FFI RAPPORT

EVALUATION OF OPTOFLOW MICROCYTE® FLOW CYTOMETER

GRAN, Hans Christian, SKOGAN, Gunnar, OLSEN, Jaran Strand

FFI/RAPPORT-2002/00772

FFIBM/136

Approved
Kjeller 15 February 2002

Bjørn Arne Johnsen
Director of Research

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FORSVARETS FORSKNINGSINSTITUTT
Norwegian Defence Research Establishment
P O Box 25, NO-2027 Kjeller, Norway

FORSVARETS FORSKNINGSINSTITUTT (FFI)
Norwegian Defence Research Establishment

P O BOX 25
N0-2027 KJELLER, NORWAY

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE
(when data entered)

REPORT DOCUMENTATION PAGE

1) PUBL/REPORT NUMBER	2) SECURITY CLASSIFICATION	3) NUMBER OF PAGES
FFI/RAPPORT-2002/00772	UNCLASSIFIED	23
1a) PROJECT REFERENCE	**2a) DECLASSIFICATION/DOWNGRADING SCHEDULE**	
FFIBM/136	-	

4) TITLE

EVALUATION OF OPTOFLOW MICROCYTE® FLOW CYTOMETER

5) NAMES OF AUTHOR(S) IN FULL (surname first)

GRAN, Hans Christian, SKOGAN, Gunnar, OLSEN, Jaran Strand

6) DISTRIBUTION STATEMENT

Approved for public release. Distribution unlimited. (Offentlig tilgjengelig)

7) INDEXING TERMS

IN ENGLISH:	IN NORWEGIAN:
a) Bacteria	a) Bakterier
b) Flow cytometry	b) Flow cytometri
c) Detection	c) Deteksjon
d) Identification	d) Identifikasjon
e)	e)

THESAURUS REFERENCE:

8) ABSTRACT

Optoflow Microcyte® flow cytometer has been evaluated with regard to its user-friendliness and ability to detect different types of bacteria. *Bacillus cereus* and *Escherichia coli* are tested at different concentrations and in presence of various contaminants such as wheat flour, icing sugar, baking powder, table salt, melted snow and river water. ToPro^{TM}-3 has been used as fluorescent dye. The tested bacteria show characteristic and reproducible fluorescence intensity vs scatter plots, which are little influenced by contaminating compounds except for very high concentrations of table salt. The observed detection limits are in the order of 10^4 organisms/ml. The may need to be improved for example by selecting other fluorescent dyes and/or different sample preparation techniques. The flow cytometer is characterised by easy handling, small size and an apparently sturdy construction. The contribution of this system in detection of micro organisms is depending on to what extent it is possible to establish a comprehensive library of various types of bacteria, effect of living conditions and potential interfering particulate materials and chemicals.

9) DATE	AUTHORIZED BY	POSITION
	This page only	
15 February 2002	Bjørn Arne Johnsen	Director of Research

ISBN-82-464-0593-4

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE
(when data entered)

CONTENTS

EVALUATION OF OPTOFLOW MICROCYTE® FLOW CYTOMETER

1 INTRODUCTION

As a result of contacts between Optoflow AS and Norwegian military- and civil defence authorities, Norwegian Defence Research Establishment, Division for Protection and Materiel has been asked by Optoflow AS to undertake a feasibility study of Microcyte® flow cytometer. The Microcyte® flow cytometer has been evaluated with regard to its user-friendliness and ability to detect and identify different types of bacteria. Flow cytometry is an established technique that is widely used in hospitals and pharmaceutical industry (1, 2). To our knowledge, use of flow cytometers of the kind that Microcyte® represents, is new in the field of detection of biological weapons.

2 EXPERIMENTAL

2.1 Preparation and measurements on test samples

2.1.1 General

The selected sample set consisted of *Bacillus cereus* and *Escherichia coli*, water from the rivers Leira and Nita, wheat flour, icing sugar, melted snow and samples which have been used in

Sample	**Mixed with**	**Fluorescent stain ToPro™-3**
Bacillus cereus	Log phase	With/without
Bacillus cereus	Stored culture (1 month at 4 °C) and spores	With
Bacillus cereus	Baking powder	With
Bacillus cereus	Table salt	With
Bacillus cereus	Icing sugar	With
Bacillus cereus	Wheat flour	With
Bacillus cereus	River water (Leira, Nita)	With
Bacillus cereus	Melted snow	With
Escherichia coli	Log phase	With/without
Escherichia coli	Baking powder	With
Escherichia coli	Table salt	With
Escherichia coli	Icing sugar	With
Escherichia coli	Wheat flour	With
Escherichia coli	River water (Leira, Nita)	With
Escherichia coli	Melted snow	With
SIBCA	Various samples	With

Table 2.1 Overview of samples categories and sample combinations.

connection with a Sampling and Identification of Biological and Chemical Agents (SIBCA) round robin test arranged by NATO. A far-red fluorescent stain, ToPro^{TM}-3, was added. This stain has the property that it labels DNA in cells that are membrane permeant. An overview of sample categories and sample combinations is shown in table 2.1. Samples of environmental pollution and non-bacterial samples were also tested alone with and without presence of ToPro^{TM}-3 to obtain a measure of the amount of auto-fluorescence and ability to bind fluorescent stain.

2.1.2 Bacteria

Bacillus cereus and *Escherichia coli*, which were selected as main test organisms, were cultivated at 30 °C over night in Nutrient Broth and Liquid Broth respectively. Broth composition is given in table 2.2. Both cultures were (30 ml) were centrifuged for 5 minutes at 2000 x g before resuspension in 30 ml distilled and sterile filtered H_2O. The concentration of bacteria in each suspension was measured in a Bürker Türk count chamber (0.05 mm layer depth) to be $1.2 \cdot 10^8$ bacteria/ml (*Bacillus cereus*) and $1.0 \cdot 10^9$ bacteria/ml (*Escherichia coli*). From these suspensions dilution sequences with final sample concentration in the range from $10^6 – 10^3$ bacteria/ml were produced.

Nutrient broth	**Liquid broth**
Peptone 5g/l	Tryptone 10g/l
Meat extract 3g/l	Yeast extract 5g/l
Distilled water	Sodium chloride 5g/l
	Distilled water

Table 2.2 Broth composition. Broth solutions were autoclaved after adjustment to pH = 7.0.

Mixing 50 µl of bacteria suspension with 50µl sterile filtered water followed by mixing with 100µl ethanol made the actual sample suspensions. Each sample was incubated for 5 minutes at room temperature followed by addition of 800 µl Solution A and 10 µl ToPro^{TM}-3 and a final incubation of 1 minute prior to the measurements.

2.1.3 Samples of environmental pollution and non-bacterial samples

Samples of environmental pollution

Samples of environmental pollution from river water and melted snow were prepared in the same way as for bacteria. That is, 50 µl of sterile filtered water and then 100 µl of ethanol were added to 50 µl of sample followed by 5 minutes incubation at room temperature and addition of 800µl of Solution A and finally – in case of fluorescent staining – addition of 10 µl ToPro^{TM}-3 and 1 minute further incubation.

In cases where samples of bacteria were mixed with samples of environmental pollution the sterile filtered water was replaced by the sample containing bacteria.

Non-bacterial samples
Before use, non-bacterial samples were dissolved by mixing 1 gram of sample in 10 ml sterile filtered water followed by filtration through a 63 µm filter. Wheat flour and baking powder did not dissolve in water and was, as a consequence, diluted further with water in the ratio 1:10 and decanted. The amount of wheat flour and baking powder in these samples was for this reason unknown.

2.1.4 Bacteria mixed with samples of environmental pollution

These samples were prepared by first adding a volume of 50 µl of environmental pollution and then 100 µl of ethanol to 50 µl of bacteria sample. This was followed by 5 minutes incubation at room temperature, addition of 800µl of Solution A and 10 µl of ToPro^{TM}-3 and, finally, 1-minute incubation.
The final concentrations of bacteria in the samples before measurements were $6.1 \cdot 10^4$ and $6.1 \cdot 10^3$ bacteria/ml in case of *Bacillus cereus* and $5.0 \cdot 10^4$ and $5.0 \cdot 10^3$ bacteria/ml in case of *Escherichia coli*.

2.1.5 SIBCA-samples

Each sample was prepared by adding of 100µl ethanol to each 100µl sample followed by 5 minutes incubation before addition of 800µl of Solution A. Where ToPro^{TM}-3 was to be applied to the samples, 10 µl of the dye was added followed by 1 minute incubation before starting the measurement.

An overview of the concentrations of each of the SIBCA-samples is presented in table 2.3. The samples were diluted 10 times before testing.

SIBCA nr	**Organism**	**Consentration**
SIBCA A	*Yersinia pestis*, La Paz strain, vegetative	3,7 x 10^7 cfu/ml
SIBCA B	*Coxiella burnetii*	2,1 x 10^7 ID_{50}/ml
SIBCA C	Blank, PBS-buffer	
SIBCA D	*Bacillus anthracis*, vollum strain, spores	8,7 x 10^6 cfu/ml
SIBCA E	VEE-virus, Vaccine strain TC83	7,5 x 10^8 $TCID_{50}$/ml
SIBCA F	Vaccinia virus, Lister strain	6,7 x 10^6 cfu/ml
SIBCA G	*Vibrio cholerae*, Inaba strain	1,1 x 10^6 cfu/ml
SIBCA H	*Brucella melitensis*, biovar 1	2,9 x 10^7 cfu/ml
SIBCA I	*Coxiella burnetii*, 9 mile phase I, m/diesel smoke	3,6 x 10^6 ID_{50}/ml
SIBCA J	*Bacillus anthracis*, vollum strain, spores, m/diesel smoke	8,7 x 10^6 cfu/ml
SIBCA K	*Francisella tularensis*, Schu 4	5,2 x 10^6 cfu/ml

Table 2.3 Overview of SIBCA-samples presenting type of organism and concentration in original sample before incubation and addition of ethanol and Solution A (and ToPro-3 + final incubation)

2.2 Instrument settings, calibration and test/cleaning-procedure

2.2.1 Settings

The flow rate of the instrument was calibrated on a daily basis using the supplied MICROCYTE® calibration kit. This kit contained an aqueous dispersion of $1x10^6$ mono disperse polystyrene particles per millilitre. The flow rate was set to 0.5 µl sample/second. Each sample was measured during a period of 100 seconds corresponding to a sample volume of 50 µl.
Before using the fluorescence detector the instrument was calibrated using the MICROCYTE® FL control kit, which was supplied with the instrument. The control kit consisted of two bottles containing particles of polystyrene, one with high and one with low fluorescence intensity. Instrument gain for both scatter and fluorescence intensity was set to a value of 4.
When measuring particle size (scatter) alone (without employing the fluorescence detector) the instrument was set to calculate the mean value of the last ten measurements.

2.2.2 Test- and cleaning procedure

Test and cleaning procedures were carried out as described in instrument operating instructions and cleaning kit instructions.

3 RESULTS

All results are presented as fluorescence intensity vs. scatter plots. Scatter is a measure of particle size and is increasing from left to right in the plots. Gain has been kept constant during the tests. All plots are therefore comparable. Plots obtained from dilution series, a culture stored at 4 °C for one month and spores of *Bacillus cereus* in presence of ToPro™-3 are shown in figure 3.1A and 3.1B. Dilution series of *Escherichia coli* in presence of ToPro™-3 is shown in figure 3.2. The observed plots of the two bacteria differ in both location and shape.

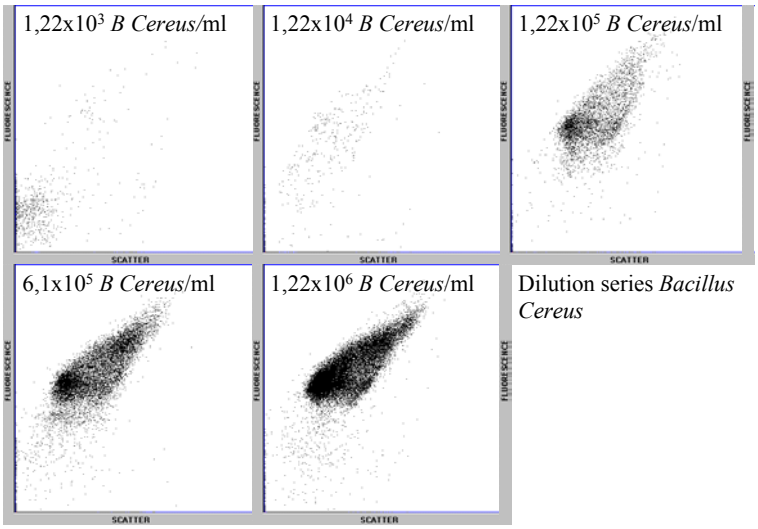

Figure 3.1A Fluorescence intensity vs scatter plots of dilution series of Bacillus cereus. Concentrations are shown in each plot

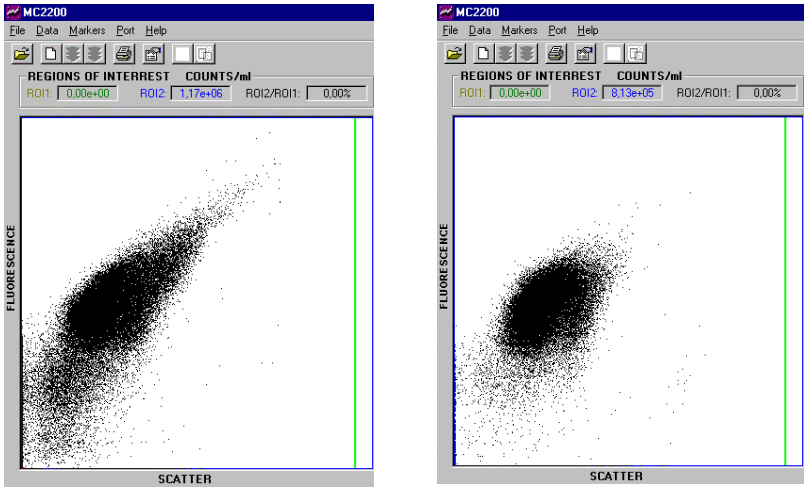

Figure 3.1B *Fluorescence intensity vs scatter plots of stored culture (left) and spores of Bacillus cereus*

When looking at the stored culture and spores the shape as well as location in the plot has changed somewhat. In particular, the plot of the stored bacteria culture of *Bacillus cereus* has expanded into a region of lower fluorescence intensity and smaller size.

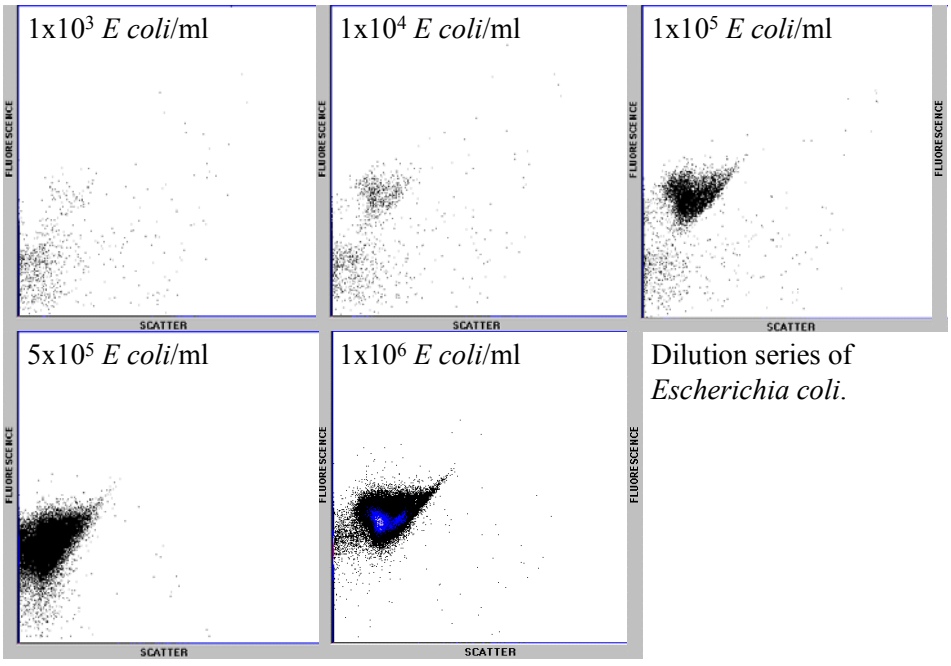

Figure 3.2 *Fluorescence intensity vs scatter plots of dilution series of Escherichia coli. Concentrations are shown in each plot*

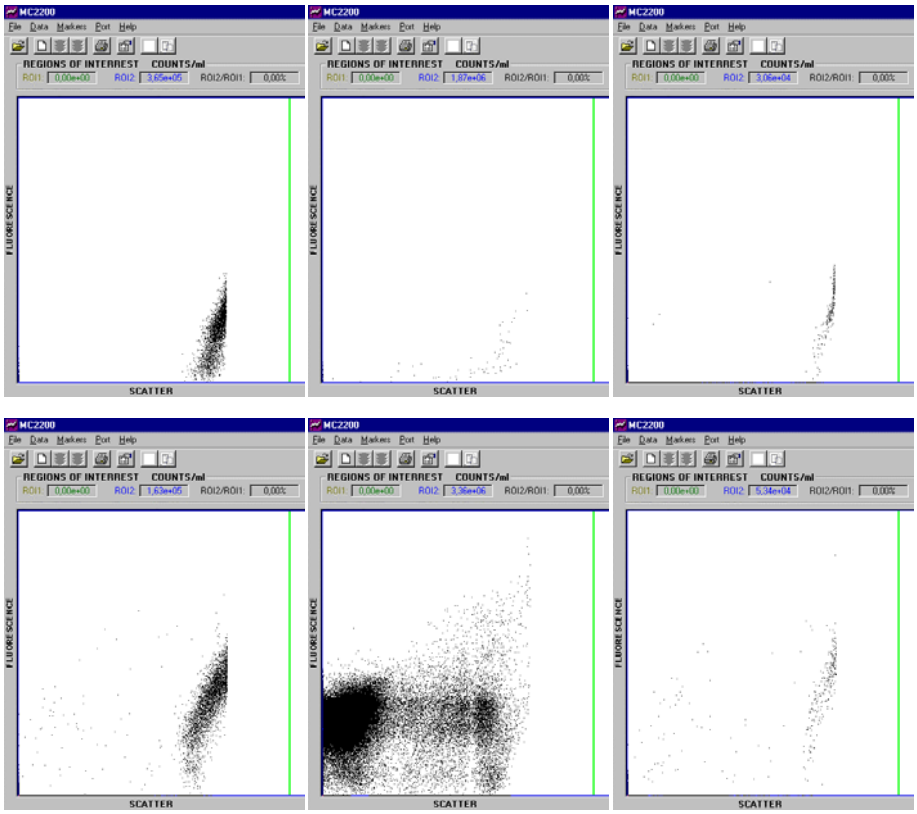

Figure 3.3 *Fluorescence intensity vs scatter plots of non-bacterial samples. From left, baking powder, wheat flour and icing sugar. Upper row with absence of ToPro™-3 and lower row with presence of ToPro™-3*

Systematic information is however lost at concentrations below $1.2 \cdot 10^5$ bacteria/ml in the case of *Bacillus cereus* and at concentration $1.0 \cdot 10^3$ in the case of *Escherichia coli*.

Plots obtained from non-bacterial samples both with and without presence of ToPro™-3 are shown in figure 3.3. Baking powder, wheat flour and icing sugar do to various extents bind to ToPro™-3. This is particularly so in the case of wheat flour. The main areas of higher observed signal intensity for baking powder and icing sugar seem to overlap in the same region in the lower right quadrant. Wheat flour also show an area of higher intensity in the left lower quadrant of the plot, which may coincide with position of the main signal from *Escherichia coli*.

Figure 3.4 presents measurements performed on water samples from the rivers Leira and Nita. A weak pattern is observed in water from Leira River. Practically no signal is observed in the sample from Nita River. Both samples show presence of material that bind to ToPro™-3.

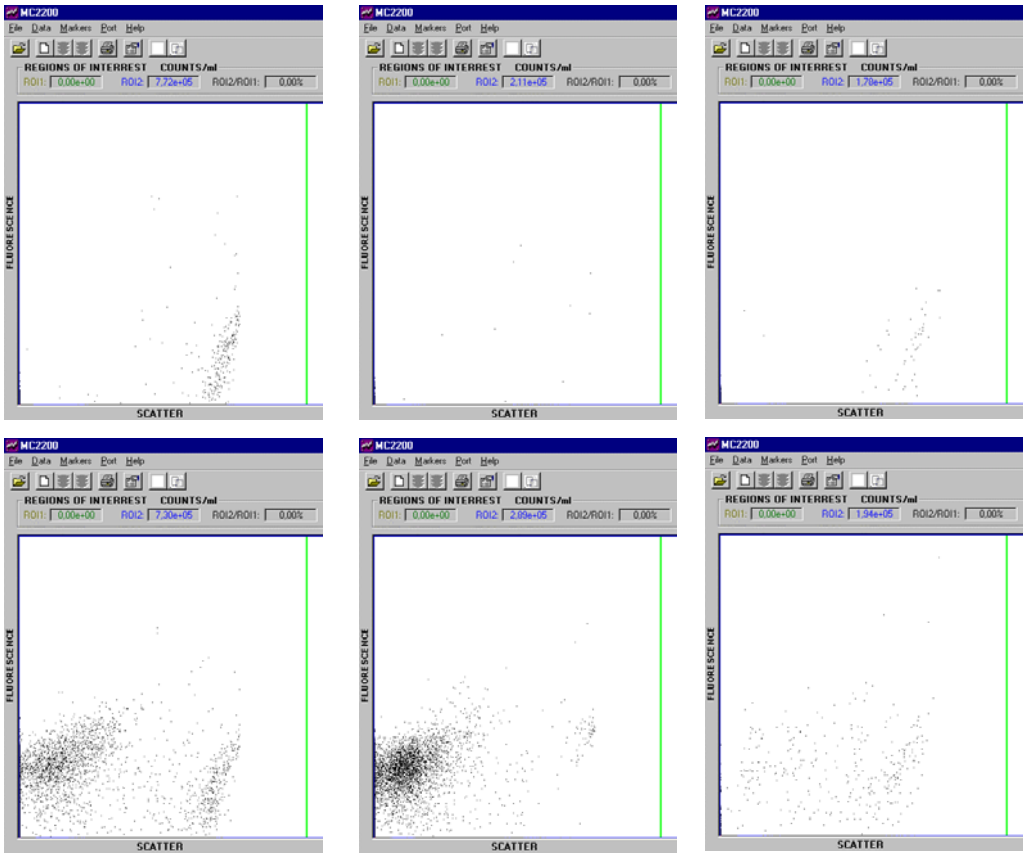

Figure 3.4 *Fluorescence intensity vs scatter plots of samples of river water. Upper row from left:Leira river, Nita river and melted snow with absence of ToPro™-3. Lower row from left: Leira river, Nita river, and melted snow in presence of ToPro™-3.*

Figures 3.5A and B and 3.6A and B show fluorescence intensity vs scatter plots from samples of bacteria mixed with non-bacterial samples and samples of environmental pollution. Addition of sugar, melted snow, baking powder or river water does not seem to have effect on the observed signal from *Escherichia coli* at higher concentrations. The signal from wheat flour does overlap with *Escherichia coli*, however, and may cause problems in recognizing the bacteria. Presence of table salt significantly alters the location and shape of the original pattern obtained from *Escherichia coli* at a concentration of $5.0 \cdot 10^5$ bacteria/ml.

Similar observations are made for *Bacillus cereus* with the exceptions that wheat flour does not only overlap and thus visually confuses the signal from *Bacillus cereus* but also moves it from its original location in the plot.

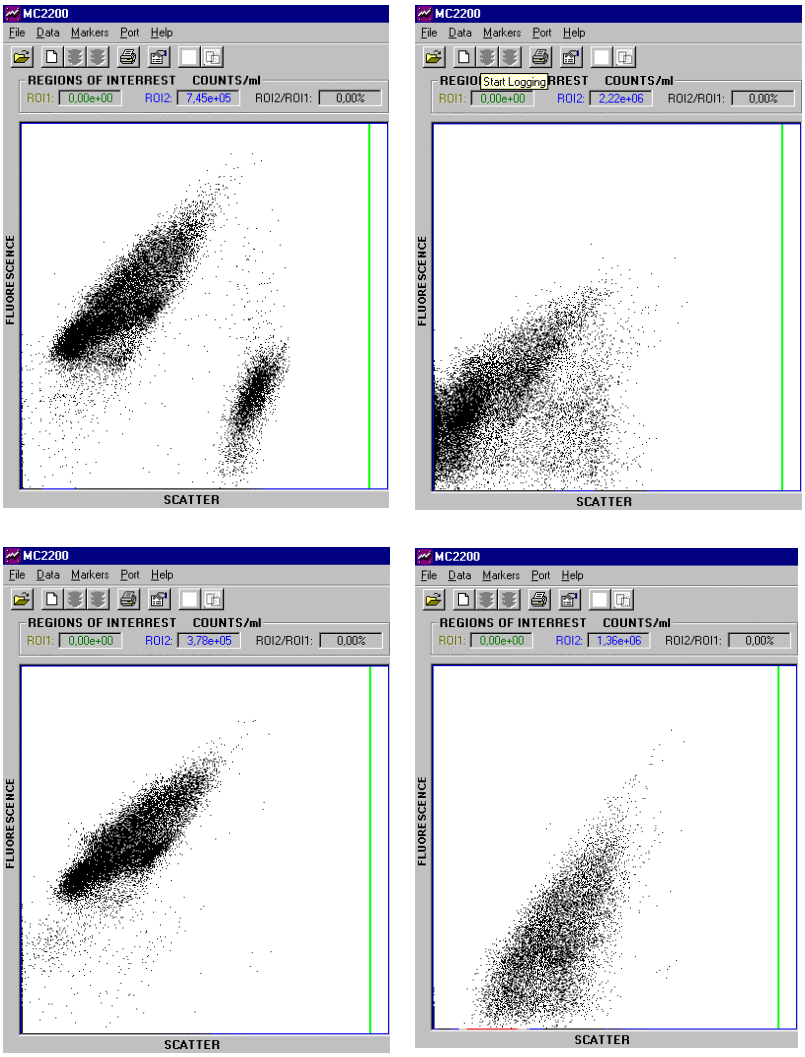

Figure 3.5A Fluorescence intensity vs scatter plots of samples of Bacillus cereus bacteria mixed with non-bacterial samples. Baking powder (upper left), wheat flour (upper right), sugar (lower left) and table salt (lower right). ToPro™-3 was added to the samples before acquisition of the plot

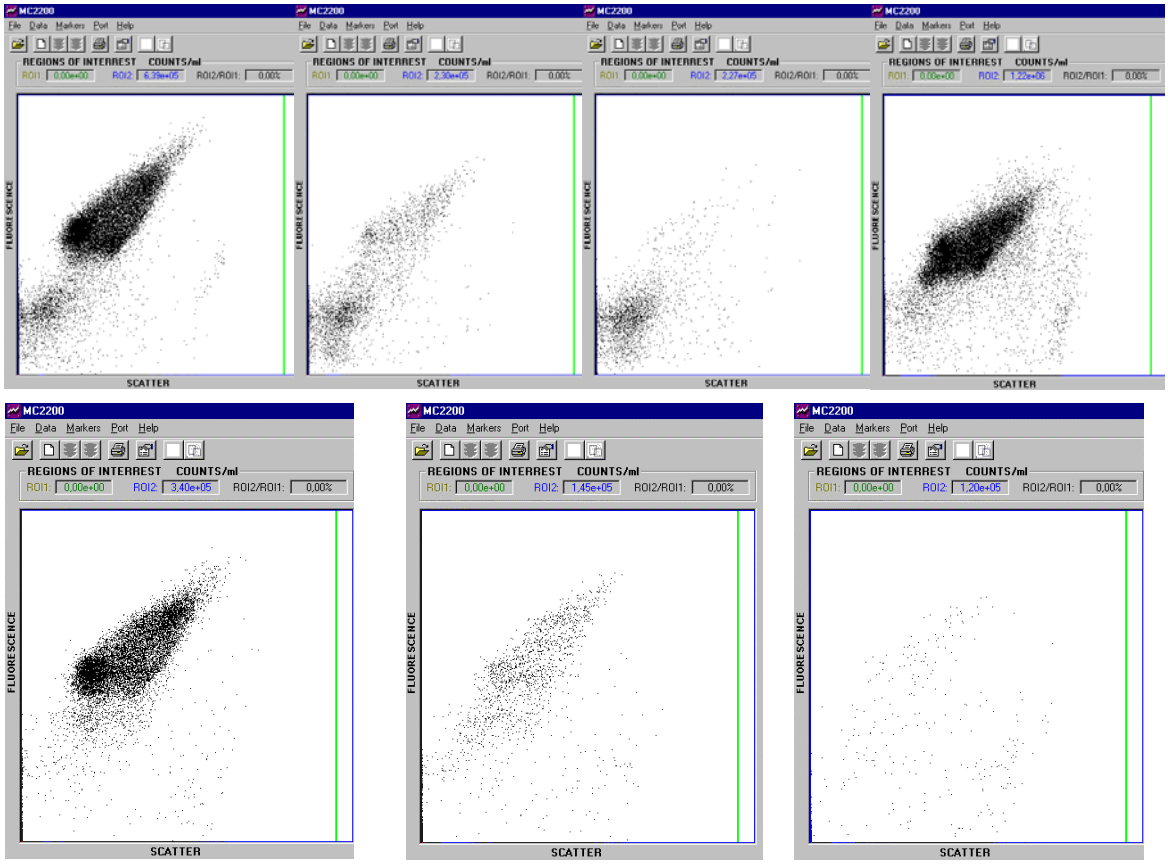

Figure 3.5B *Fluorescence intensity vs. scatter plots of samples of Bacillus cereus bacteria mixed with melted snow and river water. From upper left to upper right: Nita river, bacteria concentrations $6.1 \cdot 10^5$, $6.1 \cdot 10^4$ $6.1 \cdot 10^3$ and Leira river concentration $6.1 \cdot 10^5$. Lower row from left: melted snow, bacteria concentrations $6.1 \cdot 10^5$, $6.1 \cdot 10^4$ $6.1 \cdot 10^3$. ToPro^{TM}-3 was added to the samples before acquisition of the plot*

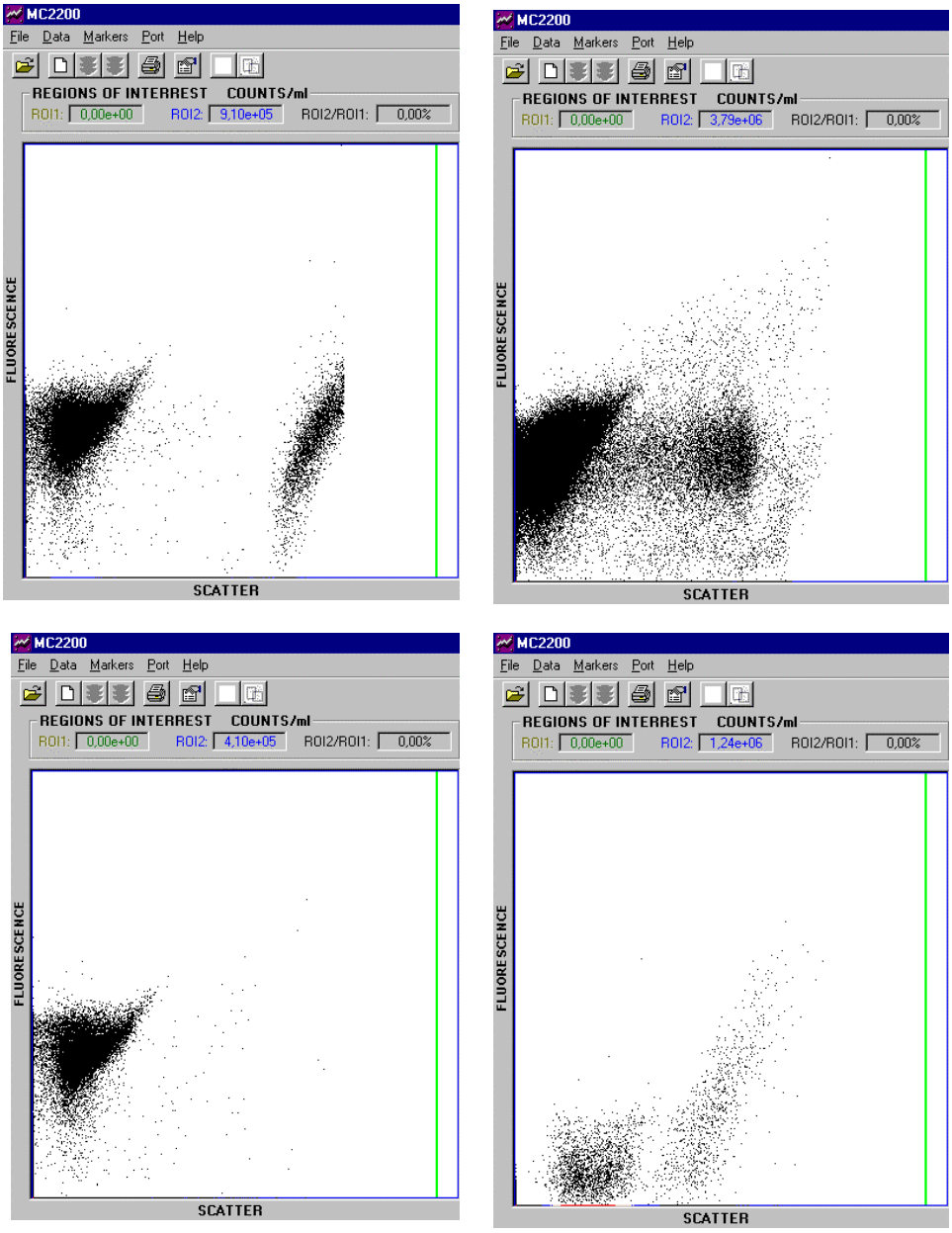

Figure 3.6A *Fluorescence intensity vs. scatter plots of samples of Escherichia coli bacteria mixed with non-bacterial samples. Baking powder (upper left), wheat flour (upper right), sugar (lower left) and table salt (lower right). Bacteria concentration $5.0 \cdot 10^5$. ToPro^{TM}-3 was added to the samples before acquisition of the plot*

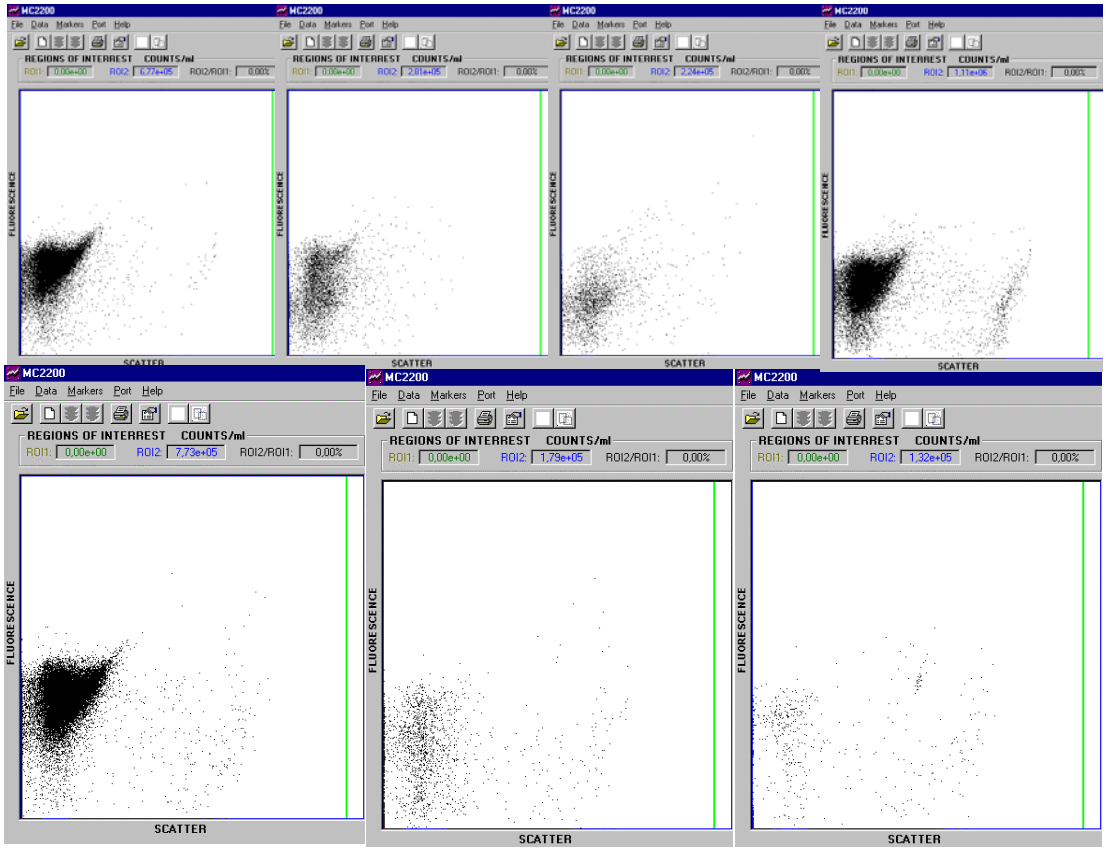

Figure 3.6B *Fluorescence intensity vs. scatter plots of samples of Escherichia coli bacteria mixed with melted snow and river water. From upper left to upper right, Nita river bacteria concentrations $5.0 \cdot 10^5$, $5.0 \cdot 10^4$ $5.0 \cdot 10^3$ and Leira river, concentration $5.0 \cdot 10^5$. Lower row from left, bacteria mixed with melted snow, bacteria concentrations $5.0 \cdot 10^5$, $5.0 \cdot 10^4$ $5.0 \cdot 10^3$. ToPro™-3 was added to the samples before acquisition of the plot*

Figure 3.7 shows plots obtained from SIBCA samples. The plots obtained from samples of *Yersinia pestis* and *Bacillus anthracis* and a mixture of both may relatively easily be recognized in the way they are made available in the SIBCA samples. Addition of diesel smoke will make the picture somewhat more obscure, however. Plots from analysis of the remaining SIBCA samples show only weak signals with less useful information.

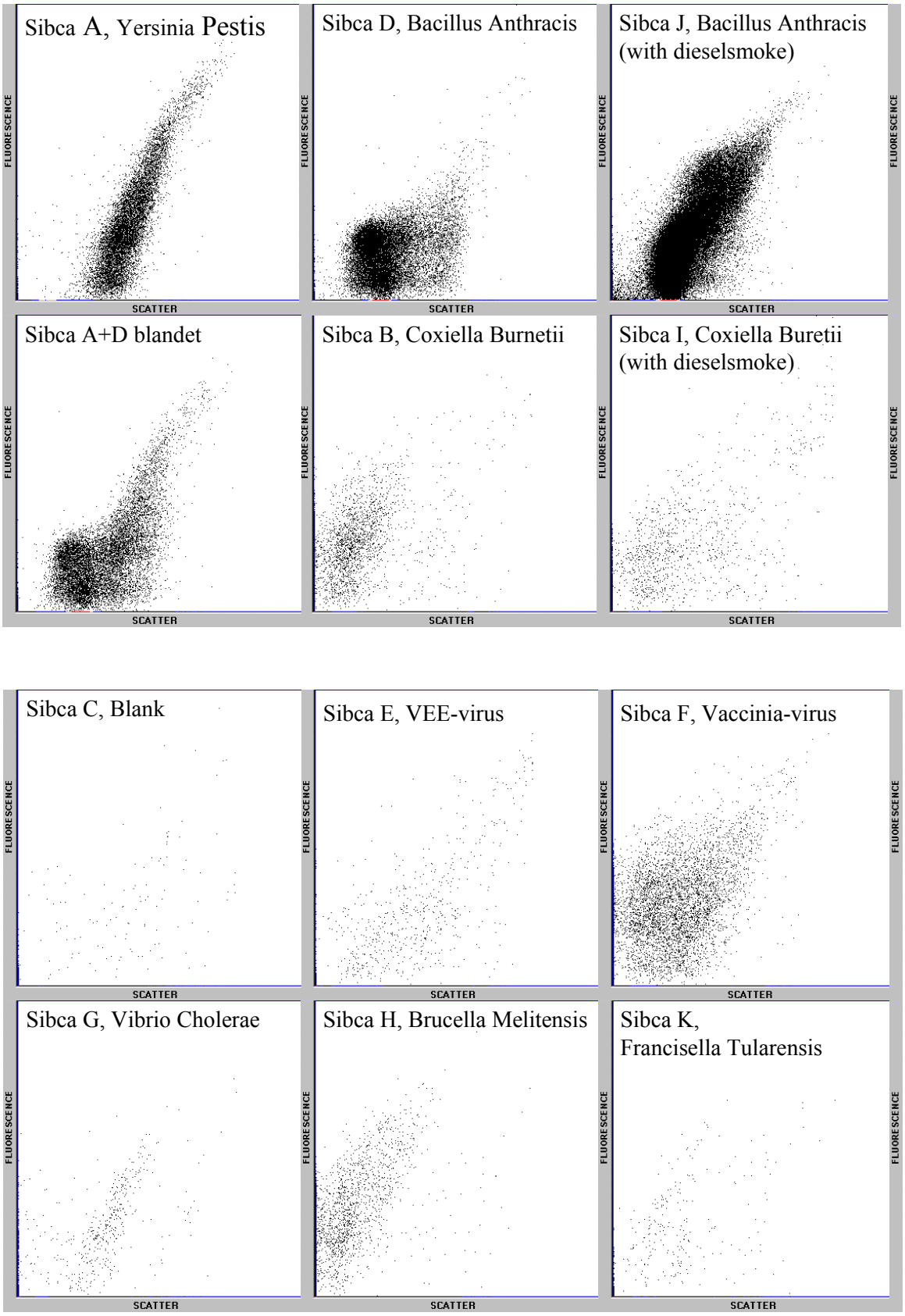

Figure 3.7 Fluorescence intensity vs. scatter plots of samples of SIBCA samples

4 INSTRUMENT DESCRIPTION AND EVALUATION OF USER-FRIENDLINESS

The Optoflow Microcyte® flow cytometer may be operated from a PC using Microsoft Windows 95 or NT or from the front panel of the instrument itself. Emphasis has been put on operating simplicity and robustness. The instrument is based on a 635 nm, 5mW, diode laser, and avalanche photo diodes for forward light scatter and fluorescence intensity detection. Power supply alternatives are internal 12 V, 2.5 Ah, rechargeable NiCd battery, 12 V battery pack (or car battery) or 100 – 200 V mains adapter. Fluorescence sensitivity is 103 – 105 molecule equivalents of Cy-5. Light scatter sensitivity allows measurement of particles size between 0.4 and 15 µm. The instrument is based on a closed flow cell, 0.25 mm x 0.25 mm.

Due to the simplicity of the Microcyte flow-cytometer, only a short briefing was necessary before the tests were started. The software leaves only few choices to be made by the operator.

After a sample was made ready for measurement, the operator placed it in the sample holder and pressed the run button. At the end of each sampling the capillary tube had to be flushed before the next sample was analysed.

All daily maintenance and calibration was easy to carry out, due to the few parameters the operator had to allow for. The instrument and its software seemed reliable in use and did not give any kind of error messages during the measurements.

At present, and depending on the type of application, an operator with some knowledge in biology or related fields should be preferred for both running and in particular for interpretation of the results.

5 DISCUSSION

5.1 Measurements

Measurements on dilution series show that bacteria of type *Bacillus cereus* give reproducible patterns at concentrations from $1.2 \cdot 10^5$ bacteria/ml and beyond. *Escherichia coli* give reproducible patterns from $1.0 \cdot 10^4$ bacteria/ml and higher. The patterns obtained from these two types of bacteria are located well apart and may therefore be distinguished from each other. Patterns obtained from stored (1 month) *Bacillus cereus* bacteria are located to the same area as bacteria a log phase culture, but show an expansion into the lower left quadrant, indicating occurrence of smaller particles and reduced ability to bind ToPro™-3. The observation may be explained by an increased resistance to osmotic stress due to nutrient limitations, which in turn may reduce the size of the bacteria. Plots from spores of *Bacillus cereus* show a somewhat narrower fluorescence intensity and size distribution. It should be noted that environmental samples normally would contain bacteria exposed to reduced nutrient conditions compared to the log phase condition provided in the laboratory.

ToPro™-3 was added to the samples in order to distinguish between non-bacterial and bacterial matter. This originates from the fact that ToPro™-3, due to ethanol treatment of the cells, is

allowed to migrate through the cell membrane and bind selectively to DNA. It is normally reckoned as a good assumption that ToPro™-3 does not bind to non-bacterial matter.

Although some auto-fluorescence is observed, patterns obtained from baking powder and icing sugar support this assumption. Wheat flour, on the other hand, shows a significant ability to bind to ToPro™-3. The reason for this may be presence of DNA as wheat flour is an organic material or that ToPro™-3 binds chemically to suitable sites or is trapped in fine pores in wheat flour where the fluorescence capability of the dye is maintained.

Fluorescence intensity vs. scatter plots acquired from samples of *Bacillus cereus* and *Escherichia coli* contaminated with non-bacterial matter show that patterns remain virtually unchanged in presence of baking powder and sugar. Wheat flour and table salt, however, tend to move the observed patterns from both types of bacteria from their original positions, with the exception of wheat flour and *Escherichia coli*. Reduced fluorescence intensity observed in presence of table salt may be explained by the fact that high osmolality (saturated solution) may tend to reduce the bond strength between DNA and dye. Verification of this was beyond the scope of this work.

Measurements performed on samples of environmental pollution from Leira river and Nita river show a minor component of auto fluorescent matter. With addition of ToPro™-3, patterns appear, which may correspond to organic small sized particulate matter with low ability to bind dye or small amounts of other organisms that may bind to ToPro™-3. In addition to this, free DNA exists in nature and may contribute to a background signal. Melted snow contains very little material that contributes to fluorescence intensity.

Mixing of bacteria and environmental pollution from river water and melted snow does not seem to have any significant influence on patterns from *Bacillus cereus* and *Escherichia coli*.

5.2 Applicability

5.2.1 Laboratory use

The Microcyte® flow-cytometer should be considered as an interesting supplement to existing techniques for detection of various kinds of bacteria. Sample preparation follows already established procedures. Its size combined with simple handling and robustness makes it suitable for mobile laboratories. Introduction of the system will, however, require that a comprehensive library of plots various types of bacteria and potential interfering particulate materials and chemicals be established. Such a library should also include information about the life situation and age of the organisms as is indicated by plots from the stored (1 month at 4 °C) culture and spores of *Bacillus cereus* bacteria.

5.2.2 Automatic surveillance applications

The arguments of size, simplicity of handling and sturdy construction may also apply when considering the instrument as part of an automatic surveillance system. Such an application raises the needs, however, for development or accommodation of an automatic sampling system as well as an automatic data analysis and warning system.

5.2.3 Detection limit

Measurements carried out on the dilution series of *Bacillus cereus* and *Escherichia coli* show that the detection limits of the Microcyte® flow cytometer is in the order of $10^3 – 10^4$ organisms/ml. This corresponds to $10^6 – 10^7$ organisms/l. The number of organisms that will amount to an infectious dose varies considerably from organism to organism. The infectious dose of *Bacillus antracis* as regards gastrointestinal anthrax for example is typically in the order of 10^4 and upwards (3). The detection limit for *Bacillus antracis* has not been tested in this report. Assuming that it is similar to the limit observed for *Bacillus cereus* and *Escherichia coli,* one litre of water near the detection limit will contain two to three orders of magnitude more than the infectious dose.

Possible ways to remedy this are 1) to boil down in order to increase the concentration (which, however, may cause release of DNA from many gram negative bacteria and subsequent changes to the observed plots), 2) to insert larger sample volumes into the flow cytometer or 3) to use filtering or extraction techniques. The latter techniques also have the advantage that it is possible to increase the relative amount of bacteria in relation to the background signals.

The situation is different for air sampling, where the amount of organisms to liquid medium may be controlled, and thus should represent a smaller problem.

The detection limit of the instrument itself is <10/ml. Thus the observed detection limit is a result of the reagent being used, and may be improved by choosing other, more selective reagents sample preparation techniques.

5.2.4 Separation of mixes of different organisms

A common characteristic of the samples that have been tested in this work is presence of significant quantities of only one or two organisms and interfering compounds. In real-life samples, many types of organisms, living conditions and interfering compounds may contribute to obscure the picture. This may be so also in cases where one organism – in a given situation – may give a significantly higher signal contribution than the rest of the sample. Mathematical or statistical methods may be one way to improve extraction of useful information from plots of more complex systems.

6 CONCLUSION

The evaluation of the Optoflow Microcyte® flow cytometer shows that:

- Bacteria of type *Bacillus cereus* and *Escherichia coli* give reproducible patterns at concentrations from about·10^4 to 10^5 bacteria/ml and beyond
- Tests indicate that the age of the bacteria may alter the shape of the observed plots
- It is possible to distinguish between the tested bacteria of type *Bacillus cereus*, *Escherichia coli*, *Bacillus anthracis* and *Yersinia pestis*
- The detection limit for detection of *Bacillus cereus* and *Escherichia coli* is about 10^4 organisms/ml
- Fluorescence intensity vs. scatter plots acquired from samples of *Bacillus cereus* and *Escherichia coli* contaminated with non-bacterial matter show that patterns remain virtually unchanged in presence of baking powder and sugar. Wheat flour and table salt, however, tend to move the observed patterns
- Mixing of bacteria and environmental pollution from river water and melted snow does not seem to have any significant influence on patterns from *Bacillus cereus* and *Escherichia coli*
- Introduction of the system will require that a comprehensive library of various types of bacteria and potential interfering particulate materials and chemicals should be established
- When carrying out measurements on water samples, some measures have to be taken in order to adapt the system to required detection limits. This may include boiling down in order to increase the concentration, insertion of larger sample volumes into the flow cytometer, filtering or use of extraction techniques

Further survey should be carried out to get an overview of the effects of living conditions and age of the organisms as well as the complexity of samples often found in natural samples. With this in place together with a combination of small size, easy handling and apparent sturdiness, the Optoflow Microcyte® flow cytometer should be an interesting supplement for detection and identification of microorganisms.

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DISTRIBUTION LIST

FFIBM **Dato:** 15 februar 2002

RAPPORTTYPE (KRYSS AV)	RAPPORT NR.	REFERANSE	RAPPORTENS DATO
X RAPP ☐ NOTAT ☐ RR	2002/00772	FFIBM/136	15 februar 2002
RAPPORTENS BESKYTTELSESGRAD		ANTALL EKS UTSTEDT	ANTALL SIDER
Unclassified		70	23
RAPPORTENS TITTEL		FORFATTER(E)	
EVALUATION OF OPTOFLOW MICROCYTE® FLOW CYTOMETER		GRAN, Hans Christian, SKOGAN, Gunnar, OLSEN, Jaran Strand	
FORDELING GODKJENT AV FORSKNINGSSJEF		FORDELING GODKJENT AV AVDELINGSSJEF:	
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