultivated, and are harvested as food or animal feed, or for their unique molecules that are used in pharmaceuticals, cosmetics, and in food supplements. The “green gold” is also seen as a potential source of biofuel. Microalgae were subjected to concentration by the Trilobite® chip in this work.

2.2.1. Oil-utilizing bacteria

Various microorganisms are able to break down oil by using the hydrocarbons that constitute crude oil as source of carbon, and still more can metabolize hydrocarbons as co-substrates. Both fungi and algae are known to degrade hydrocarbons, but bacteria are the major hydrocarbon degraders (Head et al., 2006). Microorganisms linked to degradation of crude oil are mostly associated with the oil quality being reduced. Hydrocarbon-utilizing bacteria primarily degrade the most available molecules of the oil, such as small alkanes and small aromatic compounds, leaving the large and polar molecules (resins and asphaltenes) that constitute heavier oil. The bacteria are positioned on the oil surface; here they produce surfactants that help break the oil down to smaller droplets, increasing the surface area and accessible “food” (Figure 2). Specialised enzymes produced by the bacteria break down the hydrocarbons.

There are, however, some bacteria that are able to break down large oil compounds to smaller molecules, and, hence, contribute to upgrading heavy oil into lighter oil (Shibulal

et al., 2014). Regulation of transcription may give an immediate response due to sudden environmental input (Moran et al., 2013). If starvation occurs due to the primary source of food suddenly disappearing, bacteria may switch to another group of genes in order to use other kinds of food that may be available. Such a change could be a switch from metabolising lighter oil hydrocarbons to being able to utilize heavier hydrocarbons of oil. Such an alteration is likely to be associated with the immediate initiation of transcription of specific new genes that had not been previously expressed.

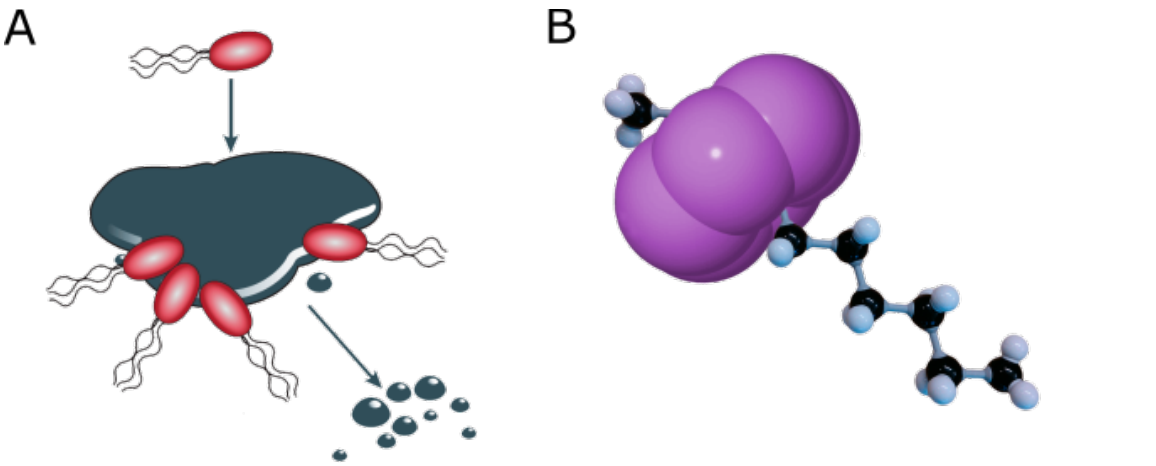

Figure 2 Bacteria that "eat" oil do so by adhering to the surface of an oil droplet (A). The oil is broken down from the surface, and an emulsion of smaller droplets forms as bacteria excrete surfactants. Enzymes break the bonds in the hydrocarbon chains (B) and convert hydrocarbons into simpler molecules that are further metabolised by the cells.

In this initial work a bacterial consortium of four oil-utilizing bacteria were studied. The bacteria used in my research were gram-positive and considered to have potential for improving or upgrading heavy oil. However, confidentiality agreements prevent me from publishing the strains, names and sequences of the bacteria investigated.

2.2.2. Protozoa

Protozoa are unicellular eukaryotic, motile, and colourless microorganisms that lack cell walls. Protozoa are phylogenetically diverse, and appears in several lineages in the Eukaryotic tree. Within this large and diverse group, we find flagellates (mastigophora), amoebae (sarcodina), ciliates (ciliophora), and apicomplexans (sporozoa). The Apicomplexa is a large phylum of obligate parasitic protozoans. As the name partly implies, the sporozoans form sporozoites, analogues to true spores, used in their

transmission stage of life. The apicoplast is an organelle unique to apicomplexan parasites, resulting from secondary endosymbiosis. It is non-photosynthetic, but functions in fatty acid biosynthesis (McFadden, 2011). The apicoplast apparently has a role that is essential to the parasite, as removal of it has been shown to result in parasite 'delayed death' (He et al., 2001). Therefore, this organelle has been target for parasiticidal drugs (Fichera & Roos, 1997; McFadden, 2011). The apicomplexan phylum includes *Cryptosporidium* and *Toxoplasma* both of which were used in my studies.

The flagellates is a broad group of protozoa that are characterised by their flagellae, and are found in different branches of the phylogenetic tree. The diplomonad *Giardia* is found in this group.

2.2.2.1. Cryptosporidium

Cryptosporidium are coccidian parasites in the apicomplexan phylum. More than 27 species of *Cryptosporidium* are known (Moore et al., 2016), and due to their morphological similarities, molecular methods are needed to identify the different species and subtypes (Ryan et al., 2014). The various *Cryptosporidium* species infect most mammals, as well as fish and reptiles (Table 1). Some *Cryptosporidium* species have a narrow host range, however, several are considered to be zoonotic; that is, they may infect different species as hosts. Around 20 *Cryptosporidium* species have been reported in humans, but *C. parvum* and *C. hominis* are the two *Cryptosporidium* species that are most often associated with human infection. *C. hominis* is largely only infectious to humans, although isolated occurrences in other animals have been detected. Calves are probably the main host of *C. parvum*. However, they are also capable of infecting other species of animal, including domestic animals (particularly lambs) and humans.

Table 1 Examples of Cryptosporidium *species and their hosts. Adapted from Ryan et al. (2014)*

Species	Major hosts	Reported in humans
C. muris	Rodents	Yes, numerous reports
C. molnari	Fish	No
C. canis	Dogs	Yes, many reports
C. hominis	Humans	Yes, most common species in humans
C. melegridis	Birds (turkeys) and humans	Yes, commonly reported
C. parvum	Ruminants and humans	Yes, commonly reported
C. serpentis	Snakes and lizards	No
C. ubiquitum	Ruminants, rodents, primates	Yes, commonly reported

Cryptosporidium is one of four most common pathogens responsible for severe paediatric diarrhoea in developing countries, together with *Shigella*, rotavirus and enterotoxic *Escherichia coli* (Kotloff et al., 2013). An estimated 64 million symptomatic infections occur each year worldwide (Torgerson et al., 2015). Symptoms include watery diarrhoea, dehydration, weight loss, abdominal pain, nausea and fever. Less than 100 instances are reported each year in Norway (2013: 31, 2014: 70, 2015: 86), but these numbers are likely to be underestimates as this infection is seldom considered by general practitioners in Norway (https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/kryptosporidiose---veileder-for-hel/#forekomst-i-norge).

In 2010-2011, two large waterborne *Cryptosporidium* outbreaks struck Östersund and Skellefteå in Sweden. These outbreaks caused infection of 45 % of the Östersund population (27,000 infected), and 28 % of the Skellefteå population (20,000 infected), and was caused by oocysts contaminating the drinking water (Rehn et al., 2015; Widerström et al., 2014). Fatigue, headache, abdominal pain and diarrhoea were reported to be post-infection symptoms several months after the initial outbreaks (Rehn et al., 2015).

The largest reported cryptosporidiosis outbreak happened in Milwaukee, USA in 1993, where more than 400 000 people were infected. This outbreak was also caused by

oocysts transmitted by drinking water, and passing through a water treatment plant (Mackenzie et al., 1995).

In its transmission stage, *Cryptosporidium* are encapsulated in thick double-walled structures called oocysts. The oocysts are tough structures that protect the parasite in the harsh environment outside its host (Robertson et al., 1992). The oocysts are 4-6 µm, and slightly oval or spheroidal (Skotarczak, 2009). They infect their host by being ingested, and the sporozoites hatch out and infect the epithelial cells in the small intestines of the host, where replication (both sexual and asexual) occurs (Figure 3). One of the major transmission routes of *Cryptosporidium* is by water, but contaminated foods may also carry oocysts. As the oocysts are infectious upon excretion, self-reinfection is possible, as well as direct hand-to-mouth infection. The infective dose is low, and human infection studies suggest a median infectious dose of 10-30 oocysts, although this varies with species and genotype (DuPont et al., 1995; Okhuysen et al., 1999).

Genome sequencing has revealed that the apicoplast is lacking in *C. parvum* (Abrahamsen et al., 2004). The mitochondria is degenerate, and lacks the citric cycle and cytochrome-based respiratory chain, hence the *Cryptosporidium* relies on the host for nutrients (Abrahamsen et al., 2004). Medicines that are used for other related parasites, are not effective for *Cryptosporidium* because of the lack of target genes currently identified in this organism (Ryan & Hijjawi, 2015). Hence, there is no completely effective vaccine or drug for *Cryptosporidium* at present.

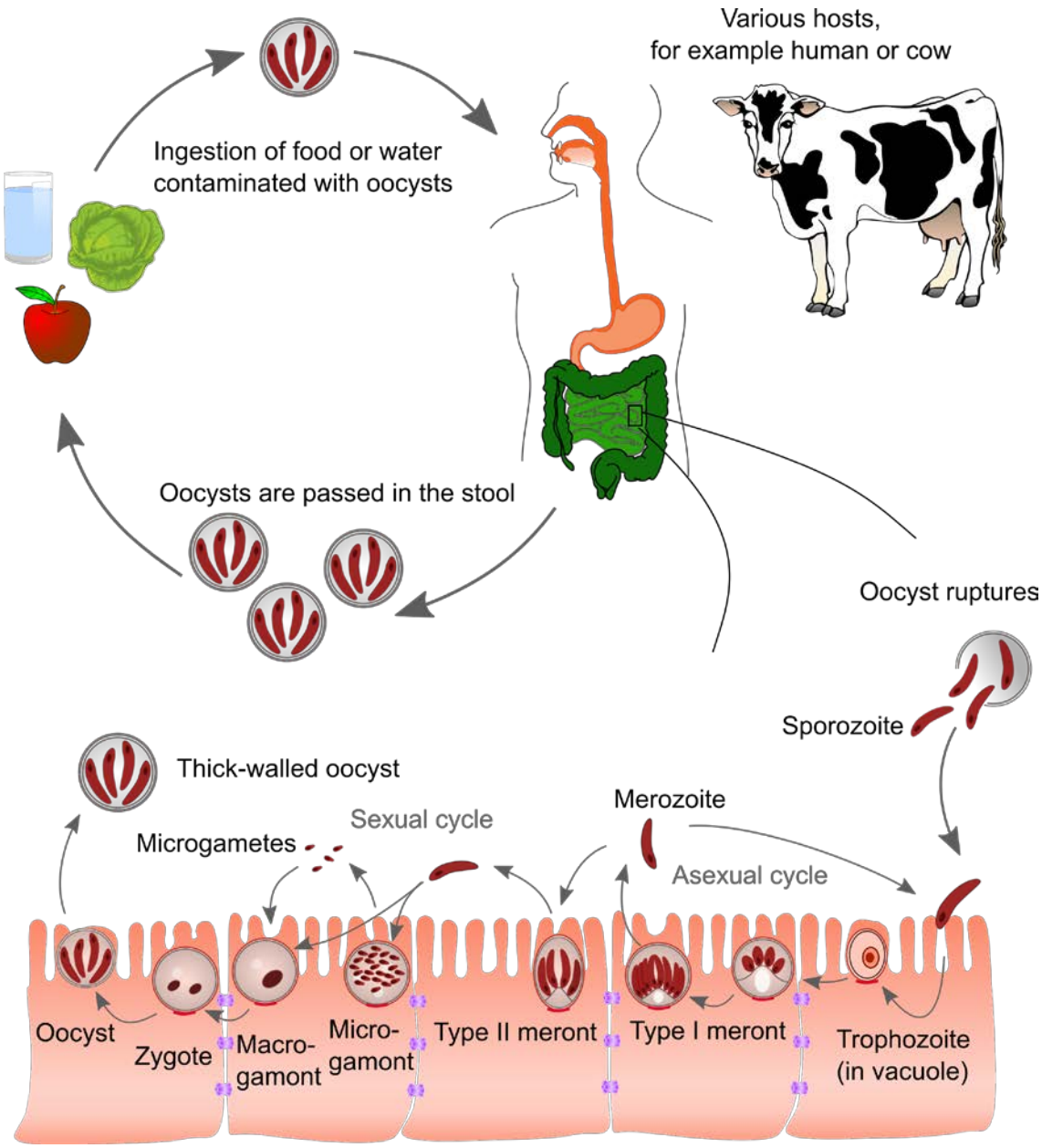

Figure 3 Life cycle of Cryptosporidium. Cryptosporidium *spreads as oocysts in the environment, and are ingested by potential hosts through contaminated drinking water and food, or recreational water. In the small intestines, the oocysts experience change in environment and rise in temperature, which leads to excystation (escape from oocyst). Four sporozoites emerge from each oocyst and infect enterocytes (epithelial cells in the small intestine). The enterocytes create a parasitophorous vacuole that embraces the sporozoite. The vacuole is intracellular, but extracytoplasmic, so the parasite is not completely "inside" the cell. The sporozoite also creates an actin-rich adhesive disc. The sporozoite develops into a trophozoite that feeds on the host. As a meront, the parasite reproduces asexually, producing and releasing merozoites. Type 1 merozoites infect new cells and develops into new trophozoites or meront type 2. Meront type 2 develop into the macrogamont or microgamont, responsible for sexual reproduction. Microgametes, released from microgamonts, fertilize macrogamonts, creating diploid zygotes. A zygote differentiates into four haploid sporozoites, which become encapsulated by an oocyst wall. The resulting oocysts can then be released into the intestine and carried away with the hosts' faeces. The oocysts are immediately infectious upon excretion (Lendner & Daugschies, 2014).*

2.2.2.2. Toxoplasma gondii

Toxoplasma gondii is another apicomplexan obligate parasite. *T. gondii* is the cause of toxoplasmosis, and worldwide, an estimated 30-50 % of the human population are infected (Flegr et al., 2014). For most people, toxoplasmosis is asymptomatic or may give flu-like symptoms, but in severe cases can lead to mental illness or even be fatal for immunocompromised individuals. It has been associated with blindness and eye problems, even in the non-immunocompromised. The greatest risk from *T. gondii* is from infection of previously uninfected pregnant women, in which the parasite may move to the foetus, resulting in abortions or serious birth defects (Shapiro et al., 2010). In animals, *T. gondii* is also associated with reproductive problems, being a cause of "abortion storms" in sheep.

A large outbreak of toxoplasmosis occurred in British Columbia, Canada in 1995. A municipal water system was probably the infection source that led to 100 acute infections (Bowie et al., 1997). According to Norwegian regulations, toxoplasmosis has not been a reportable infection to the Norwegian Institute of Public Health (FHI) from 1995. Before this, approximately 30-40 cases were reported each year (https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/toksoplasmose---veileder-for-helsep/#forekomst-i-norge). A recent serological survey among pregnant women in a particular region of Norway, indicated that *Toxoplasma* exposure is relatively rare among the Norwegian population (Findal et al., 2015), with a seropositivity of 9.3 %. This does mean, however, given the ubiquitous nature of Toxoplasma in animal populations, including in Norway, that there is vulnerability for exposure among the most vulnerable groups (pregnant women and the immunocompromised).

The sexually produced transmission stage of *Toxoplasma* is, as with *Cryptosporidium*, oocysts, and water has the potential to act as a major transmission medium (Karanis et al., 2013). The parasite can also be transferred by eating undercooked meat (Dubey & Jones, 2008), and in some countries, particularly, for example, France, where the cuisine culture promotes consumption of lightly cooked meat, this is considered to be the most important transmission route. Direct contact with cat faeces, for example when cleaning the house cat's litterbox, is another potential transmission route; however, it should be noted that freshly excreted oocysts are not infectious, and therefore daily cleaning of a

litterbox should limit exposure to infectious oocysts. Gardening, however, may result in exposure to infectious oocysts that have been buried with cat faeces, and the potential for transmission of *Toxoplasma*, among other pathogens, is one reason for ensuring that children's sandboxes are kept covered when not in use.

Toxoplasma oocysts are slightly oval and 10-12 µm in diameter. The oocyst provides good protection for the parasite against tough environmental stress; the two-layered oocyst walls are as rigid as common plastic materials. If the inner layer is chemically broken down (by bleach), the thicker inner layer still provides protection (Dumetre et al., 2013). The lifecycle of *T. gondii* is complicated, but felids are the definitive hosts, and nearly all warm-blooded animals can act as intermediate hosts (Dubey & Jones, 2008; Shapiro et al., 2010). A simplified lifecycle is outlined in Figure 4.

Although there is only one species of *Toxoplasma* in the genus, *T. gondii*, different subtypes have been identified with different virulences. Whereas the subtype in Europe is considered relatively mild, that in South America is considered to be particularly virulent, causing symptomatic disease even in immunocompetent hosts. These different sub-types cannot be identified by morphology, and molecular methods are necessary.

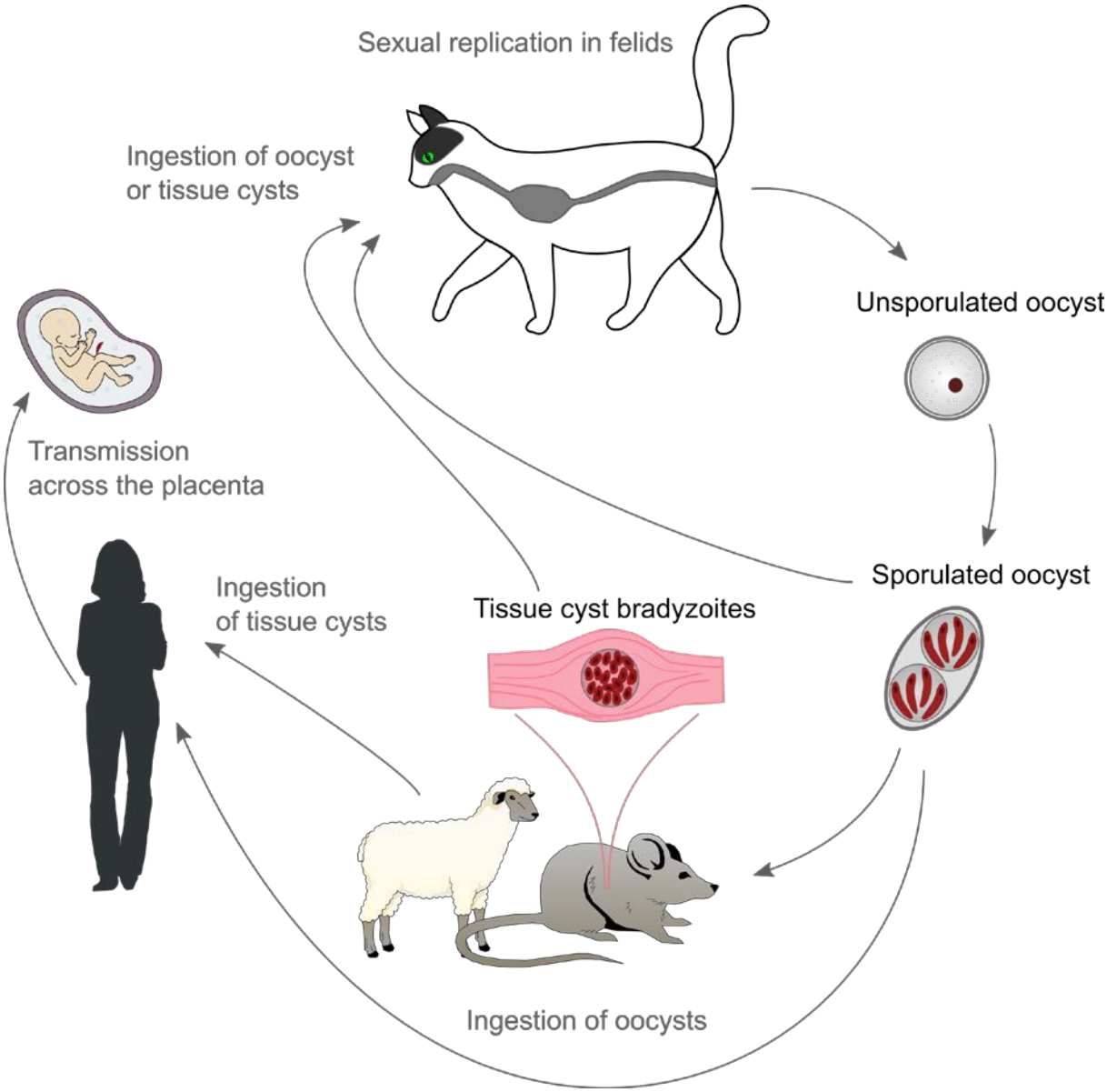

Figure 4 Simplified lifecycle of Toxoplasma. *Felids are the definite host, and sexual replication occurs only in these animals. Sexual replication results in the formation of unsporulated oocysts that are shed in the faeces. Unsporulated oocysts develop into sporulated oocysts in the environment over a period of 1-5 days, which is temperature dependent. When oocysts are ingested by other animals, the sporozoites are released from the oocyst, and invade the cells of the intestinal epithelium. There, they differentiate into tachyzoites, which spread throughout the body via the blood stream. They can invade all other host cells with the exception of erythrocytes and can develop to form tissue cysts, or invade neighbouring cells.* Toxoplasma *spreads through oocysts, or if undercooked or raw meat with tissue cysts is eaten. A clinically important route of transmission is transplacental, across the placenta to the developing foetus.*

2.2.2.3. Giardia duodenalis

The diplomonad *Giardia duodenalis* is found in the group of binucleated flagellates. *G. duodenalis* is responsible for giardiasis, a disease with an estimated 280 million symptomatic human infection each year (Lane & Lloyd, 2002). *Giardia* is the most common enteric protozoan pathogen for humans and animals (Monis et al., 2009), and has the potential to be zoonotic, transferring between animals and humans, depending on genotype (Ankarklev et al., 2010). Symptoms of giardiasis include watery diarrhoea, epigastric pain, nausea, vomiting, and weight loss, and occur 6-15 days after infection. Children and immunocompromised people are (often) most vulnerable to symptomatic infection, but as illustrated from outbreak data, immunocompetent adults can also suffer symptomatic infection.

In Norway, approximately 200-300 cases of giardiasis are reported each year (http://www.msis.no). In 2004-2005, an outbreak of giardiasis infected at least 1500 people in Bergen. This number is based on diagnosed infection, and the actual number has been estimated to be 4 to 5 times greater (Robertson et al., 2006). The outbreak was probably caused by cysts from an infected person in a sewage leakage into Svartediket, which is a source of drinking water.

As with *Cryptosporidium*, transmission of *Giardia* follows the faecal-oral route, most infections are carried through water, but cysts can also be found on the surface of foods (Baldursson & Karanis, 2011; Ganz et al., 2015; Slifko et al., 2000). The parasite survives as a thick-walled cyst outside its host, where they can survive weeks to months. The cysts are oval, measuring 8-12 x 5-7 µm (Skotarczak, 2009), and are surrounded by 0.3 µm thick walls (Adam, 1991). The infective dose for humans is 10-100 cysts (Rendtorff, 1954). When ingested, trophozoites excyst from the *G. duodenalis* cysts and colonise the surfaces of the small intestine, but do not invade the cells. Here in the lumen of the intestine they replicate asexually by binary fission (Figure 5). The colonisation of the surfaces of the small intestine, resulting in villous atrophy and crypt hyperplasia, along with effects on tight junctions, are responsible for many of the symptoms of giardiasis, including diarrhoea and steatorrhoea.

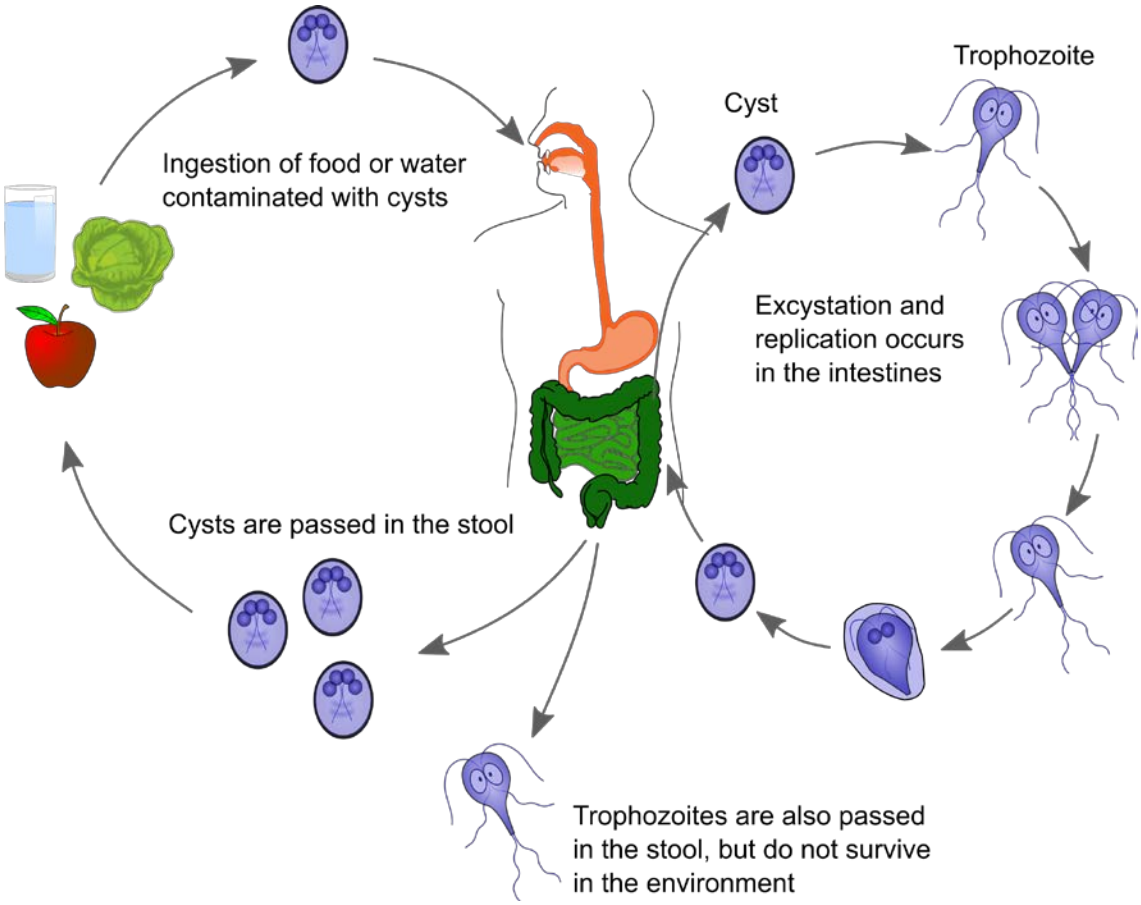

Figure 5 Life cycle of Giardia. Giardia *spread in the environment and infect hosts as cysts. When cysts are ingested and reach the small intestine of its host, they open (excyst) and give rise to four trophoziotes (Bernander et al., 2001). Trophozoites attach to the intestinal epithelium and replicate by asexually by longitudinal division. When trophozoites approach the lower part of the small intestines, they start to encyst. Mature cysts are excreted in the faeces, and are immediately infectious upon excretion.*

The *Giardia* genome has a very limited metabolic repertoire, as many microaerophilic organisms do (Morrison et al., 2007). It lacks mitochondria, peroxisomes and typical Golgi apparatus. Instead of complete oxidation of carbohydrates to CO_2 and water, these molecules are fermented. All enzymes involved in carbohydrate metabolism are localized in the cytoplasm, and simple sugars (glucose) is the primary energy source (Adam, 1991). However, this binucleated flagellate has mitosomes, an organelle type likely derived from mitochondria (Ankarklev et al., 2010). It is impossible to distinguish between the various different *Giardia* assemblages (genetic groups) from each other based on morphology. However, there is extensive heterogeneity across the *G. duodenalis* species, and assemblages have shown to be so different, also with regard to host-specificity, that they might actually be separate species (Jerlstrom-Hultqvist et al., 2010; Monis et al., 2009).

Assemblages A and B are mostly associated with human infection, but have been identified in other animals (Cacciò & Ryan, 2008).

2.2.3. Microalgae

Microalgae are microscopic plants, mostly single-celled, living in water. They increase their biomass through photosynthesis, driven by sunlight. The possible area of use for these water-plants is vast; they are grown for biofuel, animal and fish feed, natural pigments, and for use in cosmetics and nutritional supplements, among others. Microalgae cultures are readily grown in bioreactors; in closed or open tanks, or in vertical tubes, where space is limited, and they can be grown in freshwater or seawater, depending on the species. Even wastewater can be used as growth medium, which saves both valuable freshwater and recycles nutrients (Zhu et al., 2013). Microalgae require water, light, CO_2, and some nutrients to grow, and they are capable of growing 10-50 times faster than land plants (Wan et al., 2015).

One of the main challenges in microalgae cultivation is successful and efficient separation of the algae from the large volumes of water when they are harvested. Dewatering is the most expensive and demanding stage in microalgae production. The ability of the Trilobite® chip to separate microalgae from water was investigated for three different unicellular algae: one cryptomonad, and two diatoms. All three species were provided by Dag Altin and were grown at NTNU/Sintef SeaLab in Trondheim. There they are used as live feed for various aquatic organisms in research projects at the facility, for instance in production of oyster larvae. These species are commonly used in aquaculture.

2.2.3.1. Rhodomonas baltica

Rhodomonas baltica (Figure 6) is a non-toxic cryptomonad microalga, approximately 7-8 µm in diameter and flexible. This species of marine alga is mostly used as live feed for other marine organisms, such as copepods (small crustaceans) (Carotenuto et al., 2012)), blue mussels (Handå et al., 2012), and other bivalves (Sejr et al., 2004).

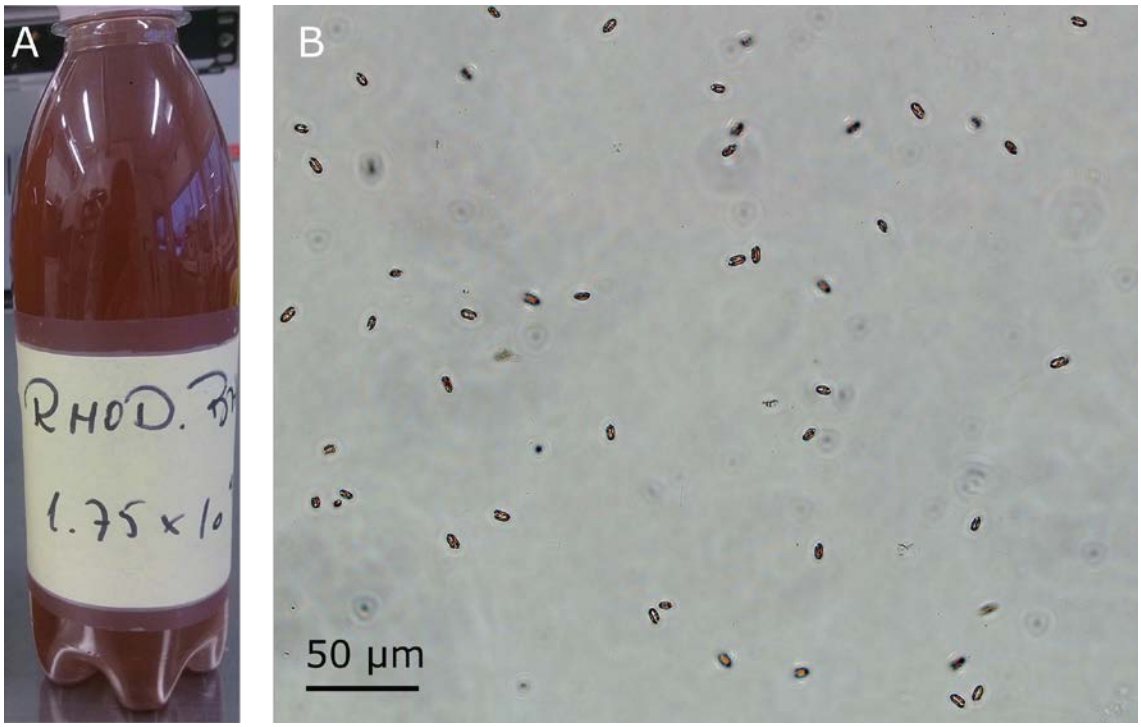

Figure 6 Culture of Rhodomonas baltica *in a plastic bottle (A) and as seen by light microscopy at 100x magnification (B)*

2.2.3.2. Thalassiosira weissflogii

Thalassiosira weissflogii (Figure 7A) is a diatom; this alga has walls that contain silica that make the cells rigid. The cells of *T. weissflogii* are normally between 10 to 20 µm in diameter, but can range between 5 and 32 µm. It is shaped like a short cylinder. Although *T. weissflogii* is considered as a marine algal species, it is also found in brackish water, rivers, and other freshwater, at many locations wordwide. *T. weissflogii* is non-toxic and widely used as feed for larvae and other zooplankton in aquaculture.

2.2.3.3. Chaetoceros sp.

Chaetoceros sp. (Figure 7B) is also a diatom, with smaller cells than *T. weissflogii*. The cells are cylindrical in shape and are about 5 µm in diameter. As with the other two algal species, *Chaetoceros sp.* is commonly used as live feed for bivalves and zooplankton.

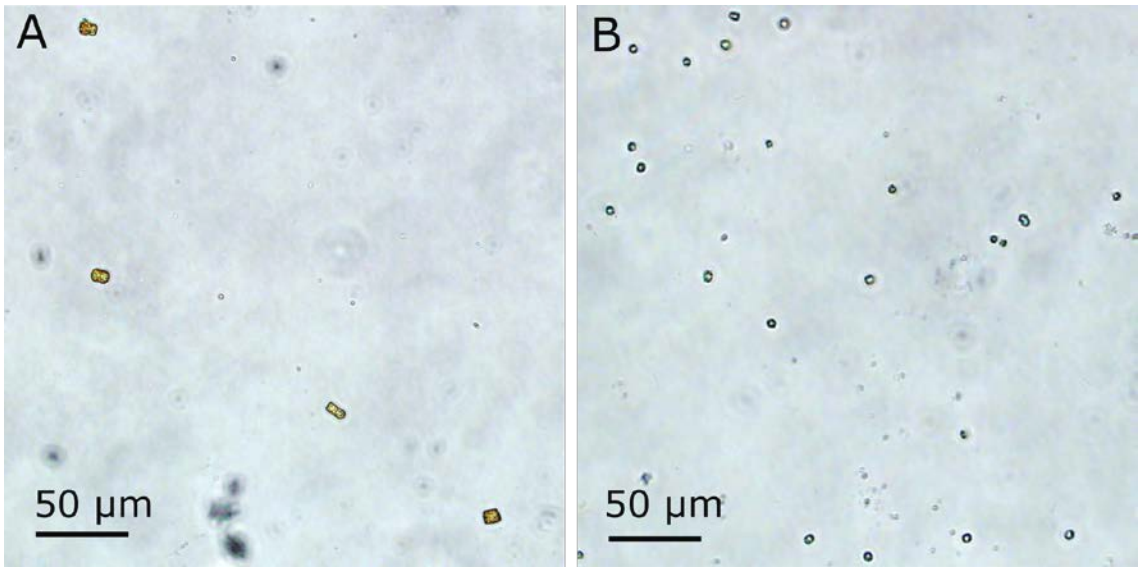

Figure 7 Microscopic view of the diatoms T. weissflogii *(A) and* Chaetoceros *sp. (B) at 100x magnification*

3. Current technologies for particle separation and analysis

Biological particles – viruses, bacteria and other microorganisms, are found everywhere, in waters, oceans, distributed in air, and in our bodies. Some particles are densely distributed, while other are scattered. There are many reasons for wanting to separate and analyse biological particles, for instance to identify potentially contaminating particles that may be found in the environment or in other places.

In clinical settings, it may be necessary to separate and detect harmful particles or organisms in blood samples, or to separate different types of cells. This may involve separation of white and red blood cells, cancer cells, bacteria, viruses or other pathogens or foreign matter. Separation of the target particle from other blood components is often required, and also concentration of the target, in order to obtain reliable results in downstream analyses.

When screening for harmful or contaminating organisms in the environment, these need to be highly concentrated from samples in order to be detected. After separation and concentration of such biological particles, we still need sensitive analyses for detection, especially when screening for waterborne pathogens, such as parasites; these can have considerable negative impacts, even when their numbers are low, and that can be transmitted in critical locations such as drinking water.

More densely distributed biological particles might also still need to be concentrated, for instance for bio-industrial purposes, such as dewatering of cultured microalgae. Such cultures do not normally require analyses but if, for instance, a bioreactor is infected by a contaminant, then it might be useful to analyse samples to gain knowledge of how to manage possible unwanted contaminants, like another competing or predatory microorganism.

In water treatment, removal of suspended solids and all particle types is required in order to provide safe drinking water, and wastewater requires similar treatments in order to protect the recipient (fjords, lakes and oceans) from our wastes. The environment itself

also seems to need removal of contaminants, for instance micro-plastics (< 1 mm plastic particles) contaminating the oceans.

3.1. Separation techniques

Separation techniques are used to purify or enrich particles and can be either passive or active: passive techniques rely on internal characteristics and can use filters, inertial forces, and adhesion mechanisms to separate particles, whereas active techniques apply external forces, and can include techniques that use acoustic, electric magnetic or optical fields (Wyatt Shields IV et al., 2015).

Centrifugation is a simple and much-used technique that is based on gravitational forces to separate particles from fluid. Conventional centrifugation is carried out in batches, and is not a continuous method. However, continuous flow centrifugation is also an available technique, and has been used for recovery of *Cryptosporidium* oocysts (Swales & Wright, 2000). Membrane filtration is also simple and much used, but is not continuous, as the filter collects particles and clogs after some time.

Many separation techniques require some sort of labelling of the particles, such as fluorescent labelling or with beads. Fluorescent-based particle separation depends on fluorescent dyes or probes to stain the particles. The labelled particles can then for example be detected and sorted using a focused laser beam and detector in a laminar stream (e.g. flow cytometry, particularly fluorescence-activated cell sorting (FACS), in which the specific fluorescent characteristics and light scattering characteristics of individual cells are used as a basis to separate a heterogeneous mixture of cells into different groups).

Bead-based separation depends on properties like size, material, and surface-binding capacities to, for instance, magnetic or paramagnetic beads. Magnets, acoustics, or electrokinetic mechanisms can then be used to sort the bead-labelled particles (Wyatt Shields IV et al., 2015).

Label-free separation is an attractive alternative, as particles are sorted based on their intrinsic properties without needing to apply an external label, which may introduce further anomalies. Such methods rely on exploiting one or more pre-defined

characteristics of the particles or cells, such as size, elastomeric (both viscosity and elasticity) properties, or electric charge. Electric methods like electrophoresis or dielectrophoresis are based on charge or polarisation of particles.

Microfluidics relies on how fluids and particles behave and how they can be controlled or engineered at the micrometre scale, mostly ten to hundreds µm (Zhang et al., 2016). Particles suspended in fluids can be manipulated in straight or curved channels with or without additional structures. Microfluidic techniques offer tuneable and controlled flow and miniaturisation of separation systems. They also provide the possibility of integration into Lab-on-a-chip (LOC) devices, so that concentrated particles can be analysed directly. The advantages of using microfluidics as a tool is not only the potential for being highly integrated and automated component of a system, with good portability due to the small size, but also the possibility of using fewer samples and less reagents necessary, thereby saving on costs, rapid analysis, and that in some systems a high sensitivity and resolution has been established.

Although precise particle manipulation and high focusing efficiency are advantages that are offered by active focusing technologies, there are also some disadvantages, including the relatively complicated fabrication of introducing external fields. In this section, I briefly present some examples of microfluidic methods that do not rely on any external force fields.

3.1.1. Microfiltration

Microfiltration is a commonly used technique for separation and collection of particles in the 0.1-10 µm scale. It is mostly carried out over a porous membrane or various filters. The membrane prevents particles that are larger than the pore size from crossing the barrier, but smaller particles and molecules can accompany the fluid across the membrane (Figure 8A). Microfiltration as a separation technique is well developed and has been implemented at the large scale. However, it is prone to particle deposition, fouling, and concentration polarisation (Dijkshoorn et al., 2017).

Microfiltration is often carried out as cross-flow filtration, to avoid clogging. In contrast to dead-end filtration, the flow runs parallel to the filter, rather than perpendicular to it

(as in dead-end filtration). Cross-flow filtration is also referred to as tangential-flow filtration. It has for instance been used as a tool in dairy production (Saboya & Maubois, 2000), and for harvesting microalgae that produce polyunsaturated fatty acids, with the potential for biodiesel production (Santos-Sánchez et al., 2016).

3.1.2. Inertial focussing

In inertial microfluidics, both inertial and viscous forces operate, and inertial focussing exploits these characteristics in order to sort particles inside micro-channels. Inertial migration is a phenomenon where randomly distributed particles suspended in a fluid that flows through a straight channel arrange themselves at equilibrium positions after a sufficient distance has been travelled (Zhang et al., 2014). Two forces contributes to this positioning of particles: the parabolic velocity profile of the fluid induces shear lift towards the channel walls (dragging particles away from the channel centre), and the walls induce lift towards the centre of the channel (forcing particles away from the channel walls) (Jimenez et al., 2017). Different particles therefore will have different positions downstream in the channel than at the inlet, and can thereby be sorted by directing them into different outlets at the end of the channel (Figure 8B).

The flow-rates of these types of systems are relatively high (up to ml/min), and as the channels have no additional structures, the risk of clogging is low.

In curved channels, an additional force called Dean drag appears. This force is dependent on flow-rate, and pushes the particles closer to the inner wall.

This principle of inertial focussing has recently been used to sort waterborne pathogens in a spiral channel with flow-rates up to 1.5 ml/min (Jimenez & Bridle, 2016; Jimenez et al., 2017). Other suggested applications have been both clinical (e.g. detection of tumour cells and leukocyte imaging (Martel et al., 2015)); and more wide-ranging (Xiang et al., 2016).

3.1.3. Deterministic lateral displacement

Deterministic lateral displacement is a microfluidic particle-separation technique where particles are separated based on their size through an array. This array consists of

obstacles arranged in an asymmetric pattern, with each row in the array shifted slightly horizontally (Huang et al., 2004). Particles are led in a laminar flow through the array of posts for sorting by size (Wyatt Shields IV et al., 2015). There are two transport modes through the array: particles that are smaller than a critical diameter follow a zigzag mode through the array, while larger particles follow a displacement mode. Particles following the zigzag mode move through the array in a zigzag pattern, and stay in one lane, and particles that follow the displacement mode are displaced every *n*-th row, and move away from the particles that follow the zigzag mode as they move through the array (Figure 8C). By differing the gap sizes, various particles of different sizes can be separated in the same channel (Huang et al., 2004). The technique mainly separates particles based on size, however, it can also be used to sort particles according to shape and deformability (Beech et al., 2012).

Huang et al. (2004) reported a resolution of approximately 10 nm, and flow speed up to 400 µm/s, and that the method works better at higher flow-rates, as there is less change of particles being thrown off the path by diffusion. The flow-rates used for deterministic lateral displacement are usually low (0-1 µl min^{-1}). Higher flow-rates can be obtained in when sorting larger particles or altering the geometry of the obstacles in the array, up to 10 ml min^{-1} have been reported (McGrath et al., 2014). This technique has been suggested, in particular for preparation of human leucocytes samples prior to flow cytometry (Civin et al., 2016); separating viable from non-viable mammalian cells (Tottori et al., 2016); separation of blood cells (Holmes et al., 2014); and circulating tumor cells (CTCs) from blood (Karabacak et al., 2014).

3.1.4. Hydrodynamic filtration

Hydrodynamic filtration is based on channel design and flow control (Pamme, 2007). A flow with suspended particles is pumped through a main channel. Branching channels are distributed along the main channel (Figure 8D). Some of the fluid exits through these branching channels, reducing fluid and leading the smaller particles closer to the walls of the main channel. Further downstream, small particles also escape through branching channels, whereas larger particles cannot enter these branching channels and will be carried further down the main channel. Each time particles pass a branching channel,

they become closer to the wall, until they are aligned with the wall, and can be carried with the flow out at the next branching channel. Using this principle and technique, smaller particles can be separated from larger ones. The resolution is typically 1 µm or less, and with relatively high flow rates (10 cm s^{-1}) (Pamme, 2007).

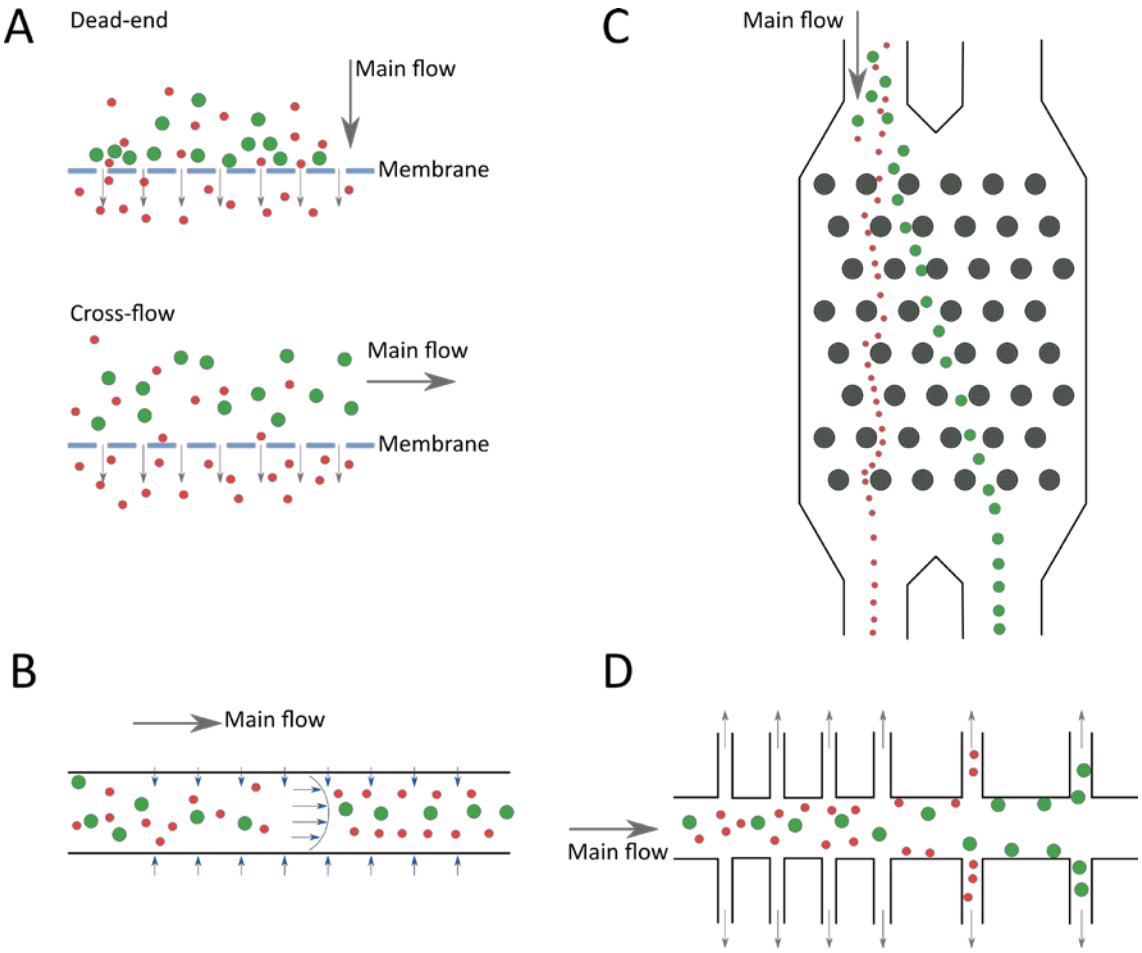

Figure 8 Simplified overview of some of the available microfluidic techniques: microfiltration (A), inertial focussing (B), deterministic lateral displacement (C), and hydrodynamic filtration (D)

In my work, I wanted to concentrate waterborne parasites before analysis, and to concentrate microalgae as part of harvesting. For both these two types of biological particles, water reduction is the main issue. However, for parasites high recovery efficiency is also very important. For microalgae, however, high recovery is not that critical, it is more important to achieve high removal of water.

In my research, the Trilobite® microfluidic chip was tested for these purposes. The chip is made to sort and concentrate particles in a continuous and non-clogging mode at

relatively high flow-rates. As a microfluidic chip, it also has the potential for being incorporated into LOC devices.

Compared to the other passive microfluidic separation technologies mentioned here, the Trilobite® chip does not currently have a resolution that goes as low as the nanometre range. However, the flow-rates though the chip may be higher than for inertial focussing, deterministic lateral displacement, and hydrodynamic filtration. The chip is less prone to clogging than dead-end microfiltration, but has a greater risk of clogging than, for instance, inertial focussing because of the complicated structures within the channel for the latter. Fine-tuning the chip and flow-rate for particular individual samples should improve this situation.

3.2. Analysis techniques

Analyses of biological particles may involve investigation by microscopy or molecular methods that target proteins (antibodies, enzymes), surface molecules, or nucleic acids. There are numerous different analysis methods, but here I will concentrate on nucleic acid-based methods, as these can offer very specific and sensitive analyses. Selected sequences are amplified to create vast numbers of copies. This allows for very sensitive detection of the target. Especially in point-of-care testing, testing for the relevant nucleic acids may be a sensitive, robust, and cost-efficient choice of analysis (Niemz et al., 2011).

Within nucleic acid analyses, there are also many techniques available. In the following section I highlight some of the most commonly used techniques (Table 2).

3.2.1. PCR

Polymerase chain reaction (PCR) (Mullis, 1990) is by far the most used method for amplification of nucleic acids. It is well developed, relatively cheap, and control of the conditions needed is relatively easy. A thermal cycler is needed, in addition to primers, deoxyribonucleotide triphosphates (dNTPs), *Taq* polymerase, and an appropriate buffer.

PCR is based on cycles of increasing and decreasing of temperature. The reaction therefore requires a thermostable DNA polymerase (*Taq* polymerase), as this enzyme is not denatured at high temperatures. A temperature cycle is as follows: dsDNAs are

separated at approximately 95 °C. The temperature is then lowered to around 68 °C to allow primer annealing to the target on the newly separated strands. As the temperature is increased to about 72 °C, the *Taq* polymerase elongates the primers and creates the complementary strand, resulting in dsDNA. The cycle then repeats, and the target sequence will be exponentially amplified for each cycle.

DNA is the target molecule for PCR, but RNA can also be amplified using reverse transcriptase PCR. This requires an additional step to convert the RNA to DNA, by a reverse transcriptase enzyme. Genomic DNA also needs to be removed from the sample to avoid contamination that could lead to amplification of unwanted sequences. For this task, DNase enzymes are used.

One disadvantage of PCR is linked to its sensitivity, as contaminating DNA may also be amplified if the primers are insufficiently specific. The *Taq* polymerase may insert the incorrect nucleotide, and it lacks the ability to proofread its newly synthesised strand. Longer products have, therefore, a higher risk of errors.

3.2.2. LAMP

Loop-mediated isothermal amplification (LAMP) is an isothermal technique for amplifying nucleic acids that is based on strand replacement. The reaction is carried out at 65 °C, so there is no need for a thermocycler. As for PCR, the target molecule is DNA, but RNA can be amplified by including a reverse transcriptase step. The reaction requires a strand replacing DNA polymerase (*Bacillus stearothermophylus* (*Bst*) DNA polymerase) and four primers that recognise six distinct sequences at the target DNA, in addition to dNTPs and buffer. By the use of inner and outer (or “bumper”) primers and strand replacing by the *Bst* DNA polymerase, the target sequence is amplified. The products are stem and loop molecules of various lengths and inverted repeats of the target sequence (Notomi et al., 2000).

LAMP can amplify a few copies of target to 10^9 in less than one hour, and detection can be achieved through measuring increased turbidity; alternatively, intercalating dyes can be used to provide a fluorescent signal or a visible colour change can be obtained using dyes such as SYBR green.

LAMP is a relatively cheap method, not only because of the lack of need for a thermal cycler, but also because signal detection does not require expensive instrumentation. For example, a SYBR green colour change can be detected with the naked eye, although a simple colour reader may be not only more accurate for borderline cases, but can also be used for relative quantification. Furthermore, because of the six distinct sequences recognised by the primers, LAMP is also highly specific (Notomi et al., 2000). The complexity of the six required sequences primer can also be a disadvantage, as is makes primer design complicated (Gill & Ghaemi, 2008).

3.2.3. RCA

Rolling circle amplification (RCA) is another isothermal amplification method (Lizardi et al., 1998). Circular DNA is the target molecule for this method. A DNA polymerase with strand displacing properties unwinds dsDNA and uses the circular DNA as template to synthesise a new strand. As DNA polymerase moves around the circular template, the newly synthesised strand is displaced. The product is a long, single-stranded DNA (ssDNA) with tandem repeats of the target sequence (Goo & Kim, 2016).

Some variations of the method have been developed, like ligation-RCA (L-RCA), branched RCA (BRCA), and hyperbranched RCA (HRCA).

L-RCA is based on a probe that forms a padlock when it bonds with the target sequence, and is ligated to form a circular DNA template. This newly formed circular DNA is then available for amplification by RCA. The padlock probe can also be used to target RNA, such as microRNA (miRNA), making this technique available for RNA detection (Jonstrup et al., 2006). Detection down to 1-10 fM of target molecule.

BRCA uses two primers; P1 initiates the reaction, and P2 hybridizes to the synthesised ssDNA, resulting in dsDNA product.

RCA can generate 10^9 copies of a target sequence in 90 minutes (Lizardi et al., 1998). One great advantage is that it can be used for detection of single mutations, as the method is less prone to amplification errors than PCR, due to the high proofreading property of the enzyme (Fakruddin et al., 2013). A limitation of RCA is that it requires a small circular target (Zanoli & Spoto, 2013).

3.2.4. SDA

Strand displacement amplification (SDA) is also an isothermal amplification method. As the name implies, the method is based on strand displacement, and is driven by strand displacing DNA polymerase. The reaction requires four primers (two bumper primers (B1 and B2), and two SDA primers (S1 and S2)), a strand displacing DNA polymerase, a BI restriction enzyme, in addition to dNTPs and buffer). S1 and S2 also contain a recognition site for the BI restriction enzyme. One of the four dNTP in the SDA reaction contains a phosphorothioate modification. This modification makes the synthesised strand resistant to cleavage by the restriction enzyme used in SDA (Walker et al., 1992).

The reaction is initiated by S1 binding to the target sequence, and elongation. B1 binds upstream from the S1 site, and elongation of B1 results in strand-displacement of the newly synthesised S1 strand. The S2 and B2 primers hybridise to the S1 product, B2 binds upstream of S2, resulting in displacement of the S2 product when DNA polymerase elongates. A dsDNA forms when S1 binds to the S2 product and is elongated by DNA polymerase. At each end of this dsDNA now has the BI binding site. As the strand with modified dNTP is resistant to cleavage, the dsDNA is not cleaved by the restriction enzyme, but nicked at one strand. With one strand nicked, DNA polymerase displaces the existing strand as it elongates in the exponential phase of the amplification (Hellyer & Nadeau, 2004).

SDA is very rapid, it can replicate the target 10^{10}-fold in 15 min. However, as for RCA, SDA functions best on short targets. The restriction enzyme may also recognise its binding site at other positions than the attended target, which can be a limitation (Hellyer & Nadeau, 2004).

Table 2 Overview of some common amplification techniques for nucleic acids

Technique	Target molecule	Temperature	Enzymes	Primers	Amplification principle
PCR	DNA	Varying ~50-95 °C	*Taq* polymerase	Two	Heat separation of double strands
LAMP	DNA	Isothermal ~65 °C	*Bst* DNA polymerase	Four (six binding sites)	Strand displacement
RCA	circular DNA	Isothermal ~65 °C	Ø29 DNA polymerase	One, or Two (branched)	Strand displacement
SDA	DNA	Isothermal 37 °C	DNA polymerase, Restriction enzyme	Four	Strand displacement
NASBA	RNA	Isothermal 41 °C	T7 RNA polymerase, RNase H AMV reverse transcriptase	Two	Alternating between RNA and DNA

3.2.5. NASBA

Nucleic acid sequence-based amplification (NASBA) was the amplification method of choice in my work. As opposed to most amplification techniques, the main target molecule of NASBA is RNA. This makes NASBA a good choice for amplification and analysis of transcripts, as they are used directly in the reaction without any need for extra reverse transcriptase step or removal of genomic DNA. NASBA was chosen both because it was already integrated into the LOC system of my original project, but also as it seemed to be a promising tool for direct amplification of transcripts (mRNA), and thereby also indicated that it could be an appropriate approach for determining the viability of the cells. NASBA is further described in greater detail in section 4.2.

4. Technologies used in my work

Three main technologies were assessed during my PhD work: the Trilobite® technology (section 4.1) for micro-particle separation; NASBA for amplification and detection of nucleic acids (section 4.2); and the POCNAD lab-on-a-chip technology (section 4.3).

4.1. The Trilobite® technology

The Trilobite® technology is intended for separation and concentration of particles in different kinds of fluids. The technology was first developed through the master Project of Eirik Bentzen Egeland with Tao Dong as main supervisor (Dong et al., 2009; Dong et al., 2011), and Eirik subsequently decided to commercialise his work by forming a company around this technology, with the support of Vestfold University College. The company, Trilobite Microsystems AS, currently works with purification of different fluids with a special focus on water treatment for industrial use. Areas within the industry currently targeted include ballast water treatment for the shipping industry and water treatment for aquaculture and process industries. The key element in this technology is a three-layered microfluidic chip with microstructures that concentrate and separate particles from a fluid. The intention is that is technology will be used for purification of water or other complex fluids, as well as sorting and classification of particles from fluids. Currently, the technology is able to separate particles down to 2 µm in diameter and there is ongoing research to take this down to 100 nm.

4.1.1. Chip principle

The Trilobite® chip (Figure 9) is a microfluidic chip that can be scaled up and down depending on the size of the particles to be separated.

In traditional dead-end filtration, fluid pass through pores in a filter (bed or membrane) and particles larger than the pore size accumulate onto the filter. In cross-flow filtration, the flow is tangential to the filter and particles are not accumulated on the filter surface, as in dead-end filtration. The method on which the design of the chip is based, works on a hydrodynamic principle and a cross-flow effect, in which suspended particles are continuously concentrated and/or separated as the liquid flow runs through it. The fluid

that contains particles enters the inlet at one end of the chip (blue arrow in Figure 9), and exits at two outlets at the other end of the chip. At one outlet, referred to as the *concentrate*, concentrated particles are collected (red arrow in Figure 9); and at the other outlet, referred to as the *permeate*, a purified fraction is collected (green arrow in Figure 9). The purified fraction contains no, or only small particles, depending on the gap size of the structures within the chip.

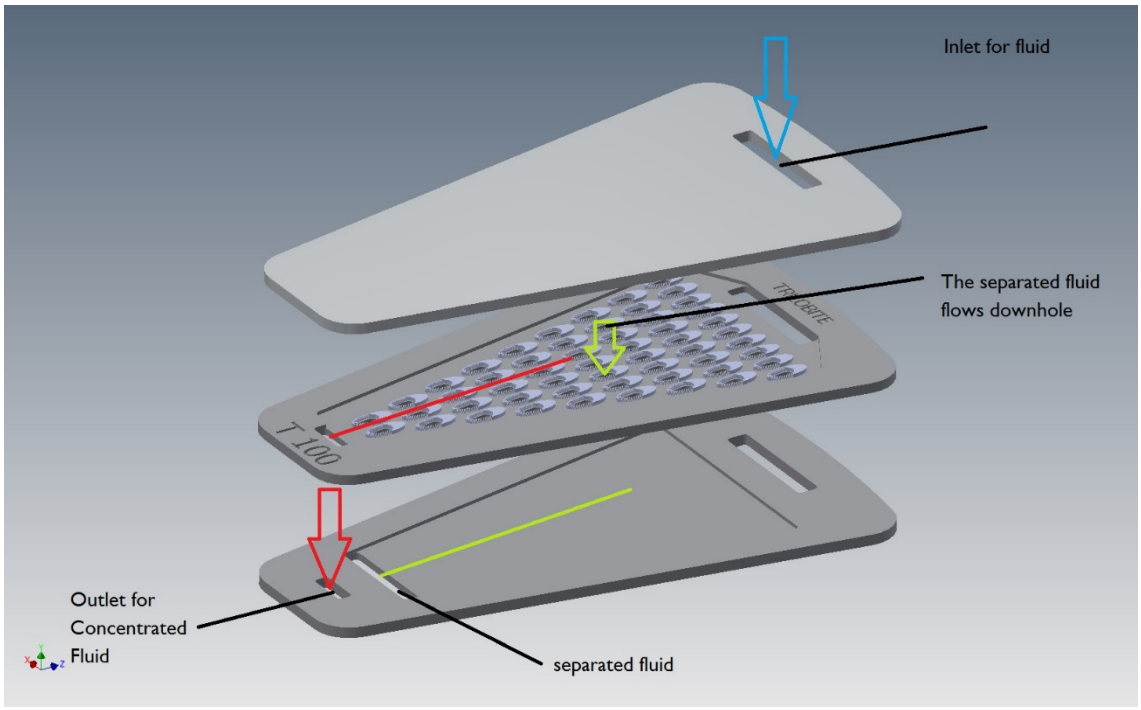

Figure 9 The Trilobite® chip. The inlet fluid enters at one end of the chip, and is pumped through the chip, which contains a field of microstructure units. Small particles flow through the units to the underlying layer and out through one outlet, while larger particles are carried with the flow through the field and out through another outlet. Figure provided by Trilobite Microsystems AS.

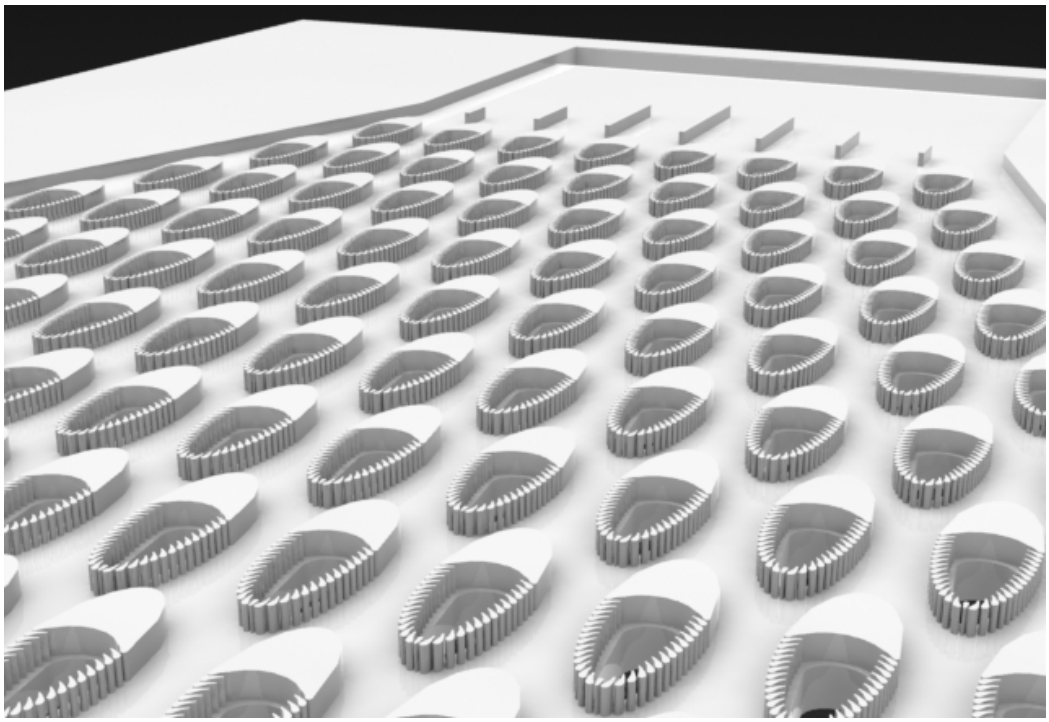

Figure 10 Inside the Trilobite® chip is a field of microstructure-units that resemble the extinct marine arthropods, trilobites. Figure provided by Trilobite Microsystems AS.

Within the upper layer of the chip is a field of microstructure-units (Figure 10). These micro-units are the key structures for particle separation. The units resemble the extinct trilobite marine animal in shape, hence the chip name. Each unit consists of a solid "head", and several turbine blades, or "feet", along the rest of the body (Figure 11). Inside the trilobite unit, a hole leads down to the underlying layer. The flow through the chip is unidirectional, and meets the "head" of the trilobite unit first, and then flows along the body. Some fluid then flows between the blades ("feet") and into the hole (as in a cross-flow), while the remainder will flow past the unit, and forward through the field of the upper chip layer. The gap size between the blades of the trilobite unit defines the size of the particles that are separated out. Particles smaller than the gap size (5 µm in this work), can pass through the gaps and into the layer underneath. Particles larger than the gap cannot enter between the blades, but flow past outside the units. Unlike in a dead-end filter, this principle allows for continuous treatment, without clogging.

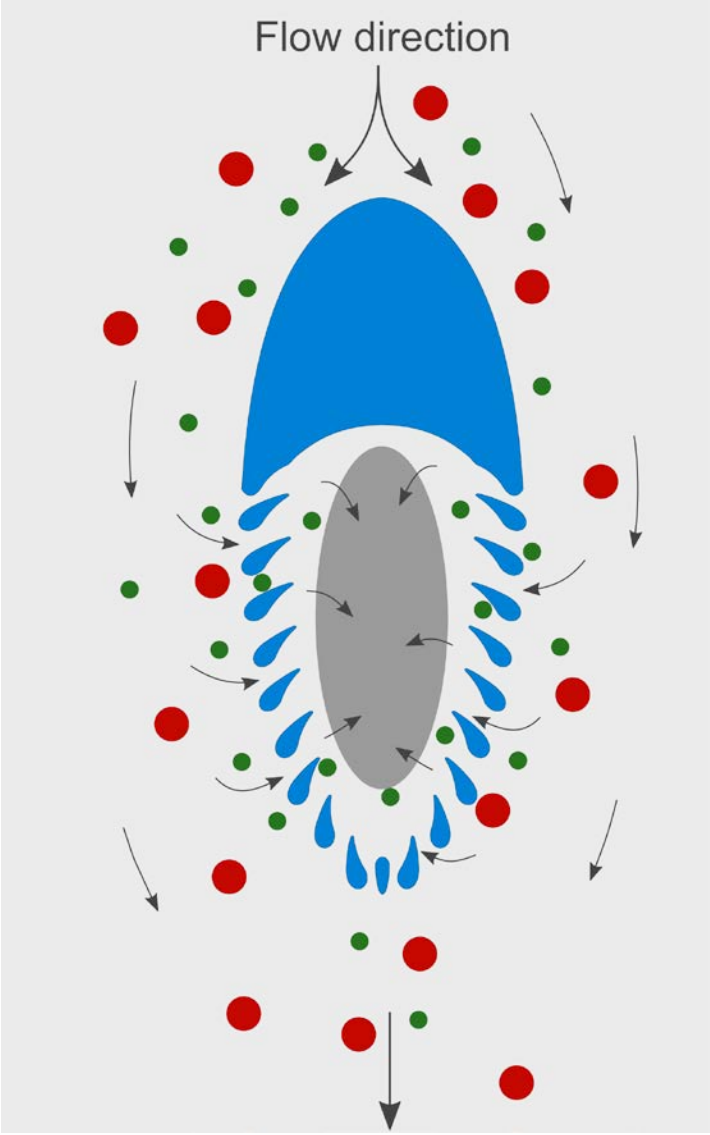

Figure 11 The Trilobite® micro-unit, principle for concentration and separation. The gaps between the blades, or "feet", of the unit are defined (5 µm in this project). Particles smaller than the gap size (green) can flow between the blades; while larger particles (red) cannot and follow the flow direction forward.

4.1.2. Potential uses of the Trilobite® chip

The Trilobite® chip allows for continuous concentration and separation of particles. Membrane filters, in comparison, are based on dead-end filtration, and need to be routinely rinsed or replaced. In theory, the Trilobite® chip can be used for continuous treatment of a fluid without requiring any replacement, as there is no dead-end that accumulate particles within the chip.

4.1.2.1. Concentration of particles

The ability of Trilobite® chips to concentrate and separate particles is of clear application for many biological samples. Often when screening environmental samples for contamination, the situation is that there are relatively few particles, often biological, that are suspended in large volumes of water or other media and should be detected or quantified. In sample preparation, the sample volume usually need to be reduced prior to analysis to enable detection, but the method should ensure that the target particles are not lost in the process. Filtration, sedimentation, or centrifugation are common methods for concentrating particles in these kinds of samples. By collecting the *concentrate* fraction from the Trilobite® chip, the chip can be used for concentration of such particles.

When water samples are being analysed for unwanted or harmful pathogens, such as cysts or oocysts of waterborne protozoa, the samples need to be concentrated before they are analysed.

Other applications might be directed towards more useful products, such as microalgae, that are used for a variety of purposes from aquaculture to cosmetics. Microalgae offer nutritional value as alternative sources of protein, carbohydrates and lipids to both humans and animals (Spolaore et al., 2006). Microalgae are also good alternatives of raw material for biodiesel compared to land plants (Mata et al., 2010), and many produce useful pigments (Spolaore et al., 2006). The potential roles for microalgae in a world requiring sustainable production seem to be only just being understood.

When microalgae are harvested, large quantities of water need to be removed. This is one of the major costs in harvesting of microalgae. The Trilobite® chip has the potential to be used as a first step in a dewatering process.

Currently, the chip is not applicable for testing on bacteria and viruses, as the gaps between the blades in the trilobite units cannot currently be made small enough and accurately enough to separate these extremely small (less than one µm) potential pathogens. However, in the future, with advancing technology for production of micro- and nanostructures, it may be possible to make the size of the gaps between the blades any size, with accuracy, and the chip might then also be applicable for these targets.

The nature of the chip makes it suitable for continuous sample collection, for example by incorporating the system in inlet- and outlet pipes in a water treatment plant for continuous water sampling, to concentrate any possible particles. Furthermore, the chip can be assembled into larger complexes, containing multiple trilobites in multiple layers, and up-scaled to handle large volumes.

4.1.2.2. Purification potential of the chip

While concentration focuses on the *concentrate* outlet fraction, the *permeate* is of interest when purification of fluids is needed. The *concentrate* becomes the "waste", and the *permeate* is valuable, for instance in recycling of water. Purification of industrial process water is also an area of interest, which is currently under investigation by Trilobite Microsystems AS. The technology can be applied to cleanse water of unwanted particles, before releasing it into the environment or prior to further purification. Purification of recycled water in a washing facility for trailers is also being tested by the company at the moment as a part of the industrial process water treatment.

Growth media used in algal cultivation is often a valuable resource that should be recycled when algae are harvested. If the chip is incorporated in a bioreactor system for algal cultivation, it has potential to concentrate algae for further harvesting in the *concentrate*, and recycling growth media in the *permeate* by reintroducing it to the algae bioreactor.

4.1.2.3. Separation potential of the chip

Particles of different sizes may be separated using the chip by defining a gap size as a cut-off value. Particles that are larger than the gap size end up in the *concentrate*, whereas particles that are small enough to slip between the blades of the trilobite units can be carried out into the *permeate*. This ability to separate larger particles from smaller particles has several potential uses, especially within aquaculture.

Separating red blood cells (erythrocytes) and white blood (leucocytes) cells in blood is a challenge in medical samples. This has been attempted using the Trilobite® chip by the company (Eirik, pers. comm.), but, to date, has not been successful. This is because blood cells are very flexible, being able to push their way through the small capillary blood vessels that are 5 to 10 µm in diameter. This means that they are also able to push their

way through the blades of the trilobite units. This indicates that the rigidity of the particles for separation or concentration is an important and critical factor. Preservation media could potentially make the cells more rigid, but that might also affect downstream analyses. When Trilobite® chips with a narrower gap (down to 100 nm gaps are planned) in the units are produced, these studies would be worthwhile attempting again.

4.2. NASBA

Molecular biological methods provide approaches for specific and sensitive detection of microorganisms that are not offered using traditional techniques. Molecular methods can be used to detect proteins and nucleic acids (DNA and RNA), and do not depend on cultivation or microscopy as required by more traditional detection techniques. Molecular methods tend to be more specific than traditional detection techniques, being able to distinguish between species or strains of organisms that are too similar to be told apart by appearance alone, and can also be used to identify specific features of organisms such as viability.

Nucleic acid sequence based amplification (NASBA) is a molecular technique for amplification of nucleic acids (Compton, 1991). RNA is the main target molecule for NASBA, and enables very sensitive detection. NASBA has been reported to be as sensitive as RT-PCR, or more, with detection limits down to one cell (Mahony et al., 2001; O'Grady et al., 2009). The technique is isothermal and the optimal temperature is 41 °C. This temperature is well below the melting temperature of DNA; double-stranded DNA are not separated and therefore, does not interfere with the reaction (Simpkins et al., 2000). This is one of the advantages of NASBA over reverse transcriptase PCR (RT-PCR); there is no need to remove genomic DNA from the samples in NASBA. By including molecular beacons in the NASBA reaction mix, the amplification product (or amplicon) can be detected in real-time, as is described in section 4.2.2.

Some of the advantages and disadvantages of NASBA are described in greater detail in Paper 3: From research lab to standard environmental analysis tool: will NASBA make the leap?.

4.2.1. The NASBA reaction

Three enzymes are involved in the NASBA reaction: T7 RNA polymerase, RNase H, and avian myeloblastosis virus reverse transcriptase (AMV RT). Nucleotide triphosphates, two specific primers, and buffer are also needed in the NASBA reaction. The forward primer has a promoter sequence for T7 RNA polymerase at its 5' end. An initial 65 °C denaturation step can be include to denature secondary structures in RNA molecules. The rest of the reaction is kept at 41 °C. The NASBA reaction consists of a non-cyclic phase followed by a cyclic phase. The steps-wise reaction is presented in Figure 12, and the steps are as follows:

In the non-cyclic phase,

1) the forward primer hybridizes to the RNA template,
2) and AMV RT creates a complementary DNA (cDNA), resulting in an RNA:DNA hybrid.
3) RNase H degrades the RNA in this hybrid, leaving single-stranded cDNA.
4) The reverse primer hybridizes to the cDNA,
5) and AMV RT elongates the strand,
6) resulting in a double-stranded DNA (dsDNA) including the T7 promoter region.

In the cyclic phase, the T7 RNA polymerase transcribes mRNA molecules from the now active promoter, with up to 100 copies in each transcription step (Compton, 1991).

7) The reverse primer binds to these mRNAs, and AMV RT elongates cDNAs from the 3' end.
8) The RNA of these RNA:DNA hybrids is broken down by RNase H.
9) The forward primer binds to the remaining DNA molecules, AMV RT elongates from the primer to create dsDNA, and the cycle repeats (from 6).

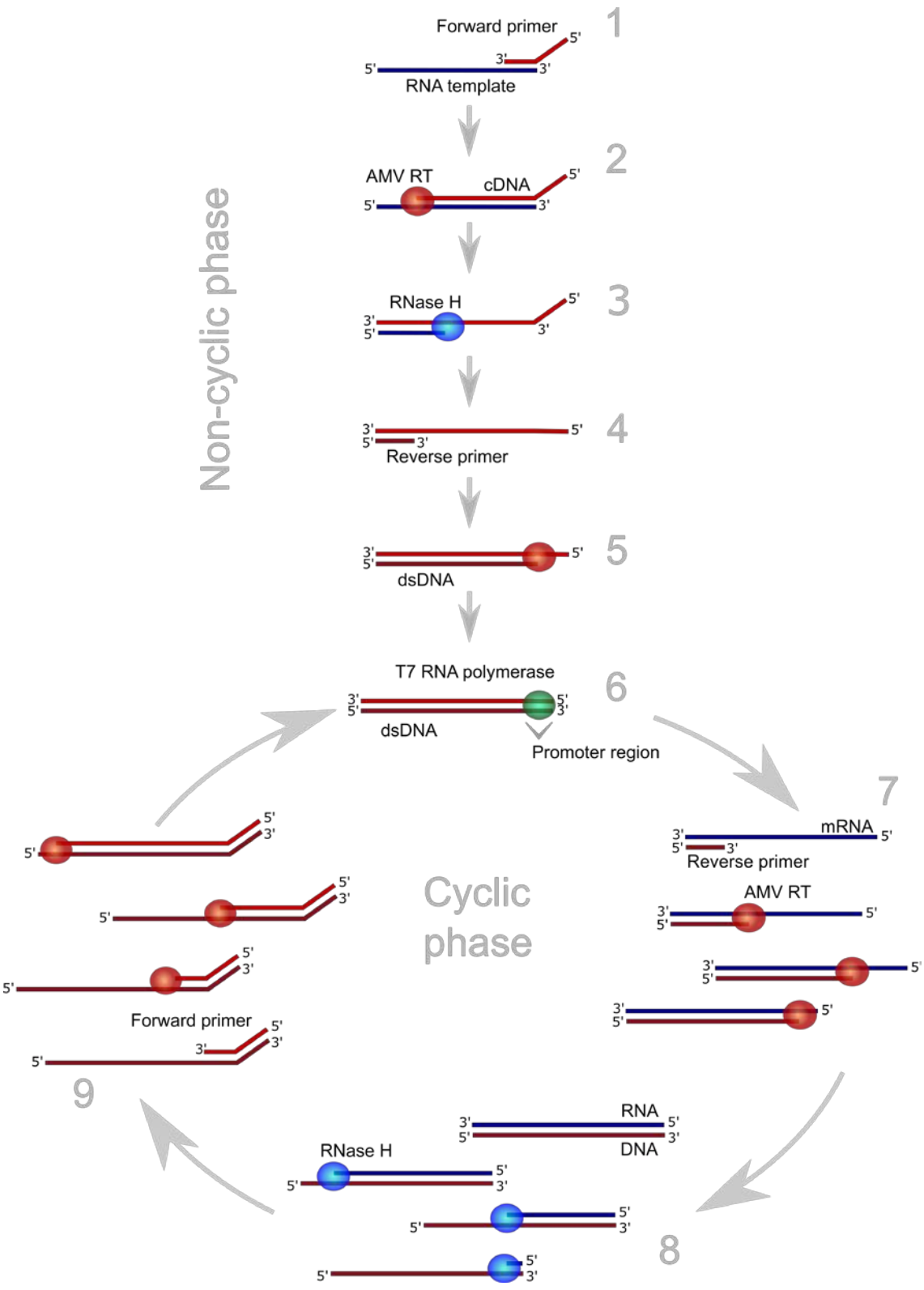

Figure 12 NASBA reaction, non-cyclic and cyclic phase. For details, see text

4.2.2. Molecular beacons

Molecular beacons are the most commonly used tool for real-time detection of amplicons during NABSA amplification. Molecular beacons consist of a fluorophore for visual signal, a quencher to act as a light switch, a probe sequence (loop) to bind the target, and two complementary stem sequences (6-8 bases) on each side of the probe sequence. The fluorophore is bound to the 5' end, and the quencher at the 3' end. With no amplicon to bind to, the beacon takes on a stem-and-loop structure, as seen in Figure 13A**.** Due to the complementarity of the stem, the fluorophore and the quencher are located next to each other in this position. When the fluorophore is excited by UV-light, the energy is transferred to the quencher and given off as heat.

In the presence of a nucleotide target sequence, the probe sequence will anneal onto the complementary strand. This will separate the strands of the stem and open the loop structure, as seen in Figure 13B. As the fluorophore and the quencher now are separated, the fluorophore will emit detectable light when excited with UV light (Tyagi et al., 1998; Tyagi & Kramer, 1996).

When the probe sequences bind to the antisense RNA amplicon in a NASBA reaction, real-time monitoring of the reaction can be achieved. As more amplicons are produced, molecular beacons will anneal and increase the light signal. This signal can be plotted as fluorescence over time, and produce a sigmoidal curve. As both amplification and detection then takes place in the same tube simultaneously, there is no need to open the tube, thus the risk of contamination is diminished (Leone et al., 1998).

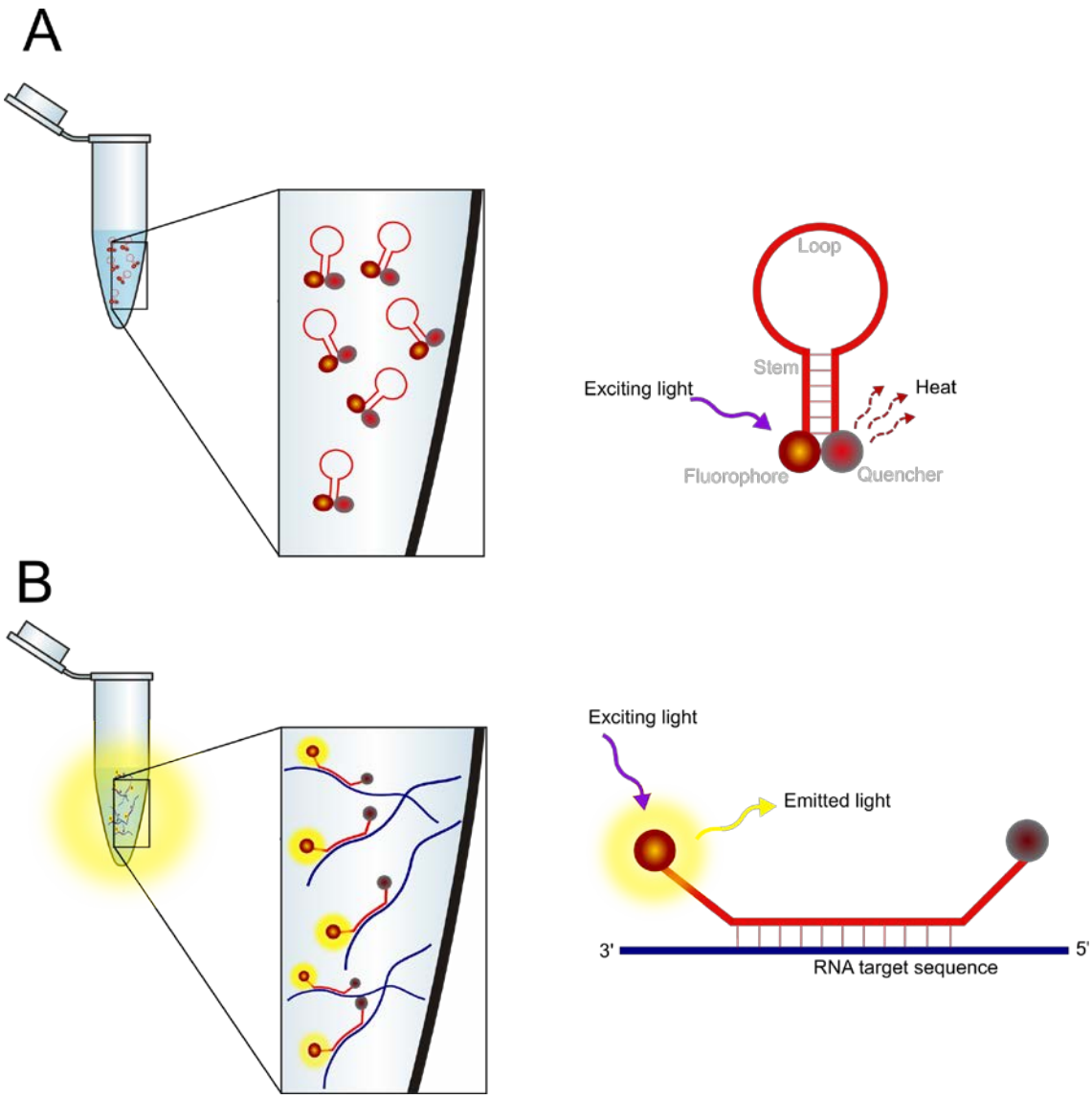

Figure 13 Molecular beacons bind to the amplicon(s) and report its presence. With no complementary sequence to bind to, the probe is in a closed stem-and-loop conformation (A), and there is no fluorescence. With a complementary sequence, the probe will bind onto it and open up (B). In this conformation, the fluorophore will fluoresce when exposed to UV light.

4.3. Lab-on-a-chip

Lab-on-a-chip (LOC) is a term used for small devices in which miniaturised versions of laboratory processes can be conducted. One or more laboratory protocols that are normally performed in the lab are transferred to, and carried out in, chips that are often made from plastic, silica, or glass, hence the name lab-on-a-chip. These systems first started emerging in the 1980s and 1990s, and have since become more common. They have been used for chromatography, extraction of DNA and RNA, and for detection of

various biological molecules (Liu et al., 2014; Okumus et al., 2016; Reinholt et al., 2014). Even coral-on-a-chip has been developed for *in vivo* study of coral physiology (Shapiro et al., 2016).

As the chip systems are miniaturised, the reagent volumes used are also reduced, which is an economic advantage. The chips are mostly microfluidic. LOC systems generally require less manual handling than conventional laboratory techniques, and some systems are fully automated (van Oordt et al., 2012). This greatly reduces the risk of contamination, and presents a potential approach for bringing analyses into the field, thus avoiding the problems, costs, and time elements associated with sample collection followed by transfer to the lab, often many miles away, for analyses. Obtaining results more rapidly and while in the field may be useful for directing additional sampling or implementation of appropriate and timely interventions. The more automation included in such a system, the easier it becomes to operate, and may also be used by non-trained personnel.

In the initial stages of my PhD project, I was involved in the development of a total analysis LOC system, called POCNAD.

4.3.1. The POCNAD system

The Point-of-care Nucleic Acid Detection (or Diagnosis) (POCNAD) system is designed to be a total-analysis system. Although there are many microfluidic and minimalized systems for sample (pre-)treatment, isolation of nucleic acids, or analysis of nucleic acids, there are very few systems available that attempt to combine all these steps in a single microsystem.

The objective of the POCNAD system is to be a fully automatic LOC system for nucleic acid detection, based on Boom extraction and real-time NASBA, and provide analysis results by the touch of a button (Figure 14). The intention of the POCNAD system is that it would be able to include all the steps from raw sample to final result:

- Sample pre-treatment (filtration, concentration and preservation, if necessary)
- Extraction of nucleic acids (using the Boom extraction method)
- Amplification and detection of RNA (using NASBA)
- Result processing

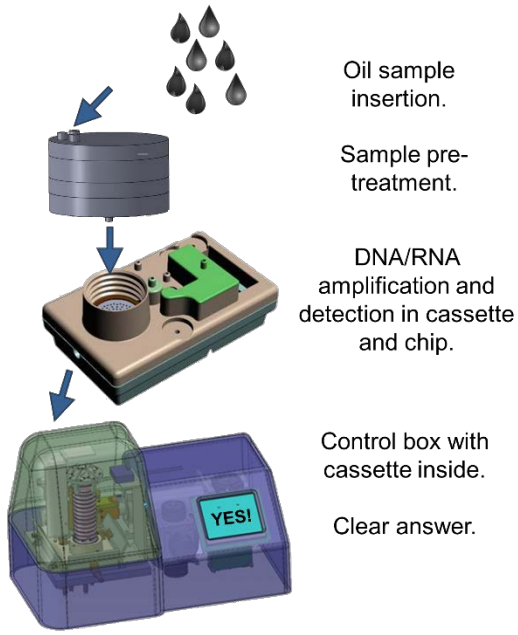

Figure 14 Overview of the sample pre-treatment and analysis system. The raw sample would be inserted in the pre-treatment device, containing the Trilobite technology, and the processed sample transferred directly to a cartridge containing a microfluidic chip for analysis. The cartridge- and pre-treatment system should be inserted to, and controlled by an instrument (box).

This system was initially developed for detection of human papillomavirus (HPV) in cervical swab samples. The first chips were made in silicon and glass, with 10-nl or 50-nl reaction chambers for NASBA (Gulliksen et al., 2004). Next generation microchips were made of cyclic olefin copolymer (COC), and 10 parallel NASBA reactions in 80-nl chambers could be run simultaneously (Gulliksen et al., 2005). This greatly reduced the reactant volumes needed; conventional NASBA is normally run in 20 µl reaction volumes. The early automatic platform consisted of two separate chips; one for sample preparation and nucleic acid extraction and one for amplification and detection by real-time NASBA (Gulliksen et al., 2012). Primer- and probe mix, and enzyme mix were freeze-dried onto the reaction chambers in the NASBA chip, and the reaction volume for NASBA on chip was 740 nl per reaction chamber. Reagents were loaded onto the chip by a custom made fluidic system driven by a syringe pump built into the POCNAD instrument that controlled the processes in the chips. The instrument also contained heating elements and an optical detection system for real-time fluorescence detection.

At the starting point of the project in which my research was involved, this system had been organised into a disposable plastic cartridge, enclosing a microfluidic chip (Figure

15, left). The top of the cartridge stored the necessary reaction reagents, and the bottom of the cartridge was used as to hold waste. The reaction chamber for nucleic acid extraction was also located in the top part of the cartridge, and the NASBA amplification and detection were placed inside the microfluidic chip. A prototype control instrument had also been developed to control the processes within the cartridge-chip (Figure 15, right). The intention was that this system should be able to analyse samples for up to 16 different targets in 8 separate reaction chambers, using multiplex real-time NASBA.

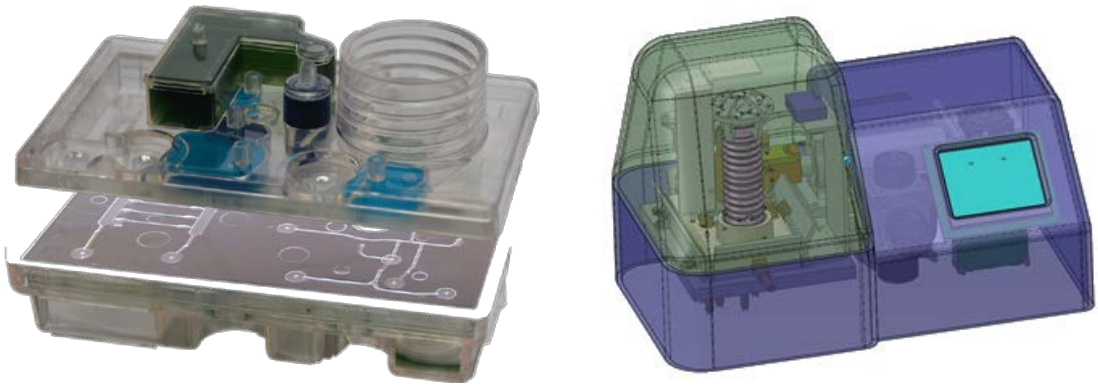

Figure 15 The disposable parts of the POCNAD system included a microfluidic chip enclosed by a cartridge storing reagents and waste (left). The cartridge was to be placed into a control instrument to run the process and analyses (right).

4.3.1.1. The POCNAD cartridge

The POCNAD cartridge (Figure 16) measured approximately 120 x 75 mm, and the height ranged from 35 mm, up to 55 mm at the highest area. It was injection moulded in medical-level acrylonitrile butadiene styrene (ABS) and acrylonitrile styrene (AS), or polymethyl methacrylate (PMMA). The cartridge was designed to be fitted into a control instrument (section 4.3.1.3). The top part of the cartridge had chambers for storage of reagents, and an inlet transition onto which a sample container or pre-treatment system could be screwed. The bottom part of the cartridge is a container for waste, and had a transition point to connect to the instrument's pump.

This section describes the principles of system, assuming that all processes functioned optimally. In practise, however, the processes were not that streamlined and not particularly easily performed.

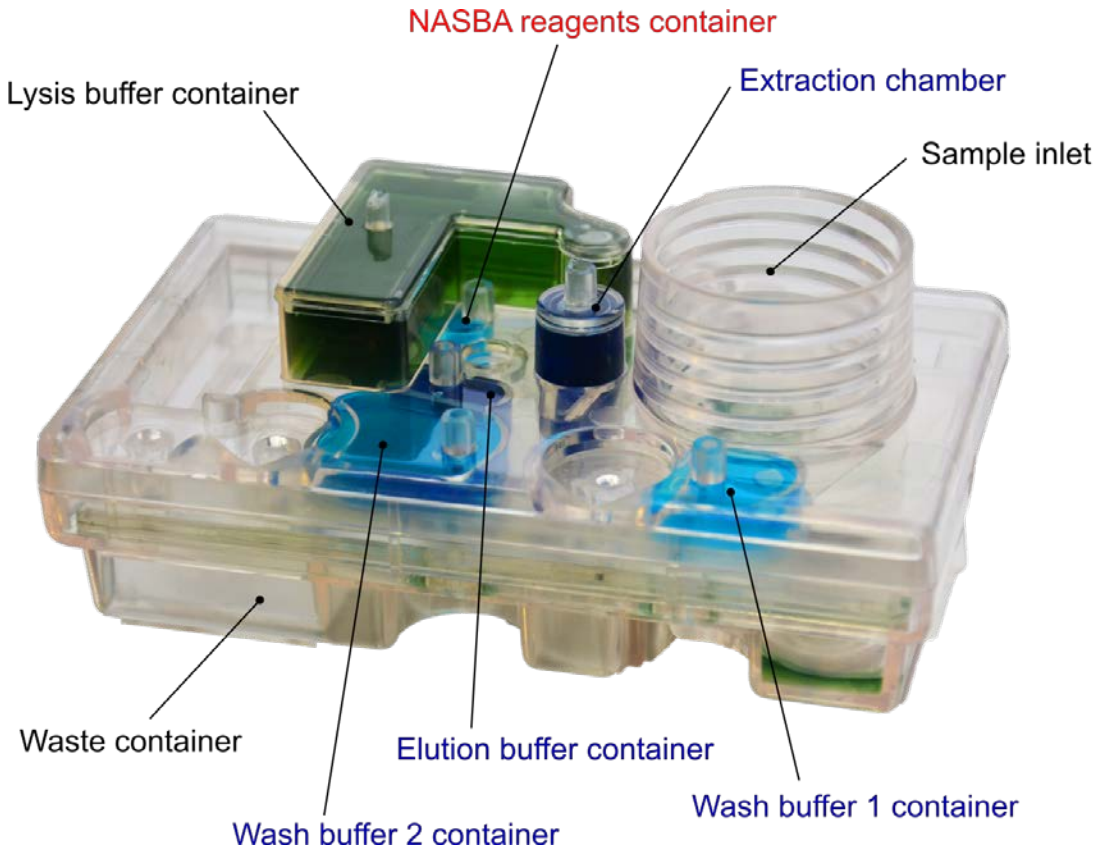

Figure 16 The POCNAD cartridge. The containers with reagents for nucleic acid extraction are indicated by blue text; the container with reagents for NASBA is indicated by red text.

The sample inlet could be connected to a simple sample container, or a more complex pre-treatment system. Lysis buffer could be pumped from the lysis buffer container to the sample container (sample inlet). Transportation of all liquids between the containers was through the microfluidic chip inside the cartridge (section 4.3.1.2.). After lysis, the sample could be pumped to the extraction chamber, where nucleic acid extraction occurred. The extraction method was based on Boom extraction using magnetic silica beads. A controllable magnetic component of the instrument was placed underneath this chamber, and could be raised and lowered in order to capture the silica beads. Wash buffers and elution buffer stored in their respective containers were pumped into the extraction chamber to wash and elute the nucleic acids, and waste was pumped into the waste container. Eluted nucleic acids could then be pumped into the chip to carry out the NASBA amplification and detection.

4.3.1.2. The POCNAD chip

The microfluidic chip placed inside the cartridge contained micro channels for transportation of sample and reagents from the containers in the top cartridge (section 4.3.1.1), and contained reaction chambers for the NASBA assay. The chip measured 90.5 mm x 65.5mm and was 1 mm thick (Figure 17). The pre-NASBA and NASBA chambers were 2 µl, and the extraction chamber was 1 ml. Chips were fabricated by injection moulding in polycarbonate (PC) and cyclic olefin copolymer (COP), and sealed by PCR film (100 µm, Thermo Scientific, Epsom, Surrey, UK) on both sides.

NASBA reagents with primers and probes were stored as freeze-dried spheres in the pre-NASBA chambers. The enzyme mix for NASBA was stored as freeze-dried spheres in the NASBA reaction chambers.

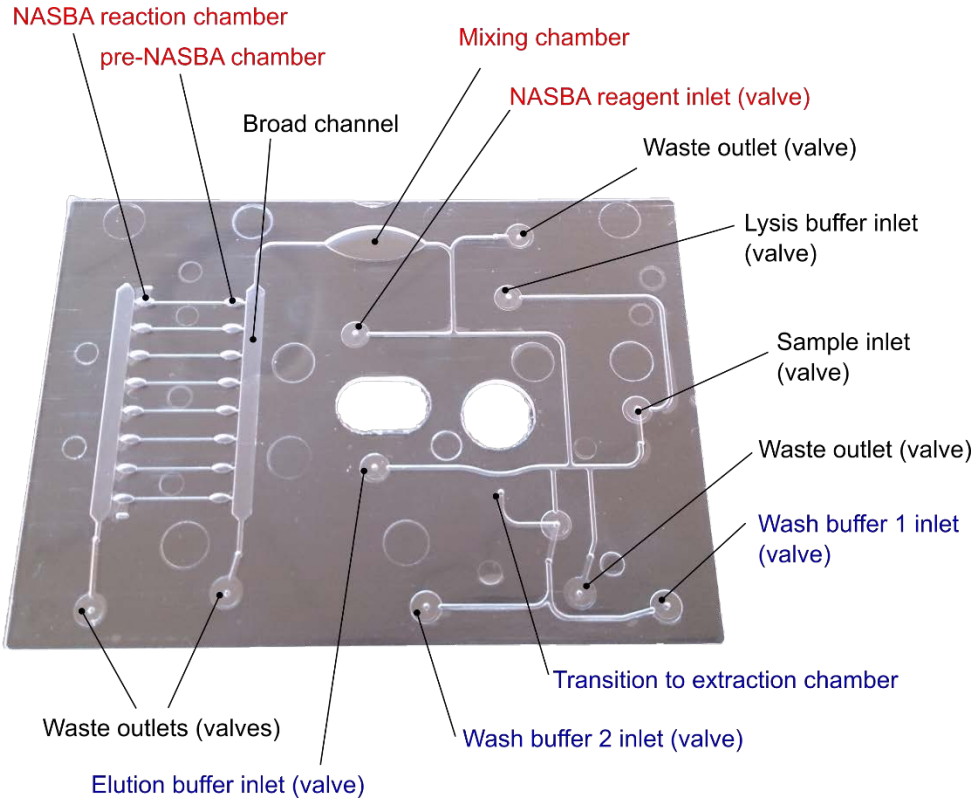

Figure 17 The POCNAD microfluidic chip. The valves and transition points involved in nucleic acid extraction are indicated by blue text; the valves, transition points, and chambers involved in NASBA are indicated by red text.

The chip was equipped with 11 valves involved in control of the flow direction in the micro channels. COP valve disks of 40 µm thickness (IK4-Ikerlan, Arrasate-Mondragón, Spain) were placed at the valve positions (Figure 18). When pushed down, the valve discs sealed

the hole in the middle of the valve. These holes led the fluids from the different containers in the cartridge into the chip. The valves were in the open position when the valve discs were released. By opening and closing these valves in different patterns, the flow of liquid through the microfluidic could be directed and controlled.

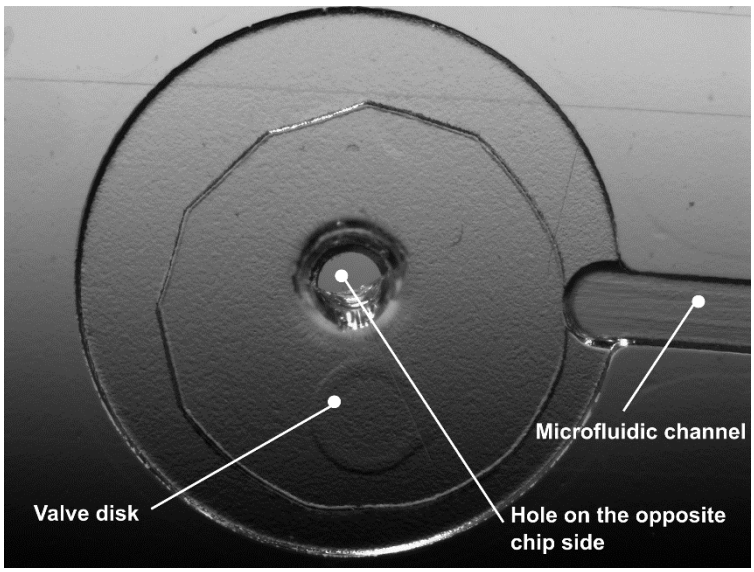

Figure 18 The valves in the microfluidic POCNAD chip.

The four waste outlets valves had hole-openings at the underside of the chip, leading liquid to the waste chamber in the cartridge. All the other valves had holes on the topside of the chip towards the sample inlet and reagent containers from the top part of the cartridge.

The NASBA amplification and detection took place in the POCNAD chip. Nucleic acids that were extracted in the cartridge's extraction chamber were transferred to the mixing chamber in the chip and mixed with NASBA reagents from the NASBA reagent container. The mix was then distributed in the broad channel before filling the pre-NASBA chambers. Freeze-dried primers and probes would then dissolve. A heating element placed under the pre-NASBA chambers would heat up to 63 °C for efficient primer annealing. The reaction mix would then be transferred to the NASBA reaction chambers. Here, the freeze-dried enzymes would dissolve, and the real-time NASBA reaction would run. A heating element placed under these chambers would keep the reaction temperature of 41 °C. Any emerging fluorescent signal from molecular beacons binding to the amplicon

would be read by a multi-pixel photon counter (MPPC) within the POCNAD instrument, and analysed in custom made software.

4.3.1.3. The POCNAD instrument

A prototype instrument for automatic control of the processes in the cartridge and chip had been developed. The housing of the prototype depicted in Figure 19 contained the following components:

- a frame to hold the disposable cartridge
- a pump to control the microfluidic flow in the chip
- pin actuators to open and close valves in the chip
- a magnet that could be moved up and down, to capture magnetic beads in the extraction of nucleic acids
- heating elements for maintaining temperatures of 63 °C and 41 °C for the NASBA reaction
- multi-pixel photon counter (MPPC) and optical fibres, with associated motor for fluorescence reading of the real-time NASBA
- Display and connection to a computer with the custom-made controlling software/ graphical user interface (GUI)

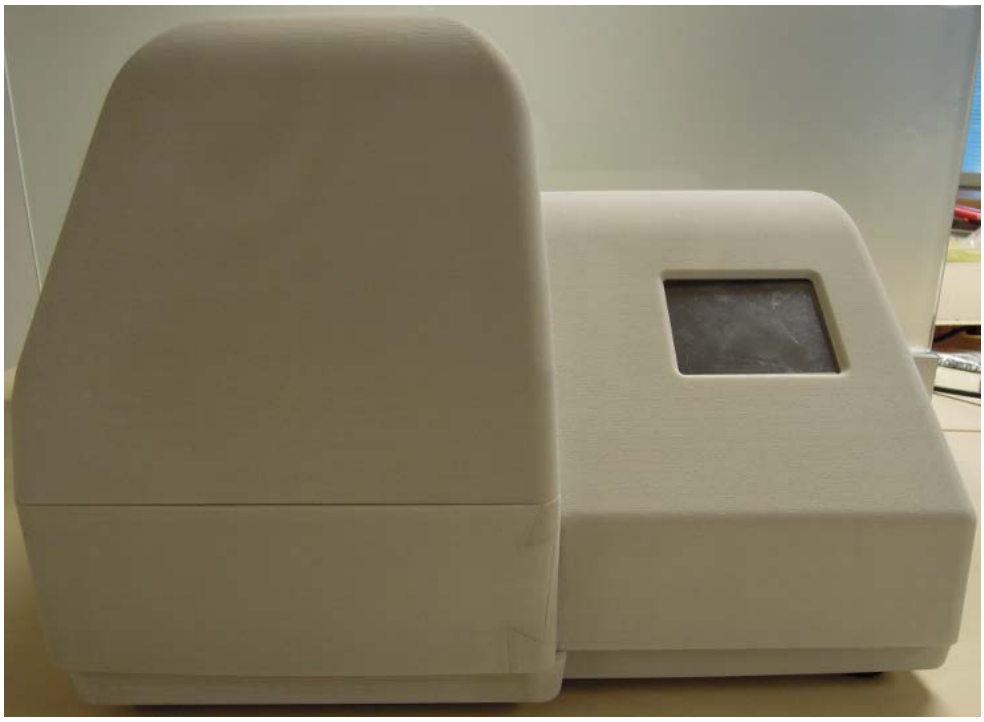

Figure 19 Housing of the POCNAD instrument. Source: EU project no 262243

The cartridge was placed in a frame in the POCNAD instrument (Figure 20) for the processes to be controlled by the instrument components.

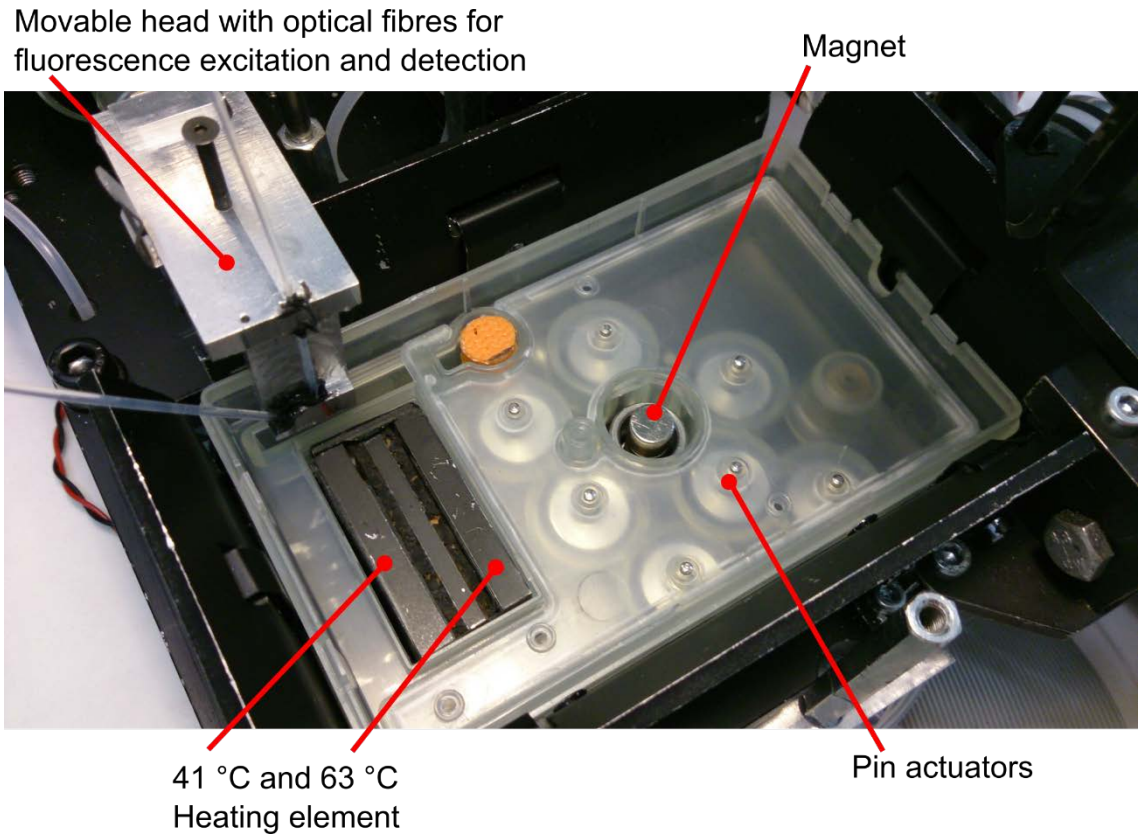

Figure 20 The cartridge frame in the POCNAD instrument, here seen with the bottom part of the cartridge in place.

The pumping system consisted of a peristaltic pump, two pinch-valves, two T-connectors and corresponding tubing. The tubing was connected to the cartridge at a fixed transition point in the cartridge frame, and onto the top of the extraction chamber. The maximum capacity of the pump was approximately 3.4 ml min^{-1}.

Pin actuators were mounted in and onto the cartridge frame; seven actuators under (Figure 20), and four for the waste valves on top. These were controlled separately to control the opening and closing of the valves in the NASBA chip. A blunt pin at the tip of the actuator pushed down on the valve discs to close the valve, and released to open the valve.

A magnet was also installed in the cartridge frame (Figure 20). This could be controlled up and down, and, at its top placement, the magnet was at the underside of the extraction chamber, capturing the magnetic silica beads used in the nucleic acid extraction. The magnet was lowered during the wash steps of the extraction protocol.

Two heating elements were installed in the instrument: one to be placed onto the extraction chamber, and heated during the elution step of the protocol; and one in the

cartridge frame, to hold the NASBA reaction temperatures. The heating element in the frame consisted of two separate heating zones; one maintaining the temperature in the chip's pre-NASBA chambers at 63 °C, and one keeping the temperature in the NASBA reaction chambers at 41 °C.

The optical system consisted of a transmitter and a receiver, each equipped with an optical fibre (Figure 21). The optical fibres were mounted on the head of the motor that led the fibre ends above the NASBA reaction chambers in the chip for real-time fluorescence detection.

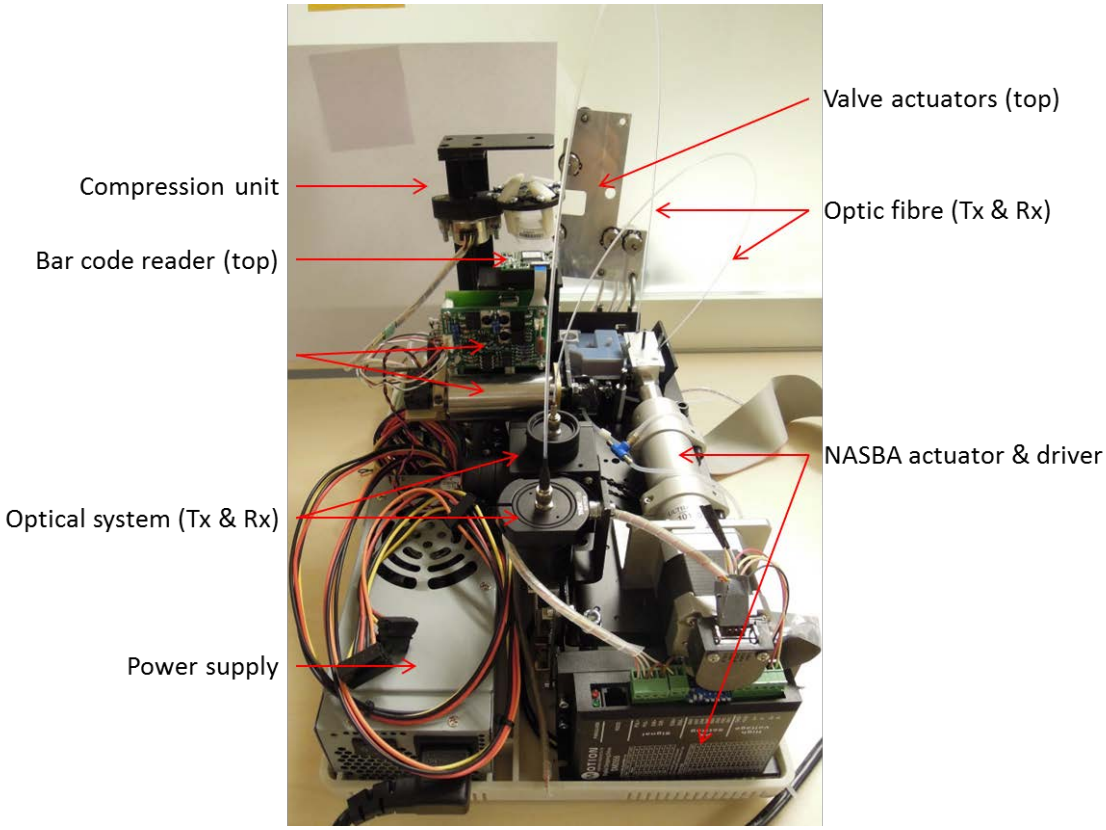

Figure 21 The inside of the POCNAD instrument. The bar code reader and compression unit was not used in this project. Source: EU project no 262243

All the components were controllable using a GUI installed in a computer connected by a USB cable. Real-time graphs of NASBA assays were presented in the GUI on the computer, and the associated data were automatically saved in a txt. file. Further details on the GUI are provided in Appendix E, section 11.5.4.

4.3.2. My contributions in the POCNAD project

The section of the POCNAD project in which I was involved had as its initial goal to use the Trilobite technology and the POCNAD system in succession for the detection of strains of the oil-utilizing consortium of bacteria described in sections 2.2.1 and 5.2.1.1. (Figure 22). An optimal system would be able to handle a raw sample containing oil and bacteria, and the oil droplets would then be separated from the bacteria in the Trilobite® chip. The bacteria would then be analysed using the POCNAD system for real-time NASBA assays with primer sets specifically designed for the various bacteria. The focus of this project regarding the POCNAD system was directed towards the application of the cartridge and chip that had already been developed. However, as I report in my results, issues linked to the POCNAD instrument made the testing challenging.

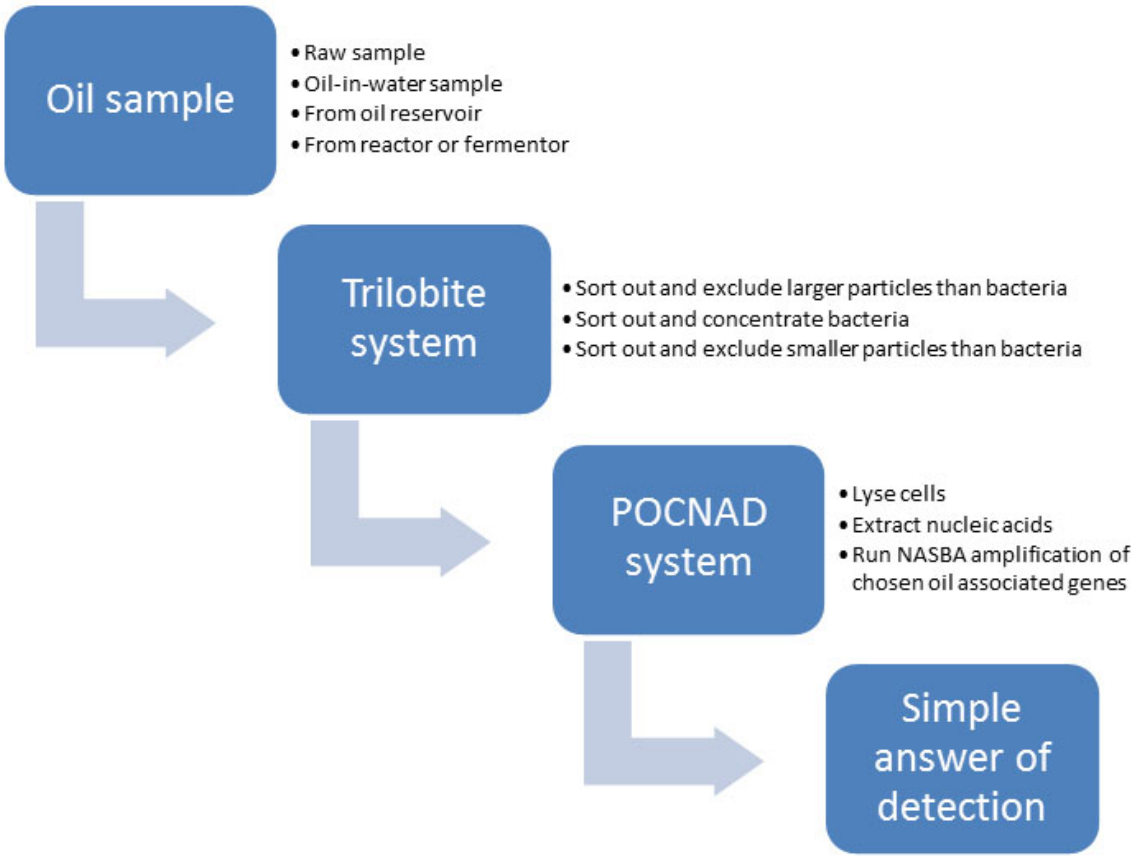

Figure 22 Flow-chart of the initial objectives: sample treatment and analysis of oil-utilizing bacterial consortia through the Trilobite® chip and the POCNAD analysis system.

5. Methods

5.1. Testing the Trilobite® chip for biological applications

In my work, the Trilobite® chip was first tested with rigid plastic particles in order to provide "proof-of-concept". The chip was further examined for its capacity to handle real biological micro-particles. Dewatering of microalgae suspensions and concentration of cysts or oocysts of waterborne parasites were investigated as real-life examples of where the chip could have an important role. In all these experiments, Trilobite® chips with 5 µm gaps between the blades (or "feet") were used.

5.1.1. Experimental setup of Trilobite® chip

The chip used for testing was fabricated in silicon and glass at Micronit microfluidics (Enschede, The Netherlands). The whole chip measured 30 mm x 60 mm. The main channel within the chip measured 10 mm x 30 mm, and the field of separation units were located in the middle of the main channel, in a 10 mm x 10 mm area, as seen in Figure 23. This field contained 113 trilobite separation units distributed in nine rows. Each unit was 1200 µm long and 500 µm wide, and the gaps between the blades were 5 µm wide.

Figure 23 The Trilobite® chip made in silicon and glass. The two inlet holes and channels are seen at the right, and the two outlet channels and holes for the concentrate fraction are seen at the left. The outlet for the permeate *fraction is at the back of the chip (not seen here). Picture provided by Micronit microfluidics. The main channel is 30 mm long, 10 mm wide and ~500 µm high.*

Micronit microfluidics (Enschede, The Netherlands) also provided a framework (Fluidic Connect Pro) to place the chip and connect to the associated tubes without leakage (Figure 24). The chip was placed in the framework and connected to the tubes.

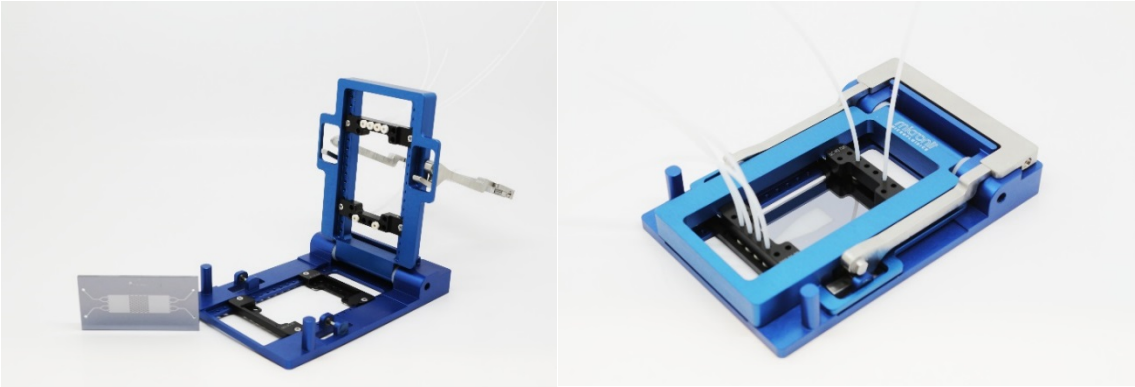

Figure 24 Trilobite® microfluidic chip and Fluidic Connect Pro framework from Micronit microfluidics (left). When the chip is placed in the framework, the inlet and outlet tubes are connected to the chip leak-free (right). Pictures provided by Micronit microfluidics.

Two different setups for pumping were used: one with a syringe pump and one with a peristaltic pump. With a syringe pump, the chip-treated fluid volume was limited by the size of the syringe. A peristaltic pump allowed (more) continuous chip-treatment, and increased volume. The setup using a peristaltic pump was used for experiments with *Toxoplasma gondii* oocysts, the other experiments were carried out using a syringe pump.

For the syringe pump setup, a plastic syringe (10 ml or 20 ml, Omnifix Luer Lock, B Braun, Melsungen, Germany) was filled with suspension, connected to the tubes from the chip framework and fitted onto a syringe pump (Aladdin AL-1000 syringe pump, World Precision Instruments, Inc., FL, USA), as seen in Figure 25. Each of the two outlet fractions (*concentrate* and *permeate*) were led out of the chip and framework through two tubes. The outlet fractions were collected in 15-ml tubes. The tubes were weighed before and after collection to calculate the volumes. Particles were assumed to have approximately the same density as water (Uduman et al., 2010), and that 1 g = 1 ml.

The parameters for these tests were mainly determined by the limitations of the syringe pump. The chip was rinsed between each test.

Enumeration of plastic particles and microalgae were carried out as follows: From each sample tube, counting of particles within a volume of 6.4×10^{-5} ml in a counting chamber

was performed in 72 parallels. The counts were carried out manually by placing the counting chamber (Assistant Neubauer improved counting chamber, Glaswarenfabrik Karl Hecht KG, Germany) under an Olympus IX51 inverted microscope (Olympus Corporation, Tokyo, Japan) connected to a computer with Cell^B imaging software (Olympus Corporation, Tokyo, Japan). The particle concentration (particles ml^{-1}) in the samples was calculated from these counts.

Enumeration of protozoan parasites are described in section 5.1.4.1 for *Giardia* cysts, and in Appendix D, section 11.4.3 for *T. gondii* oocysts.

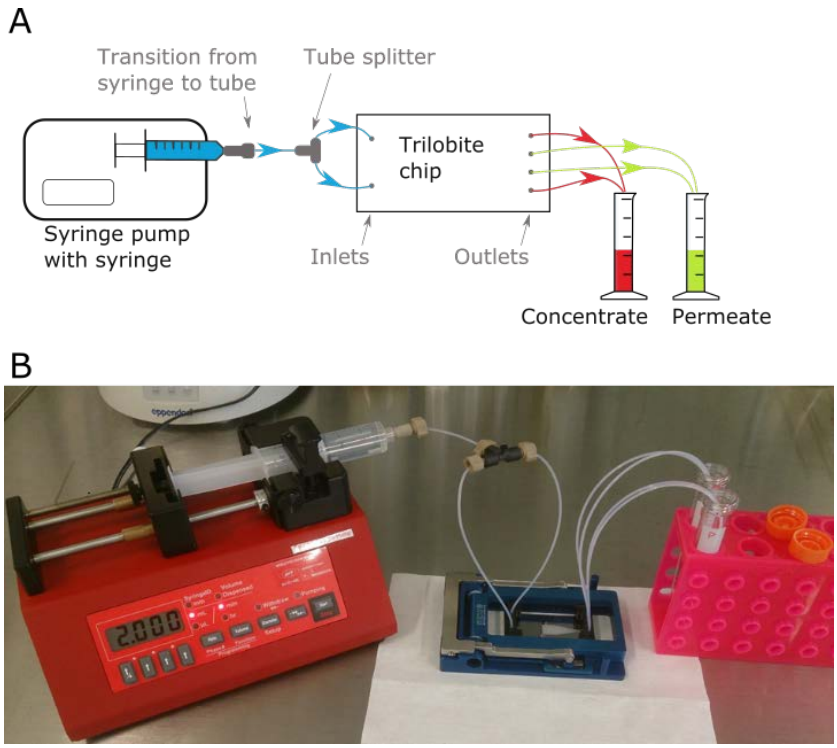

Figure 25 Schematic (A) and photographic (B) presentation of the experimental setup with a syringe pump.

In the experimental setup with a peristaltic pump (as used in the experiments with *T. gondii* oocysts), a 403/VM2 Watson Marlow pump (VWR) was used to pump the water samples though the Trilobite® chip (Figure 26). The chip was inserted in the frame system depicted in Figure 24, and the micro tubes were connected to larger tubes that were led through the peristaltic pump. The volume that was pumped through the chip was limited by the capacity of the peristaltic pump, with a flow rate of approximately 3 ml min^{-1}. The outlet fractions were collected in 50-ml tubes, and the collected *concentrate* was run

once more through the chip. The chip was rinsed twice by running 4 ml 0.01 % Tween 80 water through the system, in order to capture any oocysts remaining in the tubes.

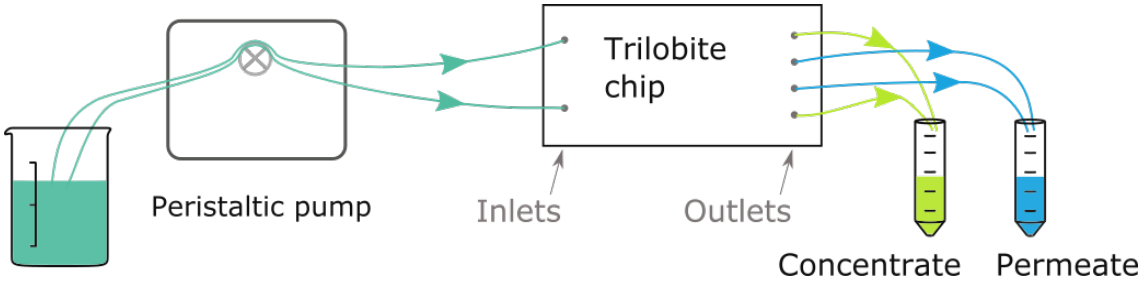

Figure 26 Experimental setup for chip treatment with a peristaltic pump

5.1.2. Proving the concept of the Trilobite® chip

In order to provide a "proof-of-concept" of the Trilobite® chip, suspensions containing plastic particles were pumped through the chip.

Spherical plastic particles with a 10-µm diameter (Spheromers® CA 10, Microbeads AS, Skedsmokorset, Norway) were suspended in deionized water to which was added 0.05 % Triton X-100 (VWR International AS, Oslo, Norway) or seawater that had been filtered sequentially through a 50 µm and 0.2 µm filter.

The effect of different particle concentrations and different flow rates were examined. The test parameters are summarized in Table A 1 (Appendix A). Each test was run four times, and one inlet sample, four *concentrate* samples, and four *permeate* samples were produced from each test.

The extents to which the particles were concentrated in these tests were determined from the concentration factor (CF), which was calculated according to Equation 1:

$$CF = \frac{Cc}{Ci} \qquad \text{Equation 1}$$

Where Ci is particle concentration in the inlet (particles ml^{-1}), and Cc is particle concentration in the *concentrate* fraction (particles ml^{-1}).

Efficiency was used as a measure of how well the chip was able to direct the incoming particles into the *concentrate* fraction. If all particles that were pumped into the chip ended up in the *concentrate*, then the efficiency would be 100 %. If all the particles ended up in the *permeate*, the efficiency would be 0 %. It should be noted that the efficiency is independent of how many times the particles were concentrated. Efficiency was calculated according to Equation 2:

$$efficiency = \frac{Cc\ x\ Vc}{Ci\ x\ Vi}\ x\ 100\ \% \qquad \text{Equation 2}$$

where Vc is the volume of the *concentrate* fraction (ml), and Vi is the total volume pumped through the chip from the inlet (ml).

The calculated particle concentrations of the samples were compared with the expected particle concentration of the *concentrate*, given an efficiency of 100 %. The expected concentration of the *concentrate* was calculated according to Equation 3:

$$expected\ Cc = \frac{Ci\ x\ Vi}{Vc} \qquad \text{Equation 3}$$

Graphs and statistical tests were done in Graphpad Prism 6. One-way ANOVA (with Tukey's test) was used for statistical analyses, α=0.05.

5.1.3. Trilobite® chip for dewatering of microalgae cultures

The Trilobite® chip was investigated for dewatering of three algae; the cryptomonad *Rhodomonas baltica* (NIVA 5/91), and the diatoms *Thalassiosira weissflogii* (CCAP 1085/18) and *Chaetoceros* sp. (CCAP 1010/3). The experimental setup for chip treatment was the same as for the proof-of-concept tests with 10 µm plastic particles, described in section 5.1.1 and in Figure 25. Four parallels were run in each test.

The cryptomonad microalgae *Rhodomonas baltica* have flexible cells that are 7-8 µm in diameter. Because the cells are flexible, it was expected that they might slip between the gaps of the trilobite units. Therefore, various chemicals were examined as fixatives, with

the objective of making the rigid versions of the algal cells. Isopropanol, ethanol, acetone, and the methanol-based PreTect TM were used as fixatives. Cultures of *R. baltica* (2.86 x 10^9 algae ml^{-1}) were mixed with each fixative in Eppendorf tubes at a 1:1 ratio. Spherical plastic beads (10 µm) were added to each tube for size indication. The fixation attempts were evaluated under a light microscope.

R. baltica tests in the Trilobite® chip were run with living algae and algae fixed with PreTect TM (PreTect AS, Klokkarstua, Norway). *Chaetoceros* sp. was also fixed for comparison. Algal suspensions were made by mixing cultures with seawater that had been sequentially filtered through a 50 µm and a 0.2 µm filter. Flow rate was set to 2 ml min^{-1} and 20 ml algae suspension was run through the chip in each parallel. Algal inlet concentrations are shown in Table 3. Concentration factor, efficiency, and expected concentration were calculated according to Equation 1, Equation 2 and Equation 3, respectively.

Table 3 Inlet concentrations of algae tests.

Test	Algae	Cell concentration (cells ml^{-1})	Alive or fixed
1	*R. baltica*	116 536	alive
2	*R. baltica*	286 675	fixed
3	*Chaetoceros* sp.	483 941	alive
4	*Chaetoceros* sp.	480 252	fixed
5	*T. weissflogii*	51 649	alive

Chaetoceros sp. cells were measured to examine differences in cell sizes in the two outlet fractions, as this diatom is close in size to the gaps within the chip (5 µm).

5.1.4. Trilobite® chip for concentration of water-borne parasites

In a collaboration with a fellow PhD student, Jemere Bekele Harito, we investigated the Trilobite® chip's ability to concentrate low numbers of parasitic oocysts in water samples.

Experiments were carried out using cysts of *Giardia duodenalis*, and oocysts of *Toxoplasma gondii*.

5.1.4.1. Chip concentration of Giardia duodenalis *cysts*

Attempts were made to concentrate *Giardia duodenalis* cysts in water samples using the Trilobite® chip with the experimental setup with a syringe pump (Figure 25) described in section 5.1.1.

G. duodenalis cysts were isolated from dog faeces using zinc sulphate flotation. Faeces (10 g) and tap water were mixed distributed into 50-ml tubes. The tubes were centrifuged and the supernatant discarded. The pellet was vortexed, and resuspended in $ZnSO_4$ solution. The tube was then carefully filled with water and centrifuged. The upper layer and the interface pipetted out and collected in a new 50 ml tube. The isolated cysts were washed several times, and stored at 4 °C. A detailed protocol is described in Appendix D, section 11.4.2.

Enumeration of *Giardia* cysts was carried out by drying and fixing 50 µl sample onto a microscopic slide with wells, and IFAT staining using monoclonal antibodies (MAB) for *Giardia* (AquaGlo G/C direct, Waterborne Inc. New Orleans, LA; USA). Cysts were counted using a fluorescence microscope (Leica DM LB, Ortomedic, Oslo, Norway).

Suspensions were prepared by suspending 200 cysts in 10 ml distilled water with 0.05 % Triton X-100 and 0.01 % Antifoam A (Fluka 10794), and added to a 10-ml syringe. The samples were run through the chip between one and four times.

5.1.4.2. Chip concentration of Toxoplasma *oocysts*

Oocysts of *Toxoplasma gondii* were provided by fellow PhD student Jemere, and had originally been provided by JP Dubey, US Department of Agriculture. Jemere also performed the majority of the counting procedures, and the control experiments. Purified oocysts were suspended in distilled water or 2 % sulphuric acid and stored at 4 °C.

In this experiment our intention was to achieve more continuous chip treatment than had been tested in the previous experiments with *Giardia* cysts. Instead of using a syringe

pump, which is limited by the syringe volume, a peristaltic pump was used to pump water samples through the system.

All enumerations of oocysts were carried out using KOVA Glasstic Slide 10. A detailed protocol is described in Appendix D, section 11.4.3. Pre-made 50 ml-suspensions containing an estimated 0, 500, or 1000 oocysts, were pumped by a peristaltic pump through the chip as described in section 5.1.1 and shown in Figure 26.

Control samples were prepared in the same manner as the samples being chip-treated: 50-ml suspensions were prepared containing 0, 500, or 1000 oocyst in 50-ml centrifuge tubes. The oocysts of the control samples were concentrated by centrifugation at 3000 rpm (1550 x g) for 10 min (Rotanta 460, Hettich, Tuttlingen, Germany), and discarded of supernatant down to a volume of 1 ml.

The chip-treatment was compared with a traditional method for concentration using a filter membrane. It should be noted that the volume pumped through the chip (50 ml) was much lower than the volumes used in membrane filtration, due to limitation set by the chip and the peristaltic pump.

For membrane filtration, suspensions of 10 L spiked with 0, 500 or 1000 oocyst were prepared in low-density polyethylene (LDPE) containers. Each suspension was pumped through a filter membrane (142mm cellulose acetate, 2.0 µm pore size, Millipore), held by a stainless steel filter holder (142mm, Millipore) at 1.5 L min^{-1} (Varmeca, Watson Marlow, Heco, Oslo, Norway), as shown in Figure 27, in order to collect the oocysts.

The LDPE container was rinsed with 2 L deionized water and the washings were pumped through the membrane filter. The filter was carefully removed from the holder by tweezers and rinsed with 25 ml membrane elution buffer (US.EPA. 1997 Method 1622 Draft), and the eluate was collected in a 50 ml-centrifuge tube. The filter was immersed in 1% Tween 80 in a high-density polyethylene (HDPE) beaker and sonicated for 3 min (35 kHz, Transsonic 310, Elma, Singen, Germany), and the fluid transferred to the 50 ml centrifuge tube. The tubes were refrigerated until centrifugation.

All treatments were carried out in triplicate.

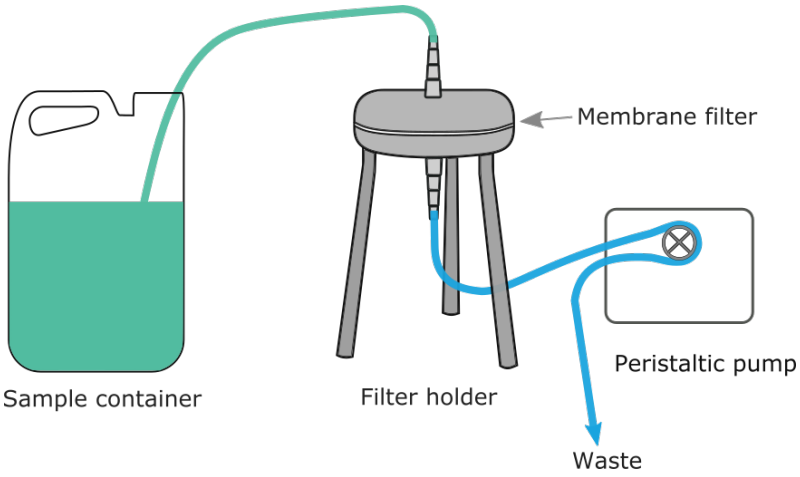

Figure 27 Filtration setup for membrane filtration of Toxoplasma *oocysts. This is currently a standard method to concentrate oocysts in water samples, and was used for comparison to the chip treatment.*

After collection, all samples (chip treated and membrane filtered) were centrifuged at 3000 rpm (1550 x g) for 10 min (Rotanta 460, Hettich, Tuttlingen, Germany), and discarded of supernatant down to a volume of 1 ml.

Oocysts were counted in KOVA Glasstic Slide 10, and recovery of oocysts was calculated according to Equation 4:

$$R = 100\,x\,\frac{n}{T} \qquad \text{Equation 4}$$

where R is percentage recovery; n is the number of oocysts counted; and T is the number of oocysts spiked.

Statistical analyses were conducted using the Kruskal-Wallis test (nonparametric one-way ANOVA) for the 500 oocyst samples; and (parametric) one-way ANOVA for the 1000 oocyst samples with a 50 % significance level (α=0.05). Each treatment was carried out in six parallels.

5.2. Developing NASBA assays during my research

During the course of this research, NASBA assays were designed both for detection of oil-utilizing bacteria and for detection of the waterborne protozoan pathogens *Cryptosporidium parvum*, *Cryptosporidium hominis*, and *Giardia duodenalis*.

The basis for primer- and probe design is described first below, and then the experimental tests of the designed primer sets are described.

5.2.1. Primer and probe design

Primers and probes suitable for NASBA were designed using Beacon Designer 8 (Premier Biosoft, Palo Alto, CA, USA) and were ordered from SBS Genetech Co. (Bejing, China) for the protozoan targets or Biosearch Technologies, Inc. (Petaluma, CA, USA) for the bacteria targets.

5.2.1.1. Designing primers and probes for oil-utilizing bacteria

A consortium of four bacterial strains (designated A, B, C and D) that were able to degrade heavy oil were used in the initial research project. The aim was to develop a sensitive, rapid, and reliable method to monitor the presence of these strains in a bioreactor. Customized primers and molecular beacon probes for real-time NASBA were developed for these strains based on 16S sequences and three different genes. The three genes had been reported to be differentially expressed (personal communication) in the strains when they were grown with heavy oil, compared with when they were grown with the more readily available carbon source, acetate. These genes were designated *m*, *n,* and *o*. Sequences for 16S and all three genes were available for strain A, and primer sets were successfully designed for all four targets. Strains B and C had some phenotypical differences, but the 16S and gene sequences were not distinguishable, and the primer sets designed targeted both strains.

Based on the 16S sequence from strain D, it was not possible to design probes designed with the available software, and sequences for genes *m*, *n* and *o* were not available for this strain. This strain was nevertheless tested using the other primer sets (Table 4).

Table 4 Primer and probe sets for oil-utilizing bacterial strains A, B, and C. No suitable primer set was found for strain D. Each primer set included a forward primer (P1), a reverse primer (P2) and a molecular beacon (MB).

Primer set	Target	Strain(s)	MB fluorophore	MB quencher
A-16S	16S rRNA	A	FAM	BHQ-1
BC-16S	16S rRNA	B, C	CAL Fluor Red	BHQ-2
A-m	gene *m*	A	FAM	BHQ-1
BC-m	gene *m*	B, C	CAL Fluor Red	BHQ-2
A-n	gene *n*	A	FAM	BHQ-1
BC-n	gene *n*	B, C	CAL Fluor Red	BHQ-2
A-o	gene *o*	A	CAL Fluor Red	BHQ-2
BC-o	gene *o*	B, C	FAM	BHQ-1

5.2.1.2. Designing primers and probes for Cryptosporidium

Primers and probes were designed to target transcripts of the MIC1 gene in *C. parvum* and *C. hominis*. The MIC1 gene in *C. parvum* (CpMIC1) has an intron, and the gene transcript consists of two exons (Figure 28). The MIC1 gene in other *Cryptosporidium* species does not have a second exon, and transcripts consist of only one exon, similar to the exon 1 of CpMIC1. Based on this information, a NASBA assay could be designed that could be used to distinguish *C. parvum* from other *Cryptosporidium* species, as has previously been done for PCR (Webber et al., 2014). One primer set, named "C.par MIC1", was designed to target the second exon of CpMIC1, and another, named "C.hom MIC1", was designed to target the first (and only) exon in *C. hominis* MIC1 (ChMIC1). The exon 1 sequences in both species are very similar, and the primer set targeting exon 1 detects both *C. parvum* and *C. hominis*. Primer and probe sequences are shown in Table B 1 (Appendix B).

Further details of this work is described in Paper 2: Real-time Nucleic Acid Sequence-based Amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts.

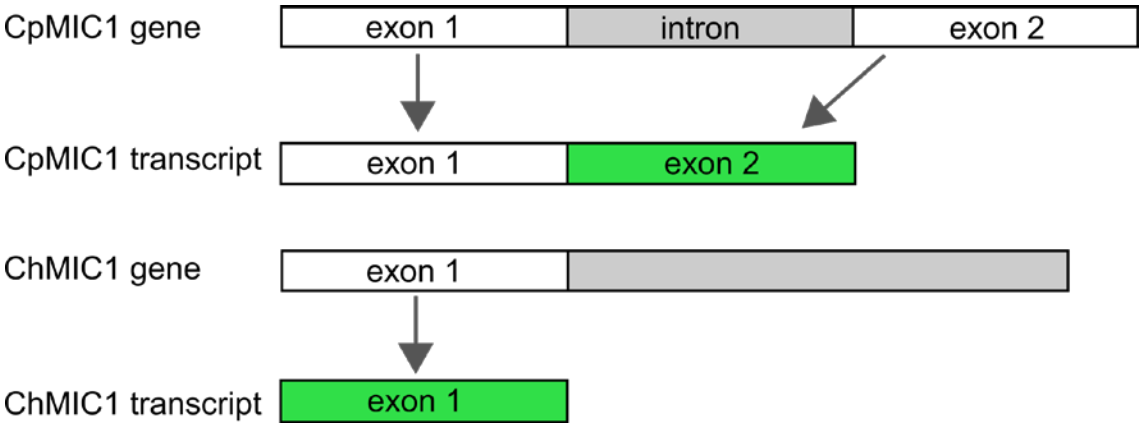

Figure 28 The MIC1 genes and transcripts in C. parvum *and* C. hominis*. Grey areas indicate sequences that do not become part of the mRNA, and green areas indicate exons targeted by the NABSA primer sets.*

5.2.1.3. Designing primers and probes for Giardia duodenalis

Primer sets were designed to detect assemblages A, B, and E of the protozoan flagellate *Giardia duodenalis*. These primer sets were based on heat shock protein 70s (Hsp70).

The aim of this work was to detect and distinguish assemblages (A, B and E) of *G. duodenalis* using NASBA with primers targeting Hsp70. Primers and probes for the primer sets "Hsp70 Giardia A" (targeting assemblage A), "Hsp70 Giardia B" (targeting assemblage B) and "Hsp70 Giardia E" (targeting assemblage E) were developed as described in section 5.2.1. Primer and probe sequences are shown in Table B 2 (Appendix B).

The primers and probes were examined *in silica*, and the primers were checked for specificity in PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The primers only matched their own target sequences when cross-checked against the target sequences of the two other primer sets, and checked against the Reference RNA sequences (Refseq mRNA) database.

Probe sequences were checked by blastn against the Refseq mRNA database. Within a cut-off expect value of 1, no other matches than the target were found for Giardia A MB and Giardia B MB. Within this cut-off expect value for Giardia E MB, three matches were found (Two versions of (predicted) *Papilio machaon* (the butterfly "swallowtail") heterogeneous nuclear ribonucleoprotein U-like protein 1 (Query cover 86 %, E-value 0.12) and a (predicted) *Brassica napus* (oilseed rape plant) uncharacterized mRNA (Query cover 82 %, E-value 0.48)).

Secondary conformation of the probes were predicted using the web-based tools developed by Reuter and Mathews (2010) and (Gruber et al., 2008). All probe sequences (Giardia A MB, Giardia B MB, and Giardia E MB) were predicted to fold into the desired stem-and-loop structure.

5.2.2. NASBA reading and detection criteria

For all real-time NASBA tests, the NucliSENS easyQ® Basic Kit (Biomérieux, Lyon, France) was used according to the protocol. Real-time NASBA readings were carried out in a Bio-Tek FLx600 reader with KC4™ Data Analysis software (BioTek Instruments, Inc., Winooski, VT, USA), or in a BioTek Synergy 2 reader with Gen5 software (BioTek® Instruments, Inc.). The samples were kept at 41 °C, and analysed every two minutes for two hours by investigating fluorescent signal. The probes used had the fluorophore FAM (abs 494 nm, em 518 nm) or CAL Fluor Red 610 (abs 590 nm, em 610 nm). For FAM, a 485/20 excitation filter and a 528/20 emission filter were used. For CAL Fluor Red 610, a 575/15 excitation filter and a 620/15 emission filter were used.

The results of NASBA readings were normalised towards the negative control. Samples were interpreted as positive if they fulfilled three criteria: (1) they reached a threshold value 1.2 times the negative control (Landry et al., 2005); (2) they had a sigmoidal curve; and (3) they had an increase in relative fluorescence by a factor of 1.7 or more. A sample was regarded negative if it did not meet all these criteria.

5.2.3. Real-time NASBA for oil-utilizing bacteria

The bacterial strains described in section 5.2.1.1 were grown separately and as a consortium with acetate, heavy crude oil, or both, as the carbon source provided for metabolism. Nucleic acids were isolated using the NucliSens® miniMAG® (Biomérieux, Lyon, France), according to their protocol. To ensure lysis of the gram-positive bacteria, 5 minutes of ultrasonic exposure in a M12 ultrasonic bath (FinnSonic Oy, Lahti, Finland) were included during the lysis step with the NucliSENS Lysis Buffer. Vigorous vortexing after addition of silica beads from the NucliSens® kit also assisted with lysing the cells. Real-time NASBA was run as described in section 5.2.2. The primer sets described in section 5.2.1.1 (Table 4) were used for real-time NASBA assays of the strains. Samples

were collected from all strains grown with only acetate, and with only heavy oil in order to examine the primer sets' ability to distinguish between strains, and whether they could provide information on growth conditions (heavy oil vs acetate).

5.2.4. Real-time NASBA for *Cryptosporidium*

The NASBA primer sets developed as described in section 5.2.1.2 were tested on *C. parvum* oocysts isolated from calf faeces by salt flotation (a detailed protocol is described in Appendix D, section 11.4.1.), and *C. hominis* oocysts provided by the UK *Cryptosporidium* Reference Unit (Singleton Hospital, Swansea, UK). These primer sets targeted the MIC1 transcripts in *C. parvum* and *C. hominis*. Oocysts (100-µl samples) were activated at 37 °C for 30 min, and then 600 µl lysis buffer added and boiled for 1 h. To each sample, 7.5 µl proteinase K (18 mg/ml, Roche, Penzberg, Germany) was added and incubated at 60 °C for 3 h or at 50 °C overnight. Proteinase K was inactivated at 95 °C for 10 min before nucleic acid isolation. RNA from the oocysts was isolated using the NucliSens® miniMAG® (Biomérieux, Lyon, France), according to their protocol, or a modified protocol based on this. In the modified protocol, magnetic silica beads from G-Biosciences (Geno Technology, Inc., USA) were used, new wash buffer 1 (prepared by mixing 70 % ethanol and 30 % NucliSENS lysis buffer), and new wash buffer 2 (prepared by mixing 70 % ethanol and 30 % RNase-free water). Low RNA yield and negative NASBA results were experienced after replacing the silica beads and wash buffers 1 and 2 in the protocol. Investigation experiments showed that the RNA material was washed out during the wash steps of the protocol. The volumes of wash buffer in each wash step were reduced, and the number of wash steps. With these changes to the protocol, the yield was increased and positive NASBA results were again obtained. Further details can be found in Paper 4: Washed away; minimising RNA losses during isolation. Real-time NASBA was run as described in section 5.2.2.

5.2.5. Real-time NASBA for *Giardia duodenalis*

Giardia cysts assemblage B (genotype H3) were ordered from Waterborne Inc. (New Orleans, LA, USA). *Giardia* cysts were also isolated from dog faeces by zinc sulphate

flotation, as outlined in section 5.1.4.1**,** and described in Appendix D**,** section 11.4.2. The cysts were stained with a fluorescein-isothiocyanate (FITC)-labelled monoclonal antibody against the *Giardia* cyst wall (Waterborne Inc., New Orleans, USA), and enumerated by fluorescence microscopy (Leica DM LB, Ortomedic, Oslo, Norway). The cyst concentration in the purified sample was 2.3 x 10^4 cysts ml^{-1}, and the cyst concentration in the genotype H3 isolate was 4.4 x 10^4 cysts ml^{-1}.

Cysts (100-µl samples) were activated at 37 °C for 30 min, and then 600 µl lysis buffer added and boiled or treated at 95 °C for 1 h. To each sample 7.5 µl proteinase K (18 mg/ml, Roche, Penzberg, Germany) was added and incubated at 60 °C for 3 h or at 50 °C overnight. Proteinase K was inactivated at 95 °C for 10 min before nucleic acid isolation. RNA from the cysts was isolated using the NucliSens® miniMAG® (Biomérieux, Lyon, France), according to their protocol, or a modified protocol based on this. Real-time NASBA was run as described in section 5.2.2. Cysts from the infected dogs were analysed with primer sets for assemblages A, B, and E (Hsp70 Giardia A, Hsp70 Giardia B and Hsp70 Giardia E), as described in section 5.2.1.3. *Giardia* cysts of assemblage B (genotype H3) were analysed with the primer sets Hsp70 Giardia A and Hsp70 Giardia B.

5.3. The POCNAD system

In order to function correctly, the cartridge and chip had to be leak-proof. In order to seal the junctions between the chip and the cartridge, different sized O-rings were used. In addition, glue, the silicone paste Chemplex 750 (Otto Olsen, Lillestrøm, Norway), and the grease paste gleitmo 805 sealants (Otto Olsen) were tested as extra sealants on the O-rings.

A graphical user interface developed by CDD Ltd. (Athens, Greece) for the POCNAD instrument was used to control the instrument components.

5.3.1.1. Nucleic acid extraction in the POCNAD

Extraction of nucleic acids was carried out in the extraction chamber in the top part of the POCNAD cartridge, and the magnet in the POCNAD instrument was used to capture the magnetic beads. Reagents from the NucliSens® miniMAG® kit (Biomérieux, Lyon,

France) were used for extraction. *E. coli* cultured in Luria Broth (Miller, Fisher Scientific) were used as sample material in this control test. Washing and elution were carried out manually, using syringes with syringe needles. A detailed protocol is described in Appendix E, section 11.5.1. The heating element for the extraction chamber did not reach the required temperature, and was not used in the elution step of the protocol.
NASBA was run on the eluted nucleic acids using the primers ColNasF1 and ColNasR1, and the probe ColBeac-1 (Heijnen & Medema, 2009). Real-time NASBA on the extracted RNA was run as described in section 5.2.2.

5.3.1.2. Preparation of chips with reagents

NASBA reagents were freeze-dried as spheres for storage in the POCNAD chip. Two types of spheres were prepared: NASBA reagent mix with primers and probes (placed in the pre-NASBA chambers in the chip), and enzyme mix (placed in the NASBA chambers). The primer sets BC-m, Grm neg 16S (Zhao et al., 2009), targeting gram-negative bacteria, and primers PLK1 and PLK2 v1.4 with molecular beacon 16S GrP MB (Zhao et al., 2009), targeting gram-positive bacteria were chosen, and NASBA reagents and enzymes from the NucliSens® Basic kit (Biomérieux, Lyon, France) were used. Spheres were made in both 3-µl and 500-nl volumes, and the 500-nl spheres were placed in the chip. Detailed protocols of the freeze-drying and preparation of POCNAD chips with spheres are described in Appendix E, sections 11.5.2. and 11.5.3.
Chips were prepared with primer/reagent spheres containing BC-m in chambers 1-3; Grm neg 16S (Zhao et al., 2009) in chambers 4-6, and PLK1, PLK2 v1.4 + 16S GrP MB (Zhao et al., 2009) in chambers 7-8. For testing purposes, chips were prepared with primer/reagent spheres in the NASBA reaction chambers, and enzymes were added through the liquid reagent/sample mix. These last chips were used in these test experiments.

5.3.1.3. Assessment of freeze-dried reagents and enzymes

The freeze-dried reagents were tested off-chip with positive RNA samples. A rehydration solution we named Solution 1 was prepared by mixing 100 µl DMSO (90 %), 100 µl sorbitol (2.25 M), and 100 µl KCl (0.42 M). Solution 1 was intended for use and storage in the NASBA reagent container in the POCNAD cartridge.

PCR tubes were prepared with one 3-µl primer/reagent sphere, 5 µl RNA sample and 10 µl Solution 1, or three 500-nl primer/reagent sphere, 2.5 µl RNA sample and 5 µl Solution 1, each in three parallels. The tubes were heated at 65 °C for 2 min. Three 3-µl enzyme spheres were dissolved in 15 µl RNase free water, and 5 µl was transferred to each sample tube prepared with 3-µl primer/reagent spheres. Nine 500-nl enzyme spheres were dissolved in 7.5 µl RNase free water, and 2.5 µl was transferred to each sample tube prepared with 500-nl primer/reagent spheres. Real-time NASBA was run in a Bio-Tek FLx600 reader with KC4^{TM} Data Analysis software (BioTek Instruments, Inc., Winooski, VT, USA).

5.3.1.4. NASBA in the POCNAD

The pre-NASBA and NASBA chambers were filled by dragging the reagents though the chip using a syringe connected to the pump-connection at the bottom part of the cartridge, or by directly injecting the nucleic acid-reagents mix through the broad channel into the chambers using a needled syringe.

During the first run of NASBA in the POCNAD, samples of nucleic acids from oil-utilizing bacteria were extracted using the NucliSens® miniMAG® kit according to the manufacturer's protocol. A nucleic acid sample (40 µl) was mixed with prepared reagent mix with the A-16S or the B-16S primer set described in section 5.2.1.1. (80 µl), and enzyme mix (40 µl) from the NucliSens® Basic kit, and transferred to the chip using a needled syringe. The chip was then placed in a POCNAD cartridge, which was placed in the frame of the POCNAD instrument. The heating element of the instrument was set to 41 °C, and real-time NASBA reading was carried out for 2.5 hours by the POCNAD instrument's optical system. As a control, 20 µl of the same sample was also prepared in a PCR tube and read by a Bio-Tek FLx600 reader with KC4^{TM} Data Analysis software (BioTek Instruments, Inc., Winooski, VT, USA). Data from the .txt file made by the software were processed in Microsoft Excel.

The oil-utilizing bacteria in the consortium were the target organisms for the chip. The primer sets A-16S, BC-16S and BC-m described in section 5.2.1.1, and a general primer set for gram-positive bacteria were used for real-time NASBA in the POCNAD system (only BC-m was freeze-dried onto chips).

6. Results

6.1. The Trilobite® technology

6.1.1. Outcome of proof-of-concept

None of the 10-µm plastic particles were found in the *permeate* (the "clean" fraction) in any of the plastic particle tests. For tests with increasing inlet particle concentrations and a flow rate of 2 ml min^{-1}, the volume of the *permeate* fraction was approximately 64 %, and the *concentrate* fraction was 36 % of the total volume in all tests.

Particle concentration factors were 2.84, 2.55, 2.60, and 2.57, respectively (Table A 2). This was as expected, according to the volumes of the two outlet fractions (Figure 29A, Table A 3). There were slightly fewer particles than expected in the *concentrate*. However, this difference was not significant. Efficiency was 103 %, 92 %, 92 %, and 93%, respectively.

The flow rate had some effect on concentration; higher flow rates lead to higher recovery rates (Figure 29B). Optimal flow rate was 4 ml min^{-1}. The majority of the treated volumes ended up in the *permeate*; the volumes of the *permeate* fraction in the flow rate-tests were 72 %, 76 %, 75 %, and 75 % of the total treated volumes, for 1, 2, 4, and 6 ml min^{-1}, respectively. The concentration factors and efficiencies were 2.85 and 80 %, 3.57 and 86 %, 4.18 and 105 %, and 4.04 and 101 %, for 1, 2, 4 and 6 ml min^{-1}, respectively.

Major deviations from the theoretical outcome would have indicated production errors in the chips. The slightly lower particle concentration than expected in the *concentrate* was probably due to some particle retention in the chip. In some instances, the efficiencies were found to be higher than 100 %. This could have been due to either deviations in the counting method used, or that particles that were left from previous tests and not removed between test washing, were accumulated in the next experiment. Although the chip was rinsed in deionised water with 0.05 % Triton X-100 between each test, some particles might have been retained inside. The greater recovery observed at higher flow rates was probably due to more particles being carried through the whole chip, and not retained inside the chip. Retention of particles within the chip is affected by characteristics of the particles (size, adhesiveness, shape, flexibility), particle

concentration, and flow rate. Although plastic particles are useful for proving concept, they are relatively simple; biological cells possess a range of properties that plastic particles do not have, and may behave differently in the chip. Thus, the limitations of surrogate particles must always be borne in mind.

A larger area of trilobite units in a chip would increase the concentration factor, as a higher number of units would increase the amount of water ending up in the *permeate*.

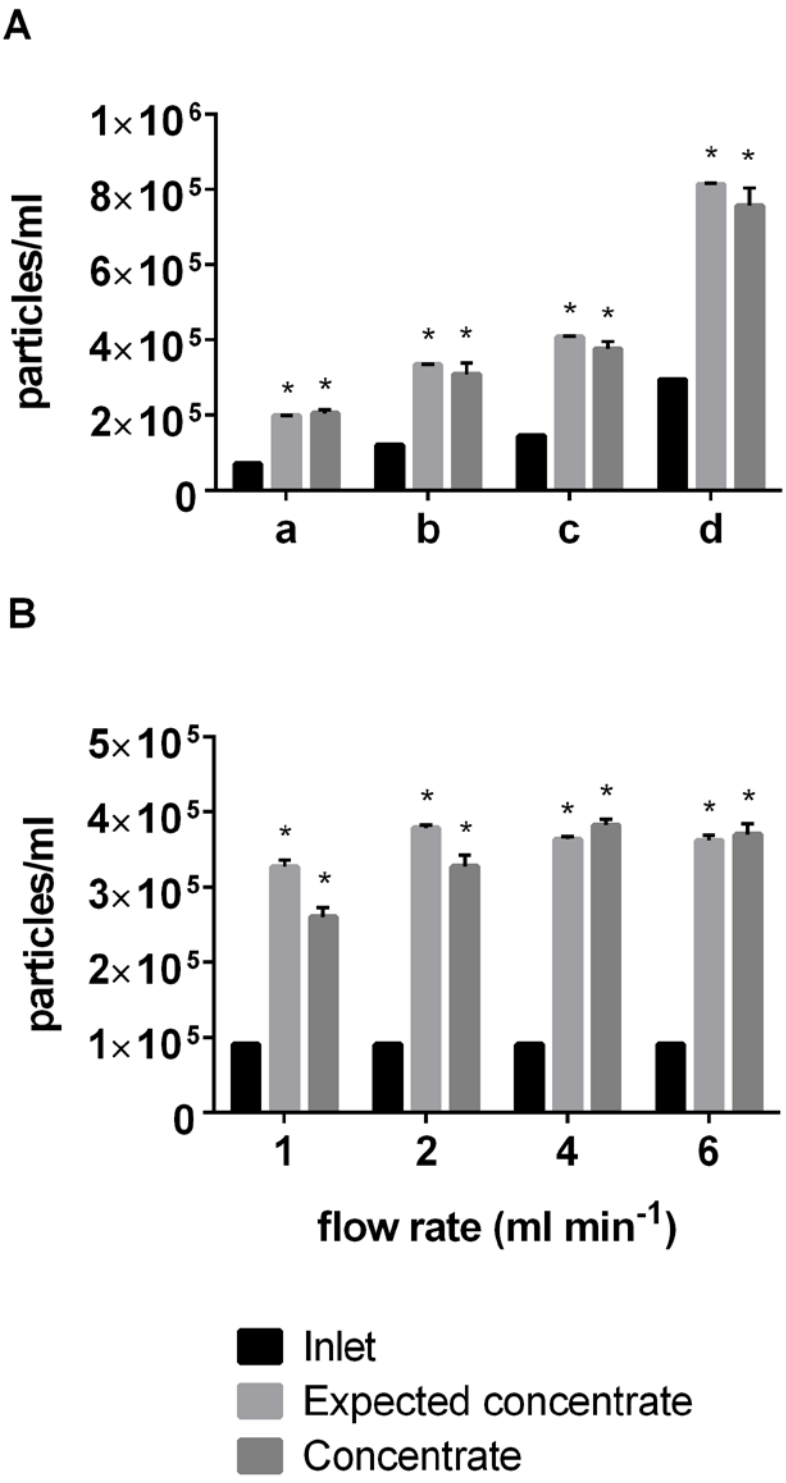

Figure 29 Particle concentrations of 10-µm plastic particles before (inlet) and after (concentrate) chip treatment, with various inlet concentrations (A) (71 615 (a); 120 443 (b); 144 531 (c); and 294 054 (d) particles ml^{-1}, respectively) and flow rates (B) (1; 2; 4; and 6 ml min^{-1}, respectively). Further details can be found in Tables A2 and A3. Standard errors of means (SEM) for concentrate and permeate are indicated. Asterisks indicate significant differences from inlet concentration.

6.1.2. Chip dewatering of *Rhodomonas baltica* suspensions

This work is described in detail in Paper 1: Continuous harvesting of microalgae by new microfluidic technology for particle separation, published in Bioresearch Technology. The following sections describe the main results, and some additional results that were not included in the article.

Fixation experiments of *R. baltica* indicated that methanol (PreTect TM) was best suited as fixative. Ethanol, isopropanol, and acetone all caused the algal cells to shrink and cell content to leak out, this created cell clusters (Figure 30A-C). Fixation with PreTect TM resulted in spherical cells (Figure 30D). The cell content did not leak, and there was little shrinking. The cells were also distributed evenly, not in clusters.

As expected, living *R. baltica* that were run through the Trilobite® chip ended up in both the *concentrate* and the *permeate* fractions (Figure 31A, Table A 4), indicating that the flexible cells had been deformed under pressure such that some squeezed through the gaps of the units in the chip. There was no significant difference in cell concentration between the two out-fractions. Fixed *R. baltica* was also treated through the chip; these cells were more rigid and spherical, but also dead. The *permeate* fraction of the fixed *R. baltica* contained significantly fewer cells than the *concentrate* fraction (Figure 31B).
The fixation increased the efficiency from 30 % to 59 %. No clogging of the chip was observed with this alga.

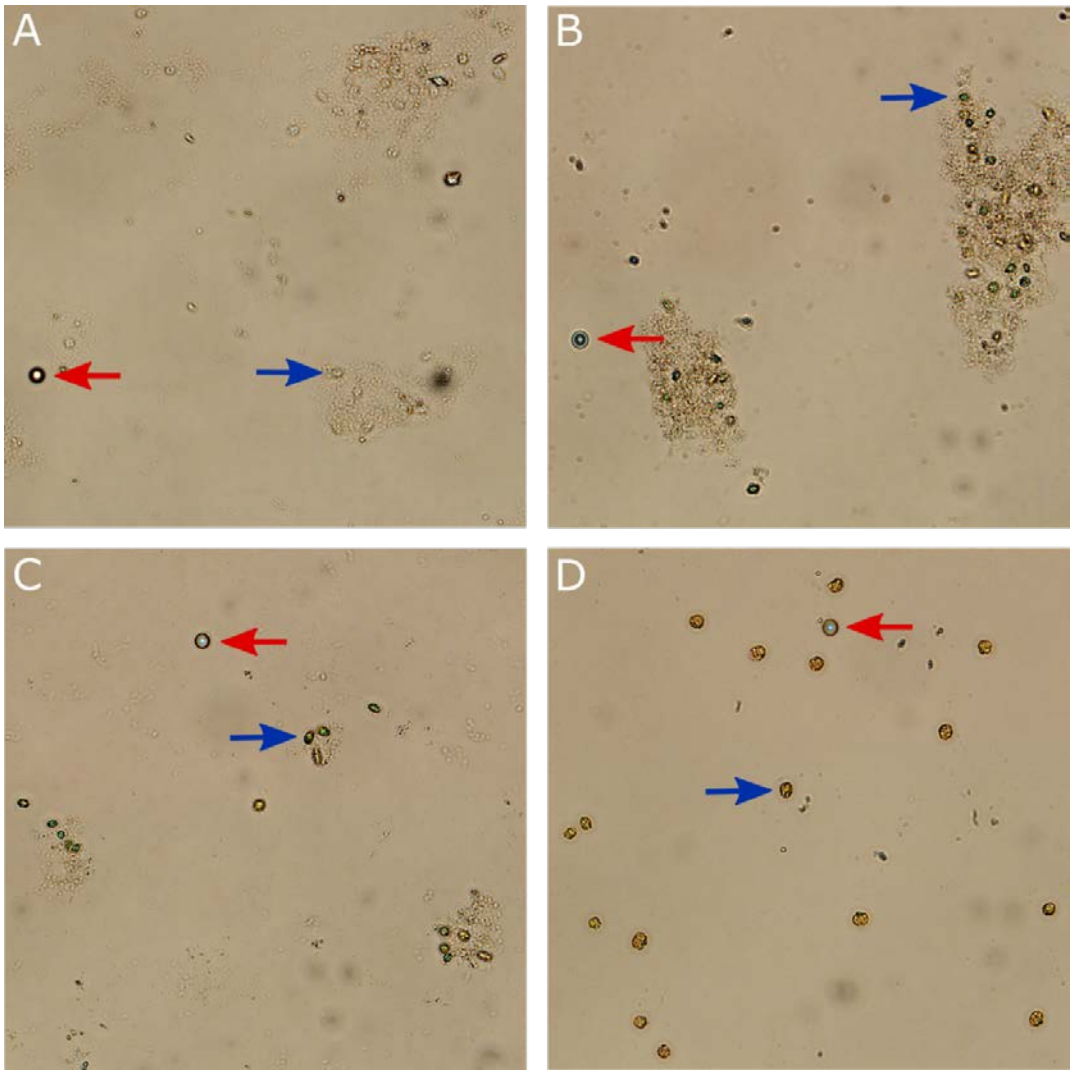

Figure 30 Fixation of R. baltica *with isopropanol (A), ethanol (B), acetone (C) and the methanol-based PreTect TM. Red arrows indicate 10-µm plastic beads, blue arrows indicate algal cells.*

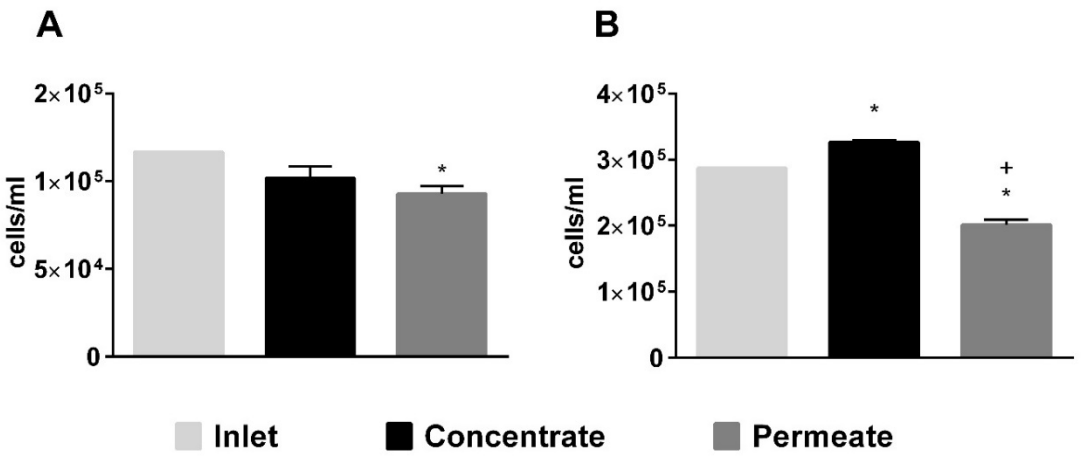

Figure 31 Cell concentrations of R. baltica *in the inlet and the two outlet fractions of the chip for living (A) and fixed (B) cells. Standard errors of means (SEM) for concentrate and permeate are indicated. Asterisks indicate significant differences from inlet concentration; plus signs indicate significant differences from concentrate concentration.*

6.1.3. Chip dewatering of *Thalassiosira weissflogii* suspensions

Unlike *R. baltica*, *Thalassiosira weissflogii* is a rigid diatom, the cells are normally 10-20 µm. Only one cell (8.6 µm in diameter) was found in the *permeate* in all four tests combined when the *T. weissflogii* suspension was concentrated through the chip. This cell might have slipped through the gaps, or it could have been transferred from the outlet tubes as a contaminant. Most cells were found in the *concentrate*; the efficiency was on average 94 %. There was a significant difference in the concentration of cells between inlet and expected *concentrate*, but not between the inlet and actual *concentrate* (Figure 32).

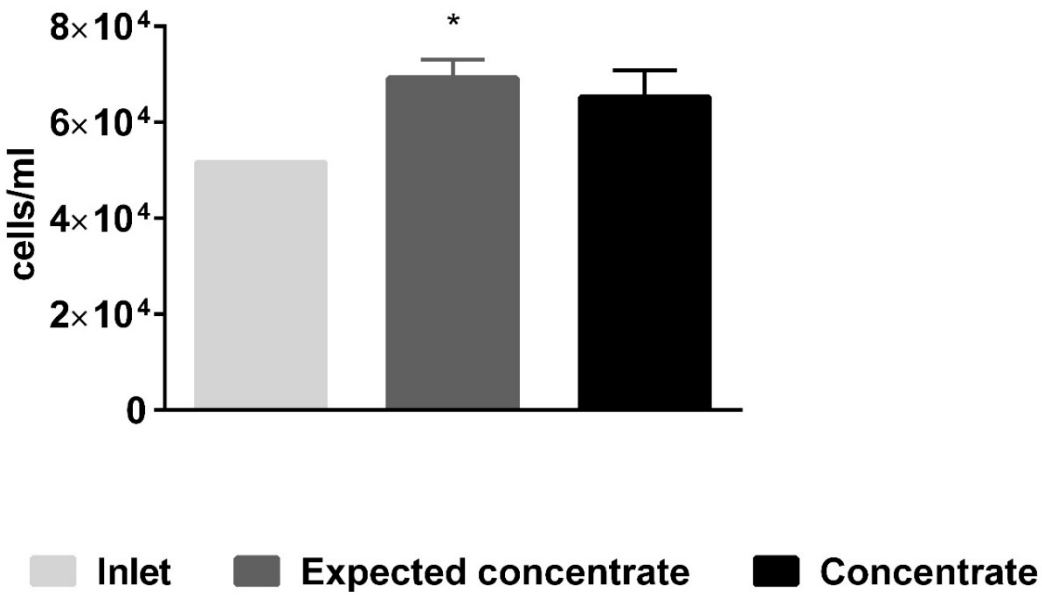

Figure 32 Cell concentrations in suspensions of Thalassiosira weissflogii*, before (inlet) and after (concentrate) chip treatment, and expected cell concentration in the concentrate. SEM for concentrate and expected concentrate are indicated. Asterisks indicate significant differences from inlet concentration.*

Most of the total volume ended up in the *concentrate* fraction, making the dewatering capacity in the chip very low. This was probably due to gelatinous matrix produced by *T. weissflogii* accumulating around the units and clogging the chip (Figure 33).

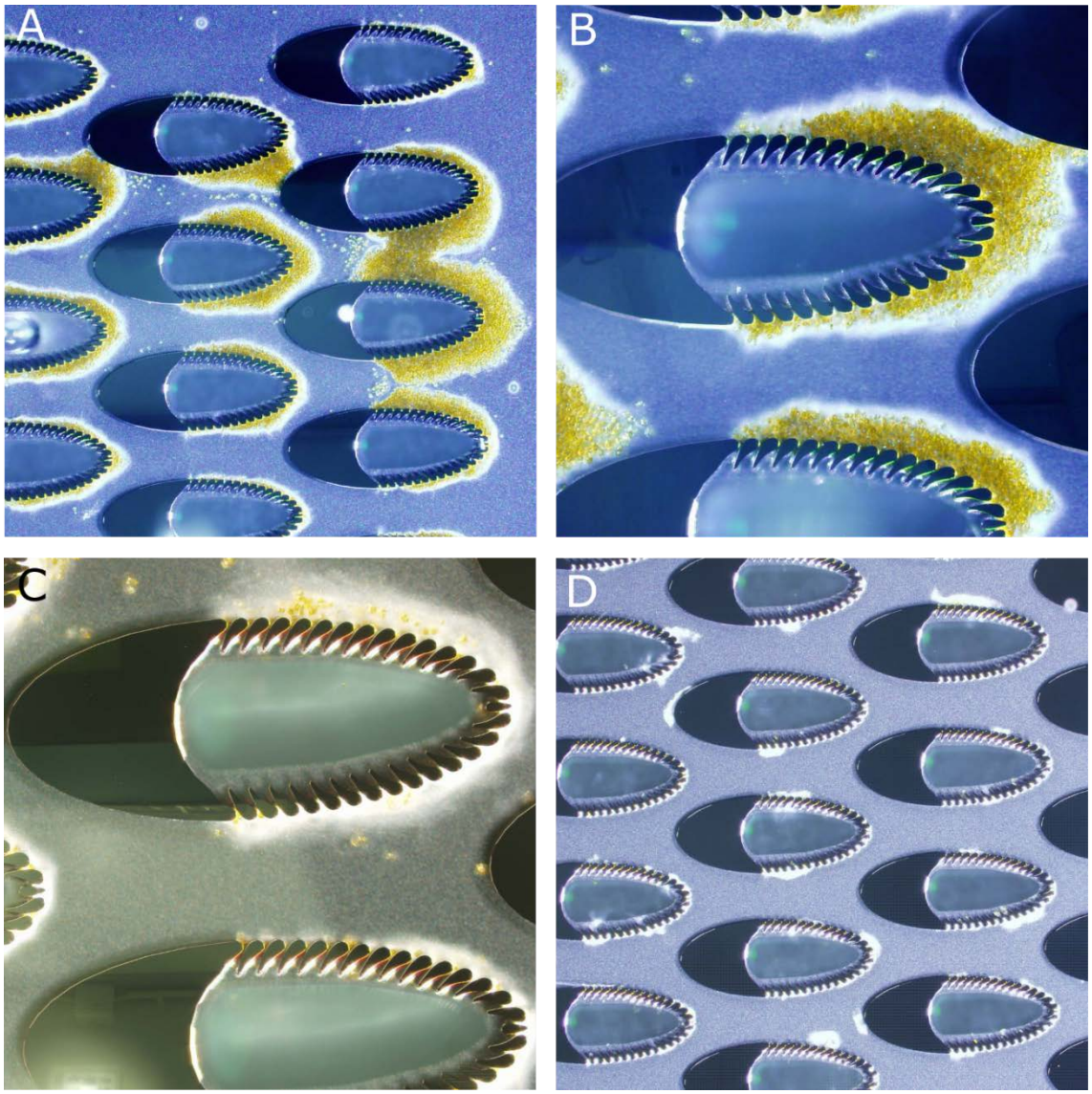

Figure 33 T. weissflogii *cells gathered around the units in the chip (A and B). Extracellular polymers remained after most of the algal cells had been washed out (C), and after further washing, the chip still had polymer residues (D).*

6.1.4. Chip dewatering of *Chaetoceros* sp. suspensions

The diatom cells *Chaetoceros* sp. are about 5 µm, and slightly smaller than *R. baltica*, but rigid. These cells were found in both outlet fractions, however, there were significantly higher cell concentrations in the *concentrate* fraction than in the *permeate* fraction and the inlet (Figure 34A). The efficiency was, on average, 58 %, and there was no observable difference between tests with living versus fixed *Chaetoceros*. Clogging of the chip was not observed.

Cell sizes in the two outlet fractions were measured, and the percentage share of large cells was higher in the *concentrate* than in the *permeate*, especially from 6-7 µm and

above. The percentage share of smaller cells, up to 4-5 µm, was higher in the *permeate* than in the *concentrate* (Figure 34B, details in Table A 5). The same pattern was observed for fixed *Chaetoceros* sp. (Table A 6).

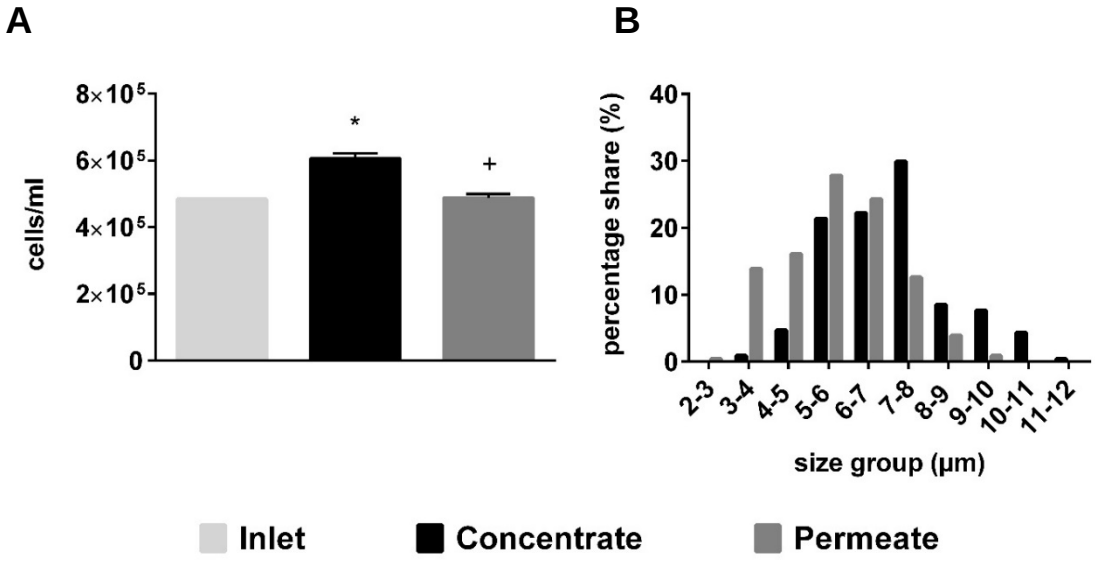

Figure 34 A) Cell concentrations in suspensions of Chaetoceros *sp., before (inlet) and after (*concentrate *and* permeate*) chip treatment. SEM for* concentrate *and* permeate *are indicated. Asterisks indicate significant differences from inlet concentration; plus signs indicate significant differences from* concentrate *concentration. B) Cell size distribution of living* Chaetoceros *sp. in* concentrate *and* permeate *fractions.*

6.1.5. Chip concentration of *Giardia* cysts

Data from counted cysts in the outlet fractions were not encouraging; very few cysts were found. Most cysts seemed to have become lost during the treatments, and could not be recovered at either outlet fraction. Salt crystals within the chip clogged the system and increased the pressure so the chip broke (Figure 35). This salt precipitation was unexpected; the cysts had been washed several times to remove any zinc sulphate residues from the cyst isolation protocol.

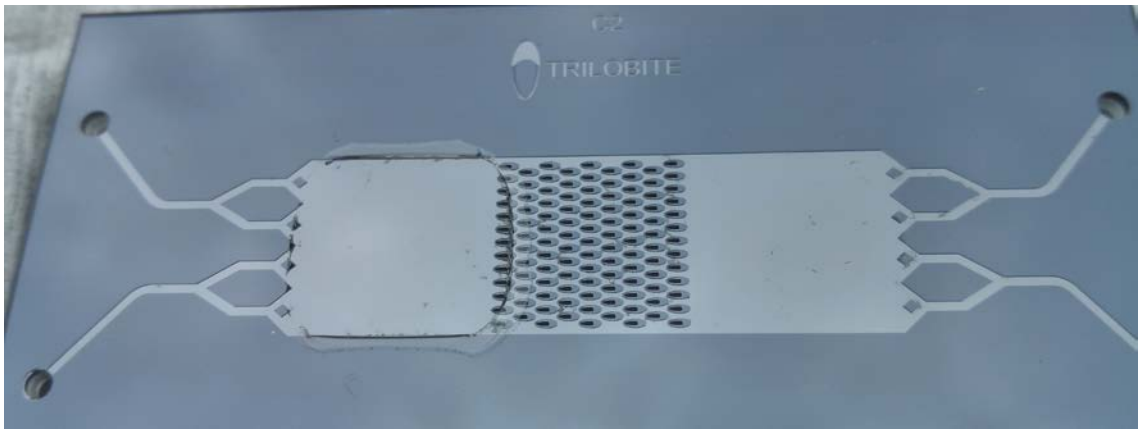

Figure 35 Broken chip due to increased pressure by accumulated salt precipitation

6.1.6. Chip concentration of *Toxoplasma* oocysts

After running the *Toxoplama* oocyst suspensions for two rounds through chip, the sample volume was reduced to 15 % of the original 50 ml-volume (by a factor of 7) to 7.5 ml. According to the results, the oocyst recovery was quite low (Figure 36 and Figure 37). The average of recoveries from 500 oocysts were 120, 181, and 360 oocysts (24 %, 36 %, and 72 %) for chip treatment, membrane filtration, and the control, respectively. The average recovery for the same treatment of 1000 oocysts were 427, 445 and 560 oocysts (43 %, 45 %, and 56 %), respectively (Table A 7). No oocysts were found in any of the eluate samples, as expected, and no oocysts were observed within the chip when the chip was checked under a microscope after treatments.

There was no significant difference between the treatments (chip treatment and membrane filtration) and the control when 1000 oocyst were tested. When 500 oocysts were tested, the chip treatment resulted in significantly lower oocyst recovery than the control. There was no significant difference between the chip treatment and the membrane-filtered samples. Large variation within the results of all treatments made it difficult to make any conclusions on the efficiency of the chip for concentration of *Toxoplasma* oocysts. Nonetheless, the oocyst recovery appeared to be lower in the chip treated samples.

Visible bubble formation occurred in the larger tubing.

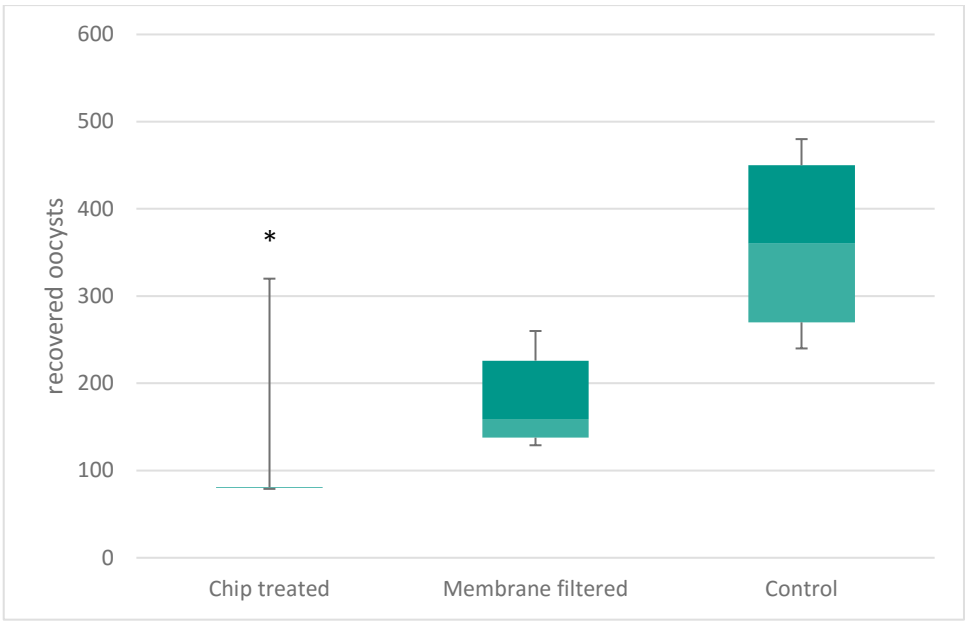

Figure 36 Box plot of recovered Toxoplasma *oocysts after chip treatment and membrane filtration of 500 oocysts. The controls were 50-ml oocysts suspensions reduced to 1 ml by centrifugation. Significant difference from the control is indicated by an asterisk.*

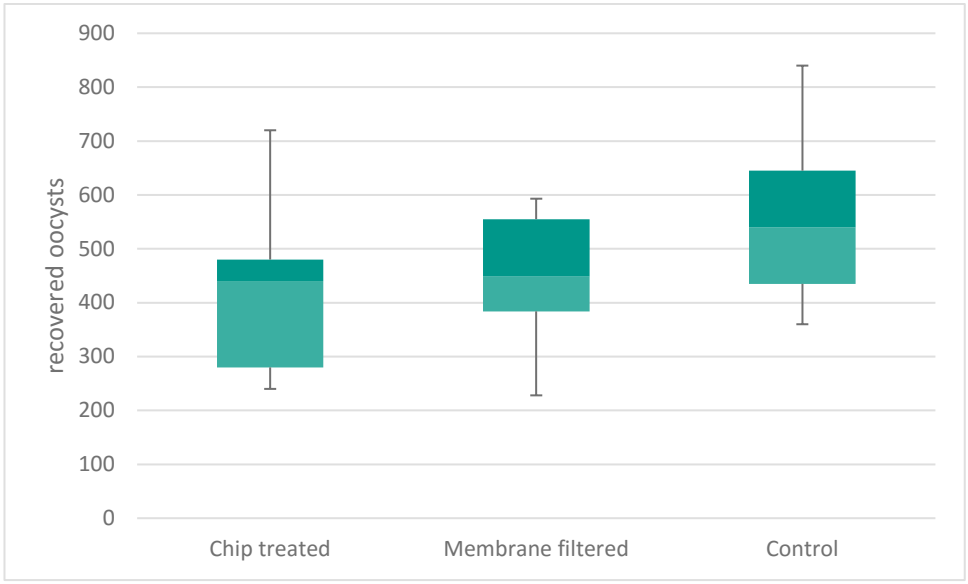

Figure 37 Box plot of recovered Toxoplasma *oocysts after chip treatment and membrane filtration of 1000 oocysts. The controls were 50-ml oocysts suspensions reduced to 1 ml by centrifugation. No significant difference between the treatments and the control were found.*

6.2. NASBA

6.2.1. Real-time NASBA for oil-utilizing bacteria

The four oil utilizing bacterial strains (sections 2.2.1 and 5.2.1.1) were grown given heavy oil or acetate as carbon source. All strains grew readily when acetate was provided as the sole carbon source, or as co-substrate together with heavy oil. When heavy oil was provided as sole carbon source, the strongest growth was observed for strain A and for the consortium. More details on growth of these bacteria are described in Appendix C, section 11.3.1.

Primer set A-m was not able to detect its target, transcripts of gene *m* from strain A, and was omitted from further work. Both primer sets A-16S and BC-16S detected all four strains. Strains A and D were not detected using the primer sets BC-n and BC-o, which could be used to distinguish strains B and C from the other strains (Table 5). Strain A could be distinguished from the other strains by using the A-n and A-o primer sets, as only strain A could be detected with these primers. BC-16S appeared to be contaminated, as positive results were also obtained with the negative control. In order to detect strain D, a unique pattern that differed from the results of the other primers was necessary. However, this was not achieved during the duration of this project.

Table 5 Outcome of detection by real-time NASBA for each strain, with the working primer sets used in this study. "+" indicates detection of the target; and "-" indicates a negative result.

strain / *primer set*	A	B	C	D
A-n	+	-	-	-
BC-n	-	+	+	-
A-o	+	-	-	-
BC-o	-	+	+	-
BC-m	-	+	+	-
A-16S	+	+	+	+

No differences were found between the results for acetate-grown and heavy oil-grown cultures. Therefore, the primer sets based on the selected genes did not provide any information on carbon metabolism.

The primer sets A-16S, BC-16S, and BC-m were chosen for NASBA testing in the LOC system POCNAD (Chapter 4.3).

6.2.2. Real-time NASBA for *Cryptosporidium*

Real-time NASBA was run on RNA isolated from *C. parvum* and *C. hominis* oocysts with the primer sets described in section 5.2.1.2. As expected, the C.hom MIC1 primer set was successful in detecting both species of *Cryptosporidium*, whereas the C.par MIC1 primer set detected only *C. parvum*, also as expected. Thus, these primer sets could be used to distinguish *C. parvum* oocysts from *C. hominis* oocysts. However, the assay did not seem not to be suitable for using as a viability assay, as transcripts from inactivated oocysts could also be detected. Positive NASBA results were obtained from the nucleic acids isolated from oocysts after both heat inactivation at 70 °C, and also after the freeze-thaw protocol. Even oocysts that had been boiled prior to RNA isolation produced positive NASBA results.

Further details of the work is described in Paper 2: Real-time Nucleic Acid Sequence-based Amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts.

6.2.3. Real-time NASBA for *Giardia duodenalis*

Real-time NASBA was run on RNA isolated from *Giardia* cysts with selected primer sets described in section 5.2.1.3. The cysts that had been isolated from the dog sample were negative for all primer sets (Giardia A, Giardia B and Giardia E).

Samples of *Giardia* assemblage B were also negative when analysed by NASBA with the primer sets Hsp70 Giardia A and Hsp70 Giardia B.

Within the time constraints of the project, we were not able to obtain suitable *G. duodenalis* genotypes for further thorough testing of these NASBA primer sets.

6.3. The POCNAD system

Major challenges were soon identified regarding the suitability of the POCNAD instrument's pump. The main problem was sealing the junctions with the cartridge, as air leakages made it impossible to create the pressure required to move the liquid through the micro channels. Thus, it was apparent that the pump was too weak to pump the liquid through the system. The pump would need to drain the air from the waste container in order to create the initial negative pressure. This was rarely achieved during tests, and the few times it was achieved, it took too long. The pump was dimensioned to pump liquid though micro channels in the chip, but not the large volumes, as in the waste container. The cartridge was connected to another pump with higher capacity to pump fluid through the chip, or manually by a syringe. The drawback of using a syringe pump was that there was no control over the valves, and, hence, no control over the flow.

The pin actuators that controlled the valves were also difficult to optimise. On some occasions these could push down too hard onto the valve, but also in other instances did not push hard enough.

Leaking was a major problem with the first batch of cartridges. These had to be glued in at several places to obtain leak-free cartridge systems. In the second batch, most cartridges were leak-free, and could be used for testing. However, when the chip was placed inside the cartridge, it bent upwards at one end. In order to seal the junction between the cartridge and the chip, different sizes of O-rings were used. However, using O-rings alone was not sufficient to achieve a completely sealed cartridge system. When the O-rings were glued on with super glue, the glue seemed to make the rubber in the O-rings more brittle and less elastic, and the O-rings were often placed in lopsided positions. This led to even more leakage. When silicone paste was used to seal the O-rings, they were still flexible, and sealed cartridge systems were achieved. Silicone paste was used for sealing throughout the rest of the tests.

When cartridges were stored for up to a week, they often leaked overnight, or the reagents had run through the chip (by capillary forces) down into the waste container.

6.3.1. Nucleic acid extraction in the POCNAD

Nucleic acids were successfully extracted in the POCNAD cartridge's extraction chamber when using the instrument's magnet to capture the magnetic silica beads. The magnet held the silica particles in place when required. However, the extraction could only be carried out manually using a syringe with a syringe needle to reach into the extraction chamber. Due to the reasons outlined in the previous section, it was not possible to pump reagents from their respective containers, and therefore the extraction process could not be achieved through automatic control using only the POCNAD instrument and the associated software.

In Paper 5: Detection of oil-utilizing microorganisms by nucleic acid sequence-based amplification in a total analysis lab-on-a-chip device, these preliminary extraction results, and the POCNAD system were described.

6.3.2. Assessment of freeze-dried reagents and enzymes

The freeze-dried primer/reagent- and enzyme spheres were tested by using them for real-time NASBA assays in a microplate reader. The 3-µl primer/reagent spheres were compact and sticky, and did not seem to have been freeze-dried successfully. Negative NASBA results that were obtained when using these spheres confirmed that they had not been prepared successfully. The 500-nl spheres gave positive NASBA results, and could be used in the chip.

6.3.3. NASBA in the POCNAD

In the first test of NASBA in the POCNAD chip, the sample mix was injected directly into the chip using needle and syringe. After the NASBA analysis was completed, the contents had leaked out of nearly all the reaction chambers. The control NASBA confirmed that the sample was positive (Figure 38), and in reaction chambers 1-3 detectable fluorescence indicated that amplification had occurred (Figure 39). However, the signal was very scattered, probably due to issues regarding the POCNAD instrument and software/GUI, or due to bubbles and decreased content in the reaction chambers. All subsequent NASBA runs also had this type of noise.

The temperature of the heating element was too high after the NASBA run. Although the temperature was set to 41 °C, a manual check with an external thermometer indicated a temperature of 44 °C, and the GUI showed a temperature of 48 °C.

The background noise in the system was relatively high, and could interfere with interpretation of the results.

When the NASBA chambers were filled by using a syringe to transfer the reagents from the NASBA reagents, then the chambers were completely filled. The NASBA results from the readings obtained using this approach indicated positive results, but the instrument's readings seemed to remain disturbed (Figure 40).

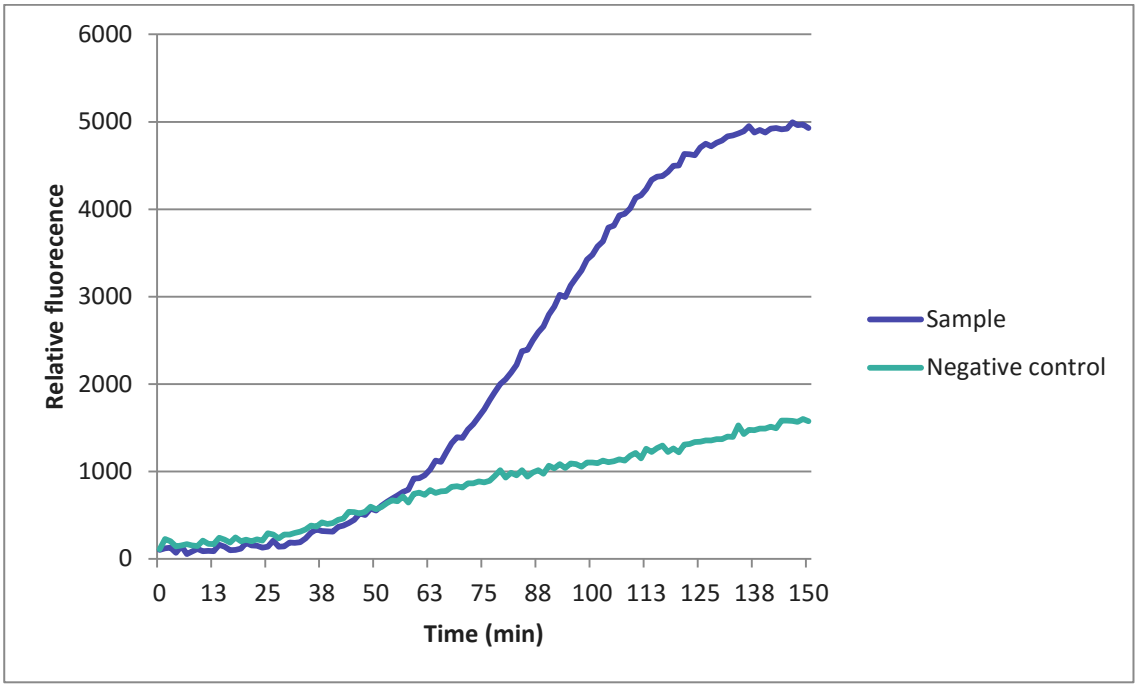

Figure 38 Control sample run as conventional real-time NASBA in the lab. Nucleic acids were extracted from strain A, and real-time NASBA was run with primer set A-16S

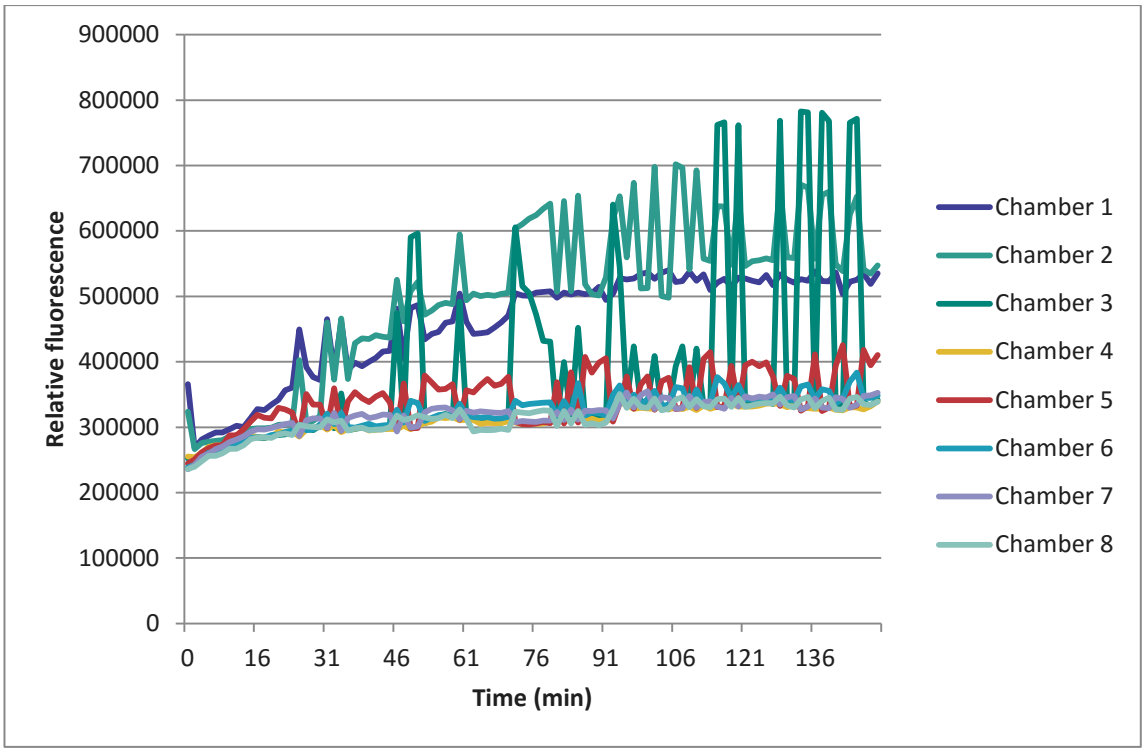

Figure 39 First real-time NASBA run in the POCNAD system. Nucleic acids were extracted from strain A, and real-time NASBA was run with primer set A-16S

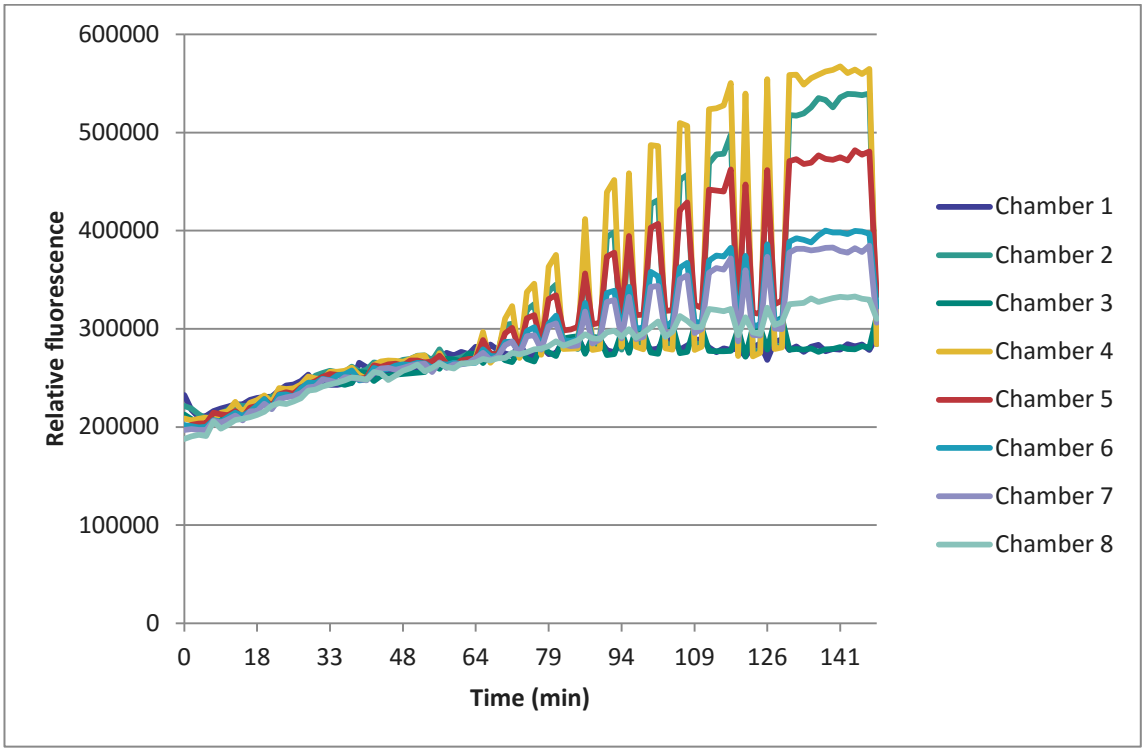

Figure 40 Scattered real-time NASBA readings in the POCNAD

Controlling the liquid flow inside the chip was very difficult. Even when the pump was stopped, the remaining negative pressure in the bottom part of the cartridge could result in the contents of the reaction chambers being sucked out. Bubble formation also occurred repeatedly in the reaction chambers during the NASBA reaction (Figure 41).

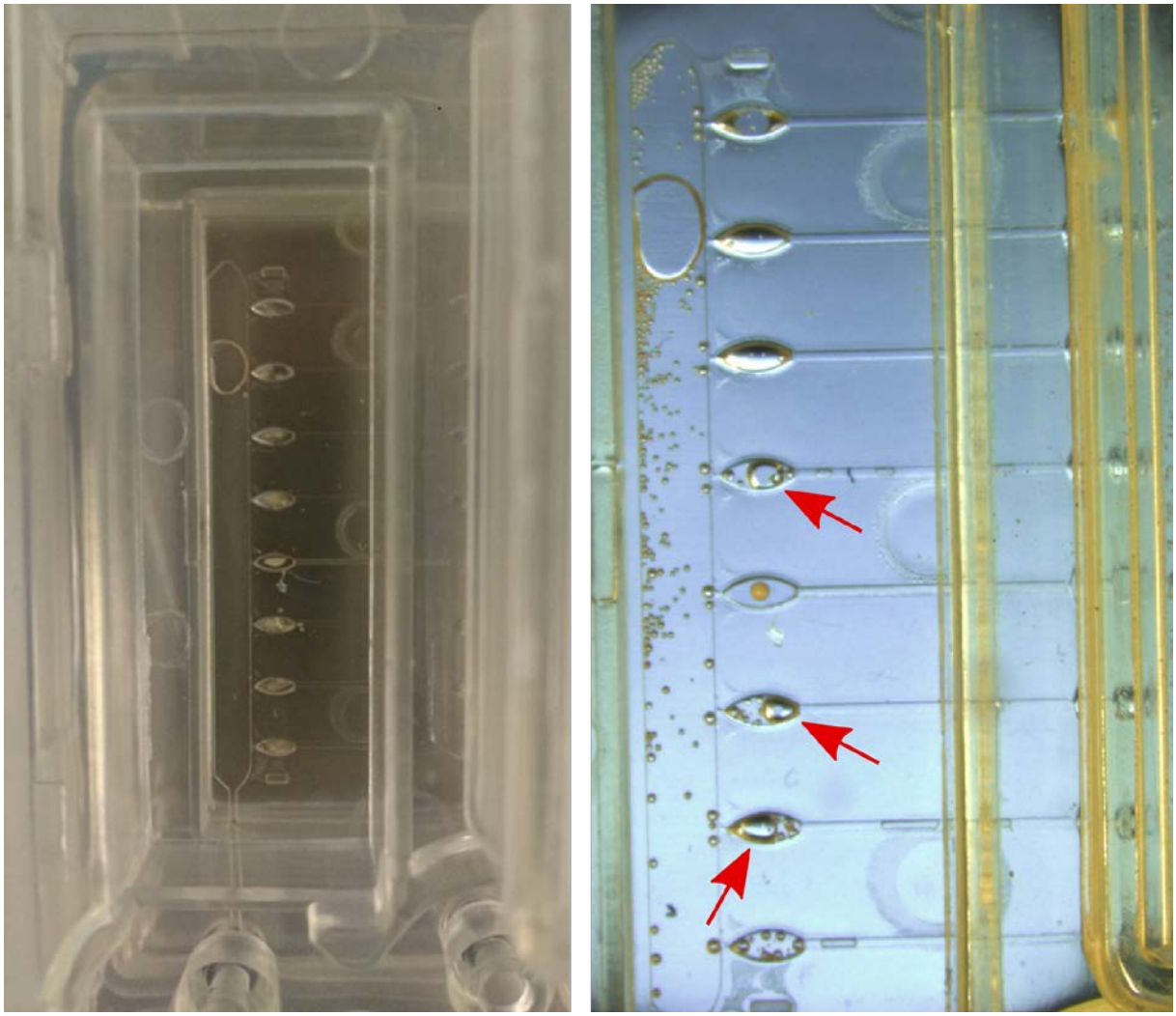

Figure 41 Bubble formation in NASBA chambers

The chips with prepared freeze-dried primers and probes inside the NASBA chambers were also tested. In the last two of these chips, using nucleic acids from the bacteria strain B, one of three chambers with BC-m primer set showed a clear positive result (Figure 42). However, in most other runs, the results were unclear and it was not possible to interpret the signal obtained. This was either because of bubble formation and scattering of the fluorescence signals, or due to high background noise. The ends of the fibre optic cables

were polished to decrease background noise, which seemed to improve the signal clarity, but not all the noise and signal scattering could be removed. Although the bacteria being used in the system were gram positive, the primer sets targeting gram-negative bacteria (Grm neg 16S by Zhao et al. (2009)) also provided positive NASBA results.

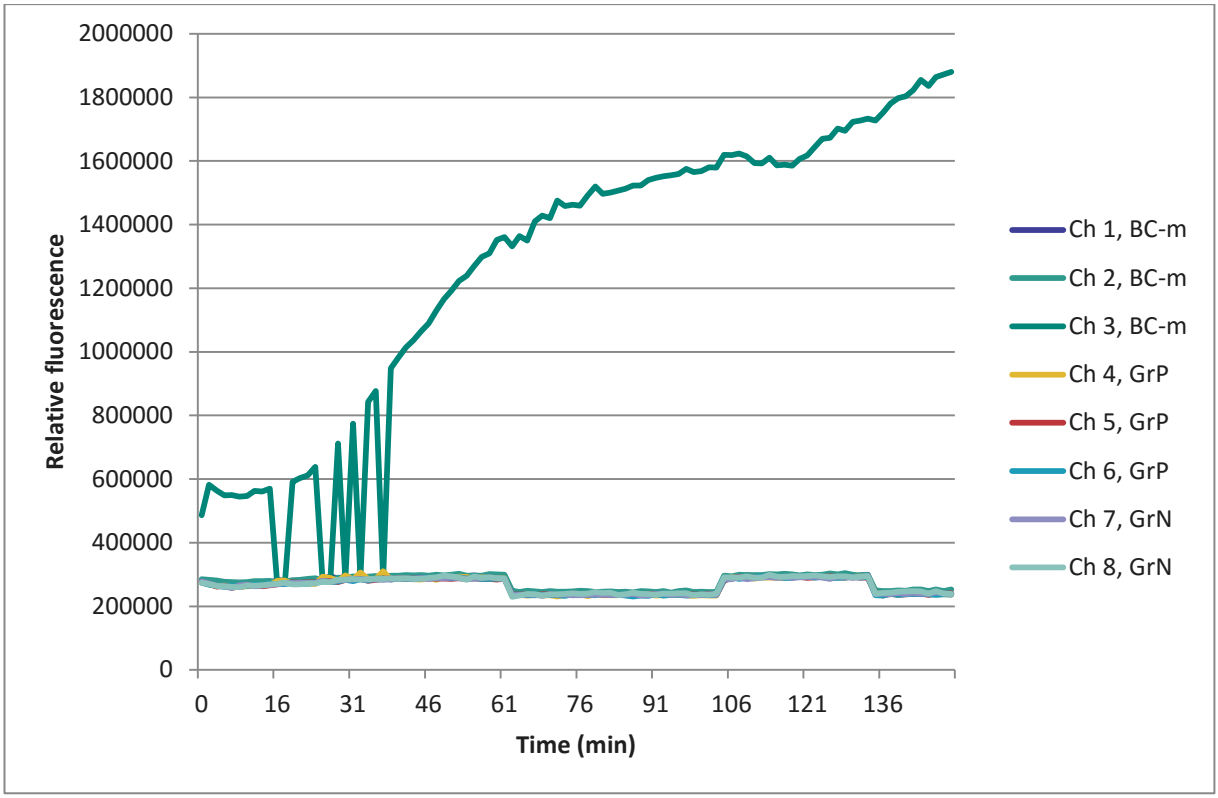

Figure 42 Real-time NASBA in the POCNAD, using a chip with primers and probes stored as freeze-dried spheres in the NASBA chambers. Chambers 7 and 8 contained a different fluorophore than chambers 1-6, and should emit no signal.

7. Discussion

7.1. Application of Trilobite® chip for biological purposes

The Trilobite® chip with 5 µm gap size was tested for concentration and separation applications towards biological particles. Over all, from a theoretical perspective, this technology should be able to offer some significant advantages, such as being able to separate particles based on size. This is obvious benefit when the intention is to separate different life stages, especially in aquaculture, where large ready-to-harvest organisms can be separated from those that have not yet reached that stage and size.

However, the experiments also demonstrated some limitations and challenges that may be encountered when working with biological particles. Most cells are not rigid structures, and flexible cells may slip through the gaps in the chip's units. It is therefore necessary to specify a gap that is small enough to minimize cells being in the *permeate*. This is also important if the chip is to be used for separation of red and white blood cells, as these are very flexible. This is a subject for further study.

Production of extracellular polymers may, as was clearly demonstrated by *T. weissflogii*, lead to clogging of the system, which greatly reduced the concentration efficiency of the chip. Different species of algae have very different properties, such as size, shape, rigidity, the ability to self-flocculate, or repel other algal cells. When choosing a suitable harvesting method for a particular alga, these properties must be taken into account.

The fluid matrix might also have a greater impact than predicted on the chip performance. For example, some salts might precipitate inside the chip, leading to clogging, increased pressure, and even breakage of the chip itself. The complex structures within the chip might also make it difficult to clean. Salt precipitation was an issue in the experiments for concentration of *Giardia* cysts, to the extent that the chip was clogged and broke. There is no obvious explanation regarding what could have caused this excessive crystallization. The zinc sulphate used for isolation of cysts and addition of antifoam A were the only factors differing from the other chip experiments. The antifoam A seemed unlikely to cause crystallization. We might speculate that increased pressuring

within the chip, or an unknown chemical reaction with the chip material, might have caused crystallization of any residue zinc sulphate.

Our experiments did not give any positive indications regarding the chip's potential with regard to efficient concentration of *Giardia* cysts. Although, experiments with previous versions of the chip indicated very high recovery efficiencies that suggested that the chip could be a feasible approach for recovering *C. parvum* and *Giardia* (oo)cysts, and as a pre-filter to improve recovery of *Cryptosporidium* oocysts from turbid water samples ((Pires & Dong, 2014; Pires & Dong, 2013), it appears that it has not been possible to replicate these experiments and obtain equivalent results. The company associated with making the Trilobite chip does not have good information on how these original experiments were conducted nor how the original high results that are reported were achieved (Eirik, pers. comm.).

In the experiments where the chip was investigated for concentration of *Toxoplasma* oocysts, the oocyst recovery was very low. Oocysts might have been lost in the tubes leading in and out of the chip system. Although efforts were made to minimize points where oocysts might become trapped, they might have accumulated at the junctions connecting the micro-tubing to the tubing through the pump. Micro-tubing is also easily blocked by particles, and these were regularly rinsed throughout the experiments. There was also the risk of leakage at these connection points, which could lead to oocyst loss. Although leakage was minimized by using hydrophobic silicone paste to seal the junctions, the oocysts could also have adhered to the silicone paste.

Bubble formation also occurred, particularly in the larger tubing. Oocysts might adhere to the bubble surfaces, leading to oocyst losses in the outlet of the system.

However, as recovery of oocysts in the control was also low, it seems that a major source of error is probably associated with the centrifugation step, where the volume was further reduced prior to oocyst counting.

Thus, it seems that the experimental design was not optimal for investigation oocyst recovery, and the actual chip treatments may not have been a major source of oocyst losses.

Due to the capacity of the peristaltic pump (approximately 3-6 ml min^{-1}) and the handling volume of one chip, the volume of the samples concentrated using the Trilobite® chip was lower than desired (0.05 L). The volume of the samples concentrated by membrane filtration was 10 L. The two methods were not directly comparable, as a smaller initial volume should result in a higher recovery efficiency. Nevertheless, compared with other microfluidic systems, 0.05 L may be considered to be relatively high. For the chip treated samples, the volumes were reduced from 50 ml to 7.5 ml by running it twice through the chip in ~10 min (a volume reduction of ~3/5 in each round). The recovery rate for 1000 oocysts was 43 %. In an inertial microfluidic separation system, Ganz et al. (2015) reduced 10 ml suspensions of *Giardia duodenalis* cysts to less than 1 ml in ~30 min by running the sample through the unit 6 times. In this system, they concentrated and detected *G. duodenalis* cysts with a recovery rate of 68.4 % for 1000 oocysts in 10 ml.

A microfluidic device to trap *Cryptosporidium* oocysts and *Giardia* cysts, and detect these with fluorescent probes/antibodies was developed by Zhu et al. (2004). The flow rate through the device was 20 µl min^{-1}, which is much lower than used in our experiments. The inlet concentrations were 1-5 x 10^3 cells in 1-5 µl fluid, and cells were trapped against a weir. They were able to capture *Giardia* at 3-4 µm gap size, and almost no *Cryptosporidium*; whereas 1-2 µm gap captured both parasites, with no parasites in the outlet.

The differences in volume of the chip treated versus the membrane filtered samples was due to the chip size as well as the pump capacity. The chip as it is now is much too small for meaningful water analysis. In the future, it might be possible to upscale the process by making larger chips, or putting several chips together into a system. In such a situation, it would be able to handle larger volumes of water.

This experiment would be improved if a smaller membrane filtration system and smaller sample volumes were used for comparison. For the chip, a pump with higher capacity would increase the treated sample volume without needing to put in an extra tube with larger diameter through the peristaltic pump. A higher-capacity pump could maintain high flow-rate even in narrower tubing, decreasing the risk of leakage and sample loss. These modifications would give better comparison of the two different methods, filtration and chip treatment.

The volume capacity of the chip that was used in these experiments is currently rather small, and the flow rate is only up to about 5-6 ml min^{-1}. Although this is relatively high compared with some other available microfluidic devices, it could not compete with the volumes handled the conventional membrane filtration (as the filtration used for comparison in the *Toxoplasma* experiments). By assembling several chips into one filtration system, volume capacities may be increased, however it is important to overcome problems at the small scale, before scaling up.

Based on our results, as a concentration device in which some losses of target are not critical (such as for concentration of microalgae), the Trilobite® chip has clear potential. However, for processes or systems when it is important that losses are minimized to very low levels (such as for water purification or detection of pathogens in water), the Trilobite® chip has considerable challenges to be overcome.

Depending on the characteristics of the biological particles used, the Trilobite® chip did not always prevent clogging as had been hoped, with the polymer-producing *T. weissflogii* especially causing clogging. According to a recent experimental characterisation of the microfluidic principles around the separation unit, the unit may work both as a dead-end filter or as a crossflow filter (Mossige et al., 2016). This relies on the pressure drop, flow-rate and Reynolds number, and, for sufficiently high flow rates, it should be possible to tune the system such that it is dominated by hydrodynamic forces, and the separation unit functions as a non-clogging cross-flow filter.

In the experiments in this project, only relatively simple equipment was available. However, with access to equipment to control and measure pressure differences in the inlets and outlets, it will probably be possible to fine-tune the chip for non-clogging separation of different particles (and with varying characteristics). There might be a need to fine-tune the chip towards each type of particle, and according to gap size and chip size. However, with better control of pressure drop, this can be achieved for the particles in question.

With this in mind, there are good prospects for developing better non-clogging Trilobite® systems in the future.

The Trilobite® chip could also be fitted for micro-systems, by directly incorporating the chip *concentrate* outlet into a lab-on-a-chip device (Chapter 4.3). If a major reduction in fluid is needed, several chips can be connected in a series, with a stepwise reduction in the volume as the sample goes through each chip. Alternatively, the fluid can be run through the chip several times. This would also enable a portable system for field work.

7.2. Potential of NASBA in microanalysis systems

Of the oil-utilizing bacterial strains that were analysed by NASBA, not all could be distinguished from one another by the assay that was developed in this work. One primer set was able to detect all strains (A-16S); two primer sets could detect only strain A; three sets were able to detect strains B and C, but no primer set could distinguish strain D from the other strains. Strains B and C could not be distinguished from each other by the use of the NASBA assay. This was due to the similarity of the genomic sequences on which the primers were based. Stains B and C were found to have similar genotypes, but were observed to have different colour phenotypes. In order to distinguish between these two strains, other gene sequences would have to be used for primer design; sequences that were different for the two strains. But this was not possible with the sequences that were given.

Strain D could be detected with the A-16S primer set, but the assay could not distinguish this strain from the others. A variety of gene sequences from strain D from which primers could be designed was not available, and distinguishing strain D would require a particular pattern of results from the other primer sets. However, this was not obtained.

The NASBA assay was not able to distinguish between when the bacteria were grown with heavy oil versus acetate. Genes *m*, *n* and *o* were reported to be differentially expressed when the strains were grown with heavy oil compared to more readily available carbon sources (third party company, pers. comm.). However, as NASBA is a very sensitive technique, targets may be detected in very low copy numbers. Thus, even if the gene expression was much lower, or higher, under heavy oil conditions, NASBA would still have been able to detect bacteria in both conditions (heavy oil vs non-heavy

oil). For such sensitive techniques, it may be that genes will only avoid detection if they are completely switched off; down-regulated genes may still be detected.

The NASBA assay that was developed for *Cryptosporidium* successfully detected *C. parvum* and *C. hominis* oocysts, and was also able to distinguish *C. parvum* from *C. hominis*. This was as expected, as the C.par MIC1 primer set targets the second exon in the *CpMIC1* gene, which is not present in *C. hominis*. In contrast, the C.hom MIC1 primer set targets the only exon in the *ChMIC1* gene, which is also the first exon in *CpMIC1*.
The assay could not, however, be used for viability measurements. *C. hominis* oocysts that were subjected to known inactivation treatment (e.g. boiling) were still detectable by the assay. mRNA is produced only in living cells and is normally broken down quickly. However, according to the NASBA results, it seems probable that mRNA is not broken down quickly in *Cryptosporidium* oocysts after inactivation. It could be argued that the inactivation methods used were insufficient to kill the oocysts, but based on knowledge of this parasite, this seems highly unlikely, to the extent of being impossible. A more plausible reason could be that the mRNA was protected from degradation by being within the oocysts (the oocyst wall protected the mRNA). An alternative explanation could be that some mRNA takes longer to break down than anticipated.
The NASBA assay for *Cryptosporidium* is further described and discussed in Paper 2: "Real-time nucleic acid sequence-based amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts."

NASBA primer sets were also designed for *Giardia duodenalis* Assemblages A, B, and E, based on the heat shock protein 70 (*hsp70*) gene. These primer sets were tested on *Giardia* cysts isolated from dog faeces, and on *Giardia* cysts assemblage B that were purchased from a supplier (genotype H3), all with negative results.
Hsp70s are among the most common chaperones, found in all domains of life. They are involved in the correct folding and activation of proteins, as well as in degradation. Hsp70s are versatile, due to their regulation by different cofactors (Kampinga & Craig, 2010). Eukaryotes have normally at least three copies of the *hsp70* gene (Morrison et al., 2001). As *hsp70* transcripts become high, particularly when the cell is exposed to stress,

such as heat shock, but also cold, UV, and other stresses, it is considered to be useful as a sensitive marker (Liang & Keeley, 2011). As mRNA is usually degenerated quickly after cell death, it has also been suggested to be a suitable marker for viability (Lee et al., 2009; Liang & Keeley, 2011). Although, as the NASBA results from the *Cryptosporidium* experiments indicated, mRNA might not always be suitable as a viability marker. Experimental study of the *Giardia* primer sets will provide useful information regarding this topic.

An extensive review of the literature suggests that NASBA has not previously been used for detection of *Giardia*; even if a reference or unpublished work has been overlooked, it is nevertheless clear that NASBA has not been extensively used for detection of this parasite. The NASBA assay that was developed here was not thoroughly tested, and it presents an exciting area for further work.

The NASBA have some obvious advantages, specifically that it is able to amplify RNA directly, so less time is used on sample preparation in the lab. NASBA is also very sensitive, which is important when screening for *Cryptosporidium* and *Giardia*. However, it should be noted that NASBA is prone to false-positive results more often than false-negative (Morabito et al., 2013). Well-tailored design of primers might overcome this challenge, but primer-design in itself might present challenges. Even if appropriate primers are found for a target sequence, it might be difficult or even impossible to find suitable probe sequences for the molecular beacon (if MBs are used for detection). When working on primer-design for the various targets in this project, many sequences could not be used because there were no probe sequences that could be used.

Qualitative NASBA can also be challenging to achieve, especially for low cell numbers. In these experiments there were no evident patterns for quantification, although it would be very interesting to explore quantitative NASBA with the primer sets developed for *Cryptosporidium* and *Giardia* in the future. Advantages and drawbacks for the NASBA method is discussed in more detail in Paper 3.

The use of NASBA for analysis of water samples and other environmental samples for contaminants is not as common or established as it is for clinical diagnostic analyses. In

Paper 3: From research lab to standard environmental analysis tool: will NASBA make the leap?, the potential for NASBA to be used for routine environmental monitoring is reviewed and discussed. As the reactants used in the NASBA reaction are often expensive, or kit-based, it is of relevance to try to decrease the reaction volume, and thereby also the costs. So-called lab-on-a-chip systems (Chapter 4.3), uses small reaction volumes in miniaturised systems, and is therefore well suited for NASBA. NASBA has also some advantages to offer these systems: it is an isothermal process, and requires only a simple heating element. In addition, it is a sensitive technique that can detect few target molecules in small volumes. Further details on the potential for integration of NASBA into LOC systems are provided in section 4.3.1.

7.3. The POCNAD system

Although this seemed to be an exciting research project, it was soon clear that the system was not at an appropriate stage of development for the testing that was planned for the bacteria.

A considerable amount of time and effort was used in attempting to make the necessary adjustments to both the cartridge and the instrument, and the project never reached a stage where consistent and reliable results could be obtained. In the following paragraphs, the problems with each of the stages, the solutions tried, and the remaining challenges are described and discussed.

The original objective was to use the Trilobite® chip for pre-concentration of bacteria prior to analysis in the POCNAD system. At the current state of development of the Trilobite® chip it is not feasible to concentrate the bacteria used in these studies; the bacteria are small, about one µm in diameter, and are often smaller when growing on a substrate of large hydrocarbons. The smallest gap between the turbine blades (“feet”) of the trilobite units is two µm, and this is large enough to let the bacteria slip through. The Trilobite® chip was therefore not tested with these bacteria. However, as bacteria that are grown in bioreactors are provided with optimal growth conditions, thereby increasing the number of cells, bacterial concentration might not be crucial. In such a case, the

Trilobite® system could be used for sorting out larger particles prior to analysis, to prevent them from clogging the downstream microfluidic system.

In its entirety, the system was very complicated, with many components. Each component provides another place for failure, particularly at junctions between the various components. This made optimisation very challenging. One important difficulty was the inability to create the negative pressure that was necessary for flow through the microfluidic channels in the chip. The large number of valves and problems in creating adequate seals at the junction points in the system made it difficult to carry out all the steps to bring the liquids through the prototype chip. Another significant and related problem was the leakage at the junctions between the chip and cartridge. Different sized O-rings were needed to ensure that the cartridge system could be sealed. Leakage in the cartridge system is a problem when storing liquid reagents for longer than a few days. In some cartridges, leaking could occur after being stored over just one night. Other were able to contain liquid for up to a week. Clearly, the system would risk reagent loss before use if they were stored for longer than this.

The NASBA results in the POCNAD instrument were unclear and difficult to interpret. This could be due to bubble formation, which led to scattering of the fluorescent signal, or due to high background noise, which could be due to the chip itself.
The positive NASBA results with the Grm neg 16S primer set, targeting gram-negative bacteria, in the chip were probably due to contamination. The primers used were very general, and should provide a positive signal with all gram-negative bacteria, and could easily have become contaminated. Positive results were also obtained with this primer set being used on negative samples, using conventional NASBA, indicating that the problem could lie outside the chip system.
For the BC-m primer set, one specific gene in the two strains B and C was targeted, and gave negative NASBA results for strains A and D. It seems unlikely, therefore, that the positive results obtained with BC-m in the chip were due to contamination issues, but instead represented positive results and a step towards improvement in the POCNAD assay.

Due to conditions beyond my control, this project was stopped before I had had the opportunity to test the whole system, and only the preliminary results described here were achieved up to that point.

My preliminary results, however, pointed out important areas of weakness in the system, and provided a strong indication that the format of the POCNAD system that I tested was too complicated, with too many weak points and thus potential sources of error.

7.4. General discussion - collecting the disparate threads

Both the different methods and the types of biological particles used in my research differed considerably. Nevertheless, the methods used can all be useful as parts of LOC systems, whether for sample preparation (Trilobite® chip), part of an analysis system (NASBA), or as an example of a full analysis system (NASBA). All these methods and systems, with their advantages and challenges (Table 6) could cooperate in a joint system to obtain analysis results from a raw sample in a portable system. The original idea of the POCNAD system was to include the Trilobite® technology in a pre-sample system that could be added into the POCNAD cassette. The NASBA amplification method, being isothermal and sensitive, was and is, a crucial part of the POCNAD analysis system.

After examining these individual components separately, I have been able to provide a better understanding of the challenges that can occur in such systems, and also which sample types are best suited for such systems.

The experiments that were carried out with the Trilobite® chip in this project, suggested that the chip was better suited for high concentration particles, like dewatering of microalgae cultures, rather than for concentrating particles that occur in low concentrations, for which efficient recovery is crucial, like recovery of protozoan (oo)cysts. Our results for these pathogens suggest that considerable improvements to minimise losses, either within the chip, and/or within the mechanism leading the environmental matrix to the chip, are essential before the Trilobite® chip can be

considered as a possible alternative method against the standard filtration methods for concentrating such organisms during routine environmental analyses.

The experiments also demonstrated that the characteristics of the tested particle, and of the medium in which the particles are suspended, affect the efficiency of the chip. Biological particles, cells, and organisms may have very different characteristics, which affects how they "behave" in the chip. This was perhaps most apparent for the diatom *T. weissflogii*, whose extracellular polymers caused blockage of the chip's structure, resulting in very inefficient dewatering. However, the chip seemed very promising for dewatering of *Chaetoceros*, and for separating cells based on their size. Although the gap size in the chip used in these tests (5 µm) was not optimal for this particular alga, a slightly smaller gap would most likely increase the concentration of algal cells. In contrast to species that are able to self-flocculate, such as *T. weissflogii*, the cells of some algal species repel one another, making harvesting with conventional methods difficult. For these types of algae, the Trilobite® chip may offer a good alternative for pre-harvesting.

The POCNAD system, in the state that was tested and developed in this work, had several challenges to overcome towards becoming a sustainable analysis system for simple analysis. The main problems were the complicated construction of both the disposable chip and cartridge, as well as the under-developed control instrument. A more developed instrument would probably provide better solutions for the disposable parts of the system as well.

Developing a fully automatic lab-on-a-chip system may reduce manual handling, decrease contamination, and limit the risk of errors caused by human factors. In such a system, the greater the extent of automatization and reduction of handling, the more complex are the requirements for the control instrument. An optimal system would be simple to use and without too many components that present sources of error. Of course, funding is also helpful to achieve these goals, but it is obviously important that proof-of-principle be explored adequately before major investment is incurred.

Table 6 Advantages and disadvantages of the techniques used in my research

Technique	Advantages	Disadvantages
Trilobite® chip	• Promising for microalgae that are difficult to harvest by flocculation and similar methods • Potential for separating cells according to size • Potential for integration into LOC systems and upscaling	• Although, theoretically, should not clog, not all biological particles are well-suited for separation • Might be difficult to clean • Not yet suitable for concentration of some critical particles (such as parasites in drinking water)
NASBA	• Sensitivity equal to or greater than RT-PCR (detection limits < 1 cell per reaction) • Specific, comparable to PCR • Shorter reaction time than PCR • Less labour intensive than RT-PCR • RNA is target, but additional cDNA treatment is not required • Not affected by (ds)DNA • No need for thermocycler, the reaction is isothermal • Decreased contamination risk with molecular beacons, reaction in closed tube	• Kits are expensive • Might be difficult to make in-house (more than PCR) • False-positives more common than false-negatives • Calculation of cell numbers by Q-NASBA might be difficult, especially of low cell numbers • Still few assays for environmental samples compared with PCR • Designing assays relies on known gene sequences
POCNAD	• Potential of offering total analysis in one system	• Complicated system with many components • Difficult to ensure leak-proof • Difficult to control properly • Bubble formation in chip chambers may distort fluorescence signal

8. Conclusions and further work – my contributions to the scientific field

In this thesis, I have set out to explore the use of different technologies and techniques for concentrating and investigating biological particles occurring in different matrices. The techniques used are relevant for the specific applications addressed (concentration or analysis) and also for the biological particles in focus in the research (microalgae, oil-utilising bacteria or waterborne pathogens). These are very different aims, with very different biological particles, and thus, despite some overlap, the techniques used also differ and have different challenges.

The Trilobite® chip was successfully used for concentration of microalgae. From my research I obtained information on which types of algae might be not so well-suited for separation in the chip, based on their characteristics, as well as useful for information regarding which types of algae that might be beneficially harvested using the chip.

The Trilobite® chip was also explored for concentration of waterborne protozoan parasites of public health significance, and tested using *Giardia duodenalis* cysts and *Toxoplasma gondii* oocysts. The results of the experiments suggested that the Trilobite® chip, in its current stage format is not appropriate for concentration of small numbers of (oo)cysts, as the sample loss was comparable to, or greater than current standard filtration methods. The reasons for the lack of efficiency could be either breakage or the experimental setup.

The use of NASBA for identifying *Cryptosporidium* oocysts, with particular emphasis on species identification, was successful, as NASBA assays for *C. parvum* and *C. hominis* were both developed and tested, and I was able to distinguish *C. parvum* from *C. hominis* using this assay. However, the results of related experiments demonstrated that these assays were not appropriate for assessment of oocyst viability.

The use of NASBA for identifying *Giardia* cysts, with particular emphasis on genotype identification focussed on development of NASBA assays for *G. duodenalis* assemblages A, B, and E. However, I was not able to carry out experimental testing of these completely, due to lack of the relevant organisms. For future work, it would be interesting to test

these NASBA primer sets properly on those genotypes for which they were designed. According to the results that were obtained when testing the primer sets, they may be very specific, and perhaps even capable of identifying *Giardia* to the sub-assemblage level. Thus, my work indicates that there is considerable potential in exploring further using this technique for *Giardia* classification.

The original objective of the PhD project, which was to develop the use of the POCNAD system, integrating both the Trilobite® chip and NASBA for environmental analysis, with particular emphasis on oil-utilizing bacteria, was not achieved, as the project was terminated prior to completion. However, some of the original sub-objectives were achieved. I was able to develop NASBA primer sets for the oil-utilizing bacteria based on the information from relevant genomic sequences. I also tested the primer sets on the oil-utilizing bacteria to the extent that I could determine those sets that would be most useful for inclusion in the chip of the POCNAD system. The primer sets were also tested on the strains under different growth conditions, but the high sensitivity of NASBA precluded me from being able to distinguish between the growth conditions. I was able to start preliminary NASBA testing of these primer sets in the POCNAD cassette before the project ended. Pre-concentration of bacteria with the Trilobite® chip could not be carried out.

My contributions are listed in Table 7.

Table 7 My contributions for improving the technologies and gaining new knowledge in this research

Application	Technologies Available	Technologies investigated in this thesis	My contribution to improving the technologies
Concentrating microalgae	Flocculation Sedimentation or flotation Centrifugation Scraping Capturing by nanoparticles Electrical processes Filtration	The microfluidic Trilobite® chip	Proposing new fields of use for the Trilobite® chip Proposing a new method of water reduction in microalgae harvesting Testing and examining the chip for three species of microalgae Proving the chip's usefulness, further insight into which types of algae the chip is most useful
Concentrating and analysing environmental samples for protozoan pathogens	Filtration Inertial microfluidic techniques Centrifugation Immunomagnetic separation	The microfluidic Trilobite® chip for concentration of pathogens	Testing and examining the chip for concentration of *Giardia* cysts and *Toxoplasma* oocysts
	PCR LAMP RCA SDA	NASBA for analysis	Developing NASBA assay for *C. parvum* and *C. hominis* Testing the NASBA assay for *C. parvum* and *C. hominis* Species-level identification of *C. parvum* and *C. hominis* by NASBA Viability testing of *C. parvum* and *C. hominis* using NASBA Developing NASBA assay for *Giardia duodenalis* assemblages A, B and E
Analysing composition of bacterial mixtures	PCR LAMP RCA SDA	NASBA	Developing NASBA assay for monitoring of a four-species consortium Testing the NASBA assay for the consortium
	Few total analysis systems	POCNAD	Adapting the analysis platform towards monitoring of the bacterial consortium by Testing and developing the compartments Preparing the chip with NASBA reagents Testing the NASBA assay in the POCNAD

One interesting area for future testing of the Trilobite® chip lies within aquaculture. Copepods are tiny crustaceans (zooplankton) commonly used as live feed in aquaculture, for example to feed cod and other marine fish. In production, the density of copepod eggs in rearing tanks become high, which increases the risk of cannibalism by the adults (Drillet et al., 2014). This limits production yield and can have a negative economic impact for the industry. Separation of eggs from adults and nauplius larvae is necessary to decrease cannibalism. The eggs of the most commonly used copepod, *Acartia tonsa*, are approximately 70-80 µm and spherical (Belmonte, 1998; Hansen et al., 2010). Newly hatched larvae are about the same size as the eggs, but they also spread out their legs, and then increase in size through six larval stages, reaching 300-400 µm. The adult *A. tonsa* is approximately 1 mm. The Trilobite® chip should be well suited for separation of eggs and (larger) larvae, especially as the size scale is relatively large. For this sort of relatively crude separation, in which it is not essential that every single organism is isolated into the correct set (as is essential for analysis of water for protozoan parasites) the chips can be made fairly readily and cheaply in plastic. I suggest (and thank Dag for the idea) that this could be tested in the near future. It could also be especially useful to incorporate the Trilobite® technology into rearing tanks for continuous harvesting, where nauplius larvae may be harvested for feed, while eggs may be returned to the rearing tank.

In future work using the Trilobite® chip for concentration of waterborne pathogens, a whole test system, and not only the chip must be optimised in order to handle large volumes of water, and also to gain higher recovery of cysts or oocysts. In such a system, one should strive to obtain as few transmission points and crevices as possible, to avoid loss of critical sample material.

The NASBA method proved to be a sensitive analysis method for the microorganisms that were targeted.

If the POCNAD system is to be developed further in the future, I would suggest simplification of the chip and cassette system. Decreasing the number of transmission points between the cassette and the chip would decrease the risk of leakage and loss of

chemicals. In addition, less complicated microchannel structures in the chip could make it easier to control the flow within the chip.

The NASBA primer sets that were designed for *Giardia* cysts were unfortunately not completely tested within the time span of the project. To the best of my knowledge, NASBA has not previously been assessed for *Giardia*, and it would be very interesting to study this assay further and determine whether a robust method for differentiating between different Assemblages could be developed. This has been the goal of many multiplex PCR systems that have been published, and the positive results with *Cryptosporidium* (for distinguishing between C. *parvum* and *C. hominis*) suggest that this is feasible. However, the results with *Cryptosporidium* also suggest the use of NASBA (and any other RNA based system) as a viability measure should be treated with caution.

The complexity of biological particles have great impact on the type of technology used for processing or analysis. Some technologies and assays may be well suited for some types of biological samples, but be inadequate for other samples. This was demonstrated in the all parts of this PhD project: in the separation technology, the molecular assay, and the complex LOC system. When aiming to developing a well-working microsystem, the properties and complexities of the biological sample must be taken into account.

9. References

Abrahamsen, M.S., Templeton, T.J., Enomoto, S., Abrahante, J.E., Zhu, G., Lancto, C.A., Deng, M.Q., Liu, C., Widmer, G., Tzipori, S., Buck, G.A., Xu, P., Bankier, A.T., Dear, P.H., Konfortov, B.A., Spriggs, H.F., Iyer, L., Anantharaman, V., Aravind, L., Kapur, V. 2004. Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science*, **304**(5669), 441-445.

Adam, R.D. 1991. The biology of *Giardia* spp. *Microbiological Reviews*, **55**(4), 706-732.

Ankarklev, J., Jerlstrom-Hultqvist, J., Ringqvist, E., Troell, K., Svard, S.G. 2010. Behind the smile: cell biology and disease mechanisms of *Giardia* species. *Nature Reviews Microbiology*, **8**(6), 413-422.

Baldursson, S., Karanis, P. 2011. Waterborne transmission of protozoan parasites: Review of worldwide outbreaks – An update 2004–2010. *Water Research*, **45**(20), 6603-6614.

Beech, J.P., Holm, S.H., Adolfsson, K., Tegenfeldt, J.O. 2012. Sorting cells by size, shape and deformability. *Lab on a Chip*, **12**(6), 1048-1051.

Belmonte, G. 1998. The egg morphology of 7 Acartiidae species: a preliminary survey of the ootaxonomy of calanoids. *Journal of Marine Systems*, **15**(1–4), 35-39.

Bernander, R., Palm, J.E.D., Svärd, S.G. 2001. Genome ploidy in different stages of the *Giardia lamblia* life cycle. *Cellular Microbiology*, **3**(1), 55-62.

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., Marion, S.A. 1997. Outbreak of toxoplasmosis associated with municipal drinking water. *The Lancet*, **350**(9072), 173-177.

Cacciò, S.M., Ryan, U. 2008. Molecular epidemiology of giardiasis. *Molecular and Biochemical Parasitology*, **160**(2), 75-80.

Carotenuto, Y., Esposito, F., Pisano, F., Lauritano, C., Perna, M., Miralto, A., Ianora, A. 2012. Multi-generation cultivation of the copepod *Calanus helgolandicus* in a re-circulating system. *Journal of Experimental Marine Biology and Ecology*, **418–419**, 46-58.

Civin, C.I., Ward, T., Skelley, A.M., Gandhi, K., Peilun Lee, Z., Dosier, C.R., D'Silva, J.L., Chen, Y., Kim, M., Moynihan, J., Chen, X., Aurich, L., Gulnik, S., Brittain, G.C., Recktenwald, D.J., Austin, R.H., Sturm, J.C. 2016. Automated leukocyte processing by microfluidic deterministic lateral displacement. *Cytometry Part A*, **89**(12), 1073-1083.

Compton, J. 1991. Nucleic acid sequence-based amplification. *Nature*, **350**(6313), 91-92.

Courties, C., Vaquer, A., Troussellier, M., Lautier, J., Chretiennot-Dinet, M.J., Neveux, J., Machado, C., Claustre, H. 1994. Smallest eukaryotic organism. *Nature*, **370**(6487), 255-255.

Dijkshoorn, J.P., Schutyser, M.A.I., Wagterveld, R.M., Schroën, C.G.P.H., Boom, R.M. 2017. A comparison of microfiltration and inertia-based microfluidics for large scale suspension separation. *Separation and Purification Technology*, **173**, 86-92.

Dong, T., Yang, Z., Egeland, E.B., Karlsen, F., Jakobsen, H. 2009. Clogging failure in microfilter for blood cell separation and its novel improvements. *2009 16th IEEE International Symposium on the Physical and Failure Analysis of Integrated Circuits*, 6-10 July 2009. pp. 759-763.

Dong, T., Yang, Z., Su, Q., Tran, N.M., Egeland, E.B., Karlsen, F., Zhang, Y., Kapiris, M.J., Jakobsen, H. 2011. Integratable non-clogging microconcentrator based on counter-flow principle for continuous enrichment of CaSki cells sample. *Microfluidics and Nanofluidics*, **10**(4), 855-865.

Drillet, G., Maguet, R., Mahjoub, M.-S., Roullier, F., Fielding, M.J. 2014. Egg cannibalism in *Acartia tonsa*: effects of stocking density, algal concentration, and egg availability. *Aquaculture International*, **22**(4), 1295-1306.

Dubey, J.P., Jones, J.L. 2008. *Toxoplasma gondii* infection in humans and animals in the United States. *International Journal for Parasitology*, **38**(11), 1257-1278.

Dumetre, A., Dubey, J.P., Ferguson, D.J.P., Bongrand, P., Azas, N., Puech, P.H. 2013. Mechanics of the *Toxoplasma gondii* oocyst wall. *Proceedings of the National Academy of Sciences of the United States of America*, **110**(28), 11535-11540.

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. *New England Journal of Medicine*, **332**(13), 855-859.

Fakruddin, M., Mannan, K.S., Chowdhury, A., Mazumdar, R.M., Hossain, M.N., Islam, S., Chowdhury, M.A. 2013. Nucleic acid amplification: Alternative methods of polymerase chain reaction. *J Pharm Bioallied Sci*, **5**(4), 245-52.

Fichera, M.E., Roos, D.S. 1997. A plastid organelle as a drug target in apicomplexan parasites. *Nature*, **390**(6658), 407-409.

Findal, G., Barlinn, R., Sandven, I., Stray-Pedersen, B., Nordbø, S.A., Samdal, H.H., Vainio, K., Dudman, S.G., Jenum, P.A. 2015. *Toxoplasma* prevalence among pregnant women in Norway: a cross-sectional study. *APMIS*, **123**(4), 321-325.

Flegr, J., Prandota, J., Sovičková, M., Israili, Z.H. 2014. Toxoplasmosis – A Global Threat. Correlation of Latent Toxoplasmosis with Specific Disease Burden in a Set of 88 Countries. *PLoS ONE*, **9**(3), e90203.

Ganz, K.R., Clime, L., Farber, J.M., Corneau, N., Veres, T., Dixon, B.R. 2015. Enhancing the Detection of *Giardia duodenalis* Cysts in Foods by Inertial Microfluidic Separation. *Applied and Environmental Microbiology*, **81**(12), 3925-3933.

Gill, P., Ghaemi, A. 2008. Nucleic acid isothermal amplification technologies - A review. *Nucleosides Nucleotides & Nucleic Acids*, **27**(3), 224-243.

Goo, N.-I., Kim, D.-E. 2016. Rolling circle amplification as isothermal gene amplification in molecular diagnostics. *BioChip Journal*, **10**(4), 262-271.

Gruber, A.R., Lorenz, R., Bernhart, S.H., Neuböck, R., Hofacker, I.L. 2008. The Vienna RNA Websuite. *Nucleic Acids Research*, **36**(suppl 2), W70-W74.

Gulliksen, A., Keegan, H., Martin, C., O'Leary, J., Solli, L.A., Falang, I.M., Grønn, P., Karlgård, A., Mielnik, M.M., Johansen, I.-R., Tofteberg, T.R., Baier, T., Gransee, R., Drese, K., Hansen-Hagge, T., Riegger, L., Koltay, P., Zengerle, R., Karlsen, F., Ausen, D., Furuberg, L. 2012. Towards a “Sample-In, Answer-Out” Point-of-Care Platform for Nucleic Acid Extraction and Amplification: Using an HPV E6/E7 mRNA Model System. *Journal of Oncology*, **2012**, 905024.

Gulliksen, A., Solli, L., Karlsen, F., Rogne, H., Hovig, E., Nordstrøm, T., Sirevåg, R. 2004. Real-Time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes. *Analytical Chemistry*, **76**(1), 9-14.

Gulliksen, A., Solli, L.A., Drese, K.S., Sorensen, O., Karlsen, F., Rogne, H., Hovig, E., Sirevag, R. 2005. Parallel nanoliter detection of cancer markers using polymer microchips. *Lab Chip*, **5**(4), 416-20.

Handå, A., Ranheim, A., Olsen, A.J., Altin, D., Reitan, K.I., Olsen, Y., Reinertsen, H. 2012. Incorporation of salmon fish feed and feces components in mussels (*Mytilus edulis*): Implications for integrated multi-trophic aquaculture in cool-temperate North Atlantic waters. *Aquaculture*, **370–371**, 40-53.

Hansen, B.W., Drillet, G., kozmér, A., Madsen, K.V., Pedersen, M.F., Sørensen, T.F. 2010. Temperature effects on copepod egg hatching: does acclimatization matter? *Journal of Plankton Research*, **32**(3), 305-315.

He, C.Y., Shaw, M.K., Pletcher, C.H., Striepen, B., Tilney, L.G., Roos, D.S. 2001. A plastid segregation defect in the protozoan parasite *Toxoplasma gondii*. *The EMBO Journal*, **20**(3), 330-339.

Head, I.M., Jones, D.M., Roling, W.F.M. 2006. Marine microorganisms make a meal of oil. *Nature Reviews Microbiology*, **4**(3), 173-182.

Heijnen, L., Medema, G. 2009. Method for rapid detection of viable *Escherichia coli* in water using real-time NASBA. *Water Research*, **43**(12), 3124-3132.

Hellyer, T.J., Nadeau, J.G. 2004. Strand displacement amplification: a versatile tool for molecular diagnostics. *Expert Review of Molecular Diagnostics*, **4**(2), 251-261.

Holmes, D., Whyte, G., Bailey, J., Vergara-Irigaray, N., Ekpenyong, A., Guck, J., Duke, T. 2014. Separation of blood cells with differing deformability using deterministic lateral displacement. *Interface Focus*, **4**(6).

Huang, L.R., Cox, E.C., Austin, R.H., Sturm, J.C. 2004. Continuous Particle Separation Through Deterministic Lateral Displacement. *Science*, **304**(5673), 987-990.

Jerlstrom-Hultqvist, J., Ankarklev, J., Svard, S.G. 2010. Is human giardiasis caused by two different *Giardia* species? *Gut microbes*, **1**(6), 379-82.

Jimenez, M., Bridle, H. 2016. Microfluidics for effective concentration and sorting of waterborne protozoan pathogens. *Journal of Microbiological Methods*, **126**, 8-11.

Jimenez, M., Miller, B., Bridle, H.L. 2017. Efficient separation of small microparticles at high flowrates using spiral channels: Application to waterborne pathogens. *Chemical Engineering Science*, **157**, 247-254.

Jonstrup, S.P., Koch, J., Kjems, J. 2006. A microRNA detection system based on padlock probes and rolling circle amplification. *RNA*, **12**(9), 1747-1752.

Jönsson, K.I., Rabbow, E., Schill, R.O., Harms-Ringdahl, M., Rettberg, P. 2008. Tardigrades survive exposure to space in low Earth orbit. *Current Biology*, **18**(17), R729-R731.

Kampinga, H.H., Craig, E.A. 2010. The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews Molecular Cell Biology*, **11**(8), 579-592.

Karabacak, N.M., Spuhler, P.S., Fachin, F., Lim, E.J., Pai, V., Ozkumur, E., Martel, J.M., Kojic, N., Smith, K., Chen, P.I., Yang, J., Hwang, H., Morgan, B., Trautwein, J., Barber, T.A., Stott, S.L., Maheswaran, S., Kapur, R., Haber, D.A., Toner, M. 2014. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nature Protocols*, **9**(3), 694-710.

Karanis, P., Aldeyarbi, H.M., Mirhashemi, M.E., Khalil, K.M. 2013. The impact of the waterborne transmission of *Toxoplasma gondii* and analysis efforts for water detection: an overview and update. *Environmental Science and Pollution Research*, **20**(1), 86-99.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F., Faruque, A.S.G., Zaidi, A.K.M., Saha, D.,

Alonso, P.L., Tamboura, B., Sanogo, D., Onwuchekwa, U., Manna, B., Ramamurthy, T., Kanungo, S., Ochieng, J.B., Omore, R., Oundo, J.O., Hossain, A., Das, S.K., Ahmed, S., Qureshi, S., Quadri, F., Adegbola, R.A., Antonio, M., Hossain, M.J., Akinsola, A., Mandomando, I., Nhampossa, T., Acácio, S., Biswas, K., O'Reilly, C.E., Mintz, E.D., Berkeley, L.Y., Muhsen, K., Sommerfelt, H., Robins-Browne, R.M., Levine, M.M. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, **382**(9888), 209-222.

Landry, M.L., Garner, R., Ferguson, D. 2005. Real-Time Nucleic Acid Sequence-Based Amplification Using Molecular Beacons for Detection of Enterovirus RNA in Clinical Specimens. *Journal of Clinical Microbiology*, **43**(7), 3136-3139.

Lane, S., Lloyd, D. 2002. Current trends in research into the waterborne parasite *Giardia*. *Critical Reviews in Microbiology*, **28**(2), 123-147.

Lee, G.-C., Nam, S.-H., Chae, J.-C., Lee, C.-H. 2009. *Giardia duodenalis*: Improved detection of viable cysts by reverse transcription-PCR of heat shock-inducible hsp70 gene. *Experimental Parasitology*, **123**(4), 377-380.

Lendner, M., Daugschies, A. 2014. *Cryptosporidium* infections: molecular advances. *Parasitology*, **141**(11), 1511-1532.

Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., Schoen, C.D. 1998. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res*, **26**(9), 2150-5.

Liang, Z.B., Keeley, A. 2011. Detection of Viable *Cryptosporidium parvum* in Soil by Reverse Transcription-Real-Time PCR Targeting hsp70 mRNA. *Applied and Environmental Microbiology*, **77**(18), 6476-6485.

Liu, W., Kou, J., Xing, H., Li, B. 2014. Paper-based chromatographic chemiluminescence chip for the detection of dichlorvos in vegetables. *Biosensors and Bioelectronics*, **52**, 76-81.

Lizardi, P.M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D.C., Ward, D.C. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nature Genetics*, **19**(3), 225.

Mackenzie, W.R., Schell, W.L., Blair, K.A., Addiss, D.G., Peterson, D.E., Hoxie, N.J., Kazmierczak, J.J., Davis, J.P. 1995. Massive outbreak of waterborne *Cryptosporidium* infection in Milwaukee, Wisconsin - Recurrence of illkess and risk of secondary transmission. *Clinical Infectious Diseases*, **21**(1), 57-62.

Mahony, J.B., Song, X., Chong, S., Faught, M., Salonga, T., Kapala, J. 2001. Evaluation of the NucliSens Basic Kit for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeaein* Genital Tract Specimens Using Nucleic Acid Sequence-Based Amplification of 16S rRNA. *Journal of Clinical Microbiology*, **39**(4), 1429-1435.

Marques, C.R. 2016. Bio-rescue of marine environments: On the track of microbially-based metal/metalloid remediation. *Science of The Total Environment*, **565**, 165-180.

Marshall, W.F., Young, K.D., Swaffer, M., Wood, E., Nurse, P., Kimura, A., Frankel, J., Wallingford, J., Walbot, V., Qu, X., Roeder, A.H. 2012. What determines cell size? *BMC Biology*, **10**(1), 1-22.

Martel, J.M., Smith, K.C., Dlamini, M., Pletcher, K., Yang, J., Karabacak, M., Haber, D.A., Kapur, R., Toner, M. 2015. Continuous Flow Microfluidic Bioparticle Concentrator. *Scientific Reports*, **5**, 11300.

Mata, T.M., Martins, A.A., Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, **14**(1), 217-232.

McFadden, G.I. 2011. The apicoplast. *Protoplasma*, **248**(4), 641-650.

McGrath, J., Jimenez, M., Bridle, H. 2014. Deterministic lateral displacement for particle separation: a review. *Lab on a Chip*, **14**(21), 4139-4158.

Monis, P.T., Caccio, S.M., Thompson, R.C.A. 2009. Variation in *Giardia*: towards a taxonomic revision of the genus. *Trends in Parasitology*, **25**(2), 93-100.

Moore, C.E., Elwin, K., Phot, N., Seng, C., Mao, S., Suy, K., Kumar, V., Nader, J., Bousfield, R., Perera, S., Bailey, J.W., Beeching, N.J., Day, N.P.J., Parry, C.M., Chalmers, R.M. 2016. Molecular Characterization of *Cryptosporidium* Species and *Giardia duodenalis* from Symptomatic Cambodian Children. *PLoS Negl Trop Dis*, **10**(7), e0004822.

Morabito, K., Wiske, C., Tripathi, A. 2013. Engineering Insights for Multiplexed Real-Time Nucleic Acid Sequence-Based Amplification (NASBA): Implications for Design of Point-of-Care Diagnostics. *Molecular Diagnosis & Therapy*, **17**(3), 185-192.

Moran, M.A., Satinsky, B., Gifford, S.M., Luo, H.W., Rivers, A., Chan, L.K., Meng, J., Durham, B.P., Shen, C., Varaljay, V.A., Smith, C.B., Yager, P.L., Hopkinson, B.M. 2013. Sizing up metatranscriptomics. *Isme Journal*, **7**(2), 237-243.

Morrison, H.G., McArthur, A.G., Gillin, F.D., Aley, S.B., Adam, R.D., Olsen, G.J., Best, A.A., Cande, W.Z., Chen, F., Cipriano, M.J., Davids, B.J., Dawson, S.C., Elmendorf, H.G., Hehl, A.B., Holder, M.E., Huse, S.M., Kim, U.U., Lasek-Nesselquist, E., Manning, G., Nigam, A., Nixon, J.E.J., Palm, D., Passamaneck, N.E., Prabhu, A., Reich, C.I., Reiner, D.S., Samuelson, J., Svard, S.G., Sogin, M.L. 2007. Genomic Minimalism in the Early Diverging Intestinal Parasite *Giardia lamblia*. *Science*, **317**(5846), 1921-1926.

Morrison, H.G., Roger, A.J., Nystul, T.G., Gillin, F.D., Sogin, M.L. 2001. *Giardia lamblia* expresses a proteobacterial-like DnaK homolog. *Molecular Biology and Evolution*, **18**(4), 530-541.

Mossige, E.J., Jensen, A., Mielnik, M.M. 2016. An experimental characterization of a tunable separation device. *Microfluidics and Nanofluidics*, **20**(12), 160.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. *Sci Am*, **262**(4), 56-61, 64-5.

Niemz, A., Ferguson, T.M., Boyle, D.S. 2011. Point-of-care nucleic acid testing for infectious diseases. *Trends in Biotechnology*, **29**(5), 240-250.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**(12), e63-e63.

O'Grady, J., Lacey, K., Glynn, B., Smith, T.J., Barry, T., Maher, M. 2009. tmRNA - a novel high-copy-number RNA diagnostic target - its application for *Staphylococcus aureus* detection using real-time NASBA. *Fems Microbiology Letters*, **301**(2), 218-223.

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L. 1999. Virulence of Three Distinct *Cryptospovidium parvum* Isolates for Healthy Adults. *Journal of Infectious Diseases*, **180**(4), 1275-1281.

Okumus, B., Landgraf, D., Lai, G.C., Bakshi, S., Arias-Castro, J.C., Yildiz, S., Huh, D., Fernandez-Lopez, R., Peterson, C.N., Toprak, E., El Karoui, M., Paulsson, J. 2016. Mechanical slowing-down of cytoplasmic diffusion allows *in vivo* counting of proteins in individual cells. *Nature Communications*, **7**, 11641.

Pamme, N. 2007. Continuous flow separations in microfluidic devices. *Lab Chip*, **7**(12), 1644-59.

Pires, N.M.M., Dong, T. 2014. A cascade-like silicon filter for improved recovery of oocysts from environmental waters. *Environmental Technology*, **35**(6), 781-790.

Pires, N.M.M., Dong, T. 2013. Recovery of *Cryptosporidium* and *Giardia* organisms from surface water by counter-flow refining microfiltration. *Environmental Technology*, **34**(17), 2541-2551.

Rehn, M., Wallensten, A., Widerström, M., Lilja, M., Grunewald, M., Stenmark, S., Kark, M., Lindh, J. 2015. Post-infection symptoms following two large waterborne outbreaks of *Cryptosporidium hominis* in Northern Sweden, 2010–2011. *BMC Public Health*, **15**, 529.

Reinholt, S.J., Behrent, A., Greene, C., Kalfe, A., Baeumner, A.J. 2014. Isolation and Amplification of mRNA within a Simple Microfluidic Lab on a Chip. *Analytical Chemistry*, **86**(1), 849-856.

Rendtorff, R.C. 1954. The experimental transmission of human intestinal protozoan parasites. II. *Giardia lamblia* cysts given in capsules. *American journal of hygiene*, **59**(2), 209-20.

Reuter, J.S., Mathews, D.H. 2010. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics*, **11**, 129.

Robertson, L.J., Campbell, A.T., Smith, H.V. 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. *Applied and Environmental Microbiology*, **58**(11), 3494-3500.

Robertson, L.J., Hermansen, L., Gjerde, B.K., Strand, E., Alvsvåg, J.O., Langeland, N. 2006. Application of Genotyping during an Extensive Outbreak of Waterborne Giardiasis in Bergen, Norway, during Autumn and Winter 2004. *Applied and Environmental Microbiology*, **72**(3), 2212-2217.

Ryan, U., Fayer, R., Xiao, L. 2014. *Cryptosporidium* species in humans and animals: current understanding and research needs. *Parasitology*, **141**(13), 1667-1685.

Ryan, U., Hijjawi, N. 2015. New developments in *Cryptosporidium* research. *International Journal for Parasitology*, **45**(6), 367-373.

Saboya, L.V., Maubois, J.L. 2000. Current developments of microfiltration technology in the dairy industry. *Lait*, **80**(6), 541-553.

Santos-Sánchez, N.F., Valadez-Blanco, R., Hernández-Carlos, B., Torres-Ariño, A., Guadarrama-Mendoza, P.C., Salas-Coronado, R. 2016. Lipids rich in ω-3 polyunsaturated fatty acids from microalgae. *Applied Microbiology and Biotechnology*, **100**(20), 8667-8684.

Schulz, H.N., Brinkhoff, T., Ferdelman, T.G., Mariné, M.H., Teske, A., Jørgensen, B.B. 1999. Dense Populations of a Giant Sulfur Bacterium in Namibian Shelf Sediments. *Science*, **284**(5413), 493-495.

Sejr, M.K., Petersen, J.K., Jensen, K.T., Rysgaard, S. 2004. Effects of food concentration on clearance rate and energy budget of the Arctic bivalve *Hiatella arctica* (L) at subzero temperature. *Journal of Experimental Marine Biology and Ecology*, **311**(1), 171-183.

Shapiro, K., Mazet, J.A.K., Schriewer, A., Wuertz, S., Fritz, H., Miller, W.A., Largier, J., Conrad, P.A. 2010. Detection of *Toxoplasma gondii* oocysts and surrogate microspheres in water using ultrafiltration and capsule filtration. *Water Research*, **44**(3), 893-903.

Shapiro, O.H., Kramarsky-Winter, E., Gavish, A.R., Stocker, R., Vardi, A. 2016. A coral-on-a-chip microfluidic platform enabling live-imaging microscopy of reef-building corals. *Nature Communications*, **7**, 10860.

Shibulal, B., Al-Bahry, S.N., Al-Wahaibi, Y.M., Elshafie, A.E., Al-Bemani, A.S., Joshi, S.J. 2014. Microbial Enhanced Heavy Oil Recovery by the Aid of Inhabitant Spore-Forming Bacteria: An Insight Review. *Scientific World Journal*.

Simpkins, S.A., Chan, A.B., Hays, J., Popping, B., Cook, N. 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Letters in Applied Microbiology*, **30**(1), 75-79.

Skotarczak, B. 2009. Methods for Parasitic Protozoans Detection in the Environmental Samples. *Parasite-Journal De La Societe Francaise De Parasitologie*, **16**(3), 183-190.

Slifko, T.R., Smith, H.V., Rose, J.B. 2000. Emerging parasite zoonoses associated with water and food. *International Journal for Parasitology*, **30**(12–13), 1379-1393.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering*, **101**(2), 87-96.

Swales, C., Wright, S. 2000. Evaluation of a continuous flow centrifuge for recovery of *Cryptosporidium* oocysts from large volume water samples. *Water Research*, **34**(6), 1962-1966.

Torgerson, P.R., Devleesschauwer, B., Praet, N., Speybroeck, N., Willingham, A.L., Kasuga, F., Rokni, M.B., Zhou, X.-N., Fèvre, E.M., Sripa, B., Gargouri, N., Fürst, T., Budke, C.M., Carabin, H., Kirk, M.D., Angulo, F.J., Havelaar, A., de Silva, N. 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 11 Foodborne Parasitic Diseases, 2010: A Data Synthesis. *PLoS Med*, **12**(12), e1001920.

Tottori, N., Nisisako, T., Park, J., Yanagida, Y., Hatsuzawa, T. 2016. Separation of viable and nonviable mammalian cells using a deterministic lateral displacement microfluidic device. *Biomicrofluidics*, **10**(1).

Tully, J.G., Taylorrobinson, D., Rose, D.L., Cole, R.M., Bove, J.M. 1983. *Mycoplasma genitalium*, a New Species from the Human Urogenital Tract. *International Journal of Systematic Bacteriology*, **33**(2), 387-396.

Tyagi, S., Bratu, D.P., Kramer, F.R. 1998. Multicolor molecular beacons for allele discrimination. *Nature Biotechnology*, **16**(1), 49-53.

Tyagi, S., Kramer, F.R. 1996. Molecular beacons: Probes that fluoresce upon hybridization. *Nature Biotechnology*, **14**(3), 303-308.

Uduman, N., Qi, Y., Danquah, M.K., Forde, G.M., Hoadley, A. 2010. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *Journal of Renewable and Sustainable Energy*, **2**(1).

van Oordt, T., Strohmeier, O., Mark, D., Zengerle, R., Eberhard, M., Drexler, J., Patel, P., Weidmann, M., Zgaga-Griesz, A., Bessler, W.G., von Stetten, F. 2012. The LabDisk – A Fully Automated Centrifugal Lab-on-a-Chip System for the Detection of Biological Threats. in: *Future Security: 7th Security Research Conference, Future Security 2012, Bonn, Germany, September 4-6, 2012. Proceedings*, (Eds.) N. Aschenbruck, P. Martini, M. Meier, J. Tölle, Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 220-223.

Walker, G.T., Fraiser, M.S., Schram, J.L., Little, M.C., Nadeau, J.G., Malinowski, D.P. 1992. Strand displacement amplification—an isothermal, in vitro DNA amplification technique. *Nucleic Acids Research*, **20**(7), 1691-1696.

Wan, C., Alam, M.A., Zhao, X.-Q., Zhang, X.-Y., Guo, S.-L., Ho, S.-H., Chang, J.-S., Bai, F.-W. 2015. Current progress and future prospect of microalgal biomass harvest using various flocculation technologies. *Bioresource Technology*, **184**(0), 251-257.

Webber, M.A., Sari, I., Hoefel, D., Monis, P.T., King, B.J. 2014. PCR Slippage Across the ML-2 Microsatellite of the *Cryptosporidium* MIC1 Locus Enables Development of a PCR Assay Capable of Distinguishing the Zoonotic *Cryptosporidium parvum* From Other Human Infectious *Cryptosporidium* Species. *Zoonoses and Public Health*, **61**(5), 324-337.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Björkholm, B., Hansen, A., Hiltula, J.I., Långmark, J., Löfdahl, M., Omberg, M., Reutenwall, C., Samuelsson, E., Widgren, K., Wallensten, A., Lindh, J. 2014. Large Outbreak of *Cryptosporidium hominis* Infection Transmitted through the Public Water Supply, Sweden. *Emerging Infectious Diseases*, **20**(4), 581-589.

Wyatt Shields IV, C., Reyes, C.D., Lopez, G.P. 2015. Microfluidic cell sorting: a review of the advances in the separation of cells from debulking to rare cell isolation. *Lab on a Chip*, **15**(5), 1230-1249.

Xiang, N., Zhang, X., Dai, Q., Cheng, J., Chen, K., Ni, Z. 2016. Fundamentals of elasto-inertial particle focusing in curved microfluidic channels. *Lab on a Chip*, **16**(14), 2626-2635.

Zanoli, L.M., Spoto, G. 2013. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. *Biosensors*, **3**(1), 18-43.

Zhang, J., Yan, S., Sluyter, R., Li, W., Alici, G., Nam-Trung, N. 2014. Inertial particle separation by differential equilibrium positions in a symmetrical serpentine micro-channel. *Scientific Reports*, **4**.

Zhang, J., Yan, S., Yuan, D., Alici, G., Nguyen, N.-T., Ebrahimi Warkiani, M., Li, W. 2016. Fundamentals and applications of inertial microfluidics: a review. *Lab on a Chip*, **16**(1), 10-34.

Zhao, Y., Park, S., Kreiswirth, B.N., Ginocchio, C.C., Veyret, R., Laayoun, A., Troesch, A., Perlin, D.S. 2009. Rapid Real-Time Nucleic Acid Sequence-Based Amplification-Molecular Beacon Platform To Detect Fungal and Bacterial Bloodstream Infections. *Journal of Clinical Microbiology*, **47**(7), 2067-2078.

Zhu, L., Zhang, Q., Feng, H.H., Ang, S., Chauc, F.S., Liu, W.T. 2004. Filter-based microfluidic device as a platform for immunofluorescent assay of microbial cells. *Lab on a Chip*, **4**(4), 337-341.

Zhu, L.D., Takala, J., Hiltunen, E., Wang, Z.M. 2013. Recycling harvest water to cultivate *Chlorella zofingiensis* under nutrient limitation for biodiesel production. *Bioresource Technology*, **144**(0), 14-20.

10. List of papers

Papers omitted from online edition due to publisher's restrictions

Paper 1

Hønsvall, B.K., Altin, D., Robertson, L.J. (2016). Continuous harvesting of microalgae by new microfluidic technology for particle separation. Bioresource Technology, 200, 360-365. doi: 10.1016/j.biortech.2015.10.046

Paper 2

Hønsvall, B.K., Robertson, L.J. 2017. Real-time nucleic acid sequence-based amplification (NASBA) assay targeting MIC1 for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts. Experimental Parasitology, 172, 61-67.

Paper 3

Hønsvall, B.K., Robertson, L.J. 2017. From research lab to standard environmental analysis tool: Will NASBA make the leap? Water Research, 109, 389-397.

Paper 4

Hønsvall, B.K., Robertson, L.J. (submitted). Washed away; minimising RNA losses during isolation. Submitted to Journal of Biomolecular Techniques

Paper 5

Hønsvall, B.K., Ezkerra, A., Gulliksen, A., Dong, T., Karlsen, F. (2013). Detection of oil-utilizing microorganisms by nucleic acid sequence-based amplification in a total analysis lab-on-a-chip device. in: 17th international conference on miniaturized systems for chemistry and life sciences, 2013, Freiburg, Germany, 27-31 Oct, 2013, Chemical and Biological Microsystems Society, pp. 1767-1769.

11. Appendix

11.1. Appendix A

This appendix contains parameters and results from testing of the Trilobite® separation chip.

Table A 1 Parameters set for the chip tests with 10 µm plastic particles

Test	Volume (ml)	Flow rate (ml min^{-1})	Particle concentration (particles ml^{-1})
1	10	2	71 615
2	10	2	120 443
3	10	2	144 531
4	10	2	294 054
5	20	1	91 580
6	20	2	91 580
7	20	4	91 580
8	20	6	91 580

Table A 2 Results of tests with plastic particles with various particle concentrations

Inlet (particles ml^{-1})	Volume *permeate* fraction (ml)	Volume *concentrate* fraction (ml)	*Concentrate* fraction (particles ml^{-1})	Concentration factor	Efficiency (%)
71 615	6.2	3.5	204 427	x 2.84	103
120 443	6.2	3.5	307 726	x 2.55	92
144 531	6.3	3.5	375 814	x 2.60	92
294 054	6.2	3.6	757 053	x 2.57	93

Table A 3 Results of tests with plastic particles and various flow rates. Inlet particle concentration was 91 580 particles ml^{-1}

Flow rate (ml/min)	Volume *permeate* fraction (ml)	Volume *concentrate* fraction (ml)	*Concentrate* fraction (particles ml^{-1})	Concentration factor	Efficiency (%)
1	14.3	5.6	260 796	x 2.85	80
2	15.1	4.9	327 094	x 3.57	86
4	15.0	5.0	382 813	x 4.18	105
6	13.8	4.6	365 992	x 4.04	101

Table A 4 Test results chip treatment of living R. baltica *suspensions through the Trilobite chip. V_i – inlet volume; C_i – inlet algal concentration; V_p –* permeate *volume; C_p –* permeate *algal concentration; V_c – volume concentrate; C_c – concentrate algal concentration; C_c exp – expected concentrate algal concentration; CF – concentration factor (of concentrate). Means of four parallels*

Test	V_i (ml)	C_i (cells ml^{-1})	V_p (ml)	C_p (cells ml^{-1})	V_c (ml)	C_c (cells ml^{-1})	C_c exp (cells ml^{-1})	effici-ency (%)	CF
1	20.2	116 536	13.1	92 719	7.0	101 508	333 684	30	0.87
2	19.7	286 675	9.5	201 226	10.2	326 009	555 066	59	1.14
3	17.2	483 941	9.3	487 522	7.9	605 469	1 050 816	58	1.25
4	19.2	480 252	10.7	509 115	8.5	647 135	1 082 956	60	1.35
5	20.1	51 649	5.0	54	15.1	65 321	68 623	94	1.26

Table A 5 Size distribution of living Chaetoceros*. The number of cells measured within each size category was counted, and percentage share was calculated accordingly.*

	Inlet		*Concentrate*		*Permeate*	
size	number	%	number	%	number	%
2-3 µm	0	0,0 %	0	0,0 %	1	0,4 %
3-4 µm	1	0,8 %	2	0,9 %	32	13,9 %
4-5 µm	1	0,8 %	11	4,7 %	37	16,1 %
5-6 µm	9	7,2 %	50	21,4 %	64	27,8 %
6-7 µm	34	27,2 %	52	22,2 %	56	24,3 %
7-8 µm	52	41,6 %	70	29,9 %	29	12,6 %
8-9 µm	16	12,8 %	20	8,5 %	9	3,9 %
9-10 µm	8	6,4 %	18	7,7 %	2	0,9 %
10-11 µm	2	1,6 %	10	4,3 %	0	0,0 %
11-12 µm	2	1,6 %	1	0,4 %	0	0,0 %
12-13 µm	0	0,0 %	0	0,0 %	0	0,0 %
total number	125		234		230	

Table A 6 Size distribution of fixated Chaetoceros. *The number of cells measured within each size category was counted, and percentage share was calculated accordingly.*

	Inlet		*Concentrate*		*Permeate*	
size	number	%	number	%	number	%
0-1 µm	0	0,0 %	0	0,0 %	0	0,0 %
1-2 µm	1	0,3 %	3	0,3 %	7	0,7 %
2-3 µm	9	2,5 %	20	2,0 %	52	5,2 %
3-4 µm	53	14,7 %	200	19,7 %	315	31,6 %
4-5 µm	78	21,6 %	199	19,6 %	232	23,2 %
5-6 µm	142	39,3 %	299	29,4 %	214	21,4 %
6-7 µm	48	13,3 %	172	16,9 %	113	11,3 %
7-8 µm	20	5,5 %	87	8,6 %	48	4,8 %
8-9 µm	7	1,9 %	16	1,6 %	15	1,5 %
9-10 µm	2	0,6 %	12	1,2 %	1	0,1 %
10-11 µm	1	0,3 %	6	0,6 %	1	0,1 %
11-12 µm	0	0,0 %	2	0,2 %	0	0,0 %
12-13 µm	0	0,0 %	1	0,1 %	0	0,0 %
total number	361		998		1017	

Table A 7 Recovery of Toxoplasma *oocysts in water samples after chip treatment with the Trilobite® chip and membrane filtration.*

	500 oocysts			1000 oocysts		
	Chip treated	*Membrane filtered*	*Control*	*Chip treated*	*Membrane filtered*	*Control*
	79	260	480	240	593	840
	80	129	480	240	228	660
	80	137	360	480	370	600
	80	140	360	400	426	360
	81	178	240	480	471	420
	320	242	240	720	583	480
Mean	120	181	360	427	445	560
StDev	98	57	107	180	138	177
Recovery	24 %	36 %	72 %	43 %	45 %	56 %

11.2. Appendix B

This part contains sequences of primers and probes designed for NASBA. The primer sets are targeting the protozoan parasites *Cryptosporidium* (primer sets were tested) and *Giardia* (primer sets were not tested with the appropriate assemblages).

Table B 1 Primers and probes used in this study, targeting MIC1 in Cryptosporidium parvum *and* Cryptosporidium hominis*. P1 = reverse primer, promoter sequence for T7 RNA polymerase underlined. P2 = forward primer. MB = molecular beacon, stem sequences underlined.*

	Name	Sequence	5' modification	3' modification
Primers	C.par MIC1 P1	AATTCTAATACGACTCACTATAGGGAGGAAGCCAAGATGACCATT		
	C.par MIC1 P2	TCATATAAACCAGAATCAGTAGGA		
	C.hom MIC1 P1	AATTCTAATACGACTCACTATAGGGCTATGACAAGGATAATATGGT		
	C.hom MIC1 P1	GATGTGGAATCTACAGGATA		
Probes	C.par MIC1 MB	CGCGATAGCAGGAGTGTATTCAACTACAATCGCG	FAM	BHQ-1
	C.hom MIC1 MB	CGCGATTGATGGATCTTGACTTGGTAGTTATCGCG	CAL Fluor Red 610	BHQ-2

Table B 2 Primers and probes used in this study, targeting heat shock protein 70 (Hsp70) for Giardia duodenalis*, assemblages A, B and E. P1 = reverse primer, promoter sequence for T7 RNA polymerase underlined. P2 = forward primer. MB = molecular beacon, stem sequences underlined.*

	Name	Sequence	5' modification	3' modification
Primers	Hsp70 Giardia A P1	AATTCTAATACGACTCACTATAGGGAGATTAACTGTCTTCTGGAT		
	Hsp70 Giardia A P2	CTGAACGATCTATTGGATGA		
	Hsp70 Giardia B P1	AATTCTAATACGACTCACTATAGGGAAACAGCATTATAATCAGACCAAT		
	Hsp70 Giardia B P2	CATATCCTCAGACGACATTA		
	Hsp70 Giardia E P1	AATTCTAATACGACTCACTATAGGGGAGGACTAATGGAGATTGTT		
	Hsp70 Giardia E P2	GATATACATGGAGACACTGA		
Probes	Hsp70 Giardia A MB	CGCGATAACTACGTGCATTGGTGGCTGAATCGCG	FAM	BHQ-1
	Hsp70 Giardia B MB	CGCGATCGAAGGGACGAACTACAAGACAATCGCG	CAL Fluor Red 610	BHQ-2
	Hsp70 Giardia E MB	CGCGATGCTGAACGATTGAAGAAGGCTCTATCGCG	FAM	BHQ-1

11.3. Appendix C

11.3.1. Growth of oil-utilizing bacterial strains

The oil-utilizing bacterial strains called A, B, C and D were grown in a Minimal salts medium separately and in consortium ABCD (consisting of all four strains) at 60 °C and 200 rpm in 250 ml baffled Erlenmeyer flasks containing 50 ml cultivation medium, in three parallels set-ups. The cultivation medium was made with and without addition of acetate ($C_2H_3NaO_2 \cdot 3H_2O$), any heavy crude oil was added directly to finalised flasks containing cultivation medium. The strains were grown in three media conditions: in medium with acetate; in medium with acetate added 2 % heavy oil; and in media lacking acetate added 2 % heavy oil. Heavy oil was heated to 60 °C and added into the flasks after inoculation. Bacterial growth was measured as optical density at 660 nm with a Synergy2 reader with associated Gen 5 software (BioTek Instruments, Inc., Winooski, VT, USA).

Cultures that were given heavy oil as sole carbon source had different growth patterns in the three parallel setups, with SD as high as 0.4 (strain A, mean OD_{660} value 0.6). The highest culture density was observed for strain A and the consortium ABCD (Figure C 1). Strains B and C did not show typical sigmoidal growth pattern, with low OD_{660} values – highest OD_{660} values for B and C were 0.18 and 0.25, respectively. Strain C peaked at 4-5 hours, declined, and peaked again after 10 hours. This was observed in all three parallels. For strain B, a less consistent pattern was observed. After 4-6 hours, the growth either inclined or declined, and after 8-10 hours the same pattern of either increasing or declining was observed. Either peaking or declining growth was also observed for strain D after 2-4 hours, before it stabilized. After 24 hours of inoculation with heavy oil, the consortium appeared as homogenous and grey, suggesting that the strains might collaborate in breaking down heavy hydrocarbon molecules.

Different growth patterns were observed with different carbon sources. All strains showed stronger growth when given acetate compared to heavy oil. When given acetate or a mix of heavy oil and acetate, classical sigmoidal growth curves were observed for all strains (Figure C 2). Strains B and C grew poorly with heavy oil as sole carbon source. Stronger growth was observed when these strains were given acetate as co-substrate in addition to oil. Strain D had more consistent growth patterns, with less difference

between growth on acetate and growth on heavy oil compared to the other strains. However, the D cultures were generally less dense than the others were.

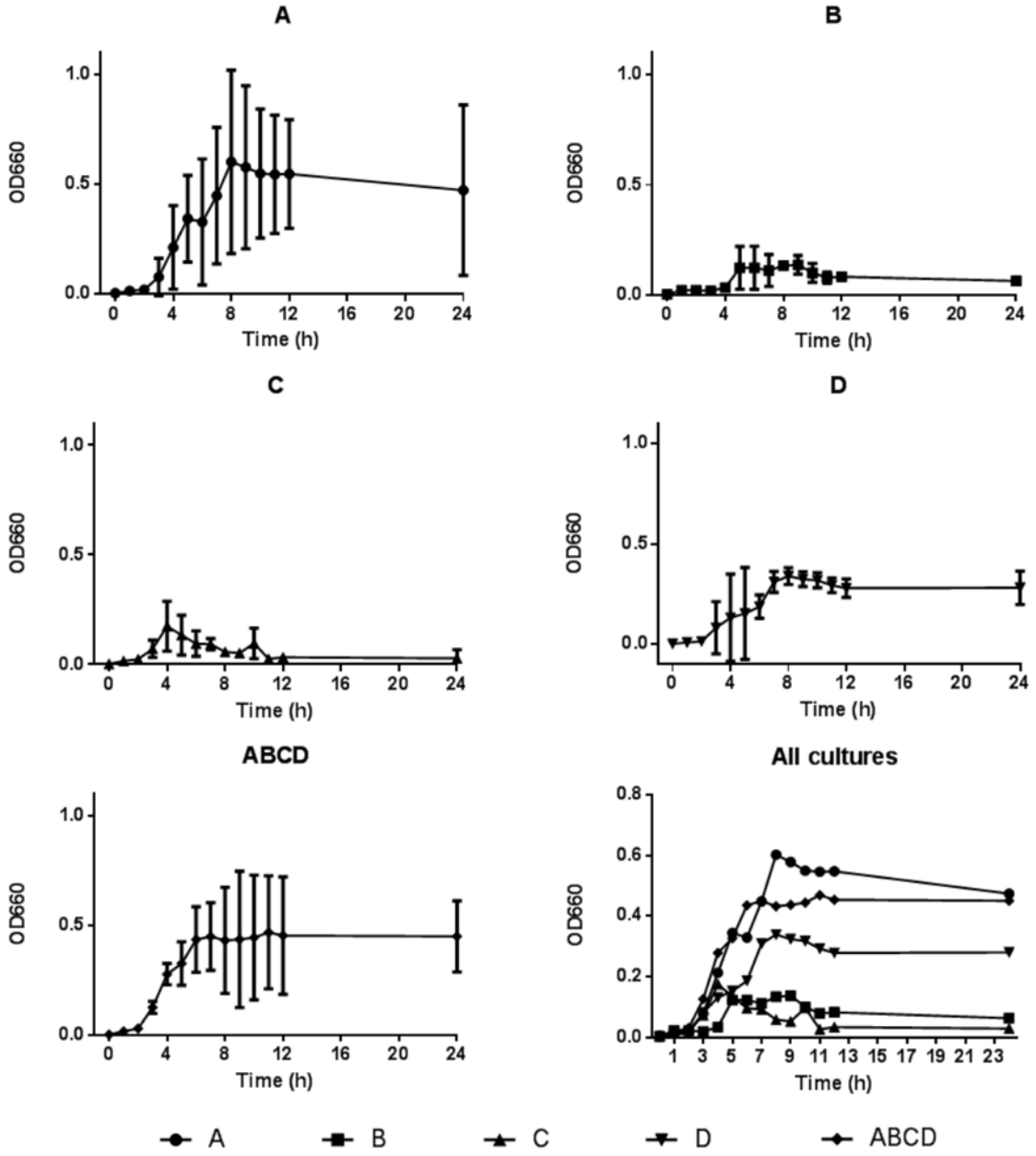

Figure C 1 Growth measured by optical density (OD660) for all strains as pure cultures and as consortium (ABCD), grown in 250 ml baffled flasks, with heavy crude oil as sole carbon source. Standard deviation (SD) is viewed as bars for A, B, C, D and the ABCD consortium. Three parallels were measured for each culture. The means of all cultures is compared together at the bottom right graph. Graphs were prepared in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA).

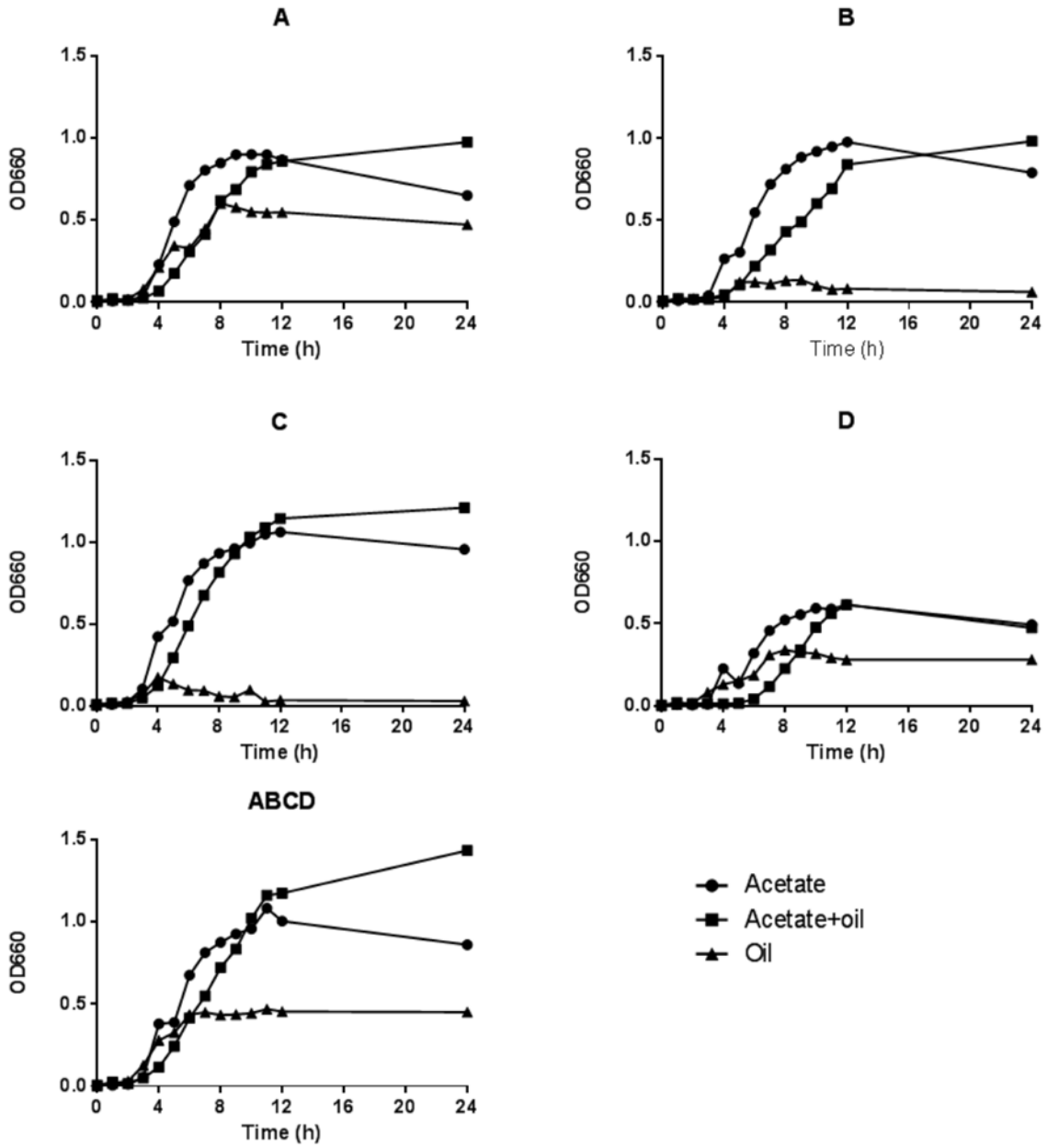

Figure C 2 Growth measured by optical density (OD660) for all strains as pure cultures and as consortium in 250 ml baffled flasks, with carbon source given as acetate only; acetate and heavy oil; and heavy oil only. The graphs display the means of three parallels.

11.4. Appendix D

This part contains methodological protocols for some standard protocols used in the parasitology laboratory.

11.4.1. Oocyst isolation from faeces sample (*Cryptosporidium*)

Before starting the isolation, check how much oocyst material you have in your sample:

Materials & reagents

Incubator
Microscope with fluorescence
Metal box with plastic rods and paper tissue
Glass slide
Cover glass
Monoclonal antibodies (MAB) for *Cryptosporidium*
Water or mounting media

Procedure

1) Do a smear of the faeces onto a microscope slide and let dry, *or*:
2) Add 5 µl sample onto a microscope slide and let dry
3) When dry, add a drop of methanol to fix the sample to the glass. Let the methanol evaporate
4) Add 5 µl MAB onto the fixed sample
5) Put the slide on top of the plastic rods in a metal box with damp tissue. Put on the box lid
6) Incubate for 30 minutes at 37 °C
7) Add a drop of water or mounting media, and cover the sample with a cover glass
8) Investigate the sample under the microscope (filter 1)

Isolation:

Materials & reagents

Fume hood
Centrifuge (Mega Star 1.6)
Vortexer

Centrifuge tubes (50 ml, 15-20 ml)
Tube racks
Pipette(s)
Water (tap water and DI water)
Membrane wash buffer (US.EPA. 1997 Method 1622 Draft)
Saturated NaCl solution

Procedure

1) Distribute your faeces sample in 50-ml tubes, preferably in the fume hood
2) Fill the tube(s) with 50 ml water and mix by vortexing
3) Centrifuge at 3000 rpm for 10 min
4) Discard the supernatant, leave approximately 5 ml pellet in the tube
5) Wash step:
    - Add approximately 5 ml wash buffer
    - Vortex to dissolve the pellet
    - Fill up the tube with water
    - Centrifuge at 3000 rpm for 10 min
    - Discard the supernatant, leave approximately 5 ml pellet in the tube
6) Repeat wash until you get a clear supernatant

It is advisable to check your sample for oocyst by MAB staining here.

7) Vortex to dissolve the pellet
8) Divide the suspended pellet in two centrifuge tubes (smaller and thinner tubes made it easier to see the interphase during the salt flotation)
    - Rinse the old tube with DI water and transfer to the new tubes to keep as much sample material as possible
9) Fill up the tubes with DI water
10) Centrifuge at 3000 rpm for 10 min
11) Discard the supernatant, as much as possible
12) Fill approximately 1/3 of the tube with saturated NaCl solution
    - Saturated NaCl solution is made by mixing 36 g NaCl in 100 ml water
13) Vortex to completely dissolve the pellet
14) Carefully fill the tubes with DI water. There should be two phases – one with salt water, and one with fresh water on top

15) Centrifuge at 3000 rpm for 10 min
16) Collect oocyst from the interphase between the salt and the fresh water with a pipette. Transfer to new tube(s)
17) Wash step:
    - Fill the tube up with DI water and vortex
    - Centrifuge at 3000 rpm for 10 min
    - Discard the supernatant (or keep, just in case)
18) Repeat wash one more time after collecting the pellets in one tube
19) Discard the supernatant, down to your desired volume

It is advisable to check your sample for oocyst by MAB staining at this stage too.

11.4.2. Cyst isolation by zinc sulphate flotation

The following protocol was used for isolation of *Giardia* cysts from faeces samples. It is advisable to check the samples for cysts material as described in section 11.4.1. Use MAB for *Giardia* instead of MAB for *Cryptosporidium*.

Materials & reagents

Fume hood
Centrifuge (Mega Star 1.6)
Vortexer
Centrifuge tubes (50 ml)
Tube racks
Pipette(s)
Water (tap water and DI water)
Membrane wash buffer (US.EPA. 1997 Method 1622 Draft)
$ZnSO_4$ solution (33 %)

Procedure

1) Mix faeces sample (10 g) and 50 ml tap water in a blender
2) Pour the mix through a 1250 µm meshed sieve into a bowl
3) Distribute your faeces sample in two 50-ml tubes, preferably in the fume hood
4) Fill the tubes with water and mix by vortexing

5) Centrifuge at 3000 rpm for 5 min
6) Discard the supernatant, leave approximately 5 ml pellet in the tube
7) Wash step:
    - Add approximately 5 ml wash buffer
    - Vortex to dissolve the pellet
    - Fill up the tube with water
    - Centrifuge at 3000 rpm for 5 min
    - Discard the supernatant, leave approximately 5 ml pellet in the tube
8) Repeat wash until you get a clear supernatant

It is advisable to check your sample for cysts by MAB staining here.

9) Vortex to dissolve the pellet
10) Add 25 ml $ZnSO_4$ solution to each tube
11) Vortex to completely dissolve the pellet
12) Carefully fill the tubes with DI water. There should be two phases – one with salt water, and one with fresh water on top
13) Centrifuge at 3000 rpm for 5 min
14) Collect cyst from the interphase between the salt and the fresh water with a pipette. Transfer to a new 50-ml tube
15) Wash step:
    - Fill the tube up with DI water and vortex
    - Centrifuge at 3000 rpm for 5 min
    - Discard the supernatant
16) Repeat wash one more time after collecting the pellets in one tube
17) Discard the supernatant, down to your desired volume
18) Stored the isolated cysts at 4 °C.

It is advisable to check your sample for cysts by MAB staining at this stage too.

11.4.3. Enumeration of *Toxoplasma* oocysts

The following protocol was used for enumeration of oocysts in the experiments using the Trilobite® chip for concentration of small numbers of *Toxoplasma* oocysts.

The procedures were carried out by fellow PhD student Jemere.

Materials & reagents

Oocyst suspension
Distilled water
KOVA Glasstic Slide 10 with Grid
Tubes
Vortexer
Centrifuge
Sodium dodecyl sulfate (SDS)

Details on the KOVA Glasstic Slide 10 with Grid are found in the instructions for use document (https://www.kovaintl.com/kca/wp-content/uploads/2014/01/DI-91064-17-final.pdf)

Procedure

1) Vortex mix the stock suspension containing *T. gondii* oocysts in a test tube.
2) Draw 1 ml and add to a test tube containing 9 mL distilled water.
3) Vortex mix very well.
4) Aspirate 1ml of the 10-fold diluted oocyst suspension and add to a new test tube containing 9 ml of 1 % SDS and mix well.
5) Allow to stand for 1 hour.
6) Centrifuge at 3000 rpm for 10 minutes and discard the supernatant fluid.
7) Re-suspend the pellet in 9 ml distilled water and repeat the centrifugation step.
8) Decant the supernatant fluid and add distilled water to a final volume of 10 ml.
9) Draw 8 µl aliquots and fill into each five wells of a KOVA Glasstic Slide 10 with Grid (Figure D 1) and place under a microscope.
10) Identify *T. gondii* oocysts using ultraviolet (UV) excitation for characteristic pale blue autofluorescence.

11) Count oocysts in all quadrants of the ruled area divided by grids (Figure D 2).
12) Estimate the mean number of oocysts in 1 µl aliquot of diluted suspension. NB: volume within grid area (ruled area) is 0.9 µl.
13) Finally, determine the average result of all five wells and use this to estimate for the required working concentrations.

Figure D 1 KOVA Glasstic Slide 10 with Grid

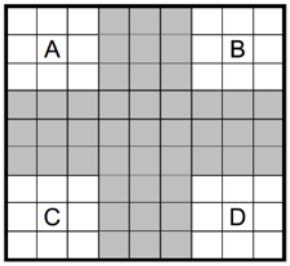

Figure D 2 Grid area in a well in the KOVA Glasstic Slide 10 with Grid

11.5. Appendix E

This part contains protocols used for experiments and preparations in the POCNAD system.

11.5.1. Nucleic acid extraction in the POCNAD cartridge

The following protocol was used for extraction of nucleic acids in the extraction chamber of the top part of the POCNAD cartridge. Reagents from the NucliSens® miniMAG® kit (Biomérieux, Lyon, France) were used, and the magnet in the POCNAD instrument was used to capture the magnetic silica beads. The extraction chamber had already been sealed at the bottom (facing the chip), and washed with RNase away and RNase free water to prevent contamination that could interfere with the reaction.

1) Transfer 1 ml sample in PreTect TM to an Eppendorf tube and centrifuge at 2,500 g for 5 min. Remove the supernatant; add 0.5 ml lysis buffer and vortex the tube. Incubate at room temperature for 10 minutes.
2) Add 50 µl silica beads to the tube, vortex and transfer the content to the Extraction chamber in the NASBA cassette.
3) Place the cassette in its holder in the prototype. Move the magnetic actuator up underneath the Extraction chamber, so the silica beads will be captured at the bottom of the chamber.
4) Discard the liquid phase, using a syringe, and move the magnet down.
5) Add 0.5 ml wash buffer 1 and mix. Move the magnet up.
6) Discard the liquid phase. Move the magnet down.
7) Add 0.5 ml wash buffer 2 and mix. Move the magnet up.
8) Discard the liquid phase. Move the magnet down.
9) Do a quick wash (15 seconds) with 0.5 ml wash buffer 3 (optionally lysis buffer). Mix, move the magnet up.
10) Discard the liquid phase. Move the magnet down.
11) Add 70 µl elution buffer (using a pipette) and incubate for 5 min. (This incubation should also include the heating element, to heat the Extraction chamber. The Biomérieux protocol states 60 °C.)

12) Move the magnet up.
13) Transfer the eluate (liquid phase) to a new labelled Eppendorf tube and store it in the deep freezer.

11.5.2. Freeze-drying of NASBA reagents

One of my fellow project co-workers carried out preparation of freeze-dried reagents.

Two types of freeze-dried reagent spheres were prepared in order to store reagents in the POCNAD chip:

- Primers, probes and NASBA reagents for the pre-NASBA chambers
- Enzymes for the NASBA chambers

Pre-NASBA sphere-mix was prepared by dissolving 2 Reagent spheres (NucliSens® Basic kit, Biomérieux, Lyon, France) in 42.43 µl nuclease free water. Of this mixture, 25.5 µl was mixed with 11.4 µl Tris-HCl (1003 mM), 15.24 µl sucrose (51 %), 10 µl primer mix (10 µM of each primer) and 2.5 µl molecular beacon (20 µM).
Enzyme sphere-mix was prepared by dissolving 4 Enzyme spheres (NucliSens® Basic kit, Biomérieux, Lyon, France) in 122.4 µl trehalose (20 %).

Reagents and enzyme spheres were prepared by dispensing 3-µl droplets and 500-nl droplets with a eVol pipette (SGE Analytical Science, Victoria, Australia) near the surface of a metal block keeping -80 °C to freeze them, then put into 3 ml vials with split stoppers (7575210 and 7576010, Labconco Corporation, Kansas City, MO, USA). The frozen reagents were freeze-dried in the Triad™ Freeze Dry System (7400030, Labconco Corporation, Kansas City, MO, USA) with the following program:
Pre freezing for 24 h until -80 °C was reached; insertion of vials with frozen reagents; ramp 1.0 °C/min to -55 °C (1h); ramp 0.5 °C/min to -10 °C (1h); ramp 1.0 °C/min to 10 °C (1h); ramp 1.0 °C/min to 20 °C (1h); ramp 1.0 °C/min to 25 °C (∞).
Vacuum was set to 0.002 mBar throughout the program.

11.5.3. Preparation of chips with reagents

The following protocol was used to prepare POCNAD chips with freeze-dried reagents. Freeze-dried spheres of primer- and probe mix, and enzyme mix were placed into the pre-NASBA chambers, and NASBA reaction chambers of the chip, respectively.

Materials & reagents

RNase away
RNase free water
Laboratory wipes
Laboratory oven (250 °C)
Sterile cabinet
Aluminium foil
Glass beakers
Forceps (pinsetter)
Metal mould for chip assembly
Roller
PCR film
Silicone sheet (slightly larger than the chips)
RNase free chips
RNase free valve disks (COP)
Freeze dried spheres of reagents and enzymes

Preparations

Wrap forceps, metal mould and the top of glass beakers in aluminium foil. Put the wrapped glass and metal equipment in an oven at 250 °C for at least 2 hour. This baking procedure will denature enzymes and, hence, make the equipment RNase free.

After baking, take the equipment out of the oven and cool it to room temperature before using it.

Open the sash in the sterile cabinet to working position and turn on the fan. When the cabinet is ready (after 3 min), wipe the inside of the cabinet down, first with ethanol, then RNase away. Wipe away residues of RNase away with RNase free water.

Place the equipment inside the cabinet, after giving it the same treatment as the inside of the sterile cabinet. As the roller is partly made of plastic and cannot be baked, wash this carefully before putting it inside the sterile cabinet. Carefully wash the silicone sheet too.

It might be convenient to have the top of a large pipette container as a box inside the cabinet. Wash this careful as well.

Procedures

Using the mould, prepare one side of a chip with valves and PCR film as described in "Bonding Protocol Slides". Lay the chip with the PCR filmed side down. Place the COP disk on the chip in their respective places.

Use the forceps to transfer spheres of reagents and enzyme spheres to the pre-NASBA chambers (Figure E 1) and the NASBA chambers (Figure E 2) in the chip, respectively. Carefully place the PCR on the chip and bond. Use the roller and silicone sheet to bond the film properly and remove bubbles.

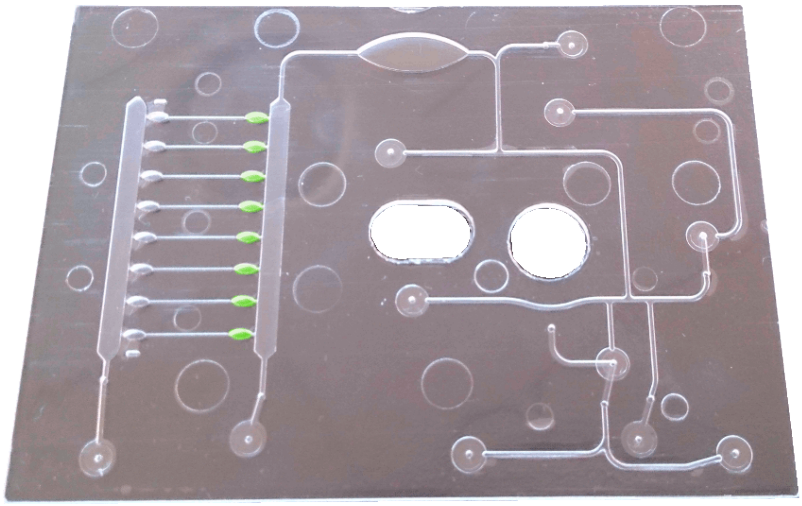

Figure E 1 Pre-NASBA chambers. Place reagents spheres inside these

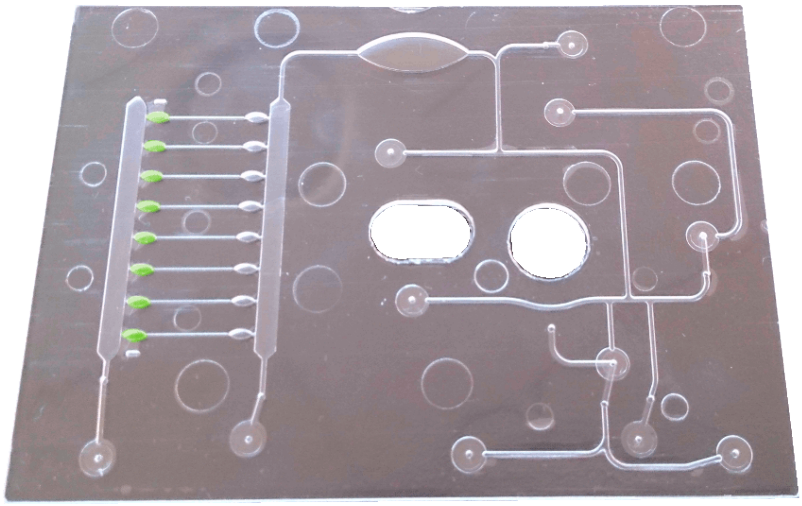

Figure E 2 NASBA chambers. Place enzyme spheres inside these.

11.5.4. POCNAD GUI

A graphical user interface (GUI), or software, to control the POCNAD instrument was developed by CDD Ltd. (Athens, Greece). The following gives an overview of the GUI applications used to control the instrument components.

Three tabs were mainly used: the configuration tab, the analysis tab, and the manual control tab.

The configuration tab (Figure E 3) included elements for connecting the GUI to the POCNAD instrument. The IP address was matched with an IP address in the LCD screen of the POCNAD instrument, and Localhost was check off in the Database section. The connection was made by clicking the Connect button in the Analysis tab. Once connected to the instrument, the Connect button changed to Disconnect, as seen in Figure E 4.

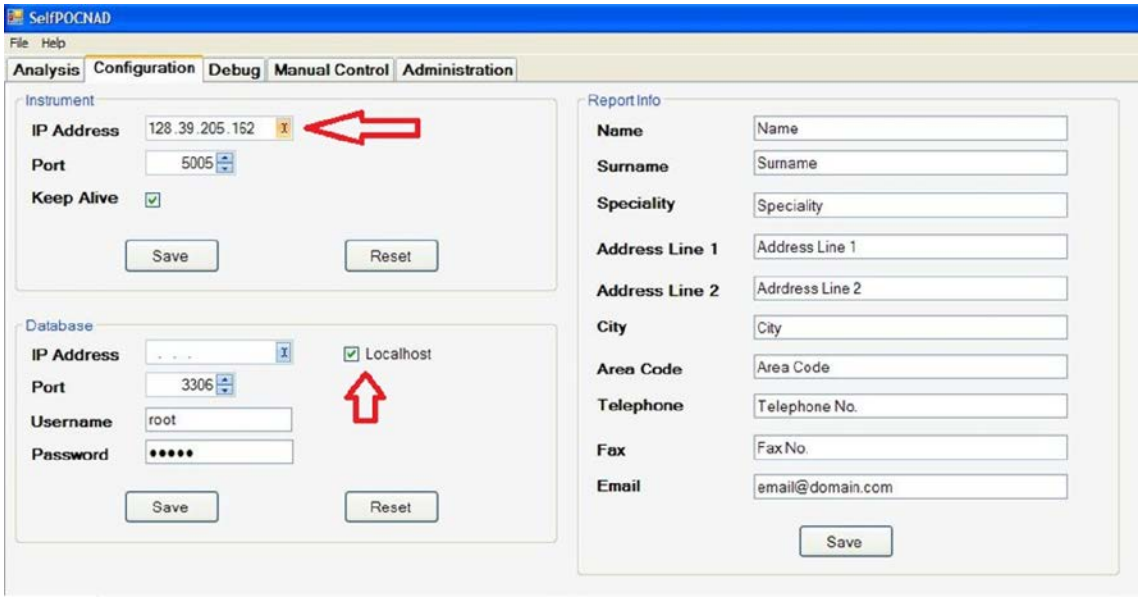

Figure E 3 The configuration tab in the POCNAD GUI

The analysis tab displayed temperature and real-time fluorescence readings of two fluorophores (FAM, ~590 nm, and CAL Fluor Red, ~470 nm). When the temperature of the different heat sources reached the required temperature, the displayed thermometers in the tab turned from red to green.

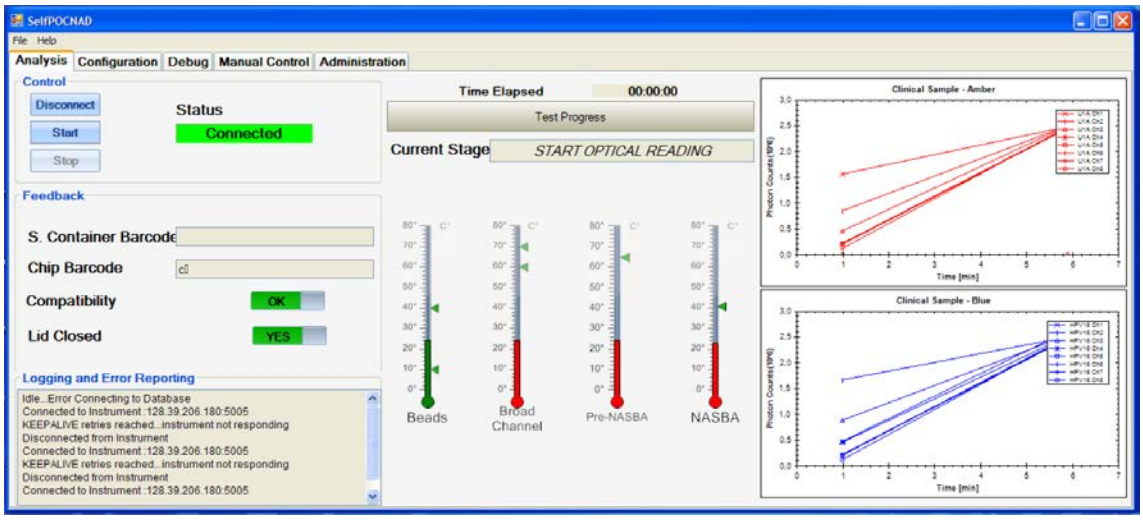

Figure E 4 The analysis tab in the POCNAD GUI

The manual control tab (Figure E 5) contained the element for control of each component in the POCNAD instrument.

MPPC controlled the motor and the optical detection system in order to read fluorescence in the chip's NASBA chambers. This could be set to read one round of all 8 chambers, or a full 2.5 h read.

Valves controlled the pin actuators that pressed down (ON) or released (OFF) the valve disks in the chip. Each pin actuator was chosen in a pull-down menu, and controlled separately.

NASBA Motor controlled the MPPC motor as well; this was used to fine-tune the position of the fibre optical cables in the optical detection system.

Pump controlled the peristaltic pump. This could be turned on and off, or turned on in a set time interval.

Actuators was used to control the movement of the magnet. A "Chip", and a "sample container" could also be chosen in the pull-down menu, but these were not used.

In the LED element, the red and blue lights in the optical system could be turned on and off, independent of the MPPC.

Thermal control controlled the heating elements in the instrument. Each could be chosen in the pull-down menu and set to the desired temperature.

The elements General, Wash 1 and Wash 2 (Figure E 5) were not used.

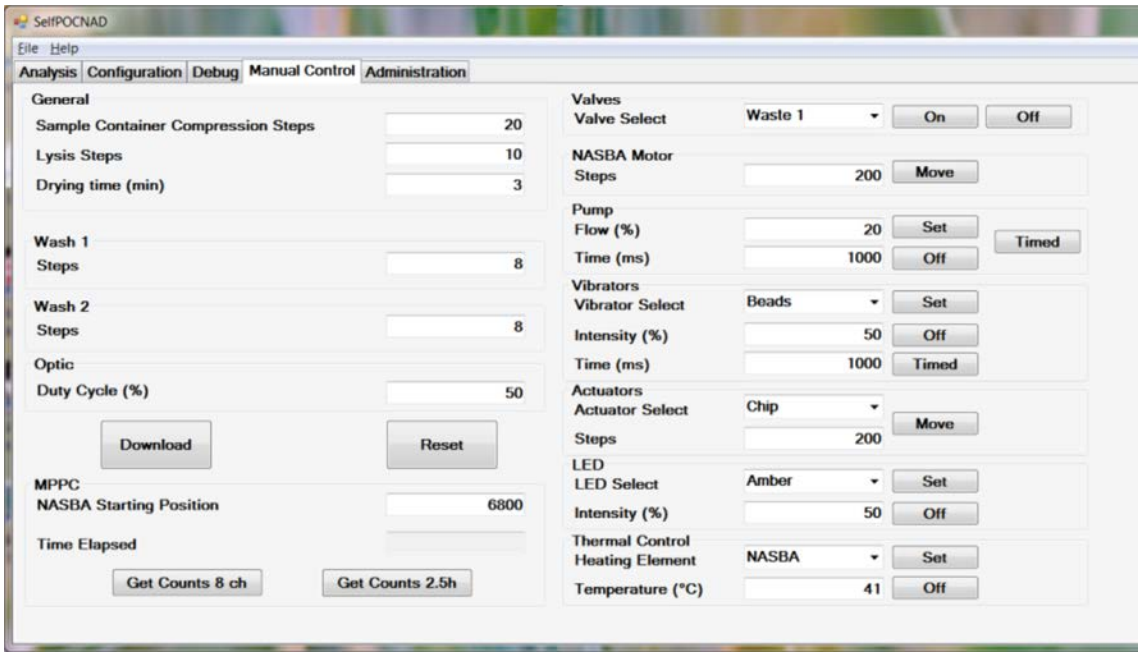

Figure E 5 The manual control tab in the POCNAD GUI

Errata

Abstract

Proved difficult → proved to be challenging

p.1

The intention of POCNAD for the analyses descried was to… → The intention of POCNAD analyses was to…

Due to other collaborations… → After discussion with other collaborators,…

p.4

The publications resulting from my work describe~~s~~ …

The Trilobite® system is focused on in Paper 1 → The Trilobite® system is presented in Paper 1

C. parvum, C. hominis → are now written in italic (*C. parvum*, *C. hominis*)

p.5

results for further publications was → were

p.10

molecular methods are need → needed

p.19

Assemblage A and B → Assemblages A and B

Figures 6 and 7

Approximate scale bars have been included in the figures

p.20

5 µm in dimeter → diameter

p.23

passive techniques use filters, inertial forces, and adhesion mechanisms to separate particles, whereas active techniques apply external forces, →passive techniques rely on internal characteristics and can use […]

p.24

Microfluidics is concerned with how fluids… → relies on

Figure 8B

The smallest particles have been moved away from the channel walls (ref. committee: “what are the small particles in B?- if they are smaller than a critical diameter they do not normally focus against the channel wall”)

p.29

may insert the incorrect ~~the~~ nucleotide

p.30

signal detection need not → does not require

p.47-48

The sample inlet… → the paragraph that was repeated twice has been removed

Figure 23

The channel length, width and height has been specified in the caption: “The main channel is 30 mm long, 10 mm wide and ~500 µm high.”

p.65

This strain was ~~project~~ ...

Figure 29

Particle concentrations of plastic particles before (inlet) and after (concentrate) chip treatment, with various inlet concentrations (A) and flow rates (B). → Particle concentrations of 10-µm plastic particles before (inlet) and after (concentrate) chip treatment, with various inlet concentrations (A) (71 615 (a); 120 443 (b); 144 531 (c); and 294 054 (d) particles ml^{-1}, respectively) and flow rates (B) (1; 2; 4; and 6 ml min^{-1}, respectively). Further details can be found in Tables A2 and A3. Standard errors of means (SEM) for concentrate and permeate are indicated. Asterisks indicate significant differences from inlet concentration.

Figure 31

The information “Standard errors of means (SEM) for concentrate and permeate are indicated. Asterisks indicate significant differences from inlet concentration; plus signs indicate significant differences from concentrate concentration.” was added to the caption.