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# Summary

We investigated the capabilities of the FilmArray gastrointestinal panel to detect bacterial and parasitical agents in contaminated river water. The FilmArray system is based upon molecular methods using a nested multiplex PCR to detect a predefined range of pathogens (virus, bacteria, parasites, yeasts, biotoxins) depending on the detection panel selected. The FDA has currently approved five of the panels for use in clinical diagnostics: respiratory – EZ and RP2; blood culture identification; meningitis encephalitis; and gastrointestinal. In addition to these panels FilmArray also has a Biothreat panel that FFI uses as part of the screening process at our CBRE laboratory. FilmArray uses a simple short sample preparation step prior to the pouch being inserted into the analytical instrument. Analysis time per sample is just 1 hour.

We wanted to compare FilmArray's gastrointestinal panel against standard methods for detecting waterborne pathogens causing gastroenteritis. We carried out all the experiments using *Campylobacter jejuni*, *Yersinia enterocolitica*, *Giardia duodenalis* and *Cryptosporidium parvum*. These agents were chosen because they are zoonotic (can infect animals and people) as well as being known causes of waterborne disease outbreaks in the Nordic region and further afield. Standard culture-based detection and identification methods for these agents require laboratory personnel with considerable experience and knowledge of the different methods used for each pathogen given that these methods are not straightforward and have long turnaround times (more than 24 hours in some cases).

We contaminated autoclaved surface water, obtained from a local river, with low and high levels of the four biological agents. We then compared detection sensitivity between the different methods. Moreover, FilmArray was able to detect the pathogens at the concentrations used in this study when samples were subject to filtration prior to analysis. However, the sample size investigated needs to be increased. FilmArray has the benefit of simpler sample preparation and shorter analysis times compared to the conventional methods tested. The next step would be to carry out further work on a wider range of sample concentrations using a sufficiently large sample size to ensure that statistically significant limits of detection could be ascertained. Another question to be addressed is whether FilmArray will work as well in the field under less ideal conditions compared to its performance on a laboratory bench. This will establish whether this analytical tool would be appropriate for use in a deployable laboratory unit to support food safety controls as well as epidemiological investigations of disease outbreaks.

FilmArray is a simple-to-use analysis system capable of simultaneously detecting multiple pathogens in complex samples. This makes it ideal for rapid screening in cases where time and or resources are limited with regard to sample preparation and analysis.

# Sammendrag

Vi har gjort innledende undersøkelser av ytelsen til multiplex PCR-systemet FilmArray med tanke på hurtig påvisning av gastrointestinale («mage-tarm») parasitter og bakterier i forurenset elvevann.

FilmArray-systemet baserer seg på gjenkjennelse av nukleinsyrer (nestet multiplex PCR) som er spesifikke for et forhåndsdefinert sett med 15–25 utvalgte patogener (bakterier, virus, parasitter). Ulike testreagenser (paneler) er utviklet for ulike diagnostiske formål og med ulike typer klinisk prøvemateriale, herunder luftveispanelet (respiratory panel), blodpanelet (blood culture identification panel), hjernepanelet (meningitis encephalitis panel) og mage-tarm-panelet (gastrointestinal panel). Videre finnes det et eget panel for påvisning av høypatogene biotrussel-agens i miljøprøver (biothreat panel) som benyttes rutinemessig ved FFIs CBRE-laboratorium.

I vår studie ble mage-tarm-panelet til FilmArray sammenliknet med konvensjonelle standardmetoder. Alle testene i studien ble utført med to bakteriearter (*Yersinia enterocolitica* og *Campylobacter jejuni*) og to parasittarter (*Giardia lamblia* og *Cryptosporidium parvum*). Agensene har flere fellestrekk: De smitter mellom dyr og mennesker (zoonotiske), de er krevende å påvise med standard metodikk og de er hyppig årsak til utbrudd av næringsmiddelbåren mage-tarm-sykdom hos mennesker.

Vi gjennomførte tester på forurenset overflatevann fra Nitelva. Vannet ble kontaminert med to ulike (høy/lav) konsentrasjoner av de fire utvalgte agensene. Deretter utførte vi sammenliknende eksperimenter med ulike metoder for prøvepreparering og analyse. Resultatene viste at FilmArray overveiende hadde like god påvisningsevne sammenliknet med de konvensjonelle metodene og ved de aktuelle konsentrasjonene som ble benyttet. En utvidet studie med bruk av flere paralleller og konsentrasjoner vil være nødvendig for å fastsette statistisk gyldige deteksjonsgrenser. Videre vil det være relevant å undersøke hvor egnet instrumentet er under mer primitive feltforhold og som del av flyttbare laboratorier i forbindelse med næringsmiddelkontroll, diagnostikk og utbruddsoppklaring.

FilmArray er et enkelt analysesystem for direkte påvisning av multiple biologiske agens i komplekse prøver ved at den integrerer prøvepreparering, DNA/RNA-ekstraksjon, amplifisering og analyse i et enkelt og lukket system. Dette har klare fordeler dersom en har behov for hurtig screening av en prøve for multiple agens og har begrenset med infrastruktur til prøvepreparering.

# Contents

# 1 Introduction

## 1.1 Background

Screening water samples for multiple pathogens can be challenging given the wide range of potential bacterial, viral and parasitic species, which can cause waterborne gastrointestinal disease. Laboratory assessments of water quality generally focus on indicator organisms as a proxy for faecal contamination. However, disease outbreaks have been reported without the detection of raised indicator bacteria (Pitkänen 2013). In the case of a suspected waterborne outbreak the pathogen may be detected in clinical cases but given the lag between infection and appearance of clinical signs, may no longer be detectable in the water (DeFraites et al. 2014). To complicate matters further some of the pathogens can enter a viable but non-culturable state (VBNC) so culturing is not possible for reliable detection of these pathogens (Pitkänen 2013; Bronowski et al. 2014). Standard methods for the screening of water samples often include culture/enrichment/purification steps which can take up to 48 hours, depending on the protocol, to provide results. BioFire FilmArray has developed a gastrointestinal panel for the rapid detection (1 hour) of 22 gastrointestinal pathogens (Figure 1.1) in clinical samples (Spina et al. 2015). This panel has to our knowledge not been validated for the screening of environmental samples.

### 1.1.1 Selection of pathogens

The choice of agents focuses on four pathogens that have been reported in suspected waterborne disease outbreaks either in Norway or abroad. We used two bacterial species (*Campylobacter jejuni* and *Yersinia enterocolitica*) and two protozoan species (*Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) and *Cryptosporidium parvum*) in this study.

| Bacteria | Viruses | Parasites |
|---|---|---|
| *Campylobacter (jejuni, coli, upsaliensis)* | Adenovirus F 40/41 | *Cryptosporidium* |
| *Clostridium difficile* (toxin A/B) | Astrovirus | *Cyclospora cayetanensis* |
| *Plesiomonas shigelloides* | Norovirus GI/GII | *Entamoeba histolytica* |
| *Salmonella* | Rotavirus A | *Giardia lamblia* |
| *Vibrio (parahemolyticus, vulnificus and cholerae)* | Sapovirus (I, II, IV and V) | |
| *Yersinia enterocolitica* | | |
| E.coli O157 | | |
| Enteroaggregative *E.coli* (EAEC) | | |
| Enteropathogenic *E.coli* (EPEC) | | |
| Enterotoxigenic *E.coli* (ETEC) lt/st | | |
| Shiga-like toxin-producing *E.coli* (STEC) stx1/stx2 | | |
| *Shigella* /Enteroinvasive *E.coli* (EIEC) | | |

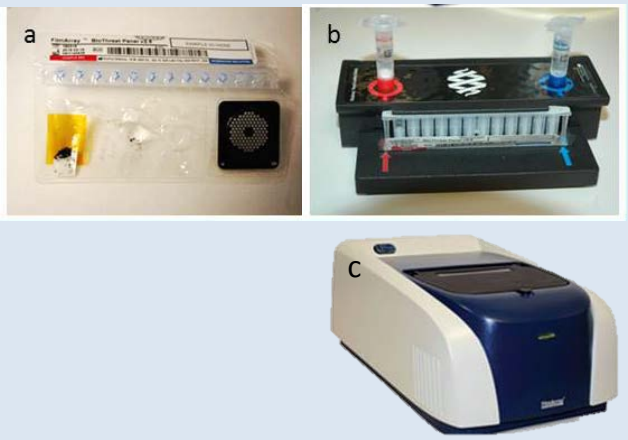


*Figure 1.1 FilmArray gastrointestinal (GI) panel showing the agents that can be detected as well as the test pouch (a), the sample preparation set-up (b) and the instrument (c).*

### *Campylobacter* spp.

*Campylobacter* are gram negative zoonotic rods that are globally one of the most common bacterial causes of food poisoning. *Campylobacter* prefers microaerophilic growth conditions within a temperature range of 30-45 ºC. According to the Norwegian Public Health Institute wild birds are common reservoirs of infection and transmission to people is often via untreated water, poultry products and unpasteurised milk (www.fhi.no). This species is capable of surviving extended periods at cooler temperatures although it does not reproduce outside of the host. Many waterborne disease outbreaks in the Nordic countries have identified *Campylobacter* as the causative agent (Pitkänen 2013; Kuhn et al. 2017; Guzman-Herrador et al. 2015) including in Røros in 2007 where a total of 1500 people were infected. Another *Campylobacter* outbreak on a military base was suspected to have originated from the base's water tower (DeFraites et al. 2014). *Campylobacter* spp. is difficult to culture from water samples and often forms viable but non-culturable (VBNC) forms (Pitkänen 2013; Bronowski et al. 2014). Mouse bioassays reveal that the VBNC bacteria are capable of reactivation in the host whereas in-vitro culturing remains unsuccessful (Baffone et al. 2006). The infectious dose during natural outbreaks and in clinical studies has been reported to be as low as 500 organisms (Robinson 1981; Kothary et al. 2001). Chlorination of the water is one measure to reduce infection levels however it is vital that the concentration of the chlorine is high enough (Richardson et al. 2007). Treatment of clinical cases relies on antibiotics with resistance testing for selecting the most suitable therapeutic (Moore et al. 2006). The Nordic standard methods for detection and

enumeration of *Campylobacter* in food and water samples are NMKL119 (culture-based) and NordVal No 017 (real-time PCR).

### *Yersinia enterocolitica*

*Yersinia enterocolitica* is a gram-negative, rod-shaped bacterium belonging to the *Enterobacteriacea* family. It can be characterized and divided into multiple serotypes based on the LPS O antigen. The main reservoir for the human pathogenic strains of *Yersinia enterocolitica* is pigs where it has been detected in the pharynx and intestines. *Yersinia enterocolitica* is psychrotrophic and capable of reproducing at refrigeration temperatures (Robins-Browne 2013) and easily survive in cold water (Terzieva and McFeters 1991).The serotypes that most commonly cause disease outbreaks in humans are O:3 (Scandinavia), O:8 and O:9 (Robins-Browne 2013). Delayed symptoms like autoimmune related arthrosis, in addition to the acute gastrointestinal symptoms, are reported (Winblad 1975).This species has been documented as the causative agent of a number of foodborne disease outbreaks and it has been detected in samples from water treatment plants (Waage *et al.* 1999). In 2014 *Yersinia enterocolitica* O: 9 was linked to a large outbreak in the northern part of Norway, with 130 reported cases, among these 114 at military bases. The source of infection was suspected to be imported lettuce (Norwegian Institute of Public Health 2014). The oral infectious dose is considered to be more than $10^8$ bacteria and studies indicate that it is capable of surviving for at least a year in water at -4°C to 10°C (Public Health Agency of Canada 2011). *Yersinia enterocolitica* is naturally resistant to penicillins and narrow-spectrum cephalosporins (Public Health Agency of Canada 2011). The Nordic standard methods for detection and enumeration of *Yersinia* in food and water samples is NMKL 117 (culture based) and NMKL 163 (real-time PCR) with 48h of selective enrichment prior to (semi)-quantitative and qualitative analytical result.

### *Giardia duodenalis*

The cosmopolitan protozoan parasite *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) is considered to be one of the most common parasitic causes of diarrhoea in humans (Halliez and Buret 2013).The largest outbreak, with 1500 confirmed cases that has been registered in Norway occurred in Bergen in 2004 when the water supplies were accidentally contaminated. Follow-up of the patients involved in this outbreak has revealed the potential for this parasite to cause long-term disease like irritable bowel syndrome and chronic exhaustion (Hanevik *et al.* 2014). The small oval *Giardia* cysts (8-10 x 7-10 µm; Gjerde 2011) can be transmitted via water, in which they can survive for months and can survive chlorination, or via food or direct contact with contaminated surfaces (Guzman-Herrador *et al.* 2015). There are multiple *Giardia* genotypes but only genotypes A and B infect humans (García-Cervantes *et al.* 2017). However these two genotypes can also be isolated from domestic animals. The infective dose is uncertain but experimental studies suggest that as few as 10 cysts can cause clinical disease (CDC 2015, Public Health Agency Canada 2015). Benzimidazoles (like albendazole) and 5-nitromidazoles

(like metronidazole) are often used to treat symptomatic *Giardia* infections (Escobedo *et al.* 2016). Norway does not require the routine testing of water for parasites (VKM 2009; Lovdata 2016). Method 1623 from the United States Environmental Protection Agency (EPA 2005) using filtration, immune-mediated separation and fluorescence staining for the detection and enumeration of *Giardia* cysts and *Cryptospordium* oocysts is used as the standard detection method in water samples.

#### ***Cryptosporidium* spp.**

*Cryptosporidium* is a protozoan parasite which can infect humans and other mammals as well as birds and reptiles (Ryan *et al.* 2014). *Cryptosporidium hominis* is the species that is generally associated with human infections however zoonotic infection with *C. parvum, C.muris, C. canis, C. cuniculus, C. felis* and *C. meleagridis* is also reported (ECDC 2014). Transmission occurs when high numbers of the small infectious oocysts (4-5µm, with 4 sporozoites) are excreted in the faeces (Gjerde 2011) and ingested by a new host who can go on to develop fever, diarrhoea and abdominal pain. There is currently no effective treatment for *Cryptosporidium*, other than symptomatic. The infection can be particularly persistent and life-threatening for immunocompromised people. The largest waterborne outbreak recorded occurred in Milwaukee during the 1990's whereby more than 400 000 people developed clinical disease and *C. hominis* was identified as the culprit (Corso *et al.* 2003). The oocysts can survive in water for a number of months (King and Monis 2007) and water treatment processes like sand filtration (due to the small size of the cysts) and chlorination are not fully effective at removing *Cryptosporidium* (Betancourt and Rose 2004). UV treatment and membrane filters are required to inactive and remove the oocysts (Betancourt and Rose 2004). Although the infective dose can be as low as 10 oocysts, the reported levels of water contamination in one outbreak was 0.19 oocysts/litre (Chalmers 2012; Puleston *et al.* 2014). Evaluation of drinking water in Norway in the late 1990's found *Cryptosporidium* and/or *Giardia* in one quarter of the samples tested (VKM 2009). The reported levels of contamination were low, just 1-3 cysts/oocysts per 10 litres. Detection of these oocysts in water uses the same standard method as for *Giardia* (EPA 1623).

## 1.2 FilmArray

### 1.2.1 Background on principles of analysis

FilmArray® is an automated system developed for the rapid detection and identification of multiple pathogens in a single sample. This is achieved by the integration of multiple steps of sample preparation and analysis in a closed system (Figure 1.2). Detection/identification is nucleic acid- based and relies on the principle of nested multiplex-polymerase chain reaction (PCR) and amplicon melt curve analysis targeting one or more agent-specific regions of the pathogen's genome. Minimal hands-on time (2-5 minutes) is required and the qualitative results are ready within approximately one hour of run time. The sample (200 µl) is mixed with the buffer solution provided in the panel. This is then added to the pouch where the entire PCR

analysis occurs without any further external input. The pouch is placed in the FilmArray® machine and the results are available after approximately an hour. The pouches contain freeze-dried reagents, which give a long-shelf life and allow them to be stored at room temperature.

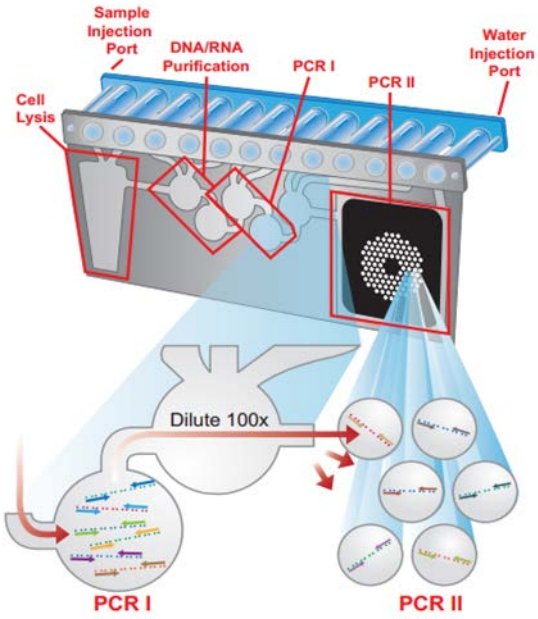


*Figure 1.2 FilmArray principle of analysis (http://www.biomerieux-diagnostics.com).*

BioFire FilmArray has developed several assays for both clinical diagnostic and biodefense purposes, such as the Respiratory, Blood culture (BCID), Meningitis/Encephalitis (ME); Gastrointestinal and Biothreat panel. The latter has been set up as a capability of FFIs integrated CBRE preparedness laboratory for the analysis of "unknown" environmental samples.

The FilmArray Gastroentestinal (GI) panel was developed by BioFire diagnostics for fast and simple detection & identification of 22 different microorganisms that cause gastroenteritis in humans (Figure 1.1) (Buss et al. 2015; Spina *et al.* 2015). The panel received U.S. Food and Drug Adminiatration (FDA) clearance in 2014. The results are reported as detected/not detected for all 22 agents.

## 1.3 Aim of study

We wanted to investigate the suitability of BioFires' FilmArray gastrointestinal panel for the rapid screening of contaminated river water for multiple pathogens. We carried out a series of pilot studies to optimize the methodology in order to enhance the sensitivity of the method. Standard culture-based methods for the detection of *Campylobacter* and *Yersinia* in food and environmental samples as well as the EPA method for the detection of the protozoan parasites

*Cryptosporidium* and *Giardia* in water (Method 1623) were used as reference methods for comparison.

# 2 Materials and methods

## 2.1 Study design

Autoclaved surface water from the local river Nitelva was spiked with four different GI pathogens (*Campylobacter, Yersinia, Giardia and Cryptosporidium*).
Two different concentrations were used:

- HIGH: $10^5$ cfu/100mL *Campylobacter* and *Yersinia* plus 100 cyst/100mL *Giardia* and 100 oocyst/100mL *Cryptosporidium*.

- LOW: $10^3$ cfu/100mL *Campylobacter* and *Yersinia* plus 50 cysts/100 mL *Giardia* and 50 oocysts/100 mL *Cryptosporidium.*

Total volume of the water sample was 200 mL. Each sample was divided into two 100 mL subsamples prior to filtering, one for the parasitological analysis and one for the bacterial analysis. Samples were analysed for pathogens:

i) prior to filtration

ii) after filtration (filter eluate)

iii) after filtration+ enrichment (bacteria)

iv) after filtration+ IMS (parasites)

The results were compared with standard methods for detection.
All samples were analysed in triplicate. Autoclaved surface water was used as negative control throughout the study. An overview of the experimental set up of the study is shown in Figure 2.1.

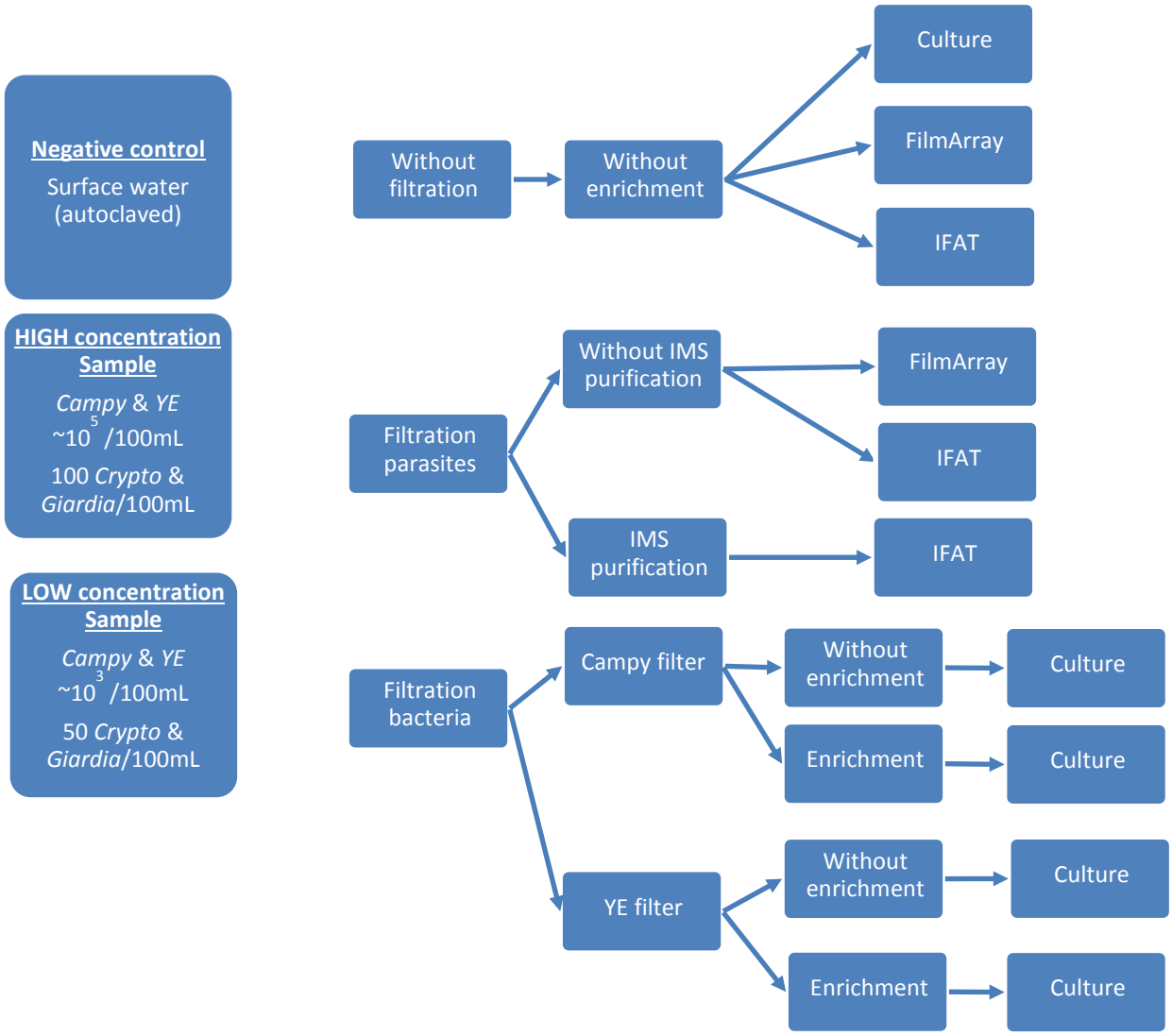


*Figure 2.1 Experimental set-up.*

## 2.2 Surface water sampling

Surface water was collected from the southern part of the river Nitelva (Lillestrøm, Akershus, Norway) where it runs through the municipality of Skedsmo. The water was dispensed in 1 L glass bottles autoclaved at 121 °C for 20 min and stored refrigerated (4-8 °C) prior to use. The recorded optical density ($OD_{500nm}$) of the water was 0.0393. The purpose of using natural surface water was to provide a water matrix that could reflect a non-optimal source of drinking water.

## 2.3 Bacterial strains

The following bacterial strains were used:

1. *Campylobacter jejuni* (44-1 Granum NVH).

2. *Yersinia enterocolitica* (VI54954, FHI1111-0495-1).

## 2.4 Preparation of bacterial stock solutions

Freeze cultures of *Campylobacter jejuni* (44-1 Granum NVH) and *Yersinia enterocolitica* (VI54954, FHI1111-0495) were plated on Colombia sheep blood agar (Oxoid ref:PB5008A) and controlled for purity. *Campylobacter* was grown under microaerophilic conditions for 48h at 41 °C while *Yersinia* was grown under aerobic conditions for 24h at 37 °C.
Stock solutions were freshly prepared for each experiment by inoculating a loop of single colony material in 1mL autoclaved (121 °C for 20 min) tap water. The concentration (cfu/mL) of the stock solution was estimated by serial dilutions and standard plate count method. The stock solutions were stored at 2-8 °C until use (< 48h). Initial experiments showed no effect of storage (≤ 48h; 2-8 °C) on viability.

## 2.5 Protozoan strains

The strain of parasites used was that provided by Waterborne Inc in their AccuSpike kits which contained 100 *Giardia lamblia* and 100 *Cryptosporidium parvum* (AccuSpike-IR, cat no. PACIR6; Waterborne Inc., New Orleans, USA).

## 2.6 Spiking water samples with pathogens

200 mL autoclaved surface water was inoculated with 2 mL of appropriate dilution of stock culture of *Yersinia enterocolitica* and *Campylobacter jejuni* to achieve a final concentration of approximately $10^3$ and $10^1$ cfu $mL^{-1}$. 200 mL surface water already spiked with the two bacterial strains at two different concentrations ($10^3$ and $10^1$ cfu $mL^{-1}$) was inoculated with the AccuSpike-IR vial (0.75 mL) to achieve a final concentration of 50 and $10^2$ cysts/oocysts 100 $mL^{-1}$ respectively.

## 2.7 Filtering, recovery and enrichment of bacterial agents

Filtration of the water samples were performed according to NMKL 119 annex B with minor modifications. 100 mL of spiked water samples were filtered through a 0.45 µm filter

(MicroFunnel, Pall P/N 4800) under vacuum assistance and then each filter bisected in to equal halves using sterile scissor and forceps (Figure 2.2). Half of the filter was inoculated and eluted in 50 mL tubes (Sarstedt) with 10 mL of PSB - **P**eptone **S**orbitol **B**ile Broth (17192 Fluka) and the other half in 10 mL of Bolton basalmedium (Oxoid ref:CM0983) with Bolton selective supplement (Oxoid, ref: SR183E) and lysed horse blood (Oxoid ref: SR0048C). The filter suspension was then agitated at 200 rpm for 15 minutes at room temperature to allow recovery of bacteria. The final concentration of recovered bacteria in the filter eluate was estimated by the colony count method. The results were compared with qPCR on DNA extracts from 1 mL of the filter eluate. Recovery rate (% cfu) from the filter was estimated to be ~50 % for *Yersinia* and ~100 % for *Campylobacter* after 15 min of incubation.


*Figure 2.2 Filtration of water using the membrane filter method and vacuum support. (Photo: FFI)*

After ~15min of recovery the filter eluate was subject to selective enrichment in order to enhance sensitivity. Enrichment of *Yersinia* was performed by further incubation of the filter eluate in enrichment broth (PSB - **P**eptone **S**orbitol **B**ile Broth) under aerobic conditions at 37°C while enrichment of *Campylobacter* was performed in Bolton broth under microaerophilic conditions at 41°C. The time for enrichment was 16h for both bacterial agents with no agitation. Microaerophilic conditons were maintained using a culturing chamber with CampyGen TM 2,5L atmosphere generating system (Oxoid). Following the enrichment step *Yersinia* was plated

in triplicate on Colombia agar with 5% sheep blood (Oxoid) and incubated under aerobic conditions at 37˚C for 21 ± 4 hours to allow growth and visible colony formation. *Campylobacter* was plated in triplicate on *Campylobacter* selective blood free agar plates (CCDA, Oxoid) and incubated at 37 ±1 °C under microaerophilic conditions for 48 ± 4 hours. A temperature of 37 ˚C instead of 41 ˚C was chosen to avoid swarming.

## 2.8 Filtration and immunomagnetic separation (IMS) of protozoa from spiked water sample

100 mL of the water sample spiked with four different agents (2 bacterial strains, 2 protozoan strains) was filtered (0.45 µm) using the same approach as described in 2.7. The filter was then placed in a 50mL conical tube for washing three times with PBS buffer. The fluid from each each washing step was collected and centrifuged for 15 minutes at 1500x g. The supernatant was removed to the 5mL level above the pellet. The pellets from each of the three washes were transferred and combined in a new 15mL falcon tube. The enrichment step used immunomagnetic separation. Dynabeads (GC-Combo IDEXX (cat.no 73002)) were added and the manufacturer's instructions followed. Any *Giardia* and *Cryptosporidium* cysts/oocysts present in the sample are trapped on the Dynabeads. Once the beads have trapped the parasites, excess fluid was removed whilst the beads were held in place using a magnet. The beads were then washed in an acid to disassociate the parasites from the beads. The samples were then transferred to microscopy slides and the acid neutralised with NaOH (1N). Once the slides had air dried, they were coloured using Aqua-Glo G/C Direct (Direct (cat.no A100FLR-1X, Waterborne Inc.) and a positive control sample was made for each round of colouring. Samples that were not enriched were placed directly on microscopy slides (50 µl) after the filtration and washing steps were completed. These too were air dried and then coloured using Aqua-Glo G/C direct. The slides were examined using a fluorescence microscope. Cryptosporidium and *Giardia* were recorded when appropriately sized structures fluoresced apple green with FITC (4-6µm *Cryptosporidium*, 8-18x5-15µm *Giardia*) and DAPI (4',6-diamidino-2-phenylindole) from Sigma (cat.no D4592)) colouring revealed bright blue internal staining with up to four nuclei according the guidelines in the EPA method (EPA 2005).

Recovery rate from the filter (N=7) was estimated to 15-60% for *Giardia* cysts and 25-68 % for *Cryptosporidium* oocysts after the IMS enrichment steps. A low recovery rate is consistent with findings from other studies (VKM 2009) but, could also reflect the limited experience of the laboratory in carrying out this method. Ideally, one would like to see a recovery rate greater than 20% and preferably 60% or higher (EPA 2005).

## 2.9 FilmArray testing

The samples were analysed by the FilmArray system according to the instructions provided by the manufacturer. Briefly, the vacuum packed FilmArray GI pouch was opened and placed in the rack provided. Analysis buffer (blue top) and sample buffer (red top) were applied to their

corresponding positions on the rack. 200 µl of the sample was added to the sample buffer, which is provided by Biofire. The contents were homogenised by inversion two to three times before adding into the pouch. The pouch was then inserted into the machine followed by the start-up of the automated analysis program with a run-time of ~1h.
Pouches were analysed in triplicate for each water sample tested. Two of the pouches, with each test run, had a best before date that had expired 18 months previously whilst one was well within its use by date at the time of the analyses. The results were recorded as detected/not detected.

## 2.10 Statistical analyses

The sensitivity, specificity, negative and positive prediction values were calculated for all the analysis methods. A receiver operator curve (ROC) was made to compare each method and a comparison of the area under the curve was made. The 95% confidence intervals were calculated for these results. Since the total number of samples analysed with each method is small (just 7 for the majority of the analyses) further statistical comparisons were not made. We did however carry out a kappa analysis (Figure 2.3) to compare the FilmArray against the enrichment/purification method (which was used as gold standard; Dohoo *et al.* 2003). We chose a statistical significance level of 5%.

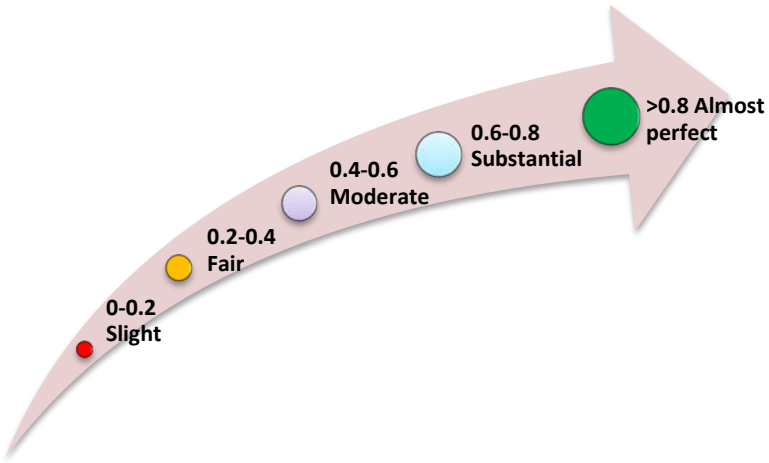


*Figure 2.3 Interpreting the level of agreement in kappa analysis, results of <0 show no agreement (Dohoo et al. 2003).*

# 3 Results

## 3.1 Detection of pathogens in surface water

A total of six river water samples (2 concentrations x3 replicates) were tested for the presence of four different pathogens using three different pre-analytical steps (filtration, enrichment, IMS purification) and three different analytical methods (culture, FilmArray, IFAT). The results have been summarized in Table 3.1 showing that FilmArray successfully detected both bacterial targets (*Campylobacter* and *Yersinia)* in 3/3 water samples containing HIGH concentrations of the pathogen without any pre-analytical treatment of the sample. This was not the case for the parasitical agents where none of the targets were detected at HIGH concentrations without pre-treatment, neither with FilmArray or the standard methods. FilmArray was unable to detect *Yersinia* at LOW concentrations in the unfiltered water sample but this was improved after applying a filtration step. When applying a pre-analytical filtration/extraction step FilmArray successfully detected all four pathogens in most of the samples except for the LOW concentrations of *Cryptosporidium.*

*Table 3.1* *Comparison of different methods for the detection of pathogens in surface water using HIGH: Yersinia $10^5$ cfu/100mL, Campylobacter $10^5$ cfu/100mL, Giardia 100cyst/100mL and Cryptosporidium 100 oocyst/mL* and LOW: *Yersinia $10^3$ cfu/100mL, Campylobacter $10^3$ cfu/100mL, Giardia 50 cyst/100mL and Cryptosporidium 50 oocysts/100mL contamination levels. All experiments were carried out in triplicate (1,2,3).*

| | ***Campylobacter*** | | | ***Yersinia*** | | | ***Giardia*** | | | ***Cryptosporidium*** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **UNFILTERED water sample** | **FilmArray** | **Culture** | **Enrichment + Culture** | **FilmArray** | **Culture** | **Enrichment + Culture** | **FilmArray** | **IFAT** | **IMS purification + IFAT** | **FilmArray** | **IFAT** | **IMS purfication + IFAT** |
| HIGH 1 | Positive | Positive | Not examined | Positive | Positive | Not examined | Negative | Negative | Not examined | Negative | Negative | Not examined |
| HIGH 2 | Positive | Positive | | Positive | Positive | | Negative | Negative | | Negative | Negative | |
| HIGH 3 | Positive | Positive | | Positive | Positive | | Negative | Negative | | Negative | Negative | |
| LOW 1 | Positive | Negative | | Negative | Positive | | Positive | Positive | | Negative | Negative | |
| LOW 2 | Positive | Negative | | Negative | Positive | | Negative | Negative | | Negative | Negative | |
| LOW 3 | Positive | Negative | | Negative | Positive | | Negative | Negative | | Negative | Negative | |
| Control water | Negative | Negative | | Negative | Negative | | Negative | Negative | | Negative | Negative | |
| **FILTERED water sample** | | | | | | | | | | | | |
| HIGH 1 | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive |
| HIGH 2 | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Negative | Positive | Positive | Positive | Positive |
| HIGH 3 | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Negative | Positive | Positive |
| LOW 1 | Positive | Negative | Positive | Negative | Positive | Positive | Positive | Positive | Positive | Negative | Positive | Positive |
| LOW 2 | Positive | Negative | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive |
| LOW 3 | Positive | Negative | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Negative | Negative | Positive |
| Control water | Negative | Negative | Negative | Negative | Negative | Negative | Negative | Negative | Negative | Negative | Negative | Negative |

### 3.1.1 FilmArray pouch expiry results

Comparison of the pouches that had expired to the results from those within date showed few differences with a few exceptions (Table 3.2).

*Table 3.2* *False negative results for Campylobacter, Yersinia, Giardia and Cryptosporidium detection with FilmArray pouches that were in date or that had expired both pre-filtration (100 mL stock solution) or after concentration with a filtration step (100 mL stock solution concentrated to 10 mL) .*

| Agent | Concentration | Out of date FilmArray pouches<br>N= 2 pouches pre-filtration and 2 post-filtration | In date FilmArray pouch<br>1 pouch pre-filtration and 1 post-filtration | Number correctly identified post filtration (*pre-filtration*) |
|---|---|---|---|---|
| *Campylobacter* | LOW | No false negatives | No false negatives | 3/3 *(3/3)* |
| | HIGH | No false negatives | No false negatives | 3/3 *(3/3)* |
| *Yersinia* | LOW | 2 false negatives (pre-filtration) | 2 false negatives (1 pre- and 1 post-filtration) | 2/3 *(0/3)* |
| | HIGH | No false negatives | No false negatives | 3/3 *(3/3)* |
| *Giardia* | LOW | 2 false negatives (pre-filtration) | No false negatives | 3/3 *(1/3)* |
| | HIGH | 2 false negatives (pre-filtration) | 1 false negative (pre-filtration) | 3/3 *(0/3)* |
| *Cryptosporidium* | LOW | 3 false negatives (2 pre-filtration and 1 post-filtration) | 2 false negative (1 pre-filtration and 1 post-filtration) | 1/3 *(0/3)* |
| | HIGH | 3 false negatives (2 pre-filtration and 1 post-filtration) | 1 false negative (pre-filtration) | 2/3 *(0/3)* |

The out of date pouches successfully detected *Campylobacter* in all the samples but did not detect low levels of *Giardia*, *Cryptosporidium* or *Yersinia* in pre-filtration samples regardless of pouch expiry date and also did not detect high levels of the parasites in the samples prior to filtration. Of greater concern are the FilmArray pouches that were unable to detect some of the agents post-filtration. Low and high levels *Cryptosporidium* were not detected in the majority of the out of date pouches whilst low levels of *Yersinia* and *Cryptosporidium* were not detected in two in date pouches, one for each respectively. To summarize, our results indicate that the expiry date of the reagents may influence the sensitivity of FilmArray when concentrations are close to the expected limits of detection such as *Cryptosporidium*. However, more than 7 replicates are needed in order to conclude with any degree of certainty.

## 3.2 FilmArray versus "gold standard" detection methods

We compared FilmArray and standard methods (culturing and IFAT) of the filtered water samples to the results after enrichment (bacteria) and purification (protozoa). The results indicate that FilmArray is just as sensitive as the standard methods at the HIGH concentrations used in this study, regardless of whether the water sample is filtered or not. However, the results were more inconsistent at LOW concentrations of the target pathogens where we observed that FilmArray was more sensitive for the detection of *Campylobacter* and less sensitive for *Yersinia* and *Cryptosporidium* than the standard methods. FilmArray was unable to consistently detect all the pathogens at the two concentrations tested in unfiltered samples, with the exception of *Campylobacter*, and thus these results are not included in the comparison.

*Table 3.3* *Sensitivity, specificity, positive (PPV) and negative (NPV) prediction values, area under the ROC curve (AUC) and kappa result of the different detection methods after filtration in which 100mL concentrated to 10mL. Kappa analysis used enrichment+ culture and purification/IFAT post filtration as the "gold standards" against which to compare FilmArray, and direct detection (culturing and IFAT) post filtration without the enrichment/purification step. The confidence intervals are not shown given the small sample size. None of the methods were significantly better or poorer at detecting the pathogens.*

| Detection Method (filter sample) | Agent | N | Sensitivity | Specificity | PPV | NPV | AUC[1] | Kappa | Kappa agreement level[2] |
|---|---|---|---|---|---|---|---|---|---|
| **FilmArray** | *Campy.* | 7 | 1 | 1 | 1 | 1 | 1 [0.6-1] | 1 | ***Perfect*** |
| | *Yersinia* | 7 | 0.83 | 1 | 1 | 0.5 | 0.92 [0.5-1] | 0.59 | ***Moderate*** |
| | *Crypto.* | 7 | 0.5 | 1 | 1 | 0.25 | 0.75 [0.3-1] | 0.22 | ***Fair*** |
| | *Giardia* | 7 | 1 | 1 | 1 | 1 | 1 [0.6-1] | 1 | ***Perfect*** |
| **Culture** | *Campy.* | 21 | 0.5 | 1 | 1 | 0.25 | 0.75 [0.5-0.9] | 0.22 | ***Fair*** |
| | *Yersinia* | 21 | 1 | 1 | 1 | 1 | 1 [0.8-1] | 1 | ***Perfect*** |
| **IFAT** | *Crypto.* | 7 | 0.83 | 1 | 1 | 0.5 | 0,92 [0.5-1] | 0.59 | ***Moderate*** |
| | *Giardia* | 7 | 0.83 | 1 | 1 | 0.5 | 0.92 [0.5-1] | 0.59 | ***Moderate*** |
| **Enrichment+ Culture** | *Campy.* | 21 | 1 | 1 | 1 | 1 | 1 [0.8-1] | - | |
| | *Yersinia* | 21 | 1 | 1 | 1 | 1 | 1 [0.8-1] | - | |
| **IMS purification + IFAT** | *Crypto.* | 7 | 1 | 1 | 1 | 1 | 1 [0.6-1] | - | |
| | *Giardia* | 7 | 1 | 1 | 1 | 1 | 1 [0.6-1] | - | |

[1]The 95% confidence interval for the AUC is given in square brackets and was calculated using MedCalc (https://www.medcalc.org/calc/test_one_proportion.php)

[2] See Figure 2.3 for further information on Kappa agreement levels.

# 4 Discussion

Our small-scale pilot study shows that FilmArray is able to provide a rapid result for the screening of water samples for multiple pathogens. However more work is needed to establish the lowest detection levels. Certainly the levels investigated in this study for *Yersinia* and *Cryptosporidium* would appear to be close to the limits of detection (LoD). These levels are still higher than reported infectious dose levels so a negative result does not necessarily rule out the pathogen. In an outbreak one would also expect to have clinical samples to test in which much higher bacterial or parasitological concentration levels could be found. Screening the water using FilmArray could give an early indication and further analysis using enrichment/purification methods for the negative samples would strengthen any negative findings. Whilst FilmArray allows rapid analysis for a wide range of pathogens unequivocal identification of the pathogen should build upon additional pathogen specific identification methods. FilmArray allows you to relatively quickly narrow the field and thus optimise further laboratory analysis time focusing on specific pathogens of interest.

Our results could have been biased by difficulties of culturing *Campylobacter* thereby giving a false estimate of the initial concentrations (cfu/mL) that were used. Our findings indicate that the amount of genetic material available, as determined by qPCR analysis, for analysis was considerable higher for *Campylobacter* compared to *Yersinia*. We suggest that the presence of campylobacters that were in a dead or in a non-culturable state resulted in the preparation of a sample solution with considerable higher level of *Campylobacter* DNA than *Yersinia* (De Boer et al. 2015). These could be claimed to be a source of false positives as they are most likely unable to cause disease. On the other hand, the presence of viable but non culturable (VBNC) bacteria could be lead to false negatives when using culture-based detection methods only. Moreover, this highlights the risks of erroneously interpreting results relying on either DNA-based or culture-based detection methods only.

Whilst the same number of *Giardia* and *Cryprosporidia* were added to the sample the size difference between these two pathogens is considerable and we suggest therefore that the *Cryptosporidium* oocysts contained relatively fewer nuclei than the larger Giardia cysts which may contain multiple nuclei (Erlandsen et al. 1994). Furthermore, we are not aware of the exact gene targets used by the FilmArray GI panel and whether this is present in multiple copies within the genome. This could also affect the sensitivity of the assay. Further measures to improve test sensitivity could include:

- Filtering a larger volume of water. In this study we investigated 100mL samples however for routine sampling of water it would be better to analyse larger volumes. Up to 10L is used as routine during outbreak investigations (VKM 2009) but the volume to be analysed can vary depending on the type of water source being tested.

- Integrate IMS -step for all pathogens after filtration and prior to analysis.

- Optimise methods to wash filter in order to avoid pathogens being captured in the filter and at the same time minimize the risk of cross contamination (Banting et al. 2016). The NMKL and EPA methods differed in how best to wash the filter and analyse the filter eluate. The bacterial methods relied on placing the whole filter into the culture medium which means that we had to carry out multiple filtrations to ensure that we could carry out analysis for four different pathogens.

# 5 Conclusions and recommendations

The FilmArray GI panel seems to be a promising tool for the simple and rapid detection and preliminary identification of multiple pathogens in contaminated river water at levels close to infectious dose. Further studies are warranted in order to investigate the performance (sensitivity & specificity) and applicability of the device under field conditions and with military relevant pathogens and matrixes, including water/food-borne viruses.

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# 7 Appendix

### Initial experimental work and method optimization

We looked at a range of different factors that could be adjusted to try and optimise the methodology and reduce the time taken to analyse each sample. Firstly we looked at the limits of detection (LoD) with and without culture steps and then we looked at how we could try to modify and combine the NMKL methods and EPA method 1623 thus allowing us only one filtration step. Finally we looked at how to reduce enrichment time without compromising sensitivity.

## 7.1 Isolation of bacterial genomic DNA and preparation of DNA stock solutions

DNA was isolated from 1mL bacterial culture. QIAamp DNA mini kit (Qiagen cat nr: 51304) according to the manufacturer's instructions. DNA concentration (ng/µL) was measured with Qubit fluorimeter (Invitrogen) and GU/ µL was calculated with the following formula based on genome size:

$$\frac{\text{GU}}{\mu\text{L}} = \frac{\text{ng}}{\mu\text{L}}\ x\ \frac{6{,}0221415\text{x}10^{23}\ \ copies/g}{\text{genome size (bp)}x660\frac{g/mol}{bp}\ x\ 10^{9}}$$

Genome size *Yersinia enterocolitica:* ~4.6 Mb

Genome size *Campylobacter jejuni*: ~1.7 Mb

## 7.2 Quantitative determination of *Yersinia* and *Campylobacter* genome copies (GU) by qPCR

In order to be able to quantify and evaluate the serial dilution effect on the concentration (GU/mL) of *Campylobacter jejuni* and *Yersinia enterocolitica* in our samples qPCR assays were established using the primers and protocols as specified in Nord Val 017 and NMKL 163. These real-time PCR analyses were not carried out in the main study where the focus was on culture and Film-Array. DNA was extracted from 1 mL liquid sample using QIAamp DNA mini kit (art.nr 51306) and analysed in triplicate using Probe Master I (Roche), primers and conditions as shown in Table 7.2. All analyses were run on the Light cycler 480 (Roche) real-time PCR instrument. In addition, serial dilutions of the spiked water sample was cultured to estimate the concentration (cfu mL $^{-1}$) at T=0, prior to filtration and subsequent enrichment and culturing steps.

*Table 7.1 The primers used for the PCR detection of Campylobacter jejuni and Yersinia enterocolitica.*

| | **Primers (Invitrogen) and probes (TIBmolbiol) (end concentration)** | **PCR conditions** |
|---|---|---|
| ***Campylobacter jejuni*** | Campy primer 1: 5'CTG CTT AAC ACA AGT TGA GTA GG 3' (440 nM) | 95°C 5min |
| | Campy primer 2 : 5'TTC CTT AGG TAC CGT CAG AA 3' | (95°C 15s, 58°C 20s , 72°C 40s) x45 |
| | (480 nM) | |
| | Campy probe: 5'FAM- TCT CAT CCT CCA CGC GGC GTT GCT GC –tamra 3' (50 nM) | |
| ***Yersinia enterocolitica*** | YE-R primer:5'CCC AGT AAT CCA TAA AGG CTA ACA TAT 3' | 95°C 5min |
| | YE-F primer: 5'ATG ATA ACT GGG GAG TAA TAG GTT CG 3' | (95°C 15s,60°C 20s , 72°C 40s) x45 |
| | YE probe: 5'FAM- TCT ATG GCA GTA ATA AGT TTG GTC ACG GTG ATC T- TAMRA 3' | |

Range of quantification: *Yersinia*: $3*10^{6}$ GU/µL – $3*10^{0}$ GU/ µL and *Campylobacter*: $2{,}2*10^{7}$ GU/µL – $2{,}2*10^{-1}$ GU/ µL.

## 7.3 Optimisation of filtration and extraction of bacteria from filter

The initial trials looking at using one single water filter for both bacteriological and parasitological analyses showed we had to rethink this strategy. The bacteriological NMKL methods describe adding the filter to the enrichment media. The parasitological washing and centrifugation method (EPA 2005) resulted in considerably lower GU/mL for the bacteria than adding half the filter directly to the enrichment medium. We therefore chose to carry out two separate filtrations: one for the parasitological and direct FilmArray analysis and one, in which the filter was cut into two pieces, for the bacteriological analyses in the further work carried out.

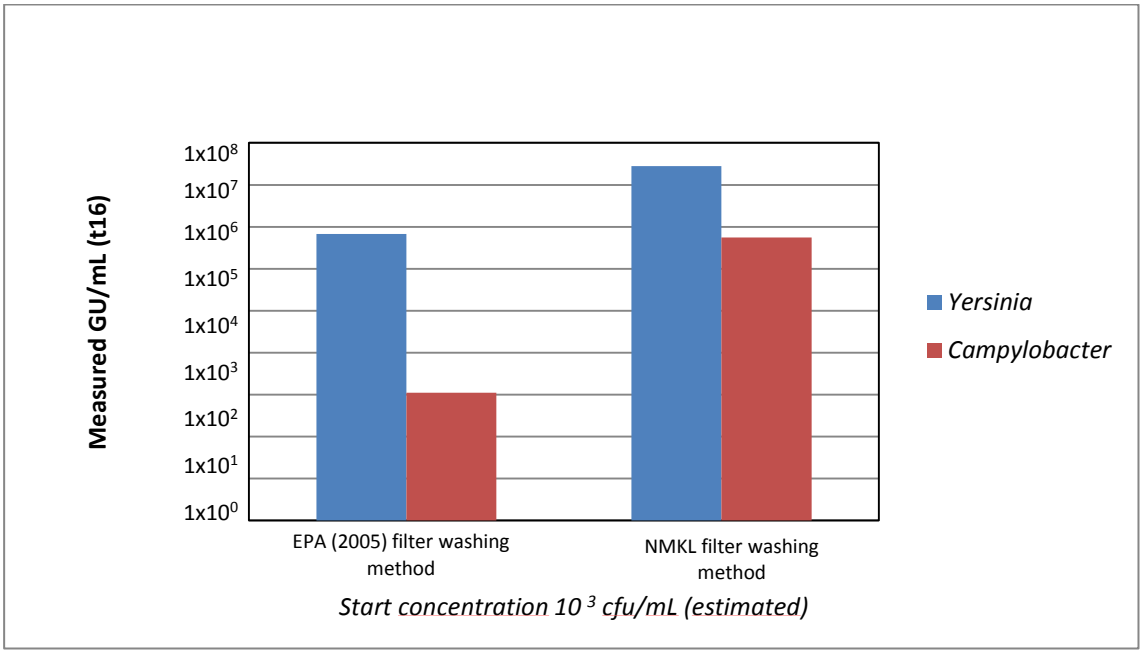


*Figure 7.1* *Detection of* Campylobacter *and* Yersinia *with qPCR in mixed sample after enrichment (T16) and two different filter washing methods. EPA method: Parasitological washing in PBS and centrifugation method. NMKL Method 2: filter cut in half and each half placed directly into the specific culture mediums for each of the bacteria investigated. DNA extraction was carried out and GU/mL calculated after the qPCR.*

## 7.4 Optimisation of enrichment time

The NMKL methods indicate an optimal enrichment time of 48 hours for the *Campylobacter* and 21 hours for the *Yersinia*. We wanted to establish what the optimal enrichment time would be for a sample containing a mixture of two bacterial species (*Yersinia enterocolitica* and *Campylobacter jejuni*). The initial culturing studies showed that 4 hours was insufficient and 24 hours more than sufficient for detecting *Yersinia* and *Campylobacter* in the water samples. We therefore investigated whether 16 hours would also be sufficient given that this would allow us to carry out filtration and set up the sample for enrichment in the afternoon and then carry out DNA extraction and PCR analysis the following morning. All the samples had a CT value <25 after the 16 hour enrichment step. We therefore opted to use 16h enrichment and not 21h or 48h as described in the NMKL methods.

## 7.5 Pilot studies with parasites and FilmArray

There was initial concern that FilmArray might not be able to detect the *Cryptosporidium* species used in the Accuspike kit. We therefore carried out an additional analysis with a faecal sample from a calf with confirmed cryptosporidiosis (courtesy of the Norwegian Veterinary Institute). FilmArray detected *Cryptosporidium* in the clinical sample from the animal. In addition to this FilmArray also detected Rotavirus. The laboratory that had donated the clinical sample was informed of the additional findings. This highlights one of the benefits of using such a panel to screen for a range of gastrointestinal pathogens. It also highlights that the detection of one or more of the agents on the panel may not necessarily confirm detection of a human pathogen. Positive findings should always be corroborated further.

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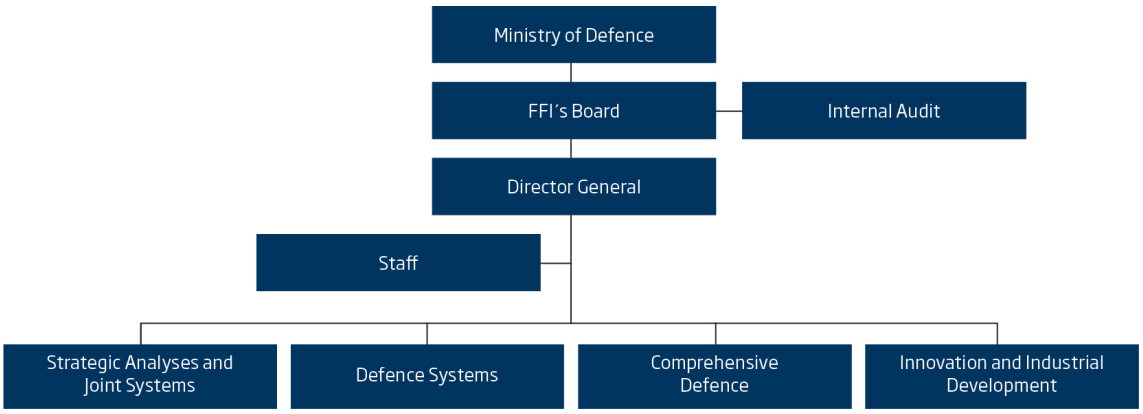

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