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Detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples with a Becton Dickinson FACSort flow cytometer.

F.M. Schets, G.J. Medema and G.D. Boschman[1)]

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This research has been performed in cooperation with BECTON DICKINSON Immunocytometry Systems Europe.

1) Becton Dickinson Immunocytometry Systems Europe, P.O.Box 13, B-9320 Erembodegem-Aalst, Belgium.

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National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands
tel. 030-749111, fax 030-742971

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SUMMARY

Current detection techniques for *Cryptosporidium* oocysts and *Giardia* cysts in water samples combine filtration of large volumes of water, concentration by centrifugation and flotation and immunofluorescence microscopy. The techniques are extremely labour-intensive and inefficient. The various steps in the sample processing procedures cause large losses of (oo)cysts resulting in an average overall recovery of about 3% in surface water samples. Microscopic interpretation of concentrates is time consuming and difficult due to the presence of debris.

We used fluorescence activated cell-sorting with a flow cytometer prior to fluorescence microscopy in order to improve our standard method. We therefore incorporated the FACSort flow cytometer prior to epifluorescence microscopy in our current detection method to purify *Cryptosporidium* and *Giardia* (oo)cysts in concentrated river water, sewage water and secondary effluent samples. The (oo)cysts were stained with FITC-conjugated monoclonal antibodies and sorted by the FACSort on the basis of fluorescence and forward scatter characteristics on a 13 mm polycarbonate membrane filter. 28 environmental samples (including 10 seeded samples) were examined with both our standard method and the method with the FACSort flow cytometer. The FACSort flow cytometer proved to be user-friendly and easy to operate and it improved the performance of the standard method. With FACSort 96% of the samples were positive for *Giardia*, the geometric mean was 8.6 cysts per liter. The standard method detected *Giardia* cysts in 86% of the samples with a geometric mean of 4.1 cysts per liter. In 22/25 samples that were examined with both methods FACSort detected 14 (range 1.2-84) times as many cysts per liter as the standard method. Differences between FACSort and standard are significant ($P<0.05$). FACSort detected *Cryptosporidium* in 74% of the samples, while the standard method detected oocysts in 64% of the samples. Geometric means were 0.95 and 0.38 oocysts per liter respectively. FACSort found 17 (range 1.1-91) times as many oocysts per liter in 15/27 samples that were examined with both methods; differences are, however, not significant. FACSort sorted (oo)cysts onto membrane filters and microscopic preparations obtained were very clean and easy to interpret, thus giving more reliable counts. When sorting was performed onto 13 mm diameter membrane filters microscopy time could be reduced with approx. 70%.

The overall recovery of both methods is relatively low. FACSort recovered 5.9% of the *Cryptosporidium* oocysts and 17.1% of the *Giardia* cysts and the standard method recovered 2.9% of the oocysts and 15.6% of the cysts. The low overall recovery is not due to the performance of the FACSort flow cytometer, for this instrument recovered 95% of oocysts present in a suspension. But FACSort as well as the standard method use the same sample processing procedure which includes various steps that cause large losses of (oo)cysts.

Recommendations are made to improve the discrimination of (oo)cysts from the debris with other monoclonal antibodies or fluorochromes, in order to overcome the need for microscopic confirmation. The recovery of the entire sample processing procedure may be improved when flow cytometry is coupled with calcium carbonate flocculation. Sorted samples containing (oo)cysts and relatively low levels of debris may be suitable for the application of techniques predicting viability which is a key characteristic in determining the impact of environmental (oo)cysts on human health. Pure suspensions of sorted environmental (oo)cysts may also be used in species-specific PCR assays.

SAMENVATTING

De huidige methode die in de Verenigde Staten, Groot-Brittannië en Nederland wordt gebruikt voor detectie van *Cryptosporidium* en *Giardia* in water bestaat uit een combinatie van filtratie, concentratie door centrifugeren en flotatie en immuunfluorescentie microscopie. De methode is zeer arbeidsintensief en inefficient. Tijdens het opwerken van de monsters gaan veel (oo)cysten verloren; dit resulteert in een gemiddelde overall recovery van ongeveer 3% in monsters oppervlaktewater. Interpretatie van de microscopische preparaten is zeer tijdrovend en bovendien wordt hinder ondervonden van het in de preparaten aanwezige debris.
In dit onderzoek werd de huidige methode verbeterd door gebruik te maken van fluorescence activated cell-sorting met een flow cytometer. Hiertoe werd de FACSort flow cytometer ingepast in de huidige methode om de (oo)cysten van *Cryptosporidium* en *Giardia* uit concentraten van rivierwater, rioolwater en secundair effluent te zuiveren. De (oo)cysten werden gekleurd met FITC-gelabelde monoclonale antilichamen en op basis van hun fluorescentie en forward-scatter eigenschappen gesorteerd op een 13 mm polycarbonaat membraanfilter. 28 milieu monsters (inclusief 10 kunstmatig besmette monsters) werden onderzocht met zowel de standaard methode als de methode met de FACSort. De FACSort bleek zeer gebruikersvriendelijk en gemakkelijk te bedienen en verbeterde de prestaties van de standaard methode. Met de FACSort was 96% van de monsters positief voor *Giardia* (geometrisch gemiddelde 8,6 cysten/l), met de standaard methode was dit 86% (geom. gem. 4,1 cysten/l). In 22/25 monsters die met beide methoden werden onderzocht detecteerde FACSort 14 (range 1,2-84) maal zo veel cysten als de standaard methode. Verschillen tussen beide methoden zijn significant ($P<0,05$). FACSort detecteerde *Cryptosporidium* in 74% van de monsters (geom. gem. 0,95 oocysten/l), terwijl de standaard methode dat in 64% van de monsters deed (geom. gem. 0,38 oocysten/l). FACSort vond 17 (range 1,1-91) maal zo veel oocysten in 15/27 monsters die met beide methoden werden onderzocht; de verschillen tussen beide methoden zijn echter niet significant. Met de FACSort werden (oo)cysten op membraanfilters gesorteerd; de verkregen preparaten waren erg schoon en makkelijk te beoordelen, waardoor de tellingen betrouwbaarder werden. Bij sorteren op membraanfilters met een diameter van 13 mm kon de tijd nodig voor microscopische beoordeling met ca. 70% gereduceerd worden.
De overall recovery van beide methoden is relatief laag. Met FACSort werd voor *Giardia* een recovery van 17,1% behaald, voor *Cryptosporidium* bedroeg de recovery 5,9%. De standaard methode vond 15,6% van de cysten en 2,9% van de oocysten terug. De lage overall recovery is niet te wijten aan de FACSort; deze vindt 95% van de oocysten in een suspensie terug. De monster opwerk procedure die voor beide methoden gelijk is kent echter een aantal stappen die een aanzienlijk verlies van (oo)cysten veroorzaken.
Er wordt een aantal aanbevelingen gedaan om het onderscheidend vermogen van de FACSort te verbeteren door andere monoclonale antilichamen of fluorochromen te gebruiken, om de microscopische bevestiging te kunnen laten vervallen. De recovery van de totale opwerkings procedure kan verhoogd worden als flow cytometrie gekoppeld wordt met flocculatie met calciumcarbonaat. Gesorteerde monsters die naast (oo)cysten weinig debris bevatten zijn mogelijk ook geschikt voor het gebruik van technieken die de levensvatbaarheid van de (oo)cysten vaststellen. Levensvatbaarheid is het belangrijkste criterium bij het bepalen van de gevolgen van (oo)cysten uit het milieu voor de volksgezondheid.

1. INTRODUCTION

Current detection techniques for *Cryptosporidium* oocysts and *Giardia* cysts in water samples consist of filtration of large volumes of water, elution of the particles from the filter material, concentration by centrifugation and flotation and staining of the (oo)cysts with fluorescein isothiocyanate (FITC) labelled monoclonal antibodies specific to the (oo)cyst wall. The stained samples are examined with epifluorescence microscopy for particles which have the morphological and fluorescence characteristics of (oo)cysts. In the United States and the United Kingdom surface water and drinking water samples are routinely examined for *Cryptosporidium* oocysts and *Giardia* cysts. Although US and UK standard methods differ in some steps of the procedure, both techniques have in common that they are labour-intensive and inefficient (1).

Sample treatment procedures in these standard methods involve various steps that cause significant losses of oocysts and cysts. Elution of the (oo)cysts from a cartridge filter recovers only 0.2 - 40 % of the (oo)cysts present, centrifugation leads to a loss of 25 - 84%, whereas cleaning of the sample by sucrose flotation causes another loss of 75% (2). Combined losses of all steps result in low recoveries of complete sample processing procedures. Wyn-Jones and Shepherd (3) reported a *Cryptosporidium* oocyst recovery of 3.0 - 16.5% (average 12.0%). They found that the overall recovery decreased to an average of 3.1% (range 0 -11.6%) when samples were cleaned by sucrose flotation. From cartridge filters that were injected with (oo)cysts, 5 of 10 laboratories recovered 10.8% of the *Giardia* cysts (range 0.8 - 22.3%) and only 4 of 10 laboratories detected *Cryptosporidium* with an average recovery of 6.7% (range 1.3 - 20.8%) (4).

The processing of one sample takes several hours and is followed by at least one hour of microscopy. Microscopic preparations are difficult to interpret and require a skilled analyst. Laboratories are searching for other detection methods to overcome these disadvantages. Some laboratories have succeeded and are now using methods that are less labour-intensive and give better recoveries. They investigate small volumes (10 l) of environmental samples using flocculation or membrane filtration coupled with flow cytometry. Flocculation with calcium carbonate recovers 60 - 80% of the oocysts present in a sample and concentration through membrane filtration using membranes with poresizes of 1.2 or 3.0 µm recovers 10 - 60% (2). Vesey *et al* (1) combined tangential flow filtration and flow cytometry and recovered over 92% of seeded (oo)cysts in 100 l samples of river and reservoir water. Regli (5) did not report recovery figures, but found that concentration of particles from water samples by flocculation with aluminium-sulphate followed by cell sorting with a flow cytometer was less time consuming, easier to perform and more specific than a reference method using filtration and direct fluorescence microscopy for the detection of *Cryptosporidium* oocysts and *Giardia* cysts.

The use of flow cytometry prior to epifluorescence microscopy largely facilitates microscopy and reduces the time required for microscopic examination of samples. In concentrated water samples stained with fluorescence labelled monoclonal antibodies, a flow cytometer is capable of separating particles with shape, size and fluorescence characteristics of *Cryptosporidium* oocysts and *Giardia* cysts from other particles. Separated particles

can be sorted onto a membrane filter or a microscope glass slide, rendering a highly purified preparate that is easy to examine by microscopy. In environmental samples, however, large numbers of particles have the morphological and fluorescence characteristics of *Cryptosporidium* oocysts or *Giardia* cysts. Flow cytometry is not yet capable of discriminating all of these particle from (oo)cysts and they are included in the sort. So flow cytometry is considered as an extra purification step and sorted particles need to be confirmed by microscopy.

Apart from increasing recovery and efficiency, flow cytometry can aid to the other methodological drawbacks of the standard methods. The purified state of sorted (oo)cysts makes it possible to analyse their viability. Viability is a key characteristic in determining the impact of environmental (oo)cysts on human health. There are several ways in which viability of (oo)cysts in purified concentrates can be determined. Viability staining procedures that use fluorescein diacetate (FDA) and propidium iodide (PI) can be applied to predict the viability of *Giardia* cysts (6). The viability of *Cryptosporidium* oocysts can be determined by using propidium iodide and diaminophenylindole (DAPI) (7). The Fluorescense In Situ Hybridisation (FISH) technique uses fluorescence labelled oligonucleotide probes against ribosomal RNA to detect the viability of cysts and oocysts. Viability determined with rRNA-specific probes appears to correlate with viability established through excystation (8).

The aim of this study was to incorporate the Becton Dickinson FACSort flow cytometer prior to fluorescence microscopy in our current method for detection of *Cryptosporidium* oocysts and *Giardia* cysts in water samples. Our current method is the method of LeChevallier (9) with minor alterations. We investigated whether the incorporation of the FACSort led to the expected detection of higher numbers of (oo)cysts in naturally contaminated and seeded samples and an increased efficiency of the current method. We examined 28 environmental samples (including 10 seeded samples) using both our current method and the method with the FACSort flow cytometer and compared the results.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sample collection

Ten river water samples and five samples from sewage treatment plants were collected using a petrol-driven centrifugal pump and filtered through wound polypropylene cartridge filters having a nominal porosity of 1 µm (Filterite, type DFT classic, length 10 inch; Memtec America Corporation, Maryland, USA). Approximate volumes of 200 l (surface water) or 10-100 l (sewage water) were collected. Three samples of surface water after reservoir storage were collected from a pressurized tap; approximate volumes of 1000-2000 l were collected.

Another six river water samples and four sewage water samples were seeded with 10 ml of a *Cryptosporidium* oocyst suspension (approximate concentration 500 oocysts per ml) and 10 ml of a *Giardia* cyst suspension (approximate concentration 300 cysts per ml). The exact numbers of (oo)cysts in the seeding suspensions were determined by fluorescence microscopy. Microscopic preparations were prepared as described below for processed water samples. After sample collection all filters and filter housings were placed on melting ice and transported to the laboratory where they were stored at 2-8 °C. Samples were usually processed within 24 to 72 h. One sterile cartridge filter was processed as a blanc sample.

2.2. Methods

2.2.1. Sample processing and staining

Filters were processed according to the method described by LeChevallier (9), with some slight modifications. Filters were cut in half lengthwise to produce separate fibers. Fibers of the inner third of the filter were placed in a Stomacher bag with 1 l washing solution (phosphate buffered saline (PBS) with 0,1% (w/v) SDS and 0,1% (v/v) Tween 80). We homogenised the filter material in a Stomacher (type 3500) for three subsequent 5-min intervals. The filter material was hand kneaded in between. After homogenisation, small portions of the fibers were hand wrung to remove the eluant water and washed in a beaker containing another 1 l of washing solution. The fibers were again hand wrung before discarding. This procedure was repeated for the middle and outer thirds of the filter material using the same portions of washing solution.

Homogenised and washed samples were centrifuged (1040 x g for 10 min at 4 °C) and combined into one pellet in two centrifugation steps. Final pellets were weighed. Weighed 1 ml fractions of the resuspended pellets were added to 19 ml of washing solution and sonicated in a Branson sonication bath (type 5210) for 10 min. The suspensions were carefully pipetted on top of 30 ml Percoll-sucrose flotation medium (specific gravity 1.10

g/ml, checked and used at room temperature) and centrifuged (1083 x g for 10 min). The upper 25 ml were aspirated, dilluted to 50 ml with washing solution and centrifuged again at 1083 x g for 10 min. The upper 45 ml were removed and the pellet was gently resuspended. Dilution to 50 ml with washing solution and centrifugation were repeated. Finally the supernatant was siphoned down to approximately 2 ml, the pellet was gently resuspended and filtered through a 25 mm polycarbonate membrane filter (1.2 µm pore size).

Monoclonal antibodies specific for *Giardia* (Giardia-Cel IF test; Cellabs Diagnostics, Brookvale, Australia) and *Cryptosporidium* (Detect IF cryptosporidium; Shield Diagnostics, Dundee, UK) were diluted 1:5 and 1:4 resp. in PBS. 125 µl of each diluted antibody was pipetted onto the filter and incubated for 30-45 min at 37 °C. The filter was rinsed with PBS and placed on a microscope glass slide, embedded in a drop of DABCO (Janssen Chimica no. 1124792)-glycerol mounting medium and covered with a cover slip. Preparations were sealed with colorless nailpolish, stored at 2-8 °C in the dark and examined for the presence of *Cryptosporidium* oocysts and *Giardia* cysts by epifluorescence microscopy, usually within 5 days.

Samples for FACSort were processed identically but supernatants were finally siphoned down to approximately 300 µl, before gently resuspending pellets. Volumes of 100 µl of each undiluted monoclonal antibody were added to the resuspended pellets, incubation was again at 37 °C for 30-45 min. After staining the suspensions were diluted to 10 ml with washing solution and centrifuged for 10 min at 1083 x g. Supernatants were siphoned down to approximately 300 µl and pellets were resuspended. Suspensions were filtered through 35 µm mesh filters to remove sand and other large particles.

2.2.2. Flow cytometry

Flow cytometric analysis was performed on a Becton Dickinson FACSort flow cytometer. The FACSort is equiped with a 15 mW Argon-ion laser operating at 488 nm. The instrument has two light scatter detectors and three fluorescence detectors. It uses an aerosol free sorting principle in which it mechanically sorts particles by capturing them from the flow stream using a catcher tube. We analysed our samples at a flow rate of 60 µl/min. The catcher tube is capable of catching particles from the flow stream at a rate of 300/sec. Even at an analysis speed as high as 20,000 - 30,000 cells per second, which is often required for samples containing huge amounts of debris particles, FACSort will effectively sort all particles specified by the sort region if put in the recovery sort mode. The sheath fluid consisted of isotonic saline (FACSFlow; Becton Dickinson cat.no. 342003). The detected signals were forward scatter (FSC) and FITC fluorescence. Forward scatter signals give information about the size of the analysed particle. Instrument controls were set in such a way that stained oocysts and cysts appeared in the top of a FSC-FITC dot plot; FSC and FITC were recorded on a logaritmic scale. Instrument settings were stored and recalled in subsequent measurements. In the FSC-FITC dot plot a rectangular sort region was defined that included both oocysts and cysts. All particles with fluorescence and light scatter characteristics similar to oocysts and cysts were sorted onto 1.2 µm 13 or 25 mm polycarbonate membrane filters. Filters were placed on microscope glass slides, embedded in a drop of DABCO-glycerol mounting medium and covered with a cover slip.

Preparations were sealed with colorless nailpolish, stored at 2-8 °C in dark and usually examined within 2 days.

Before each use the instrument was checked with CaliBRITE FITC Flow Cytometer Beads and a positive control sample containing stained *Cryptosporidium* oocysts. When using the recalled instrument settings the beads were to give narrow peaks in a dot histogram at preset places and the oocysts were to appear as a tight cluster within the sort region in the dot plot.

2.2.3. Microscopy

Microsopic preparations were examined using a Zeiss Axioskop epifluorescence microscope fitted with a x 25 and a x 100 oil immersion objective. Slides were examined for the presence of green fluorescing particles. *Cryptosporidium* oocysts were defined as spherical shapes with an apple green fluorescence of the cell wall and a diameter of 3-7 µm. *Giardia* cysts were defined as oval to spherical shapes with an apple green fluorescence of the entire particle, the fluorescence of the cell wall being brighter than that of the interior, and a size of 5-15 x 8-18 µm.

2.2.4. Statistical analysis

To evaluate differences between counts obtained with the standard method and the method with FACSort, analysis of variance was performed on log transformed counts using MINITAB (12).

3. RESULTS

When describing and discussing the results, our standard method will be referred to as 'standard', whereas the method in which the FACSort flow cytometer was used will be called 'FACSort'.

Figure 1, in which a line of equality is drawn, shows that FACSort recovered higher numbers of *Giardia* cysts than standard; differences between the methods are significant ($P<0.05$). With FACSort 24/25 (96%) samples were positive; standard detected *Giardia* in 24/28 samples (86%). In positive samples FACSort detected a geometric mean of 8.6 *Giardia* cysts per liter (min 0.009/l; max 5545/l), whereas standard detected a geometric mean of 4.1 cysts per liter, numbers ranging from 0.04 to 3795 per liter. 25 samples were examined with both methods. FACSort detected 14 (range 1.2-84) times as many cysts per liter as standard in 22 of those samples. Standard detected more cysts in 2 samples and neither method detected *Giardia* cysts in 1 sample. FACSort performed better than standard when analysing both river and sewage water and seeded samples. The improvement was particularly profound when samples with low numbers of *Giardia* cysts were examined.

Figure 2, in which also a line of equality is drawn, shows that FACSort also performed better than standard when detecting *Cryptosporidium* oocysts in both naturally contaminated and seeded water samples, although differences between the two methods were less distinct and not significant ($P>0.05$). FACSort detected *Cryptosporidium* oocysts in 20/27 samples (74%), with a geometric mean of 0.95 oocysts per liter (min 0.005/l; max 218/l). Standard detected oocysts in 18/28 samples (64%) with a geometric mean of 0.38 oocysts per liter (min 0.004/l; max 79.2/l). 27 samples were examined with both methods and FACSort detected 17 (range 1.1-91) times as many oocysts per liter as standard in 15 samples, whereas standard gave higher counts in 6 samples. In 5 samples neither method detected *Cryptosporidium* and in 1 sample the methods performed equal. For *Cryptosporidium* differences between FACSort and standard were generally maximal when seeded samples were analysed.
Neither method detected (oo)cysts in the blanc sample.

We have seen that *Giardia* cysts fluoresce brightly and most cysts in a suspension have the same fluorescence intensity. FACSort clearly separated *Giardia* cysts from other particles and debris in concentrated water samples. A suspension of fresh *Cryptosporidium* oocysts contained bright green fluorescing oocysts, however, the intensity of fluorescence was lower than that of *Giardia* cysts. We have noticed that oocysts from environmental samples vary in their level of fluorescence from very bright to very weak.

Analysis of samples resulted in dot plots in which each dot represents one sorted particle. With the chosen instrument settings dots representing (oo)cysts appear in the top of the dot plot. Figure 3 shows the position of the *Cryptosporidium* oocysts (3A) and *Giardia* cysts (3B) in the sort rectangular, when forward scatter and FITC fluorescence signals are depicted on a logaritmic scale. The figures illustrate that *Giardia* cysts are larger and fluoresce stronger than *Cryptosporidium* oocysts. Figure 3A shows that the position of the oocysts in the sort rectangular is above and to the left of most of the debris particles, but

it is obvious that a fraction of the debris is included in the sort region. Dots representing particles below the set fluorescence threshold are not shown in the dot plot.
The FACSort flow cytometer is user-friendly and easy to operate; the instrument is ready for use within 15 min. After sample processing and staining analysis of one sample with the FACSort takes 15-20 min. Rinsing of the flow cytometer between two subsequent samples and getting ready for the next sample takes 10 min.
When cysts and oocysts are sorted onto 25 mm diameter membrane filters, microscopic examination of one sample takes 45-60 min. Microscopic examination of one sample that is processed with the standard method takes at least one hour. There is only a minor gain in time when FACSort is used, but preparations are cleaner and easier to interpret thus giving more reliable counts. Preparations obtained with the standard method were usually thick and difficult to interpret. (Oo)cysts might have been obscured by debris particles and weakly fluorescing (oo)cysts were difficult to detect or might have been missed because of background fluorescence. Sorting of (oo)cysts onto 13 mm diameter membrane filters reduces microscopy time to 15-20 min.

4. DISCUSSION AND CONCLUSIONS

The use of the FACSort flow cytometer to purify concentrated water samples prior to epifluorescence microscopy has, compared to our standard method, resulted in more positive samples and higher *Cryptosporidium* and *Giardia* counts.

Microscopic confirmation of sorted particles is still necessary: in 10 river water samples we analysed with the FACSort flow cytometer, the average number of sorted particles was about 5100, while microscopic confirmation showed that the average numbers of *Cryptosporidium* oocysts and *Giardia* cysts in these samples were 2 and 55 respectively. The purified state of the sample after sorting by the FACSort makes microscopy easier, faster and more reliable.

FACSort detected higher numbers of *Giardia* cysts in 91% and more *Cryptosporidium* oocysts in 69% of the samples that were positive with both methods. Samples that were analysed with FACSort were stained in suspension in stead of on membrane filters or on glass slides. In suspension more epitopes of the (oo)cyst cell wall are exposed to the monoclonal antibodies, resulting in (oo)cysts with a brighter fluorescence which are easier to detect. FACSort separated these brighter fluorescing (oo)cysts from most other particles in concentrated water samples. Differences between FACSort and standard seemed to be maximal when samples with low *Giardia* concentrations were analysed. At low cyst concentrations the impact of masking of the cysts by debris particles is relatively high. Although FACSort performed better than standard, the difference between the methods for *Cryptosporidium* detection was not as distinct as in *Giardia* analysis. This different performance of FACSort might be due to differences between oocysts and cysts in intensity of fluorescence signals. Figure 2 shows that the difference for *Cryptosporidium* between FACSort and standard was most obvious in seeded samples. FACSort recovered large numbers of oocysts from these samples, but performed less in naturally contaminated samples. Seeded samples contained brightly fluorescing oocysts that were clearly separated from other particles by FACSort. Environmental samples contain a relatively large amount of aged oocysts. Vesey *et al* (10) found that aged oocysts fluoresce less intense than fresh ones and show more variation in light scatter characteristics. He also stated that oocysts present in the aquatic environment might show subnormal reactions with monoclonal antibodies and are thus difficult to detect. Although the FACSort sort region is set broadly around the cluster of fluorescing *Cryptosporidium* oocysts, the environmental oocysts with a weak fluorescence and abnormal light scatter characteristics may not have been included in the sort region and may therefore not have been sorted. The fluorescence signal of *Giardia* cysts is stronger than that of *Cryptosporidium* oocysts and when *Giardia* cysts loose some of their fluorescence intensity at ageing, the *Giardia* cyst cluster will get a lower position in the FSC-FITC dot plot, but most of the cysts will still be included in the sort region.

The overall recovery of both methods is relatively low and differences between methods are small. The recovery of seeded oocysts with FACSort ranges from 2.5 to 17.4 %, with an average of 5.9 %. The standard method recovered -0.4 to 8.2 % of the *Cryptosporidium* oocysts from seeded samples, the average was 2.9 %. Recoveries of *Giardia* cysts from

seeded samples with FACSort and the standard method were 1.2 to 29.7 % (average 17.1 %) and 1.9 to 44.6 % (average 15.6 %) respectively. The low overall recovery is not due to the performance of the FACSort flow cytometer, for this instrument recovered 95% of oocysts present in a suspension. But FACSort as well as the standard method use the same sample processing procedure which includes various steps that cause large losses of (oo)cysts.

Concluding, the incorporation of the FACSort flow cytometer in our standard method has improved the performance of the standard method. With FACSort higher numbers of *Cryptosporidium* oocysts and *Giardia* cysts were detected in naturally contaminated and seeded environmental samples. The efficiency of the standard method could be improved, mainly because the time required for microscopic examination of samples, which is the most strenuous part of the method, could be reduced with approx. 70%.

5. RECOMMENDATIONS

The FACSort performance in naturally contaminated water samples might be improved when other monoclonal antibodies directed to the *Cryptosporidium* oocyst cell wall are used giving brighter fluorescence. A fluorescence signal for both cysts and oocysts that is much stronger than the maximum signals that are now obtained will change the position of the cyst and oocyst clusters in the FACSort FSC-FITC dot plot in such a way that the distance between these clusters and the debris is large enough to avoid interference. If this can be achieved FACSort may develop into an (oo)cyst counting instrument without the necessity of microscopic confirmation. In this study the monoclonal antibodies directed to *Cryptosporidium* and *Giardia* were both FITC conjugated. Conjugation of these monoclonals with two fluorochromes of different colour, eg. FITC and Phyco-Erythrin, may improve discrimination of cysts and oocysts. This is a prerequisite for enumeration by flow cytometer only, without the need for microscopic confirmation.

When FACSort is coupled with another sample processing procedure like flocculation with calcium carbonate, the overall recovery can be improved. Vesey *et al* coupled flocculation with calcium carbonate and flow cytometry and reported oocyst recoveries of over 68% (11). Flocculation of samples and purification steps that follow (washing and centrifugation) are not as labour-intensive as the filtration/elution procedure. The flocculation procedure is currently under investigation at our laboratory.

Sorted samples containing *Giardia* cysts and *Cryptosporidium* oocysts and relatively low levels of debris are suitable for the application of viability staining techniques and other methods to determine viability, like excystation and the use of probes. These methods may be applied after sorting in suspension or sorted (oo)cysts on membrane filters can be used. Pure suspensions of sorted environmental (oo)cysts may also be used in PCR assays that discriminate between species.

The Becton Dickinson FACStar-plus flow cytometer can also be used for sorting (oo)cysts from environmental samples. This flow cytometer can analyse samples at higher speed and sorted particles are concentrated in a tiny droplet on a glass slide. Examination of these slides by epifluorescence microscopy will be faster and, moreover, Direct Interference Contrast microscopy (DIC) can be used to confirm the (oo)cysts. The FACStar-plus flow cytometer is more flexible because it can be fitted with various lasers, which could make it possible to use additional fluorochromes to improve discrimination or for viability staining of the (oo)cysts. However, the viability dyes that are currently used will cause a very high level of background fluorescence, because the dyes will stain all biological material in the water concentrate. Thus far, no attempts to combine fluorescence activated cell-sorting and viability staining in natural samples are reported. The FACStar-plus is a more complex instrument than the FACSort and requires more operator skills and instrument testing for proper performance.

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FIGURES

Figure 1: *Giardia* cyst concentration in water samples from various origins. Comparison of the numbers detected with and without FACSort prior to fluorescence microscopy.

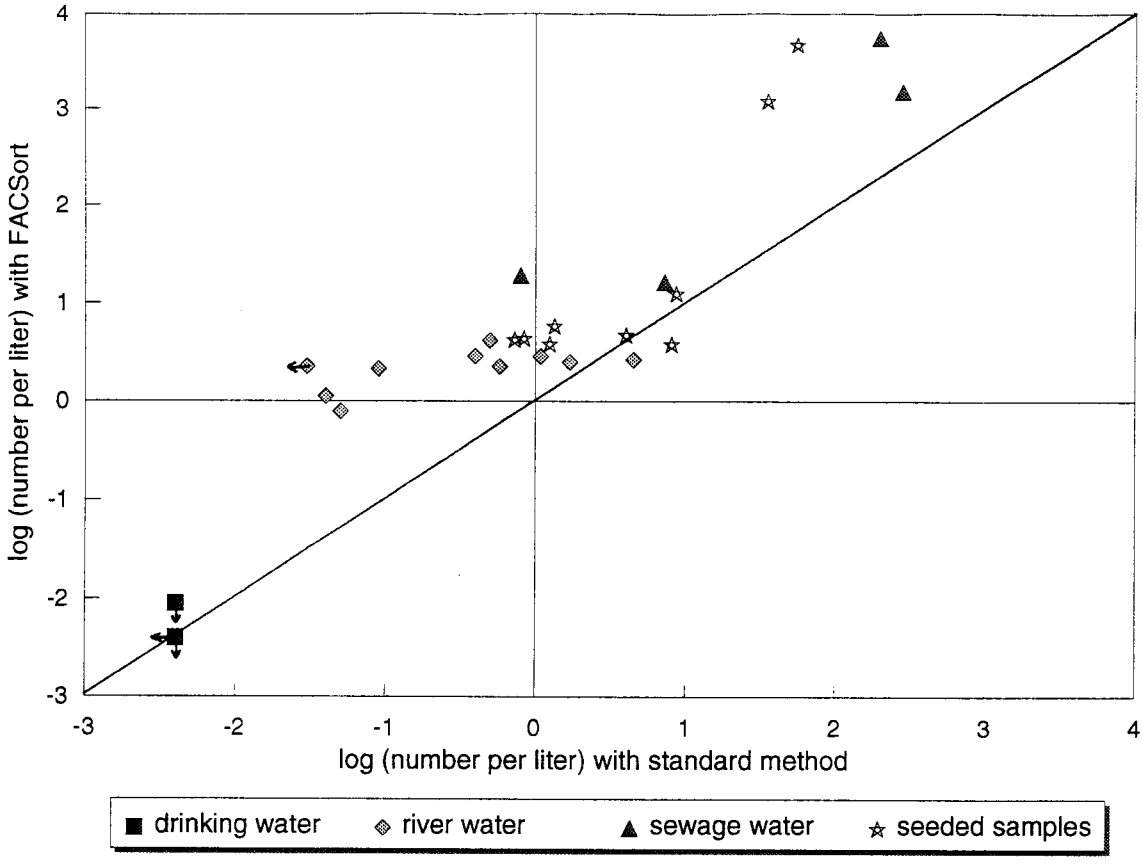


Figure 2: *Cryptosporidium* oocyst concentration in water samples from various origins. Comparison of the numbers detected with and without FACSort prior to fluorescence microscopy.

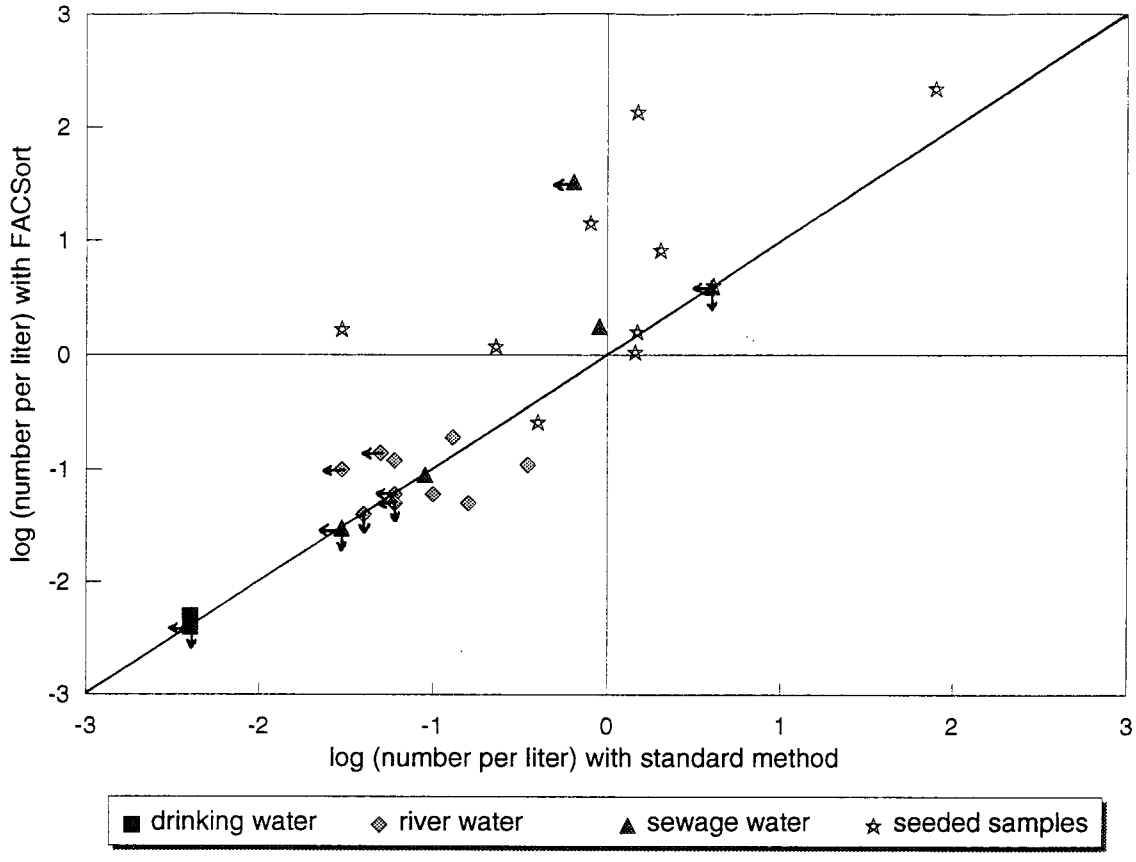
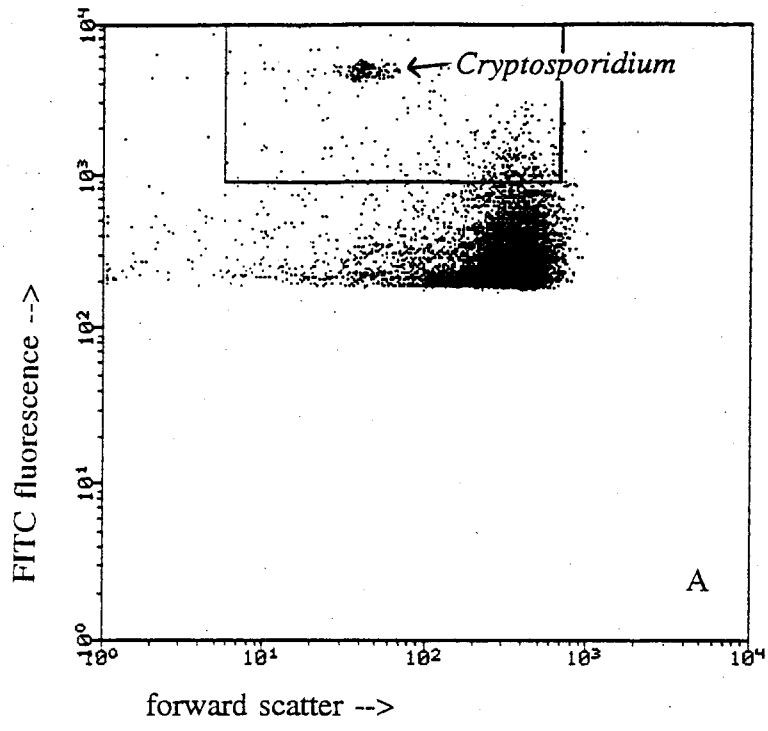
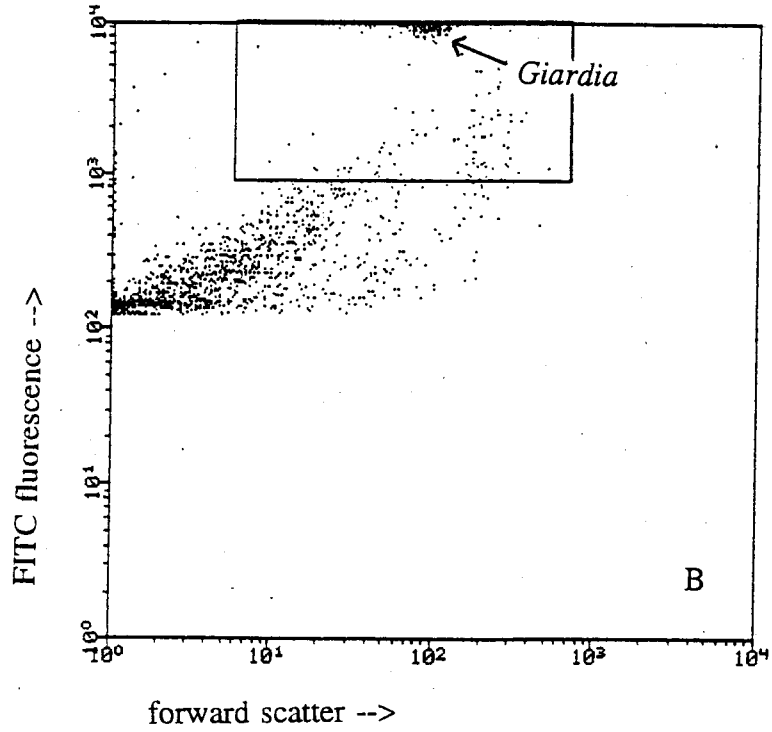


Figure 3: Forward scatter-fluorescence dot plot obtained with the FACSort flow cytometer, showing the position of *Cryptosporidium* oocysts (A) and *Giardia* cysts (B) in the sort rectangular.





SUPPLEMENTS

Supplement 1: *Cryptosporidium* and *Giardia* counts in environmental samples from various origins obtained with a standard method.

sample	sample date	sampled volume (l)	total weight pellet (g)	examined weight (g)	number Crypto-sporidium	number Giardia	Crypto-sporidium per liter	Giardia per liter
afgeleverd water WBB	310594	2395	2.97	0.54	0	0	<0.002	<0.002
influent Waalwijk	130694	11	2.96	0.90	3	11861	0.90	3546
influent Waalwijk spike	130694	10.4	3.26	0.99	250	11985	79.20	3795
Maas Keizersveer	140694	200	7.37	0.56	2	26	0.13	1.71
Maas Keizersveer	280694	200	11.20	1.08	2	21	0.10	1.09
Maas Keizersveer spike	280694	200	10.98	1.10	82	25	4.09	1.25
afgeleverd water WBB	50794	1190	4.51	0.84	0	0	<0.004	<0.004
Maas Keizersveer	190794	200	13.46	1.05	0	9	<0.06	0.58
Maas Keizersveer spike	190794	200	9.19	1.03	9	30	0.40	1.34
afgeleverd water WBB	260794	2041	9.50	1.03	1	0	0.004	<0.004
effluent Den Bosch	260794	100	3.28	1.02	0	227	<0.03	7.3
influent Den Bosch	260794	10	47.70	1.18	0	70	<4.0	283
effluent Den Bosch spike	260794	100	2.56	1.01	80	344	2.03	8.72
Maas Keizersveer	20894	200	10.44	1.01	3	1	0.16	0.05
influent Huizen	290894	50	31.72	0.98	0	308	<0.64	199
influent Huizen spike	290894	51	23.90	0.95	3	113	1.50	55.70
Maas Keizersveer	300894	204	8.23	0.99	1	1	0.04	0.04
influent Amsterdam spike	50994	50	5.90	0.89	6	266	0.80	35.30
effluent Amsterdam	50994	100	9.10	1.03	0	9	<0.09	0.80
Maas Keizersveer	130994	200	7.70	1.00	9	13	0.35	0.50
Maas Keizersveer spike	130994	200	7.42	0.97	6	22	0.23	0.84
blanc	61094	-	4.34	0.97	0	0	-	-
Maas Keizersveer	41094	201	8.94	1.00	0	9	<0.05	0.40
Maas Keizersveer spike	41094	200	6.49	1.03	46	129	1.45	4.06
Maas Keizersveer	81194	200	6.88	1.01	0	0	<0.03	<0.03
Maas Keizersveer spike	81194	200	5.93	1.02	1	25	0.03	0.73
Maas Keizersveer	61294	200	6.16	1.05	2	3	0.06	0.09
Maas Keizersveer	131294	200	11.40	1.04	0	83	<0.06	4.55
Maas Keizersveer spike	131294	200	12.81	1.03	24	130	1.49	8.08

Supplement 2: *Cryptosporidium* and *Giardia* counts in environmental samples from various origins obtained with a method in which a FACSort flow cytometer was used.

sample	sample date	sampled volume (l)	total weight pellet (g)	examined weight (g)	number Crypto-sporidium	number Giardia	Crypto-sporidium per liter	Giardia per liter
afgeleverd water WBB	310594	2395	2.97	-	-	-	-	-
influent Waalwijk	130694	11	2.96	0.90	6	-	1.79	-
influent Waalwijk spike	130694	10.4	3.26	0.99	687	-	218	-
Maas Keizersveer	140694	200	7.37	0.95	5	64	0.19	2.48
Maas Keizersveer	280694	200	11.20	1.01	0	51	<0.06	2.83
Maas Keizersveer spike	280694	200	10.98	1.01	75	70	4.08	3.81
afgeleverd water WBB	50794	1190	4.51	1.05	0	0	<0.004	<0.004
Maas Keizersveer	190794	200	13.46	1.05	0	35	<0.06	2.24
Maas Keizersveer spike	190794	200	9.19	1.04	6	132	0.26	5.83
afgeleverd water WBB	260794	2041	9.50	1.02	1	2	0.005	0.009
effluent Den Bosch	260794	100	3.28	0.97	0	483	<0.03	16.30
influent Den Bosch	260794	10	47.70	1.21	0	379	<3.94	1494
effluent Den Bosch spike	260794	100	2.56	0.91	293	442	8.24	12.40
Maas Keizersveer	20894	200	10.44	1.00	1	15	0.05	0.78
influent Huizen	290894	50	31.72	1.04	54	9090	32.90	5545
influent Huizen spike	290894	51	23.90	1.06	308	10592	136	4683
Maas Keizersveer	300894	204	8.23	1.01	0	28	<0.04	1.12
influent Amsterdam spike	50994	50	5.90	1.08	132	10766	14.40	1176
effluent Amsterdam	50994	100	9.10	1.00	0	209	<0.09	19.00
Maas Keizersveer	130994	200	7.70	1.01	3	108	0.11	4.12
Maas Keizersveer spike	130994	200	7.42	1.00	32	117	1.19	4.34
blanc	61094	-	4.34	0.97	0	0	-	-
Maas Keizersveer	41094	201	8.94	0.96	3	62	0.14	2.87
Maas Keizersveer spike	41094	200	6.49	0.98	32	142	1.06	4.70
Maas Keizersveer	81194	200	6.88	1.01	3	66	0.10	2.25
Maas Keizersveer spike	81194	200	5.93	1.02	58	145	1.69	4.22
Maas Keizersveer	61294	200	6.16	1.04	4	72	0.12	2.13
Maas Keizersveer	131294	200	11.40	1.06	1	49	0.05	2.64
Maas Keizersveer spike	131294	200	12.81	1.08	27	64	1.60	3.80

Supplement 3: Comparison of the numbers *Cryptosporidium* oocysts and *Giardia* cysts per liter in environmental samples detected with a standard method and a method in which a FACSort flow cytometer was used.

sample	sample date	Cryptosporidium per liter		Giardia per liter	
		standard	FACSort	standard	FACSort
afgeleverd water WBB	310594	<0.002	-	<0.002	-
afgeleverd water WBB	50794	<0.004	<0.004	<0.004	<0.004
afgeleverd water WBB	260794	0.004	0.005	<0.004	0.009
Maas Keizersveer	140694	0.13	0.19	1.71	2.48
Maas Keizersveer	280694	0.10	<0.06	1.09	2.83
Maas Keizersveer	190794	<0.06	<0.06	0.58	2.24
Maas Keizersveer	20894	0.16	0.05	0.05	0.78
Maas Keizersveer	300894	0.04	<0.04	0.04	1.12
Maas Keizersveer	130994	0.35	0.11	0.50	4.12
Maas Keizersveer	41094	<0.05	0.14	0.40	2.87
Maas Keizersveer	81194	<0.03	0.10	<0.03	2.25
Maas Keizersveer	61294	0.06	0.12	0.09	2.13
Maas Keizersveer	131294	<0.06	0.05	4.55	2.64
effluent Den Bosch	260794	<0.03	<0.03	7.30	16.30
effluent Amsterdam	50994	<0.09	<0.09	0.80	19.00
influent Waalwijk	130694	0.90	1.79	3546	-
influent Den Bosch	260794	<4.0	<3.94	283	1494
influent Huizen	290894	<0.64	32.90	199	5545
Maas Keizersveer spike	280694	4.09	4.08	1.25	3.81
Maas Keizersveer spike	190794	0.40	0.26	1.34	5.83
Maas Keizersveer spike	130994	0.23	1.19	0.84	4.34
Maas Keizersveer spike	41094	1.45	1.06	4.06	4.70
Maas Keizersveer spike	81194	0.03	1.69	0.73	4.22
Maas Keizersveer spike	131294	1.49	1.60	8.08	3.80
effluent Den Bosch spike	260794	2.03	8.24	8.72	12.40
influent Waalwijk spike	130694	79.20	218	3795	-
influent Huizen spike	220894	1.50	136	55.70	4683
influent Amsterdam spike	50994	0.80	14.40	35.30	1176
blanc	61094	-	-	-	-

Supplement 4: The recovery of *Cryptosporidium* oocysts and *Giardia* cysts from seeded environmental samples with a standard method and a method in which a FACSort flow cytometer was used.

sample	sample date	recovery of seeded Cryptosporidium (%)		recovery of seeded Giardia (%)	
		standard	FACSort	standard	FACSort
Maas Keizersveer	280694	2.4	2.5	*	*
Maas Keizersveer	190794	8.2	5.4	4.9	23.0
effluent Den Bosch	260794	0.9	3.8	*	*
influent Amsterdam	50994	1.0	17.4	*	*
Maas Keizersveer	130994	-0.4	3.5	1.9	1.2
Maas Keizersveer	41094	6.0	3.8	21.3	10.6
Maas Keizersveer	81194	0.1	5.4	5.1	29.7
Maas Keizersveer	131294	5.3	5.7	44.6	21.0

* sample not seeded with Giardia cysts