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Comparison of Rapid Methods for Detection of *Giardia* spp. and *Cryptosporidium* spp. (Oo)cysts Using Transportable Instrumentation in a Field Deployment

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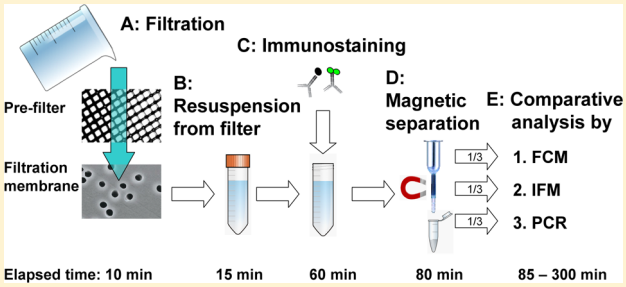
Supporting Information

ABSTRACT: Reliable, sensitive, quantitative, and mobile rapid screening methods for pathogenic organisms are not yet readily available, but would provide a great benefit to humanitarian intervention units in disaster situations. We compared three different methods (immunofluorescent microscopy, IFM; flow cytometry, FCM; polymerase chain reaction, PCR) for the rapid and quantitative detection of *Giardia lamblia* and *Cryptosporidium parvum* (oo)cysts in a field campaign. For this we deployed our mobile instrumentation and sampled canal water and vegetables during a 2 week field study in Thailand. For purification and concentrations of (oo)cysts, we used filtration and immunomagnetic separation. We were able to detect considerably high oo(cysts) concentrations (ranges: 15−855 and 0−240 oo(cysts)/liter for *Giardia* and *Cryptosporidium*, respectively) in 85 to 300 min, with FCM being fastest, followed by PCR, and IFM being slowest due to the long analysis time per sample. FCM and IFM performed consistently well, whereas PCR reactions often failed. The recovery, established by FCM, was around 30% for *Giardia* and 13% for *Cryptosporidium* (oo)cysts. It was possible to track (oo)cysts from the wastewater further downstream to irrigation waters and confirm contamination of salads and water vegetables. We believe that rapid detection, in particular FCM-based methods, can substantially help in disaster management and outbreak prevention.



INTRODUCTION

The 2010 Haiti earthquake, including the associated outbreak of cholera, demonstrated the need for reliable and rapid monitoring of water quality to prevent the spread of waterborne pathogens, especially in disaster management.[1,2] Conventional approaches for evaluating the microbial water quality based on standard plating for indicator bacteria take from 18 h to several days and methods for specific pathogen detection take days up to weeks until a result is obtained.[3−6] Furthermore, for a range of pathogens including protozoan parasites, plating methods are not applicable.[7−9]

In contrast, rapid detection methods, e.g., flow cytometry (FCM), immunofluorescent microscopy (IFM), and polymerase chain reaction (PCR) may provide results after only a few hours and can also detect noncultivable organisms. Especially in crisis situations, international intervention units would highly benefit from the capability to analyze food, clinical, and water

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samples for pathogenic organisms within hours, preferably by using conveniently transportable instrumentation. Therefore, a rapid detection approach based on immunological staining, immunomagnetic separation, and flow cytometric detection was developed,[10] which is compatible with mobile instrumentation.

Giardia lamblia and *Cryptosporidium parvum* are the major intestinal parasites in humans worldwide and among the most common reasons for diarrheal disease.[11] Large waterborne outbreaks are documented to have been caused by these pathogens, which have been classified as "neglected diseases" by the WHO.[12] Infection is maintained through the fecal-oral route by the (oo)cysts that are environmentally inert and highly robust against chlorination.[13] Agriculture and human wastewater are major sources of contamination [14] and since not only humans but also many invertebrates can be infected, there is a high potential for zoonotic transmission.[13,15] Drinking water is considered as the major cause for infections,[16,17] and (oo)cysts can be detected in over 80% of U.S. surface waters.[18] Furthermore, many infections are estimated to be associated with food, such as shellfish, fresh fruit juices, raw milk products, and raw salads.[19,20]

Low numbers of oo(cysts) can cause human infection, i.e., 10−100 for *Giardia lamblia*[21,22] and 10−1000 for *Cryptosporidium parvum*.[23] The approved standard method for oo(cysts) detection, USEPA 1623,[24] is based on filtration, purification, and microscopic quantification and is considered tedious and time-consuming with low and variable recoveries, especially for environmental water samples.[19,25] A blind survey conducted in different routine laboratories resulted in recoveries ranging from 0.8 to 22.3%, averaging 9.3% for spiked samples.[26] Thus, it is very difficult to establish a useful and cost-effective monitoring program under field conditions using this method.[27] In some recent outbreaks, typical warning signals, like elevated turbidity or coliform counts, did not show abnormalities early enough, thus these indicators cannot provide safety.[28] A typical example is the huge Milwaukee *Cryptosporidium* outbreak in 1993.[29]

Thailand is a fast-developing country with a high economic growth rate. There are extensive networks of man-made waterways that are used for trade, agriculture, flood protection, defense, waste management, and transport. The urban population is steadily increasing, but the infrastructure is not developing at the same pace. Thus major parts of domestic and commercial wastewaters are released untreated into these canals.[30] Although, the practice of recycling nutrients has economical and ecological benefits, it is opposed by the inherent infection risks. Of health concern is that these waters are used for irrigating and fertilizing rice and vegetable fields and thus contamination of agricultural products cannot be excluded. Hence, the population is highly exposed to infection risks not only when consuming contaminated foodstuffs, but also when interacting with this water.[31]

In our study, we first evaluated the performance of our pathogen concentration and purification approach and compared rapid analysis methods (FCM, IFM, PCR) for the detection of the parasites *Giardia spp.* and *Cryptosporidium spp.* with mobile instrumentation in a set up similar to humanitarian missions. Second, we tried to quantify the pathogen flow from a wastewater inlet to 100 m downstream locations where people are actually exposed (Figure S1 of the Supporting Information, SI) and to evaluate the reduction in pathogen load due to dilution effects or sedimentation. In addition, we analyzed irrigation water, salad and vegetables to evaluate consumer's risk and obtain evidence for contamination of these products by the irrigation water. For all samples, standard methods, i.e., heterotrophic plate count (HPC), turbidity, *E. coli* count, conductivity, and the flow cytometric total bacterial cell count (TCC) were included.

MATERIALS AND METHODS

Organisms. Reference *Giardia lamblia* and *Cryptosporidium parvum* (oo)cysts were obtained from Waterborne Inc. (10^7 cyst and 10^7 oocysts, New Orleans, LA, U.S.) each stored in 8 mL of sterile PBS (phosphate buffered saline, 150 mM NaCl, 15 mM KH_2PO_4, 20 mM Na_2HPO_4, 27 mM KCl, pH 7.4; Sigma-Aldrich, St. Louis, MO, U.S.) at 4 °C.

Instrument Deployment. All materials used in this study were shipped from Switzerland to Thailand by air cargo in 6 transport boxes with a total weight of 249 kg. We installed the instruments in an empty lab at the Asian Institute of Technology and tested functionality with oo(cysts) reference solutions prepared in Switzerland. Only electricity, benches, fridges, and freezers of the lab infrastructure were used.

Sampling. The field campaign lasted two weeks and 24 environmental water samples (2 L) were collected from canal water systems of the Klong Luang Municipality, Pathumthani Province, Thailand (Table S1, Figure S2 of the SI). Canal water samples were taken directly in front the wastewater inlets and from bridges 100 m downstream of the wastewater inlet (Figure S3 of the SI). A custom-made 2 L sample collector was immersed approximately 30 cm into the water. Water can enter the sampler from top and bottom and the bottom opening is blocked by a plastic ball when the sampler is removed from the water. Washed and rinsed with 0.22 μm-filtered water 1 L screw cap glass bottles were used to transport the samples. Samples were cooled by ice and transported in less than 3 h to our laboratory, where they were processed instantly. Temperatures and pH of all samples were measured directly after sampling with an hand-held instrument (EcoSense pH100, YSI Inc., Yellow Springs, OH, U.S.). Additionally, to assess the exposure for field workers as well as the exposure risk for consumers, samples were collected from salad field irrigation water connected to the canals, from freshly harvested lettuce, washed lettuce and Morning Glory (syn. *Ipomoea aquatica*, water spinach, Figure S4 of the SI).

Preparation of Salad and Vegetable Samples. From lettuce heads, leaves were removed with gloves and around 500 g of leaves were washed individually in 300 mL PBST (1 × PBS with 0.005% Tween 80 (Fluka) added). For Morning Glory, the leaves with the flower stem were taken and washed in buffer as described above. The rinsing water was then filtered and further processed like the water samples. Results are given for 200 g ("a serving") to have a basis for sample comparison (Table 1).

Table 1. (Oo)cysts Detected in the Irrigation Water, Salads and Vegetables with Either FCM or IFM

	IFM	FCM	IFM	FCM
sample description	*Giardia* cyst count		*Cryptosporidium* oocyst count	
irrigation water, 1 L	10	0	3	3
unwashed lettuce, 200 g	16.7	25	1.2	0
washed lettuce, 200 g	15.7	17.6	0	1.3
Morning Glory, 200 g	38	50	4	10

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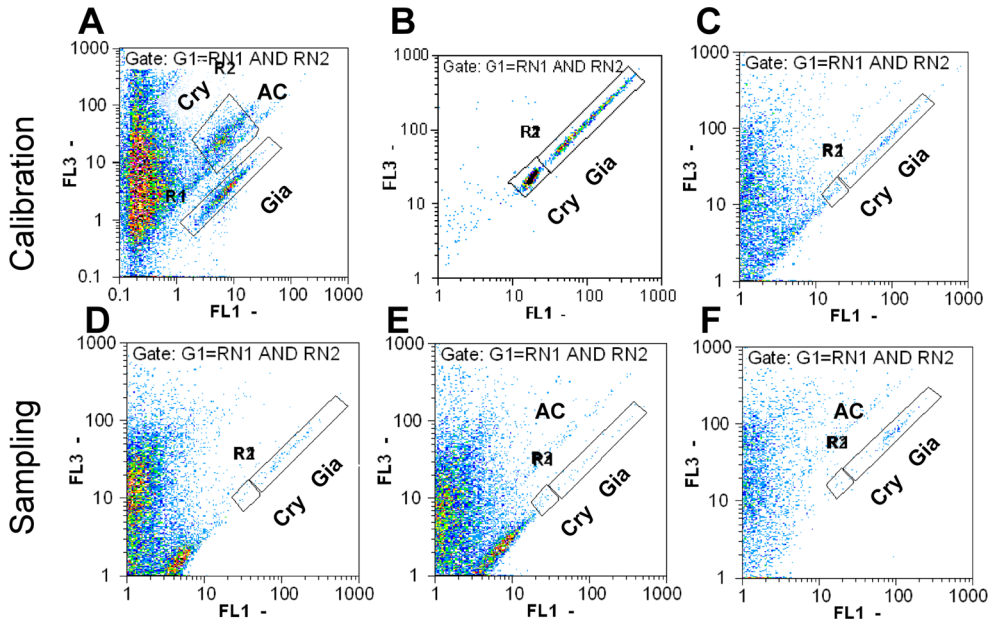

Figure 1. FCM dot plots of green (FL1 −520 nm) vs red (FL3 −630 nm) fluorescence signals. Letters in the plots represent the different clusters, where "Cry" is for *Cryptosporidium*, "Gia" for *Giardia*, and AC for a presumptive autofluorescent algae cluster (always below "AC"). All other annotations in the plots are from the original FCM software outputs that cannot be removed without modifying the images. Plot A: Spiked (oo)cysts in canal water with intended double-staining technique and appropriate gating, employing an additional red fluorescent dye for *Cryptosporidium* staining. The algae cluster interfered with the original gate for *Cryptospordium*, so a protocol without the red dye was used. Plot B: Calibration measurement with spiked cysts in PBS buffer and oocysts and single-staining, employed throughout this study. Plot C: Spiked (oo)cysts in canal water with the staining and gating presented in this work. Plots D to F illustrate the different background levels encountered in environmental samples.

Sample Processing. The protocol was adapted from Keserue and co-workers;[10] briefly, 1 L water samples were vacuum filtered through a 47 mm diameter, 30 μm nylon-net filter (Millipore, Billerica, MA, U.S.) and subsequently through a 47 mm diameter, 2 μm-pore-size polycarbonate track etch filter (PCTE, Sterlitech Corporation, Kent, WA, U.S.) and resuspended in 5 mL of sterile PBST by vortexing vigorously for 5 min. Then we added 10 μL of 10% BSA (Bovine serum albumin, Sigma, Steinheim, Germany) and 3 μL IgG rabbit polyclonal FITC-conjugated cell surface specific antibodies (A100FLK, Waterborne Inc.). Samples were incubated for 15 min at 30 °C (ambient) temperature in the dark. After incubation, 100 μL of colloidal, superparamagnetic anti-FITC MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and incubated for 30 min on ice at around 5 °C, protected from light. The extraction column (MACS MS Column, Miltenyi Biotec) was placed in the magnet and the sample was run through the column. Subsequently, the column was washed with 3 × 2 mL of 0.22 μm-filtered PBST. The column was then removed from the magnet and retained cells were eluted with 1 mL of PBS flushed through the column with the provided piston. This positive fraction was collected and split into 300 μL fractions for analysis by flow cytometry, fluorescence microscopy, and PCR.

Flow Cytometry. Flow cytometric detection was performed with a light (17 kg) and mobile (43 × 37 × 16 cm) Partec CyFlow SL flow cytometer (Partec GmbH, Münster, Germany), equipped with a 20 mW blue solid-state laser emitting light a 488 nm and a volumetric counting sample port. Optical filters were adjusted to measure green fluorescence at 520 nm (FL1), red fluorescence at 630 nm (FL3), the sideward scatter (SSC) at 488 nm, and the forward scatter (FCS) at 488 nm. The trigger was set on green fluorescence. Events were defined based on forward scatter (FCS), sideward scatter (SSC), 520 nm (FL1) and 630 nm (FL3) fluorescence. Results were presented by plotting the histograms as well as dot plots for: FL1 versus SSC, FL1 versus FL3, FL1 versus FSC, and FCS versus SSC. For quantification, we applied defined gating regions, Gate R1 for *Giardia* and gate R2 for *Cryptosporidium* (Figure 1, Figure S5 of the SI). Green and red fluorescence are emitted by the FITC and since the red signal is only a fraction of the intensity the amplification (gain) for this signal is very high. The specific instrumental gain settings used for green fluorescence, red fluorescence, forward scatter, and sideward scatter canals were 291.0, 551.0, 222.5, and 242.0, respectively. The flow speed rate was 3 μL/second, implying a counting rate of less than 500 events / second and a total duration of about 3.5 min per measurement.

Fluorescence Microscopy. From each water sample, 300 μL of the immunomagnetically enriched and purified samples were filtered onto a 14-mm diameter and 0.20 μm pore size GTPB microscopy black membrane filter (Millipore) by using a filter holder (Millipore No. XX3001240). The black membrane filter was then placed on a microscopy glass slide. After approximately 1 h of air-drying, 5 μL of antifade mounting medium (Waterborne, Inc., New Orleans, USA) was added to the stained cells on the membrane and the filter was covered with a cover slide suitable for fluorescent microscopy. The samples were examined within 5 h after collection using a very light (9.6 kg), mobile PrimoStar iLED microscope (Zeiss, Jena, Germany) at 1000× magnification. For counting, the whole filter was screened for parasites in about 30−45 min. The parasites were detected based on green fluorescence, size, and shape.

qPCR. DNA extraction and PCR protocol were adapted from Guy and colleagues.[32] Briefly, from each water sample 300

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Table 2. Recovery Experiments of the Complete Method (Filtration Resuspension, Immunolabelling, Immunomagnetic Separation, and Flow Cytometric Detection) with Surface Waters from Canals[a]

organism	sampling week	no. of environmental (oo)cysts	no. of spiked (oo)cysts	no. of recovered organisms	recovery of spiked organisms, % ± CV, %
Giardia	1	55.0 ± 15	5,525 ± 100	1,720 ± 328	30.1 ± 6.2
Giardia	2	48.3 ± 15.3	4,225 ± 79	1,300 ± 221	29.6 ± 5.6
Cryptosporidium	1	31.7 ± 7.7	7,600 ± 217	990 ± 138	12.6 ± 2.0
Cryptosporidium	2	51.7 ± 28.4	10,400 ± 505	1,400 ± 74	13.0 ± 1.2

[a]The numbers of (oo)cysts are given per liter. Recovery is given in percentage after subtraction of the environmental organisms ± coefficient of variation (CV) in %, (n = 3).

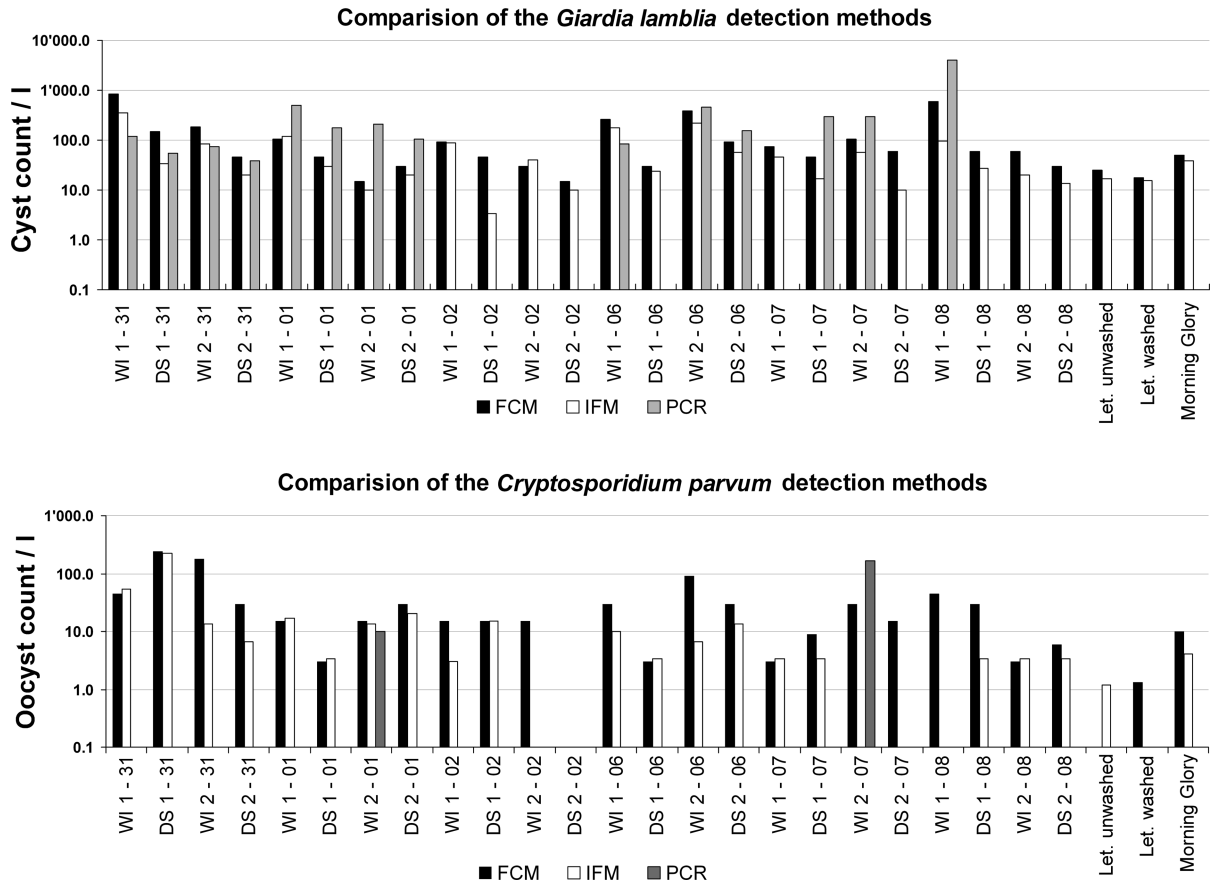

Figure 2. Comparison of the detection methods used. Top bar chart for *Giardia lamblia* and bottom chart for *Cryptosporidium parvum* quantification. The x-axis represents the sampling points with the sampling date (four sampling sites for each date; see Table S1 of the SI). For comparison, we added the salad and vegetable samples in the charts. Let. stands for lettuce.

μL of the immunomagnetically enriched and purified sample were subjected to DNA extraction using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) on the transportable (29 kg) EZ1 BioRobot (Qiagen). Modifications of the manufacturer's protocol were employed, adding a sequence of three freeze–thaw cycles. Cycles consisted of 30 min freezing at −20 °C, subsequent thawing at 90 °C and three 30 s sonication runs at a maximum value of 240 W (Elmasonic S10H, Elma GmbH, Singen, Germany). Total DNA was eluted in 200 μL of buffer whereof 5 μL was used for each PCR reaction. PCR reactions were run in doublets using the Qiagen QuantiFast PCR SYBER GREEN mix on a transportable Mastercycler realplex (18 kg, Eppendorf, Hamburg, Germany). The primers used for *Giardia* were targeted against β-Giardin P241 and for *Cryptosporidium* against COWP P702 (Mycrosynth, Balgach, Switzerland) (Table S 2 of the SI). The primer concentration was 1 μM per reaction.[32] For each PCR run a triplicate standard curve for *Cryptosporidia parvum* and *Giardia lamblia* (oo)cysts (10^{2}–10^{5}/mL) was run for control. Total time to result for our PCR approach was about 200 min.

Recovery Evaluation. Recovery was determined by FCM only, since we reported recently very good agreement for comparison of FCM and IFM counts of spiked *Giardia* cysts in tap and wastewater.[10] Thus, diluted oo(cyst) stock solution was stained with fluorescent antibodies as described above and directly counted by FCM in order to prepare the spiking concentrations. Triplicates of two liter canal water sample were divided into two one liter aliquots; one aliquot was spiked with a defined amount of (oo)cysts, then the concentration of (oo)cysts was determined in both aliquots. Thus, the recovery in % represents the fraction of recovered parasites, after subtracting the naturally occurring ones, compared to the spiking value (Table 2).

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Other Measured Parameters. The total flow cytometric bacterial cell concentration (TCC) was determined as described earlier.[33] Turbidity of the samples was measured with a Hach 2100 turbidimeter (Hach Company, Loveland, CO) according to the instructions of the manufacturer. Results are given in nephelometric turbidity units (NTU). The heterotrophic plate count (HPC) and the *E. coli* count was performed with the most probable number method according to APHA-AWWA-WPCF, Standard methods for the examination of water and wastewater, 21st edition. For the HPC, the spread plate method on R2A agar with incubation at 28 °C for 5 days was performed.[34] For *E. coli* Section 9221F was applied.[35]

Statistical Analysis. Linear regressions were performed with Microsoft Excel, Pearson correlations were computed with IBM SPSS, and box plots and Wilcoxon signed rank test were generated with Graph Pad Prism.

RESULTS

After transport and installation all instrumentation was tested successfully with precounted reference samples and/or calibration beads for precise quantification.

Water Samples. The water samples had an average temperature of 29.7 ± 2.3 °C and an average pH of 6.57 ± 0.14.

Flow Cytometry. The *Giardia* cysts could be discriminated well from background signals [10] (Figure 1). Since *Cryptosporidium* oocysts are considerably smaller than *Giardia* cysts, we originally intended to use in this study a double-staining approach to better discriminate the oocysts. Therefore, R-Phycoerythrin-labeled antibodies (A400 R-PE, Waterborne Inc.) against *Cryptosporidium parvum* were used as an additional red fluorescence emitter for the oocysts. However, in many of the canal water samples signals from an autofluorescent presumptive algal cluster overlapped with the *Cryptosporidium* signals (Figure 1, plot A) and therefore we had to refrain from using this stain in this field study. Thus, the *Cryptosporidium* cluster is smaller and more distinct but closer to the *Giardia* gate and to the background signals; hence, false-positive results from background signals in this gate cannot be excluded. Given the fact, that we had some zero oocyst counts and did detect cysts in the single digit range the number of false positives cannot be very high. The recoveries evaluated by FCM are listed in Table 2.

Immunofluorescent Microscopy. It was possible to clearly identify labeled *Giardia spp.* and *Cryptosporidium spp.* (oo)cysts based on shape, size, and FITC-fluorescence. Since the portable microscope used did not include a violet or UV light source, we were unable to visualize the distinct nuclei with the additional DAPI-staining as proposed by USEPA 1623.[24] Often fluorescent particles such as algae and supposedly fragments from destroyed oocysts were observed. The drying of the samples on the slides may have destroyed some of the oocysts.

qPCR. The linear equations of the Ct values for the controls (10^{2}–10^{5} oo(cysts)/mL) were $y(\log 10) = -0.4332x + 16.12$ ($R^2 = 0.9677$), and $y(\log 10) = -0.295x + 11.9$ ($R^2 = 0.986$) for *Giardia* and *Cryptosporidium*, respectively.

For *Giardia*, the quantification by PCR, if successful, performed well, but for almost 50% of the cases we did not obtain a result. For *Cryptosporidium* only two of the 27 samples tested showed a positive result (Figure 2).

Since the theoretical detection limit of the PCR is one (oo)cyst and given our dilution of the extracted DNA, we estimate for our approach a detection limit of 40 (oo)cysts per reaction. Thus, in a number of cases, the (oo)cyst concentration was below the detection limit of our method. However, it is most likely that in the many other cases where concentrations were above this limit, as indicated by IFM and FCM, detection was hampered by inhibitory compounds, which can be considered ubiquitous in canal waters. Such interference of inhibitory compounds in natural water samples with PCR detection is often reported in literature.[36−38]

Comparison of the Detection Methods. For *Giardia lamblia*, concentrations ranged from 3 to 347, 15 to 855, and 39 to 4,074 cysts per liter for IFM, FCM, and PCR, respectively (Medians: 32, 60, 165). Generally, PCR indicated higher cyst concentrations than the other two methods, but often gave a false negative result (13 out of 27 samples, i.e., ∼48%). FCM and IFM gave consistently similar results, though the FCM count was usually (in 93% of samples) higher (Figure 2). Because of the high false negative rate of the PCR analysis we linearly correlated only FCM vs IFM, leading to a Pearson correlation for FCM vs IFM of $r = 0.867$, $p < 0.0001$, $n = 24$ and a linearization function of $y = 0.35x + 16.2$ for the surface water samples (Figure 3).

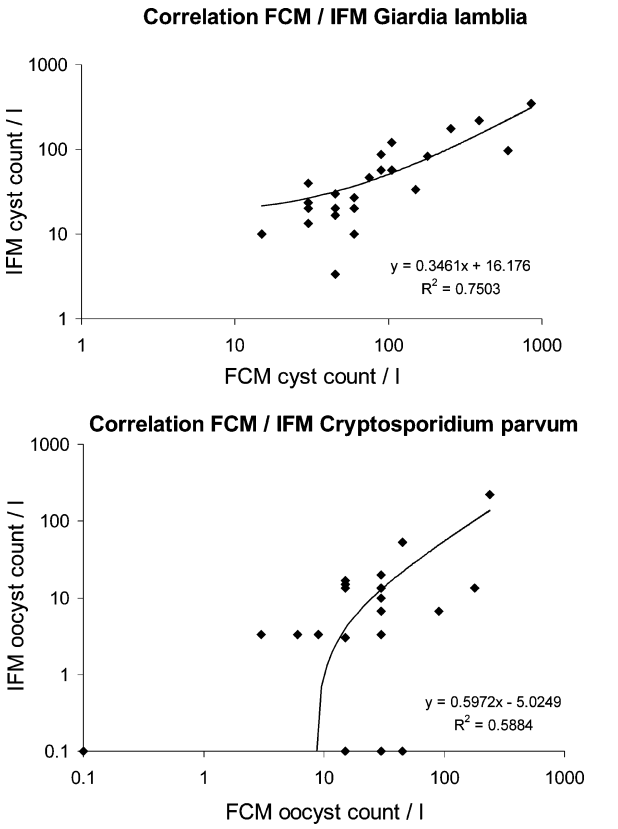

Figure 3. Linear regression for FCM versus IFM quantification.

For *Cryptosporidium parvum*, concentrations ranged from 0 to 220 oocysts per liter for IFM, and from 0 to 240 oocysts per liter for FCM detection. While PCR detection failed except for two samples, FCM and IFM did give consistently similar results with 67% of the samples with higher FCM counts. The Pearson correlation for the results from all surface water samples was $r =$

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0.767, $p < 0.0001$, $n = 24$ and a linearization function of $y = 0.60x - 5.0$ for the surface water samples (Figure 3).

Overviews of the measured (oo)cyst concentrations per sampling point and the reduction of oo(cysts) from wastewater inlet to downstreams locations can be found in the Supporting Information (Figures S6 and S7).

Vegetable Samples. We sampled irrigation water, lettuce and Morning Glory to test for contamination due to their exposure to canal water. Directly after harvesting the lettuce was briefly washed by the farmers with irrigation water to eliminate the soil prior to being sold on the market. Therefore, we took samples from unwashed and washed lettuce. The Morning Glory was directly harvested in the canal, as this plant grows on the water surface. Consistent with the assumption that the fecal contamination from the wastewater will eventually contaminate the food, we found up to 50 *Giardia* cysts and up to 10 *Cryptosporidium* oocysts in the salad and Morning Glory per 200 g of sample (Table 1).

Comparison to Other Parameters Measured. For all other measured parameters, we found high and variable values underlining the high complexity of this matrix (Figure S8 of the SI). Apart from the above-described obvious correlations between the *Giardia* and *Cryptosporidium* data obtained with different methods (FCM and IFM), we also found some with the other measured parameters. HPC and *E. coli* counts correlated well (Pearson; $r > 0.5$; $p < 0.005$) with the *Giardia* cyst load. Surprisingly, the TCC did not correlate with any other parameter, whereas the conductivity did correlate well with turbidity, *E. coli*, and HPC counts (Table S3 of the SI).

DISCUSSION

Laboratory Setup. For this 2-week mission, all instrumentation, consumables, and additional equipment was transported from Switzerland to Thailand and our lab was set up in the Asian Institute of Technology. Although based in a laboratory, we used only lab benches, fridges, freezers, and electricity to carry out the three methods. If necessary, for completely remote and independent field missions, all of these could be made available, since all of our instrumentation can be run on 12 V batteries.

Methods. Our IMS approach employed superparamagnetic particles that require a high gradient magnetic field and the passage through a column for enrichment. This approach was sufficient for our samples but obviously the performance was reduced compared to other surface water samples (average recoveries >80%, [10]), as these columns are neither designed nor optimized for surface water samples and are prone to clog very fast. Beads with stronger paramagnetic properties of the beads would be advantageous to allow a magnetic separation without a column.

IFM detection was very tedious and time-consuming, though the visual confirmation of the size and morphology is a great benefit of this method. In highly turbid samples, the major drawback is the formation of layers of particles on the filter, which can cover and obscure the fluorescent target organisms. Missing of (oo)cysts due to viewer fatigue may also be a reason for the consistently lower count of IFM versus FCM.

The poor performance of PCR to detect *Giardia* and *Cryptosporidium* in the tested water samples can be attributed to multiple factors. Since we had no possibility to restrict the elution volume of the DNA extraction (e.g., by SpeedVac) only a small fraction (5 μL of 200 μL) could be employed for a single PCR run. A further issue might be the disruption of the cells by repeated freeze and thaw cycles, which was shown to be crucial for DNA extraction efficiency. Since dry and liquid nitrogen are neither easy to transport by air cargo nor everywhere available, freezing of the samples had to be performed at −20 °C, which substantially prolonged the cell disruption process. These suboptimal conditions might have led to a lower extraction yield due to DNA degradation processes and inefficient cell disruption. Last but not least, the biological complexity of the sample matrix may impair primer specificity and lower the PCR efficiency due to inhibitors.

FCM detection was very fast and convenient and worked very satisfactory for *Giardia*. The performed single-staining approach lead to the *Cryptosporidium* cluster being very close to the background and *Giardia* cyst signals, leading to a risk of some false-positive signals. Nevertheless, the detection method might be improved by another double-staining approach that helps in discriminating, e.g., autofluorescent algae from oo(cysts), and decrease the risk for false-positive results. Furthermore, the integration of viability indicator stains, such as propidium iodide revealing oo(cyst) integrity with good correlation to in vitro excystation protocols,[39] would further improve these methods.

For assessing the pathogen flow in a water system based on the reduction of cysts by IFM and FCM we showed that both methods provide similar results (Figure S7 of the SI).

In summary, *Giardia* FCM and IFM compared sufficiently well, whereas for *Cryptosporidium* the FCM method needs improvement. Nevertheless, compared to USEPA 1623 our method performs sufficiently well, as our approach does meet the minimum criteria for USEPA 1623 that are 11 to 100% recovery of *Cryptosporidium* and 14 to 100% recovery of *Giardia*. Furthermore, two studies employing USEPA 1623 found average recoveries in high turbid samples to be 0.5−22% and 17−35% for *Giardia* and *Cryptosporidium* detection, respectively.[27,40] This underlines the potential usefulness of the here presented purification approach.

Since the successful application of PCR in highly soiled samples was demonstrated previously,[30−32] we conclude that PCR is dependent on optimal conditions that cannot be easily maintained in field campaigns. IFM has the advantage of visual confirmation and higher specificity, whereas the FCM method is more sensitive and faster, since the time per analysis takes only 4 min instead of 30 and more minutes for the microscopic counting. At the state of the art presented here, we propose to apply FCM for the initial rapid screening of water samples with IFM as a useful addition for control and confirmation in case of ambiguous FCM results.

Pathogen Concentrations. Although we did not adjust the presented data according to the determined recovery, the observed contamination with pathogens was substantial. The reduction of organisms was very variable from wastewater inlet to exposure locations and, therefore, the spatial distance from the wastewater inlet cannot provide safety (Figures S6 and S7 of the SI). Consequently, the infection risk when in contact with these waters is very high and precautions similar to when dealing with wastewater are recommended. We could demonstrate that the brief salad wash prior to distribution did not reduce the pathogen load. As the vegetables were considerably contaminated and other sources of contamination were out of the question, we could show the transfer of pathogens from the wastewater to foodstuffs.

Outlook. Rapid detection tools for microorganisms based on molecular and immunological technology can be trans-

ported by plane and set into operation within a few hours. With these new techniques and instrumentation, it is possible to analyze different environmental samples for protozoan pathogens within hours. This approach can also be adapted for the detection of pathogenic bacteria [41] and other sample matrices. In particular, FCM detection appeared to be very convenient, and given some optimizations, it allows an easy, fast, cost-effective (~30 USD vs >200 USD for USEPA 1623 consumables cost per sample [42]), and reliable monitoring with considerable potential for automation. Therefore, we believe that in the near future this detection method could be applied successfully by intervention units abroad.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of the sampling sites, methods, and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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