INTERNATIONAL MARITIME ORGANIZATION

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SUB-COMMITTEE ON POLLUTION PREVENTION AND RESPONSE
6th session
Agenda item 5

PPR 6/INF.22
11 December 2018
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REVISED GUIDANCE ON METHODOLOGIES THAT MAY BE USED FOR ENUMERATING VIABLE ORGANISMS

Analytical methods for enumerating organisms in the 10 to 50 µm size class

Submitted by the Netherlands

SUMMARY

Executive summary:	This document informs the Sub-Committee of the status of the validation of Flow Cytometry as a method for enumerating organisms, addressing the concerns raised at PPR 5
Strategic direction, if applicable:	1
Output:	1.15
Action to be taken:	Paragraph 6
Related documents:	MEPC 69/WP.8; MEPC 70/4/3; MEPC 71/4/14; MEPC 72/17; BLG 13/18; PPR 4/21; PPR 5/INF.6, PPR 5/24 and BWM.2./Circ.61

Introduction

1 At MEPC 71, document MEPC 71/4/14 (Netherlands) was referred to PPR 5, with the request to provide more information on the details and validation of the proposed methods. At PPR 5, document PPR 5/INF.6 on the Flow Cytometry (FCM) method for particle characterization and the Pulse-amplitude-modulation (PAM) method for phytoplankton activity measurement was considered. At that session the Sub-Committee decided not to add the methods to BWM.2/Circ.61 at that stage and invited the delegation of the Netherlands to submit information on the validation of these methods as one combined method, also taking into account the concerns expressed in the working group. This document presents recent developments with regard to the validation of the FCM method.

Validation of the FCM method

2 In March 2018 the validation process was started. During the course of the process, it was decided not to pursue validation of the PAM method any further, since it would not produce quantitative results. Instead, the choice was made to use the Fluorescein DiAcetate (FDA) staining method as indicator of viability.

3 The annexed document informs on the details and the validation process of the analytical method FCM for enumerating phytoplankton. FCM, in combination with the FDA method, can be used to distinguish between living and dead planktonic organisms, as well as for counting organisms in the 10-50 µm size class that do not possess chlorophyll (i.e. ciliates and small zooplankton). The FDA method is included in the list of BWM.2/Circ.61. The annexed document elaborates on the ongoing development and validation process of using FCM in combination with FDA.

4 The~~,~~ limitations of the method are addressed and information on practical aspects is included, such as costs and personnel requirements according to the requirements of the aide memoire set out in annex 6 to document BLG 13/18. Furthermore, the presence of bacteria and the potential of further developing the method to distinguish live and dead organisms is discussed as part of an ongoing validation process.

5 When the validation by the appropriate authorities is completed, the full report will be made available to the Sub-Committee for its consideration.

Action requested of the Sub-Committee

6 The Sub-Committee is invited to note the information contained in this document.

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ANNEX

Flow cytometry as an accurate method for determining plankton abundance in aquatic environments

MEA-nl B.V. project P0067
Publication 1823 v1.0

Den Oever, November 2018

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This report has been prepared under the MEA-nl Quality Management system. The Quality Management System in accordance with the ISO-9001:2015 standard of MEA-nl is certified by Lloyd's Register.

Quality Management ISO 9001:2015

Revision history

Date	Version	Remarks
08/11/2018	0.1	Initial draft
13/11/2018	1.0	Final report

This report is compiled with the utmost care by MEA-nl. The undersigned attest that the report contains no known errors, omissions or false statements.

Approved by

Project manager	Operational director
A. Hoogstraten – 13/11/2018 	F. Fuhr – 13/11/2018 

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1. Executive summary

This document reports the first phase of a thorough validation process for flow cytometry and a description of the method itself. The tests in the report are valid for counting phytoplankton by means of chlorophyll autofluorescence.
The validation of the method is an ongoing process and is continuously updated. Once all tests have been finished and all DQIs have been determined the method will be submitted as a new, reliable method for counting phytoplankton with the Dutch Accreditation Council (RvA).

The results reported here include a range of measurements that determine whether the set instrument flow rate matches the actual flow rate and the range in which the flow rate is linear. Furthermore, we determined whether the particle size and abundance affect the variability of the counts.

Through repeated size-fractionation and standardised beads measurements, we determined that analysing different size classes of organisms is always possible. Because of the increased sample volume that is analysed, as compared to microscopy and the low variability within duplicate measurements, the results are more reliable.

Finally we have reported our first, preliminary results of flow cytometric counts with FDA stained samples. While microscopic life/dead determination with FDA and CMFDA is an established method within verification testing, FDA staining combined with flow cytometry has been used in scientific experiments. We have not found a validation of the method for verification tests, which is the next logical step to take.
Preliminary results show that it is possible to accurately carry out the determination of viability for both phytoplankton and zooplankton, although the method needs to be developed further and be validated.

The validation processes will be continued and more DQIs and parameters will be determined.

2. Terms of reference

- Review the current state of the scientific status of flow cytometry and the applicability of the method as an automated method for counting planktonic organisms in different aquatic environments.
- Discuss positives and negatives of the method.
- Discuss the ongoing validation process of phytoplankton counts by flow cytometry. The procedure is based on chlorophyll autofluorescence and is only valid for phytoplankton. Validation is done according to NEN 7777+C1:2012.
- Show the first results of the validation and discuss future perspectives, including the development and validation process of a flow cytometrical method using a Fluorescein Diacetate (FDA) stain. FDA, a stain that is currently being used both in microscopy and flow cytometry to distinguish between live and dead cells.
- Report on the finished process and provide validation data for the methods in due course.

3. Introduction

3.1 Flow cytometry

3.1.1 Technical and theoretical background

In flow cytometry, a fluid sample is injected in a second carrier fluid, the sheath fluid. These two streams do not mix (laminar flow). Because of the narrow samples stream, particles are aligned and pass a laser beam one by one. Once the particle passes the laser, the light is scattered in different directions and fluorescence can be produced. This fluorescence can originate from either autofluorescence from pigments, fluorescent DNA staining or fluorogenic stains. The latter are metabolised into fluorescein molecules through the cells metabolic processes. The particle enters the flow cell, and passes the laser. When its characteristics meet the specific range which is pre-set by the user, it is recorded. In this way each single particle in the sample stream can be recorded (Shapiro 2003).

In flow cytometry, two major scatter parameters are detected.

1. Small angle scatter. This scatter is the amount of light scattered at small angles, which are between 0.5 and 5°. This scatter is also called Forward Scatter (or FSC) and is a rough estimate of cell size.
2. Large angle scatter (at large angles between 15 and 150°). It is also called Side Scatter (or SSC) and the values increase with increasing cell granularity or cell structure. It is considered for example possible to distinguish between *Emiliania huxleyi* cells with and without coccoliths, both of which have a different SSC signal (Olson, Zettler et al. 1989).

Besides scatter detectors, most flow cytometers contain different fluorescence detectors. These detectors are dependent on the type of laser used in the instrument. Although the flow cytometers used and described in this document have the possibility for the addition of two more lasers (namely: UV, 405 nm; and Red, 638 nm), currently only the blue laser (488 nm) is employed. Each of the laser has its own set of fluorescence channels. Here, we only mention the important ones, which are combined with the blue laser. These are:

1. FITC (Fluorescein Isothiocyanate) fluorescence (525/40 BP)
   FITC fluorescence is often obtained by staining the cells with either fluorescent DNA stains or fluorogenic stains that are converted in fluorescein molecules through, for example, cell metabolism.
2. PE (phycoerythrin) fluorescence (585/42 BP) PE fluorescence is characteristic for cyanobacterial cells (for example *Synechococcus* sp.(Postius, Kenter et al. 1998)) and/or cryptophytes. Most phototropic organisms possess chlorophyll *a* as the main light harvesting pigment, however some, for example the cryptophyte *Rhodomonas* sp, contain accessory pigments, such as carotenes or alloxanthin (Schlüter, Mohlenberg et al. 2000). The presence of PE fluorescence in combination with chlorophyll fluorescence with flow cytometric analysis indicates the presence of cyanobacteria and these can be distinguished from other phytoplankton groups in a PE fluorescence versus Chlorophyll fluorescence plot.
3. Chl (chlorophyll) fluorescence (690/50 BP).

3.1.2 Possibilities and challenges

Flow cytometry used to be a very expensive, and therefore not readily available method for analysing water samples. In the last decades prices have reduced significantly and the instrument has now become more affordable (Gasol and Del Giorgio 2000, Kamiya, Izumiyama et al. 2007). The most important advantage of flow cytometry, as compared to microscopy, either “normal” light microscopy or fluorescence microscopy is the amount of samples and the volume that can be analysed in a certain amount of time. Even with a rather long analysis time

of 5 minutes, 12 1 mL samples can be analysed per hour (as described later in this document). Few technicians will ever acquire this rate of enumerating organisms through microscopy, especially when larger numbers of organisms are present. Each flow cytometer has a set range of particle density for which reliable measurements can be made. For example, the flow cytometers used in the laboratory of MEA-nl (Beckman Coulter CytoFLEX) have, according to the manufacturers specifications, a nominal rate of acquisition of 30,000 events per second. At a high flow rate of 200 $\mu L.min^{-1}$ this would mean a total concentration of $9*10^6$ particles.mL^{-1}. In general, only very dense cultures of phytoplankton might reach these organism numbers. Bacteria samples could reach those abundances, however these are diluted in the preparation process, and we tend to count approximately 10^5 particles, at a lower flow rate (60 $\mu L.min^{-1}$). Microscopically counting 1 mL of sample containing these particle concentrations would be virtually impossible.

Because of the relatively large volume, and large amount of particles a flow cytometer can count, statistical reliability is increased. Furthermore, a flow cytometer does not think. It decides whether to count or dismiss a particle based on pre-set characteristics. This reduces the variability between duplicate counts. Once an analysis template has been created each sample will be analysed in the exact same way, reducing the human error factor for the analysis. Of course one has to make sure each technician operating the flow cytometer handles the samples in the same way. This can be done by implementing standard operating procedures (SOP's) which explain the relevant procedures and important steps, such as mixing and incubation times and an adequate level of training.

Most of this document will discuss validating the method and the instrument for the analysis of phytoplankton by chlorophyll autofluorescence. This however is not the only possible application of the instrument. Currently, we are using the flow cytometer for the analysis of heterotrophic bacteria in aquatic samples, which is common practise in scientific studies (Gasol and Del Giorgio 2000, Kamiya, Izumiyama et al. 2007). In order to count bacteria, it is necessary to apply one or more staining protocols. Heterotrophic bacteria do not possess chlorophyll or phycoerythrin and are therefore not distinguishable from other small particles without the use of a fluorescent stain, such as PicoGreen, SYBRGreen I or SYTOX Green (Gasol and Del Giorgio 2000).
Depending on the specifications of the flow cytometer used it is possible to enumerate virus particles as well (Marie, Brussaard et al. 1999, Brussaard, Marie et al. 2001). Furthermore, flow cytometers are used in the medical sciences for analysis of for example leukocytes and lymphocytes (e.g. (Loken, Brosnan et al. 1990)), showing the broad range of applicability of this instrument.

One of the main concerns is whether flow cytometry can distinguish between living and dead organisms. The answer to this is YES!. One needs to adopt some changes to a "normal" protocol though. When the analysis is based only on chlorophyll autofluorescence for phytoplankton or DNA staining of bacteria (the latter are usually fixed with a fixative, such as formaldehyde or glutaraldehyde) it is very difficult, maybe even nearly impossible to distinguish between living and dead cells. One way to overcome this challenge is using stains that stain either live cells (for example fluorescein diacetate) (Dorsey, Yentsch et al. 1989) or dead cells (for example propidium iodide, PI, or SYTOX green), or the use a combined staining protocol, for example FDA combined with PI staining to clearly separate the living from the dead organisms (Franklin, Adams et al. 2001).

We previously described the possibility of distinguishing between different groups of phytoplankton by looking at PE and Chlorophyll fluorescence at the same time. This is a general separation, but it does not give an indication of the amount of different species that are present. In theory, it would be possible to specify each species in a plot and create a

template accordingly (Marie, Simon et al. 2005). However this would mean each species that occurs in the water has to be cultured. One problem that arises from such an approach, is that the amount of chlorophyll fluorescence is dependent on the amount of light a photosynthetic organism receives during the growth of the cell. After the template, in which each species has been included, two problems will arise:

1. There is a lot of overlap between the species,
2. The organisms will behave differently in natural water samples thereby limiting the use of the template even further.

In order to overcome this limitation the easiest solution would be a quick scan with the microscope and determine the species that are present in the sample. For this no stain is necessary, nor should it take a significant amount of time.

Another difficulty in the analysis is counting cysts. Cysts can be formed as a survival strategy by several species of phytoplankton in adverse conditions, such as nutrient starvation or severe light limitation (Fistarol, Legrand et al. 2004). Furthermore, the cysts are often tolerant to extreme temperatures and chemicals, including fixatives and stains. Cysts are hard to kill. Although cysts are viable organisms, life/dead determination is nearly impossible. Cysts can remain dormant for extended periods of times and suddenly start to develop into a normal cell again when environmental conditions are improving (Blackburn and Parker 2005).
We have witnessed cells of *Tetraselmis* sp. dropping their flagella and become non-motile, and cells forming cysts after a prolonged period of intended neglect (Figure 1). Detecting the cysts with FDA-staining is difficult or nearly impossible. Compared to non-motile cells (yellow circles), the amount of stain in a cell is much smaller, making it difficult to see the cysts after staining (blue circles).

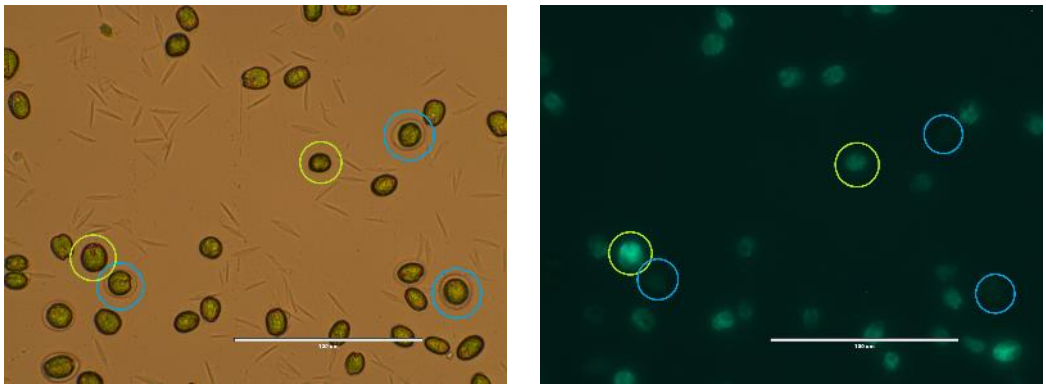


Figure 1: A FDA stained culture of Tetraselmis sp. *In the blue circles cysts can be seen, in the yellow circles non-motile Tetraselmis* sp. *cells are visible. Left: an image made with conventional light microscopy; Right: the same image made with fluorescence microscopy showing fluorescein fluorescence. Note the absence of fluorescence in the blue circles in the right picture.*

The last concern that need to be addressed is the enumeration of chain forming organisms. Most of the time the flow cytometer will analyse such chains as one, very large, single cell, because it cannot distinguish between the different cells in the chain. It is possible for some species, by gently mixing the sample, to disintegrate the chains and enumerate single cells. For some species, due to the nature and shape of the chains, it is impossible to disintegrate the chains without damaging and killing the cells (Garvey, Moriceau et al. 2007). If cells disintegrate this easily by gently mixing, such species won't survive the pressure with which the water they are in is pumped through ballast water pumps. There are some species which can withstand this amount of pressure and remain in a chain, such as *Bidulphia* sp. which are the too large to enumerate with flow cytometry. In order to address this challenge we will have to enumerate the presence and the viability of these diatoms after such treatment in order to determine the underestimation of the flow cytometry counts.

Since cyanobacteria can be distinguished from phytoplankton cells these are not interfering with the enumeration of phytoplankton. Cyanobacteria can form large chains, but the single cells are smaller than 10 μm (Olenina, Hajdu et al. 2006) and are therefore not of importance to ballast water treatment system verification testing.

3.1.3 Perspective of flow cytometry in verification testing of ballast water treatment systems through the addition of Fluorescein diacetate (FDA)

Fluorescent and/or fluorogenic stains, the latter including fluorescein diacetate (FDA) can be used in differentiating between living and dead organisms. In itself, FDA does not exhibit fluorescence. It is a nonpolar lipophilic molecule, which diffuses freely across the plasma membrane of a cell. This molecule is then hydrolysed by esterases, which are common enzymes, present in both plant and animal cells (Dorsey, Yentsch et al. 1989). The hydrolysis of FDA produces a fluorescein molecule. Since the produced fluorescein molecule is highly polar and hence hydrophilic it is trapped in cells with possess intact membranes (Jochem 1999). Because of these characteristics it can be used to determine both the metabolic activity and the membrane integrity of individual cells and organisms, which are both considered characteristics of viable cells.

Although there is scientific debate concerning the application of FDA as a an indicator of viability in marine phytoplankton (Garvey, Moriceau et al. 2007), the staining of samples with FDA is currently an accepted method for the determination and microscopic enumeration of viable cells in verification tests of ballast water treatment systems (IMO 2017).
Because of the rapid fading of the fluorescence of the fluorescein molecule it is often combined with other stains, such as CMFDA (chloro-methoxy fluorescein diacetate) which is a larger molecule and exhibits fluorescence for a longer period of time (Welschmeyer and Maurer 2011). Besides the rapid fading, the strong red autofluorescence of chlorophyll can mask the green fluorescein fluorescence, while enumerating phytoplankton organisms through fluorescence microscopy (Garvey, Moriceau et al. 2007). The chlorophyll autofluorescence can be masked with filters in order to increase the visibility of the green fluorescein fluorescence. This might however, increase the rate of the fading (Jansen and Bathmann 2007).

Flow cytometry could potentially address the fading and chlorophyll autofluorescence issues.

1. The amount of sample volume analysed by a flow cytometer, as compared to microscopical enumeration in the same amount of time is much larger. Currently we are operating the flow cytometer with a flow rate of 200 $\mu L.min^{-1}$ for a duration of 5 minutes per sample. This means, that 1 mL of sample is analysed. In theory, when a fading time of 15 minutes is assumed, 3 mL sample could be analysed. When running samples with a flow cytometer for a prolonged period of time, settling of organisms should be taken into account prior to developing a protocol for analysis.
2. A flow cytometer contains several fluorescence detectors, through which specific band widths are detected, hence reducing the interference of chlorophyll autofluorescence.
3. A third potential improvement of using flow cytometry, rather than microscopy is the threshold level of fluorescence which can be detected. The detectors in the flow cytometers are likely to be more sensitive than the human eye, which increases the sensitivity of the analysis.

3.2 Practicalities in using flow cytometry

3.2.1 Costs of equipment

Flow cytometers are available at a range of prices and types. The flow cytometer used in our laboratory costs approximately €70.000.

3.2.2 Maintenance and operating costs

A flow cytometer requires three different types on maintenance:

1. Daily maintenance,
2. Regular maintenance,
3. Yearly maintenance.

The first two types of maintenance can be performed by a trained laboratory technician, the latter is performed by a technician from the manufacturer. The maintenance and costs described in this section are an estimation, and are specific for the flow cytometers used by MEA-nl, namely the Beckman Coulter CytoFLEX (Coulter). The prices are based on previous orders of replacement parts and might vary for different countries. We mention these costs in order to give an indication of the operating costs of our flow cytometers.

We assume, also from previous experience with other flow cytometers, that most flow cytometers need similar maintenance. It is depending on the manufacturer and the model which maintenance can be performed by laboratory technicians and for which maintenance technicians from the manufacturer are required.
In this overview (Table 1) the cost of a laboratory technician is not included.

3.2.2.1 Daily maintenance

The daily maintenance of the flow cytometer includes:

1. Replacing the sheet fluid with either reversed osmosis (RO) water or commercially available sheet fluid.
2. Emptying the waste tank.

Furthermore a number of steps need to be taken prior to analysis in order to ensure proper functioning of the equipment.

These are: a daily start-up cycle which includes initialising and priming the flow cytometer. This is the warm-up cycle of the flow cytometer.
Priming the flow cell is necessary after each sheet fluid replacement, in order to remove potential air bubbles from the fluidics system.

After each day of measurements, the system needs to be cleaned, by using the pre-set daily clean procedure. During this cycle the system is cleaned with Beckman Coulter FlowClean solution (a detergent) after which it is rinsed with RO-water.

3.2.2.2 Regular maintenance

In order to ensure proper functioning of the flow cytometer, several procedures need to be followed on a regular basis. These can all be executed by trained laboratory technicians and are fairly straight-forward:

1. Once every month a deep clean cycle needs to be run. This cycle is pre-programmed in the instrument. During the Deep Clean cycle the system is cleaned with a Deep Clean solution (made with Contrad 70 solution), which thoroughly cleans the fluidics system. In case of clogging this cycle can be run once or multiple times, usually removing the clogging from the system. The Deep Clean solution needs to be replaced every six months.
2. Every six months the sheet fluid filter which removes potential particles from the fluid and the sample tubing need to be replaced.
3. The sample probe can be replaced, but this is only necessary when it is broken, or there is severe clogging in the probe. In case of severe clogging or a broken probe, it needs to be replaced.

3.2.2.3 Maintenance by the manufacturer

Beckman Coulter offers yearly maintenance packages which range between
€ 3000.- and € 9000.- (excluding taxes) per instrument. The price of the maintenance package depends on the requirements of the owner, for example, whether the cost of replacement parts are included, or whether these are excluded or offered at a discount price. Furthermore the costs are dependent on the amount of time needed for the maintenance.

3.2.2.4 Calibration of the instrument

In order to calibrate the instrument appropriately, prior to each day of measurement a Daily QC cycle should be run. This cycle is pre-programmed in the instruments software and is run with CytoFLEX Daily QC Fluorospheres. Each batch of fluorospheres has a specific download file which is used by the software in order to check the quality of both the fluidics and the optical systems.
For the verification of absolute counts FlowCount fluorospheres are available. These are uniform sized beads, and each vial contains a standardised concentration of beads.
For a quick size indication the commercially available (Poylsciences) Megabead NIST (National Institute of Standards and Technology) traceable particle size standard beads are used. Two sets of beads, with a nominal size of 10.0 μm and 50.0 μm are included in each set of measurements.
Size classification of natural samples is done through sieving samples over a 10 and a 50 μm nylon mesh sieve.

Table 1: Approximate operating costs (excluding taxes) of a Beckman Coulter CytoFLEX flow cytometer, not including the cost of employing a laboratory technician.

Maintenance/calibration	Frequency	Laboratory technician	Manufacturer technician	Costs	Cost per day (300 operating days)
FlowClean solution	1 bottle per year	X		€ 22.-	€ 1.-
Contrad 70 solution	1 bottle per year	X		€ 100,-	€ 1.-
Sheet fluid filter	2* per year	X		€ 250.-	€ 2.-
Pump tubing	2* per year	X		€ 60.-	€ 1.-
Sample probe	When necessary	X		€ 210.-	€ 1.-
Yearly maintenance	Once a year		X	€ 3000.- to € 9000.-	€ 10.- to € 30.-
Daily QC calibration	Daily	X		€ 310,-	€ 1.-
FlowCount fluorospheres	Daily, 1 bottle every 30 days	X		€ 725.- per bottle	€ 25.-
NIST traceable beads 10 µm	Daily	X		€ 350.-	€ 2.-
NIST traceable beads 50 µm	Daily	X		€ 350.-	€2.-
Total costs of operating					€46.- to € 66.-

3.2.3 Training

Personnel is trained for flow cytometric analysis in two different steps. Often the manufacturer of the instrument offers a training program specific to the instrument. It offers a good start in operating the instrument and learning the basics.
The maintenance of the Beckman Coulter CytoFLEX flow cytometers, which are used in the MEA-nl laboratory, is rather straight-forward. By using the operating manual of the instrument, a laboratory technician can easily replace the parts and fluids according to the maintenance schedule.
The basics of building a template for sample analysis are seemingly easy. For proper data analysis specific tailor-made training is necessary. In order to build a template, knowledge concerning the different fluorescence channels, scatter parameters, lasers and the characteristics of the organisms analysed is necessary.

While analysing samples, the technician should keep an eye on the instrument. Although it is running automatically, some problems can occur:

1. Clogging of the sample tube or fluidics system: Rinsing is necessary and a sample should be re-analysed.
2. Settling of particles. When this happens, the sample should be mixed and re-analysed.
3. Waste full and or/ sheath fluid empty. The machine will give an alarm, which is hard to miss. The containers need to be replaced after the measurement is finished.

All of these “problems” are easy to spot, when one pays attention to the measurement.

4. Materials and methods

4.1 Equipment

A Beckman Coulter Cytoflex flow cytometer was used for the analyses presented in this document. The flow cytometer is equipped with a 488 nm (blue) solid state laser (50 mW) and has five channels for the detection of fluorescence. With those channels, several relevant parameters can be analysed, including Fluorescein isothiocyanate fluorescence (FITC; 525/40 BP channel), Phycoerythrin fluorescence (PE; 585/42 BP channel) and chlorophyll fluorescence (Chl; 690/50 BP channel).
Besides the fluorescence channels, two more detectors are present, measuring the light scattering characteristics of particles. The Forward Scatter (FSC, small angle scatter) is generally used to get a relative size indication by measuring the diffraction of light around a cell, while the Side Scatter (SSC, 90° scatter) gives an indication of the internal complexity of a cell (Shapiro 2003).
Data were analysis with the Beckman Coulter Cytexpert software package (v2.0). The analysis of the data does not change the raw data, which are stored electronically as *.xit files (entire experiment) with a corresponding folder containing all sample data as single *.fcs files. The files contain information on size and fluorescence of each particle analysed.

4.2 Data analysis

Phytoplankton cells are separated from sediment particles by the differences in bio-optical properties. This is done at the time of sample analysis by using the pre-set analysis template. A separation between phytoplankton cells and other particles can be made based on chlorophyll and/or phycoerythrin autofluorescence, which can be detected by the light detectors in the flow cytometer. The detector used for the detection of chlorophyll autofluorescence is “Chlor 4” (690/50 nm), the detector used for phycoerythrin is “PE” (585/42 nm). The fluorescent signal of the pigment is plotted against the “Forward Scatter” (Figure 2), a relative size determination parameter. Only phytoplankton cells with a clear chlorophyll signal (Figure 2, green gate: “phytoplankton”), the threshold of which is determined through a “blank” sample filtered over a 0.2 μm syringe filter, are selected, through gating (selecting a group of particles with specific characteristics through the software package), and replotted in a dot plot in which chlorophyll fluorescence is plotted against phycoerythrin fluorescence (Figure 3). This allows for distinguishing between chain-forming cyanobacteria (containing the pigment phycoerythrin, green gate: “PE-phyto”, Figure 3) and autotrophic phytoplankton (red gate: “phyt-select” Figure 3).

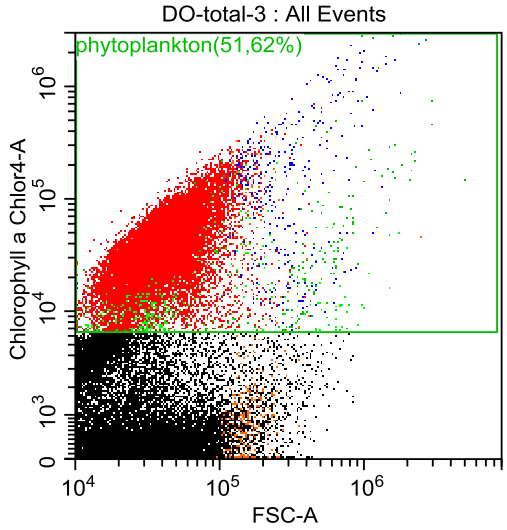


Figure 2: A typical result of a flow cytometric analysis of a water sample, in this case originating from brackish water. The threshold value for chlorophyll was determined by filtration steps through which phytoplankton was excluded from the sample. The green-gated area shows the phytoplankton based on the threshold. Below the gating the debris, consisting of sediment, dead particles and single chloroplasts of dead phytoplankton can be seen.

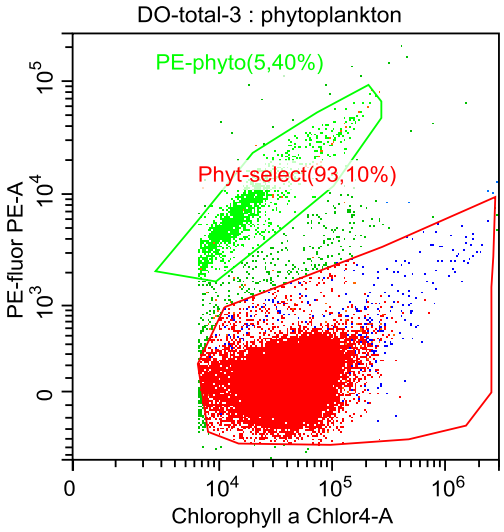


Figure 3: The phytoplankton cells are replotted in a dot plot specific for pigments. The red gate shows phytoplankton cells containing chlorophyll. The green gate shows cells containing phycoerythrin. These latter are mostly cyanobacteria, which can be chain-forming hence affecting the measured relative size.

4.2.1 Size determination

To determine the size of the organisms present in the water samples, two different size validation methods are used.
First, the commercially available Megabead NIST (National Institute of Standards and Technology) traceable particle size standard beads are used. Two sets of beads, with a nominal size of 10.0 µm and 50.0 µm are included in each set of measurements. These beads are available through Polysciences (http://www.polysciences.com). The beads give the first size indication for developing a template for data analysis (Figure 4). Since spherical beads are not a perfect representation of actual aquatic organisms, and scatter patterns are different from those of organisms, fractionated water samples are regularly analysed to validate sizing

by forward scatter. After the gates are properly placed for the right size classes, samples can be analysed and the abundance of phytoplankton in each size class can be determined (Figure 5).

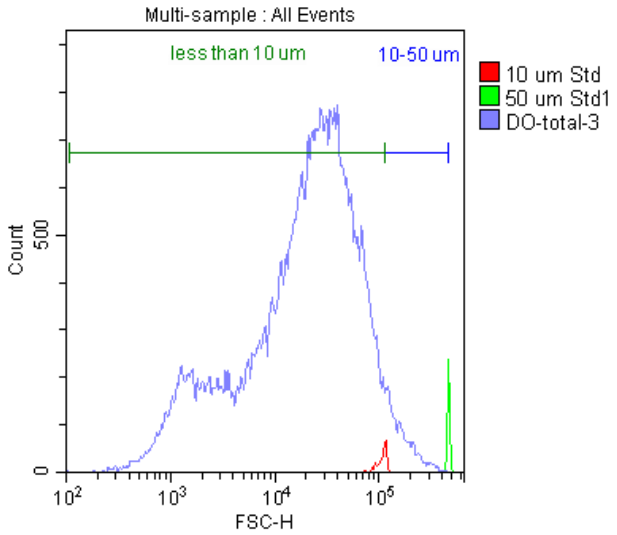


Figure 4: Frequency histogram of size distribution of particles analysed by the flow cytometer. In blue the size distribution of the complete sample, in red the 10 µm standardised beads, and in green the 50 µm standardised beads

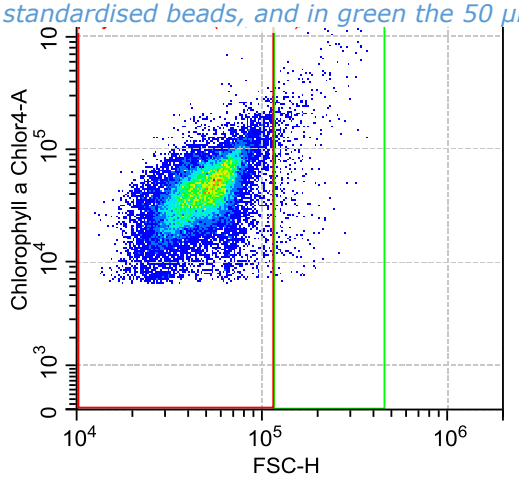


Figure 5: Phytoplankton cells from the red-gated area are plotted against the relative size. The gates are determined based on standardised beads and regular filtration of sample. The cyanobacteria are left out of the analysis, because the individual cells are smaller than 10 µm.

4.2.2 Calculations

The total counts and biological characteristics (size and chlorophyll fluorescence per cell) of phytoplankton cell are collected for each sample. The counts are converted into an absolute number according to:

$$\boldsymbol{Phytoplankton\ abundance} = \frac{\boldsymbol{x - control}}{\boldsymbol{volume\ analysed}}$$

Where:

x: the counted number of phytoplankton: number of chlorophyll auto-fluorescent cells in the size class of interest (either 2-10 µm or 10-50 µm)

control: number of chlorophyll auto-fluorescent cells in 0.2 µm prefiltered sample water in the same size class

Volume analysed: the duration of the analysis (s) * the flow rate ($\mu L.min^{-1}$) of the measurement

The data can be extracted from the .xit file to a comma separated file (csv file), after which further data analysis can be performed.

4.3 Validation of the method

MEA-nl is currently in the process of validating flow cytometry as a new automated method for counting phytoplankton. The validation is done according to NEN 7777+C1:2012 (NEN 2012). Once this validation is finalised the final report will be submitted to the Dutch Accreditation Council (RvA) and will be part of a submission containing the fully validated report of FCM in combination with FDA by the delegation of the Netherlands to PPR 7.
In order to validate the method several Data Quality Indicators (DQI's) need to be determined. The DQIs and the method with which they are determined are discussed below.

The determined DQI's are then tested against criteria set for absolute limit values. These criteria are dependent on factor p, which is calculated according to:

$$p = \sqrt{\frac{X_v^2(0.05)}{v}}$$

In order to validate the method for verification testing, three different water types need to be distinguished. The classification of these water types originates from the IMO criteria for land-based verification testing of ballast water treatment systems and are specified in Table 2 (IMO 2016, IMO 2018).
It is necessary to determine the DQI's for each water type in order to properly validate the method.

Table 2: Specification of water types according to the criteria for ballast water treatment system verficiation testing.

Water type	Salinity (psu)
Fresh	< 1
Brackish	10-20
Marine	28-36

4.3.1 Data Quality Indicators (DQI's) that need to be determined

4.3.1.1 Measurement range

Although the measurement range is specified by the manufacturer of the instrument (Beckman Coulter), it is necessary to determine several factors which possibly influence the measurement range of the instrument.

1. The flow rate of the flow cytometer can be regularly checked by a pre-programmed setting. Usually it is calibrated once a month, and after replacing the peristatic pump tubing. All flow rates can be calibrated at the once by calibrating the fast flow rate (60 $\mu L.min^{-1}$). Before entering the tube in the flow cytometer the weight of the tube with water should be measured by a calibrated analytical balance. This is recorded in the software. The flow cytometer will then run the sample and asks for the weight of the tube after the run. After the weight is entered, the flow rate is automatically determined and, if necessary corrected, by the software. In order to verify this internal procedure we determined the linearity of the flow rate by running the flow cytometer at different flow rates (10; 30; 60; 90; 120; 150 and 180 $\mu L.min^{-1}$) for five minutes with reversed osmosis water (3 mL per sample). The sample tubes were weighed before and after the analysis in order to determine the amount of volume taken up by the flow cytometer. As there is a short pre-measurement uptake of approximately three seconds, it was estimated that approximately 1% of the volume uptake was not used for the measurement, but rather for flushing the instruments fluidics system.
2. The amount of particles passing the laser at any moment in time, might influence the accuracy of the counts by the flow cytometer. Therefore a dilution series for each type of water (marine, brackish and fresh water) was performed and the difference between duplicate measurements was determined.
3. Fractionation of different samples was performed in order to determine the accuracy of the counts of particles in different sizes, thereby giving an indication of size limitation of the analysis. Again the difference between duplicate measurements was determined.

4.3.1.2 Detection limit

The detection limit (AG_{Rw}) of the analysis will be determined after the reproducibility standard deviation (s_{Rw}) has been determined, according to:

$$AG_{Rw} = 3 \times s_{Rw}$$

This detection limit will be determined for each water type separately by using natural samples and is tested against an absolute limit value according to:

$$AG_{Rw} \leq p * AG_{Rw,abslim}$$

4.3.1.3 Reporting limit

The reporting limit (RG) is determined as being greater than, or equal to the detection limit (AG_{Rw})

$$RG \geq AG_{Rw}$$

4.3.1.4 Repeatability

The repeatability of the analysis, within the laboratory, was determined by analysing eight natural samples for each water type in duplicate. Each sample was analysed on a different day. The duplicate measurements were performed on the same day by the same technician.
For each duplicate measurement the coefficient of variation (vc_r) was determined:

$$vc_r = \sqrt{\frac{\sum_{i=1}^{n}(\frac{x_{i1} - x_{i2}}{0{,}5(x_{i1} + x_{i2})})^2}{2n}}$$

Where X_{i1} is the first of the duplicate measurement, X_{i2} is the second of the duplicate measurement and n is the number of samples (eight).

From this the relative repeatability (r_{rel}) was calculated according to:

$$r_{rel} = 2vc_r\sqrt{2}$$

4.3.1.5 Reproducibility

Determining the reproducibility entails the analysis of 8 duplicate samples. However the duplicate analyses cannot be done on the same day. This imposes difficulties while using natural water samples, since plankton does not stop growing simply because one requires stable numbers, samples need to be preserved, for example with formaldehyde. Preservation might change both the forward scatter signal and the fluorescence signal, since formaldehyde will remove water from the cells in the fixation process (Marie, Simon et al. 2005).
We are currently in the process of testing the effects of formaldehyde on the plankton counts in order to be able to determine the reproducibility of natural samples.
When this proves to be impossible, the next step is to make samples in which natural water, filtered over a 0.2 µm pore size syringe filter is used to dissolve the standardised NIST sizing beads (10 µm). The dilutions can be kept in the fridge for several days, allowing for duplicate measurements on different days, thereby allowing for the determination of reproducibility.

The variation coefficient of the reproducibility (vc_{Rw}) is determined according to:

$$vc_{Rw} = \sqrt{\frac{\sum_{i=1}^{n}(\frac{x_{i1} - x_{i2}}{0{,}5(x_{i1} + x_{i2})})^2}{2n}}$$

Where X_{i1} is the first of the duplicate measurement, X_{i2} is the second of the duplicate measurement and n is the number of samples (8).

The reproducibility (Rw_{rel}) follows from this according to:

$$Rw_{rel} = 2vc_{Rw}\sqrt{2}$$

4.3.1.6 Bias

The bias of the analysis is determined by analysing reference beads: Coulter FlowCount Beads. The beads have a uniform size (10 µm in diameter) and the vials contain a standardised amount of beads.
The beads are dissolved in the three different water types in order to test for bias resulting from the different water types.
Eight samples of dissolved beads are analysed during eight days (single analysis). Furthermore a dilution series of the standardised beads needs to be analysed in order to determine whether the bias is either in absolute numbers or relatively independent of the amount of beads in the sample.
When the absolute bias (d) is independent of the concentration of beads in the sample, it can be calculated according to:

$$d_i = x_i - c_{ref,i}$$

$$d = \frac{\sum_{i=1}^{n} d_i}{n}$$

$$s = \sqrt{\frac{\sum_{i=1}^{n}(d_i - d)^2}{n-1}}$$

Where x_i is the concentration of beads measured in the reference sample, $c_{ref,i}$ is the concentration of beads in the reference sample, n is the amount of samples analysed (8) and s is the standard deviation in the experiment.

When the relative bias (d_{rel}) is independent of the concentration of beads in the sample, then it can be calculated according to:

$$d_{rel,i} = \frac{x_i - c_{ref,i}}{c_{ref,i}}$$

$$d_{rel} = \frac{\sum_{i=1}^{n} d_{rel,i}}{n}$$

$$vc = \sqrt{\frac{\sum_{i=1}^{n}(d_{rel,i} - d_{rel})^2}{n-1}}$$

Where x_i is the concentration of beads measured in the reference sample, $c_{ref,i}$ is the concentration of beads in the reference sample, n is the amount of samples analysed (8) and s is the standard deviation in the experiment.

4.3.1.7 Recovery

The recovery is determined by addition of a known concentration of standardised beads (Beckman Coulter Flow Count beads) to a sample. For each water type, eight different duplicate samples are measured on eight different days. For each duplicate, to one of the measurements known concentration of beads has been added (∆c).
First, the recovery (Tv) is determined for each duplicate pair (Tv_i) according to:

$$Tv_i = \frac{x_{c+\Delta c,i} - x_{c,i}}{\Delta c}$$

Where $x_{c+\Delta c,i}$ is the concentration of cells with the addition, $x_{c,i}$ is the concentration of cells in the original sample and ∆c is the concentration of beads added to the sample.

The recovery is then determined according to:

$$Tv = \sum_{i=1}^{n} \frac{Tv_i}{n}$$

After which the standard deviation (s) is determined:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(Tv_i - T_v)^2}{n-1}}$$

4.3.1.8 Non-linearity

The non-linearity of the analysis is comparable to the determination of the measurement range. Standardised samples (containing Beckman Coulter FlowCount fluorospheres, at four different concentrations 0,25; 0,5; 0,75 and 1* the normal concentration of 100 µL beads for 1 mL sample) are measured on eight days, after which the average (x_{gem}) and the standard deviation (s) are calculated.
Furthermore the non-linearity of the flow rate is determined by analysing the samples at different flow rates (60, 120, 180 and 200 µL.min^{-1}). For this purpose the average and standard deviation are determined again.

The non-linearity is calculated according to:

$$d_{c,model} = x_{gem} - c$$

Where c is the concentration of cells in the sample.

Also tested is (for n-1 degrees of freedom), whether this non-linearity is deviating from the value zero by:

$$d_{c,model} \leq \frac{t_{0.975}s}{\sqrt{n}}$$

When the result is compliant to this equation, there is no non-linearity present and the data quality indicators are accepted. For n≥8, $t_{0.975}$ = 2.

4.3.1.9 Selectivity

Specifically in ballast water verification testing, several natural water characteristics are adjusted in order to challenge the system that is tested.
The additions include:

1. Addition of dissolved organic carbon, in both aromatic and non-aromatic form (i.e. lignin and citric acid)
2. Addition of total suspended solids (TSS).

In order to test for the applicability of the flow cytometry method the selectivity of the method is determined after the additions. This is done by adding different concentrations of lignin, citric acid and total suspended solids (Table 3).
The concentrations of these substances were determined from previous ballast water verification tests and mimic actual additions made throughout the testing season.
The experiment is set up in the same way as the recovery experiment. The samples are analysed in duplicates, one of each duplicate is analysed with a possible interferent, while the second sample of the duplicate is analysed without the possible interferent.

The selectivity ($I_{c,i}$) is calculated according to:

$$I_{c,i} = \frac{x_{c,i}}{\Delta c_{interf}}$$

Where $x_{c,i}$ is the concentration of cells with the addition and Δc_{interf} is the difference between the duplicate measurements. The selectivity (I_c) is calculated as the average of the $I_{c,i}$ values), after which the standard deviation (s) is determined:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(I_{c,i} - I_c)^2}{n-1}}$$

Table 3: The amount of the different added substances, possibly interfering with the measurement

Lignin (mg.L^{-1})	Citric Acid (mg.L^{-1})	TSS (mg.L^{-1})
2	4	25
4	8	50
6	12	75
8	16	100
10	20	125

4.3.1.10 Memory Effect

The memory effect of the measurement mainly consists of carry-over from one analysis to the next one. Although there is a short period of backflush after each measurement and a small amount of sample is flushed through the fluidics system prior to the next measurement, the memory effect was determined in order to establish whether the instrumental settings are sufficient to prevent a memory effect.
In order to determine the memory effect, eight identical subsamples with a very low concentration of cells are measured, after which one sample with very high cell concentration, followed by another sample with very low cell concentration (x_1) (identical to the first eight samples) are measured. The average (x_{gem}) and standard deviation (s) of the first eight samples are calculated, followed by the memory effect (G) according to:

$$G = x_1 - x_{gem}$$

The memory effect is negligible when one of the according to criteria is matched:

$$G < 2s\sqrt{1 + \frac{1}{n}}$$

or

$$G < \frac{1}{3}s_{Rw}$$

4.3.2 Current status of the validation

The validation process is a process that has a minimum duration of four weeks. For a better coverage of the natural range of the samples, it is advisable to distribute the tests over a longer period of time. Due to the limited availability of trained laboratory technicians, the validation process has taken longer than expected, but progress has been made and data have been collected since early 2018, resulting in tests with a large natural variability.

Currently the process is ongoing and new data are being added on a daily basis.
Samples for several DQI's are being analysed and/or data are analysed at the moment (Table 4).

Table 4: The current status of the validation process

DQI	Sample analysis	Data analysis
Measurement range – flow rate	Finished	Finished
Measurement range – particle abundance	Finished	Finished
Measurement range – particle size	Finished	Finished
Detection limit	Work to be started	
Reporting limit	Work to be started	
Repeatability	Finished	Finished
Reproducibility	Ongoing with fixed samples and standardised samples	
Bias	Work to be started	
Recovery	Work to be started	
Non-linearity	Work to be started	
Selectivity	Ongoing	
Memory effect	Finished	Finished

4.3.3 Applicability in ballast water verification and/or environmental testing

The previously described validation process focusses on phytoplankton abundance in natural water samples. We are aware that this is not sufficient for ballast water verification testing for which the criteria do not follow taxonomic or ecologically defined groups, but are based on size classes. The size class that is of interest in relation to flow cytometry is the ≥10 µm up to 50 µm size class (Table 5, Table 6)(IMO 2016, IMO 2018).
This size class does not only entail phytoplankton, it also includes smaller zooplankton. The organisms in this size class are enumerated by fluorescence microscopy, which is a time-consuming, limited volume analysis. Samples are stained for live/dead analysis and are incubated together with a mixture of FDA (fluorescein diacetate) and CMFDA (Chloro-methoxy fluorescein diacetate), after which a small amount of volume (a minimum of 100 µL and a minimum of 200 organisms are counted) can be analysed microscopically (Denmark and Norway 2016). A time window of only 20 minutes is available to count the sample. Furthermore, the accuracy of the analysis is highly dependent on the training of the technician performing the analysis and it is therefore more prone to human errors. An automated method decreases the risks of human errors, because it has fixed threshold levels and does not deviate from its settings. Furthermore, by analysing a sample for 20 minutes through flow cytometry, the analysed volume increases significantly (in the same time, up to 4 mL sample could theoretically be analysed compared to only 100 µL by microscopy), thereby increasing the statistical reliability of the counts. One does have to take into account that cells can start to settle, which decreases the accuracy of the measurement. In the validation process for an automated method the amount of time after which settling results in an unacceptable loss of reliability should be determined. Up to now, all of the flow cytometric analyses were performed with an analysis time of 300 seconds (5 minutes, resulting in 1 mL sample analysed). During this analysis period the counts were not affected by settling (Figure 6).

Table 5: Criteria for land-based verification testing of ballast water treatment systems. Only parameters that are relevant to this document are mentioned.

Land-based verification testing				
Parameter	**Intake/discharge**	**Marine**	**Brackish**	**Fresh**
Salinity (psu)	Intake	28-36	10-20	<1
DOC ($mg.L^{-1}$)	Intake	> 1	> 5	> 5
POC ($mg.L^{-1}$)	Intake	> 1	> 5	> 5
TSS (including mineral matter) ($mg.L^{-1}$)	Intake	> 1	> 50	> 50
10 ≤ x < 50 µm ($cells.mL^{-1}$)	Intake minimum	> 10^3	> 10^3	> 10^3
10 ≤ x < 50 µm ($cells.mL^{-1}$)	Control discharge minimum	≥ 10^2	≥ 10^2	≥ 10^2
10 ≤ x < 50 µm ($cells.mL^{-1}$)	Treated discharge maximum	< 10	< 10	< 10
Other demands	≥5 species; ≥3 taxa, at intake	x	x	x

Table 6: Criteria for shipboard verification testing of ballast water treatment systems. Only parameters that are relevant to this document are mentioned.

Shipboard testing		
Parameter	**Intake/discharge**	**Criterium**
10 ≤ x < 50 µm ($cells.mL^{-1}$)	Intake minimum	≥ 10^2
10 ≤ x < 50 µm ($cells.mL^{-1}$)	Treated discharge maximum	< 10

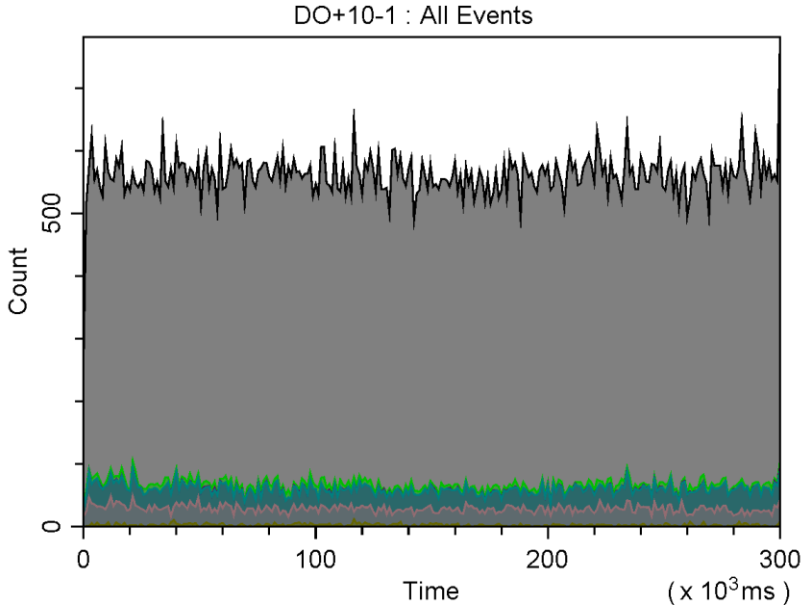


Figure 6: A flow cytometric plot of the number of counts per second versus the time counted. In this specific sample, sediment was added to test for the sensitivity of the flow cytometer in high-sediment conditions.

We are currently developing a FDA staining protocol for the live/dead analysis of natural planktonic samples. It is a time-consuming and tedious process, since at first we are comparing flow cytometric counts of viable cells with microscopic counts of the same sample to determine the differences/similarities between the two counting methods.
In this document, we present some preliminary results of FDA staining experiments with marine phytoplankton. For the first experiments we used the same final concentration of FDA as in microscopy counts, 5 μM. The experiments were done with *Rhodomonas* sp., *Tetraselmis* sp. and a freshwater sample. Both live and dead samples (heat-killed, 10 min at approximately 100 °C) were analysed with and without stain, resulting in preliminary templates for live-dead analysis.

Once the method has been fully developed, a second set of validation experiments will be conducted and data quality indicators will be reported for approval of the method.

5. Results and discussion

5.1 Data Quality Indicators that have been determined

5.1.1 Measurement range

5.1.1.1 Flow rate

The flow rate of the instrument was measured by weighing each sample before and after a run on an analytical balance. The difference between the two measurements was calculated and converted into the measured flow rate according to:

$$Measured\ flow\ rate = \frac{weight\ before - weight\ after}{5\ min} * 1000\ (\mu L)$$

The results are plotted in Figure 7. The set flow rate of the instrument matches well with the measured flow rate (R^2=0.9999). The first flow rate that has been set was 10 µL.min^{-1}. While it is not clearly visible in the plot, quite a large difference was found between the set flow rate and the measured flow rate (37%). The difference decreased rapidly to 12% at 30 µL.min^{-1} and 5% at 60 µL.min^{-1}. At the highest flow rates 220 and 230 µL.min^{-1} the measured flow rate was slightly lower (1%) than expected from the set flow rate.
As discussed before, a small amount of sample is measured before the actual measurement starts, to reduce carry-over of a previous sample. It accounts for an estimated 1% of the total volume uptake during analysis, but it is most likely depending on the flow rate. Keeping this in mind, we concluded that in the range of 60 to 230 µL.min^{-1} the calibrated flow rate in the machine corresponds very well to the measured flow rate.

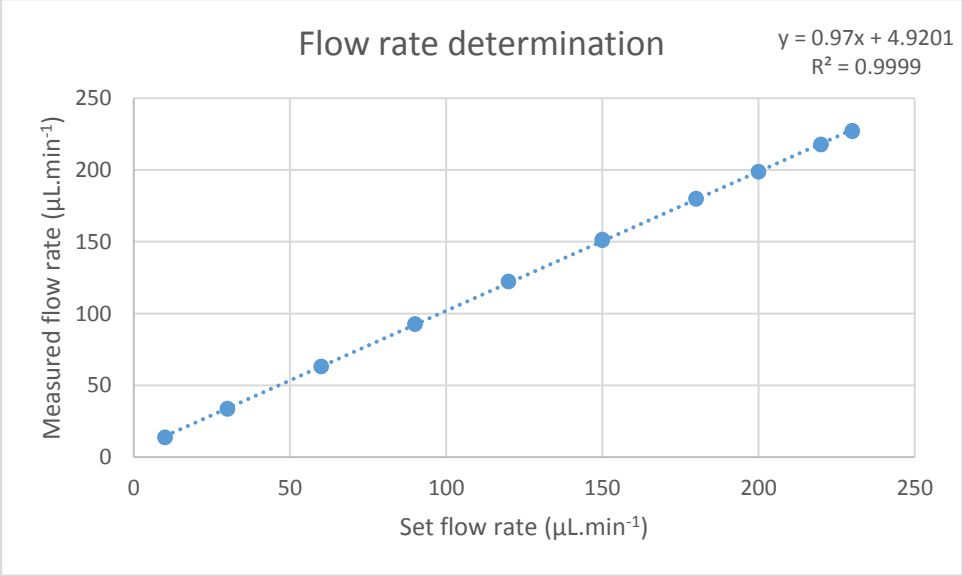
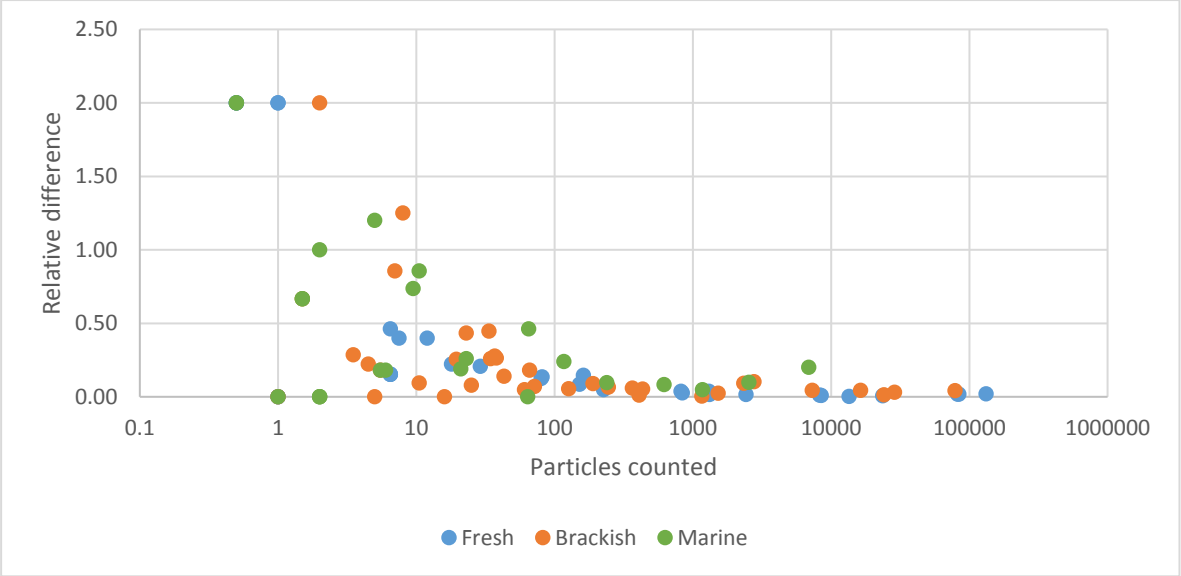


Figure 7: The measured flow rate (µL.min^{-1}), calculated from the volume of RO-water taken up by the instrument, plotted against the set flow rate.

5.1.1.2 Particle abundance

The effect of particle abundance on the reliability of the counts was determined by a dilution experiment for each type of water. Each dilution step was measured in duplicate and the relative difference between the two measurements has been determined. This was done for five different groups in the flow cytometric template: 1. Total phyto; 2. PE-Phyto; 3; Phyto-

select; 4. Phyto 10-50 µm and 5. Phyto < 10 µm. All groups showed the same pattern. First the dilution series have been well-executed, samples were well-mixed and the 10-fold dilutions were reflected in the counts. Second it became obvious that the relative difference within the duplicates increased wit decreasing counts. It proves that it is very important to count as much of the sample as possible. All samples were counted for 5 minutes at 200 µL.min^{-1}, resulting in 1 mL volume counted.
The experiment needs to be repeated with low organism numbers in order to validate whether it is possible to fulfil the requirements that are set for testing the 10-50 µm size class in verification tests of ballast water treatment systems.



5.1.1.3 Particle size

For determining whether particle size had an effect on flow cytometric counts, natural samples were filtered over four different nylon meshes, resulting in a smaller portion of particles with each increases filtration step (Table 7). It is clear that most particles are smaller than 50 µm, although it is visible that not all particles are passing the filter mesh. For example, the portion filtered over 50 µm in brackish water is smaller than that filtered over 30 µm. When it is necessary to concentrate samples, because there is a need to count higher densities of organisms, this is something to keep in mind. It would be necessary to study the recovery when concentrating samples.
From Table 8 it is obvious that the relative difference between counts is not related to particle size, therefore we can assume that particle size, apart for particles which are too large and clog the system, does not influence the counts by the flow cytometer. Some high values are recorded in the table (100%). They originate from the very low particle abundance, since the samples were filtered over 0.2 µm syringe filter and should therefore be more or less empty.

Table 7: The precentage of particles (of the total, 100 µm filtered sample) present after each filtration step.

Mesh size (µm)	Fresh	Brackish	Marine
0.2	6%	6%	0%
10	57%	52%	22%
30	78%	101%	70%
50	94%	93%	102%
100	100%	100%	100%

Table 8: The relative difference between duplicate measurements when samples are filtered over different mesh sizes.

	Mesh size (µm)	All Events	Phyto	Phyto-select	Phyto <10 µm	Phyto 10-50 µm
Fresh	0.2	2%	11%	100%		100%
	10	0%	0%	0%	17%	0%
	30	1%	0%	0%	2%	0%
	50	0%	1%	1%	3%	1%
	100	0%	0%	0%	5%	0%
Brackish	0.2	6%	11%			100%
	10	1%	0%	1%	28%	0%
	30	3%	2%	3%	1%	2%
	50	2%	0%	0%	8%	0%
	100	0%	1%	1%	9%	2%
Marine	0.2	3%	8%	100%		33%
	10	4%	2%	2%	7%	2%
	30	3%	1%	1%	2%	0%
	50	4%	1%	6%	7%	6%
	100	1%	1%	2%	4%	5%

5.1.2 Repeatability

The repeatability has been analysed from samples that mostly originated from actual shipboard and land-based verification tests. It resulted in data covering a large range of cell abundances, such as those for control intake water and for treated discharge water.
The three different water types were verified by salinity measurement with each test, according to the salinity requirements in Table 2.
The results of the repeatability tests are shown in Table 9. We have analysed the relative repeatability, since the variability is dependent on the amount of cells counted. The variability (vc_r) is relatively low.

Table 9: The relative repeatability (r_{rel}) and the variation coefficient (vc_r) of the flow cytometric measurements of phytoplankton. Phyto-select depicts the group of phytoplankton without phycoerythrin. Within this group the division is made between Phytoplankton smaller than 10 µm and Phytoplankton in the size range of 10 and 50 µm.

Water type		n	All events	Phyto-select	Phyto <10 µm	Phyto 10-50 µm
Marine	R_{rel}	8	0.16	0.34	0.32	0.98
	vc_r		0.06	0.12	0.11	0.35
Brackish	R_{rel}	8	0.11	0.12	0.09	0.77
	vc_r		0.04	0.04	0.03	0.27
Fresh	R_{rel}	8	0.04	0.04	0.06	0.12
	vc_r		0.01	0.02	0.02	0.04

5.1.3 Memory effect

Whether there is a memory effect, or carry-over, between two subsequent measurements has been determined by counting eight samples with a very low concentration; one sample with a high concentration of particles followed by another sample with a low amount of particles. The average of the first eight low samples was subtracted from the last low sample, resulting in the memory effect value: “G”.
The memory effect was checked against a criterium according to:

$$G < 2s\sqrt{1+\frac{1}{n}}$$

In all but one case (Table 12), the last low sample was even lower than the first eight samples, showing that there was no memory-effect (Table 10, Table 11).
In case a memory effect would have been found, it would easily have been solved. A flow cytometer offers the possibility of only analysing part of the data it produced. For example, when there is a 10 second carry-over or memory effect, one can gate the data accordingly and only the last 290 seconds are analysed. The same can be done when the tubing has become clogged and part of a measurement has failed. Analysing the data does not alter the raw data, therefore the decisions made in the data analysis can always be verified.

Table 10: Memory effect for fresh water samples.

Fresh water				
	Total phytoplankton	**Phyto-select**	**Phyto 10-50 µm**	**Phyto < 10 µm**
Average low	1370	763	86	676
Last low	1283	711	84	626
s	77	37	7	41
Memory effect (G)	-87	-52	-2	-50
Criterium	164	78	16	86
Passed	YES	YES	YES	YES

Table 11: Memory effect for brackish water samples.

Brackish water				
	Total phytoplankton	**Phyto-select**	**Phyto 10-50 µm**	**Phyto < 10 µm**
Average low	2325	1510	29	1581
Last low	2137	1380	20	1477
s	110	76	4	75
Memory effect (G)	-188	-130	-9	-104
Criterium	233	162	9	159
Passed	YES	YES	YES	YES

Table 12: : Memory effect for marine water samples.

Marine water				
	Total phytoplankton	**Phyto-select**	**Phyto 10-50 µm**	**Phyto < 10 µm**
Average low	183	53	4	55
Last low	164	53	3	48
s	8	9	2	9
Memory effect (G)	-19	0	-1	-7
Criterium	17	18	5	19
Passed	YES	YES	YES	YES

5.2 Preliminary FDA results

5.2.1 *Rhodomonas* sp. cultures

As mentioned in section 3.1.3, fluorescein diacetate, FDA, is a stain which transfers easily over the cell membrane, after which it is hydrolysed to fluorescein by cells which have an active metabolism.
The method is already applied both in shipboard and in land-based verification testing, in combination with CMFDA and microscopy. Automating this method would decrease the cost of the analysis, mainly because microscopy is a time consuming technique. More samples could be analysed in the same amount of time, and more accuracy is achieved by increasing the volume counted.

We have done some preliminary tests in order to establish a base-line in FDA and flow cytometry for our laboratory. For the first experiments, we have used the same concentration of FDA as has been used in the microscopic analysis, a final concentration of 5 µM.

The first species tested was *Rhodomonas* sp. The small (most cells are smaller than 10 µm) (Figure 8) Cryptophyte species can have flagella. Cryptophytes are recognised by their Phycoerythrin fluorescence. This is clearly visible in the flow cytometer measurements (Figure 9).

The results from the FDA test show that the species stains very well (Figure 10). For the samples, there was a 6 to 7 % difference (data not shown) between the total group of *Rhodomonas* sp. and the stained living group, the latter group representing the lower amount. It indicates that all living organisms were stained. Furthermore, a duplicate measurement with stained cells resulted in a very small difference of less than 1 % (10,331 and 10,284 cell.mL^{-1}). Dead cells maintain their fluorescence level, with or without FDA and do not interfere with the measurement of living cells. The results are very promising and more work will be done to continue the process of validating FDA staining methods for flow cytometry.

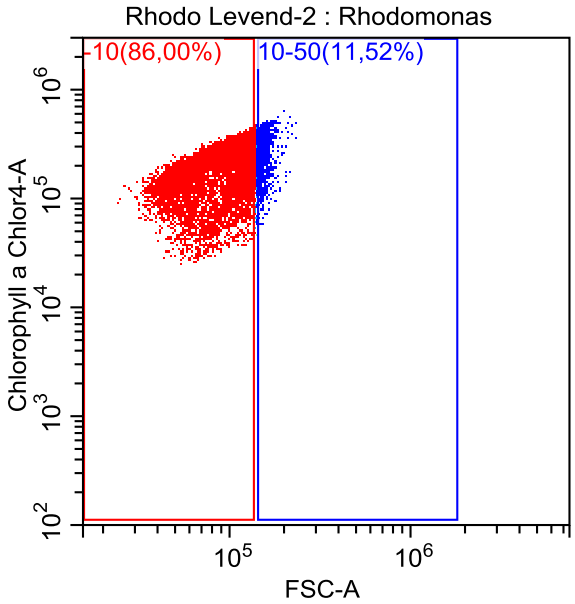


Figure 8: A size separation plot of Rhodomonas sp. In red the organisms smaller than 10 µm, in blue the cells in the 10-50 µm size range.

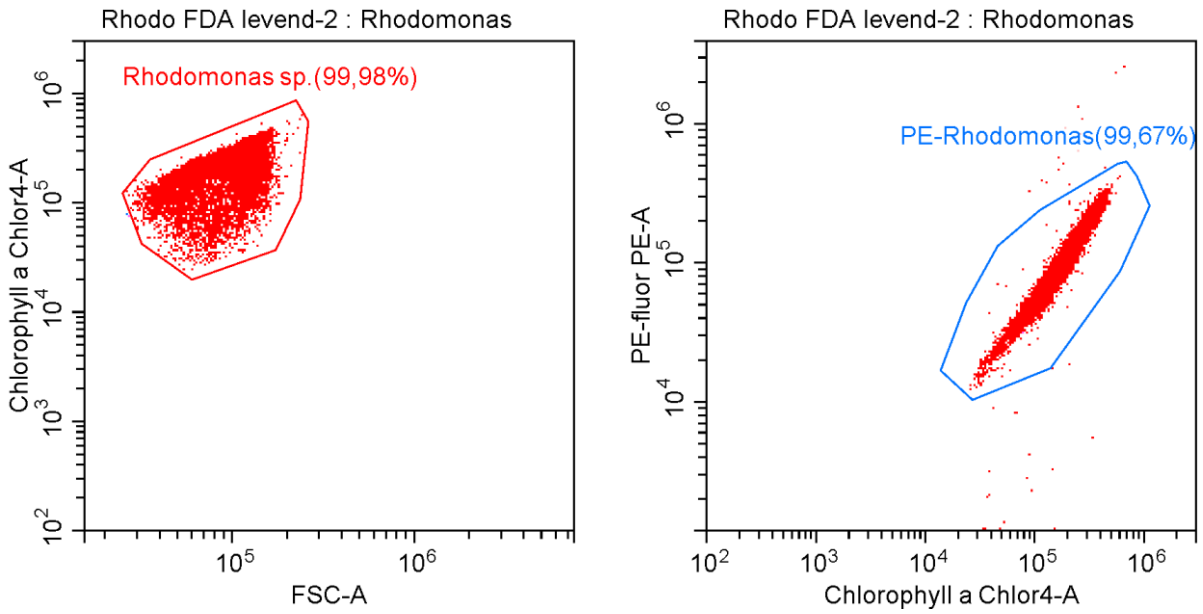


Figure 9: Typical flow cytometric plots of Rhodomonas sp. cultures.
Left: FSC signal versus chlorophyll fluorescence. Right: Chlorophyll fluorescence signal versus PE-fluorescence.

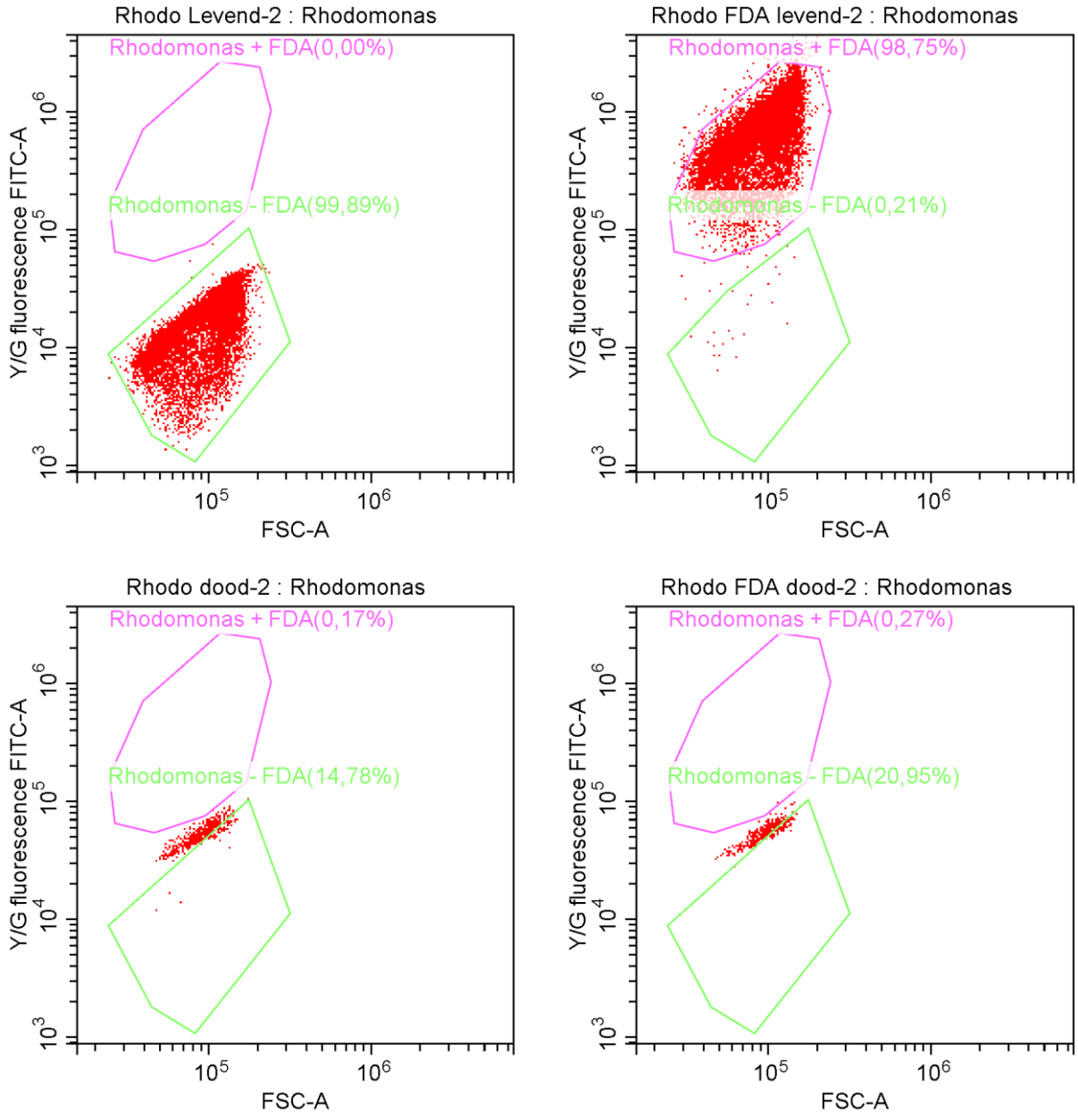


Figure 10: Flow cytometric plots of Rhodomonas sp. *cultures showing the Yellow/Green fluorecence signal (FITC) originating from the fluorescein produced by metabolic hydrolysis of FDA. Top left: a living culture, without FDA staining. Top right: a living culture stained with FDA. Bottom left: a heat-killed culture without staining. Bottom right: a heat killed culture with FDA*

5.2.2 *Tetraselmis* sp. cultures

The second test that has been performed with FDA was the staining of a culture of *Tetraselmis* sp. The species belongs to the Chlorodendrales, green algae. Just like *Rhodomonas* sp. the algae can have flagella, they can also reside as non-motile species or cysts. The first results show that the living cells stain well, however, there appears to be a discrepancy between the stained cells and the cells without stain. Only approximately 70% of the Tetraselmis counted without stain, is also stained and counted with FDA staining. Furthermore there is some overlap between stained and non-stained cells (Figure 11). The culture used for this experiment was very old and left to form cysts. As can be seen in the bottom two plots of Figure 11 the cells stained with FDA have a slightly higher count than the sample that was not stained. This might be due to the cysts, which do not die after heat treatment, but might still have some active metabolism and produce a very small amount of fluorescence. Future experiments, with and without cysts, will be carried out in order to determine whether the cells that can be seen between the stained and non-stained groups are cysts, or dying cells.

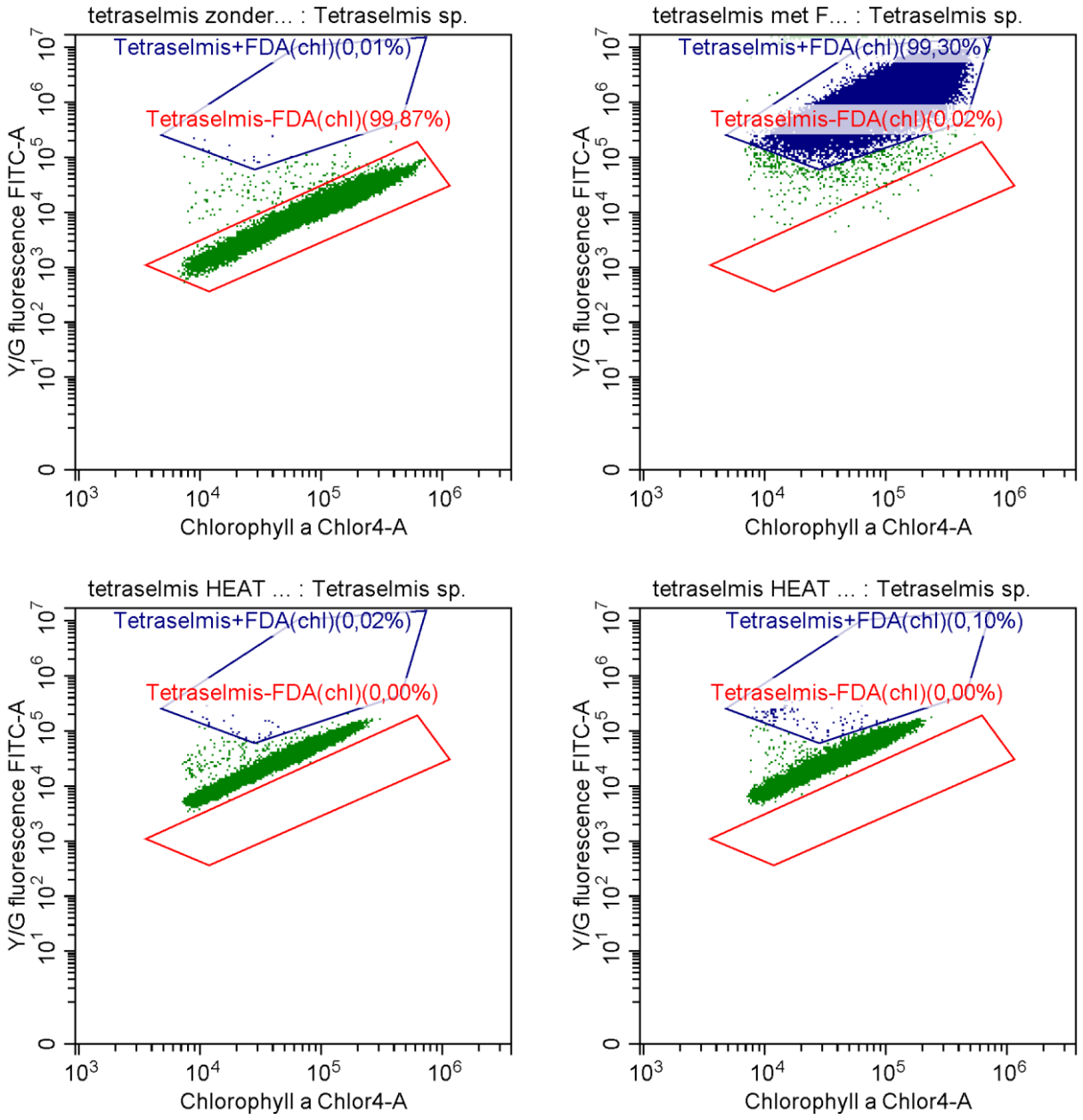


Figure 11: Flow cytometric plots of Tetraselmis sp. *cultures showing the Yellow/Green fluorecent signal (FITC) originating from the fluorescein produced by metabolic hydrolysis of FDA. Top left: a living culture, without FDA staining. Top right: a living culture stained with FDA. Bottom left: A heat-killed culture without staining. Bottom right: a heat killed culture with FDA staining.*

5.2.3 Fresh water sample

The last experiment was conducted with natural water. Unfortunately the marine and brackish water did not contain many organisms, but we got a nice result from the fresh water sample. The top two graphs of Figure 12 show the results of the FDA staining in live samples. Without stain, no visible yellow/green fluorescence is visible, but as soon as FDA is added there is a group of organisms that fluoresce. The amount of FDA-stained particles is approximately 20% larger than the phytoplankton counts in the same sample, indicating that zooplankton has been stained and is counted (data not shown).
We do recognise that only one natural sample has been tested, it is however a promising start and together with the *Rhodomonas* sp. and *Tetraselmis* sp. data it indicates that the combination of FDA and the flow cytometer is a successful one!

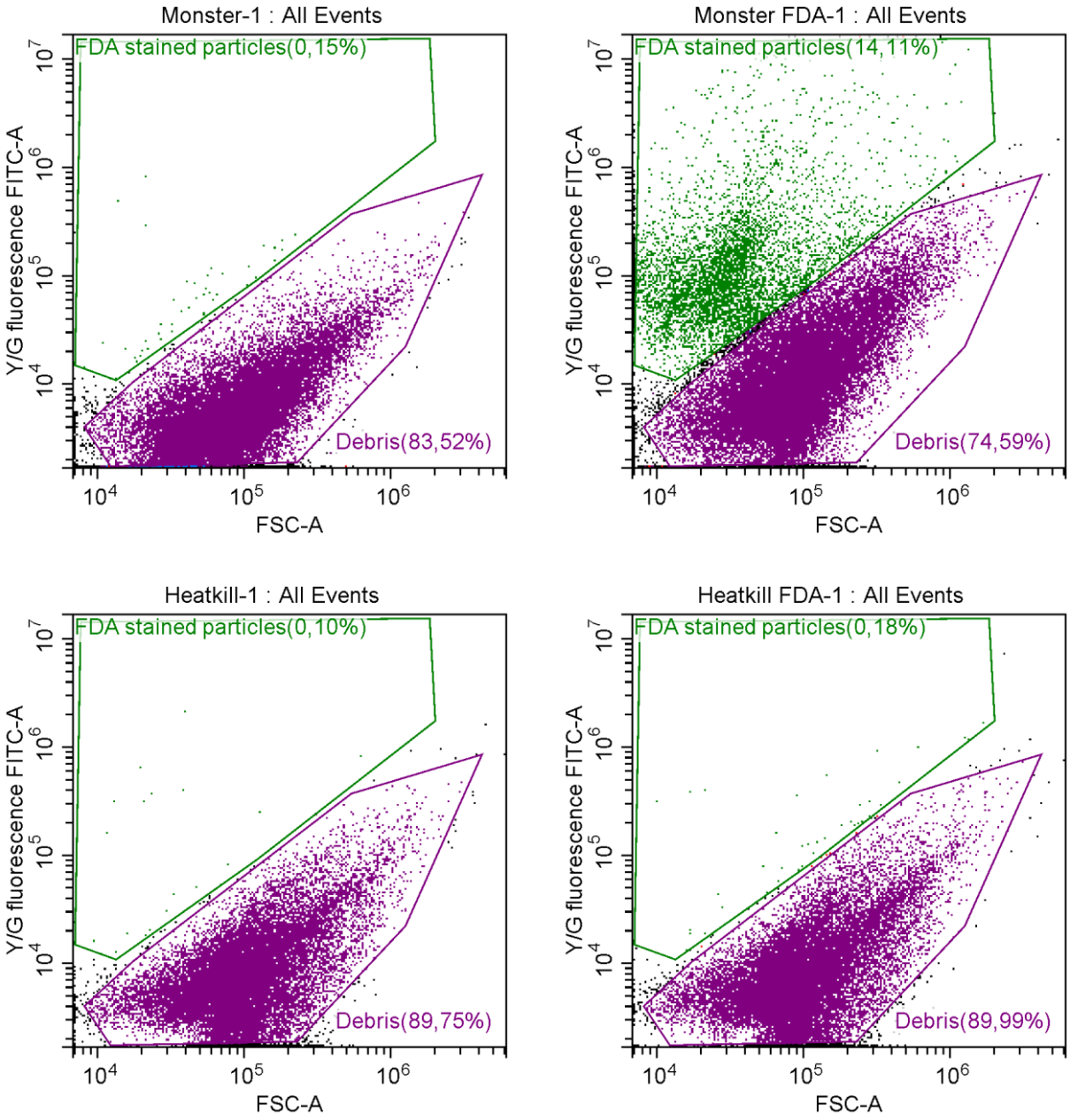


Figure 12: Flow cytometric plots of a freshwater sample. Plotted is the Forward Scatter signal (FSC) versus the Yellow/Green (FITC) fluorescence induced by fluorescein.
Top Left: Fresh sample without FDA staining. Top Right: Fresh sample with FDA staining.
Bottom Left: Heat-killed sample without FDA staining. Bottom Right: Heat-killed sample with FDA staining.

6. Quality Assurance (QA) and Quality Control (QC)

6.1 Quality assurance

As of May 2018, MEA-nl is an EN ISO/IEC 17025:2005 accredited company, accreditation number L 632. The Quality Management System is in accordance with the requirements of the ISO-9001:2015 standard.
The applied methods were mandated by the scope of the validation experiment presented here.
All analyses were performed by trained personnel.

6.2 Quality control and data acquisition

The data quality indicators (DQI's) presented in this document are determined according to NEN-7777+C1:2012.
The standard operating procedure (SOP) and flow cytometric template for phytoplankton analysis were developed prior to the start of the validation process. During validation however, depending on changes in the natural phytoplankton community, the gates in the template were slightly adjusted in order to fit to these changes. This is also described in the normal standard operating procedure (SOP) for phytoplankton flow cytometry as developed by MEA-nl (MEA-nl 2018).

6.3 Verification of test data

Size determination has been verified by regular size fractionation of natural water samples, thereby verifying the daily quick verification of the size class gating with NIST standardised size beads.
A full validation of phytoplankton and bacteria counts is in progress.

6.4 Deviations from the test plan

Due to the limited availability of trained laboratory technicians, the validation process as described in this document is taking longer than expected. However, due to this longer time span, an increased range in natural variability of the planktonic community is tested, thereby improving the value of the determined DQI's.

7. Summary and conclusion

The data and experiments reported in this document are the start of a full validation process. Although there are some difficulties, we do believe that flow cytometry is an effective and promising way forward for analysing water samples.
One of the major concerns that is always raised when speaking about flow cytometry is: How can we determine the size of the particles which are recorded? It has been stated repeatedly that the forward scatter is a relative size determination and this is definitively something to take into account. It is not only affected by the size of organisms, but also by the difference in the refractive index of the water and that of the cells that are measured (Shapiro 2003). By using natural samples and size-fractionation the sizing becomes more accurate and the use of the standardised beads, as a rapid size indication, is regularly verified throughout the process.
Repeated size-fractionation has shown that the size classes (compared to the forward scatter), as we have established them, remain constantly in the same position, when the same settings are used for analysis. Which means that even if something would go wrong and there is no sizing data for a specific experiment, data can be imported into another analysis template (old or new) and the size separation can be done afterwards.

Several possible instrumental limitations were tested, including flow rate, particle abundance and particle size. Flow rate, as specified by the manufacturer and pre-set in the instrument's software, is stable and matches the measurements. By repeating the test regularly we can monitor the instrument's stability. The instruments internal calibration of the flow rate works well, although it is limited, to 60 $\mu L.min^{-1}$. Therefore a regular check of the faster flow rates is necessary.

The dilution experiment showed that the machine does not have any problems with larger particle densities when samples are run that only contain few organisms, variability increases significantly. Since it is necessary to count low numbers in both shipboard and land-based verification testing (treated discharge) it might be necessary to increase the duration of the measurement or to concentrate the sample. Both methods have positive as well as negative sides. The positive side is the increase in the number of organisms that are counted once the duration of counting is prolonged, thereby reducing the statistical uncertainty (which according to a Poisson distribution is three for a count of 10). The same argument can be used for concentrating the sample. The negative side of such options is the loss of organisms and thereby an underestimate of the actual numbers present. When a sample is counted by a flow cytometer during a longer period of time, organisms can start to settle and counts are significantly reduced. Concentrating the sample will also lead to a loss of organisms, which do not pass through a filter or that are killed in the process.
Both increasing the analysis time and concentrating the samples are two techniques that should be tested and validated before they can be used with flow cytometry.

We have shown that the repeatability has been good, although it needs improvement for samples with low organism numbers. No memory effect has been detected. This is due to the programming of the flow cytometer. After running a sample, there is a short backflush cycle, which is followed by a short flush with the new sample, significantly reducing the carry-over of previous samples. Sometimes phytoplankton can be sticky and some carry-over can occur; when this happens, it is easily solved by removing the first 20-30 seconds of data from the data analysis, which does not alter the raw data, but merely increases the reliability of the analysed data.

The live/dead determination with FDA and CMFDA is an established method within verification testing. By incorporating flow cytometry in the method, with its high sensitivity, accuracy and speed, it is possible to improve the analysis method. The preliminary experiments with FDA staining reported in this document are promising. For both cultures tested and the natural water

sample, a clear fluorescein signal was obtained. In the natural water sample the amount of FDA stained particles was larger than the amount of chlorophyll fluorescent particles in the same sample, indicating that the zooplankton is stained and counted as well. The experiments will be continued and the method will be optimized after which it will be thoroughly validated.

8. References

8.1 List of references

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8.2 List of figures

8.3 List of tables

9. Annexes

9.1 Tables Particle abundance

Table 13: The results of the dilution experiment. The first table shows the dilution of the fresh water sample, the second table shows the dilution of the brackish water sample and the third table shows the results of the dilution of marine water. Note that the dilution series of the fresh water sample ranged from low concentrations to high concentrations, as opposed to the other two water types.

Fresh water	Phytoplankton	PE-phyto	Phyto-select	Phyto 10-50 µm	Phyto < 10 µm
1E-07	6	0	1	0	1
1E-07	7	0	1	0	1
1E-06	5	0	0	0	0
1E-06	8	0	1	0	1
1E-05	9	0	2	0	2
1E-05	6	0	0	0	0
1E-04	16	0	7	0	7
1E-04	20	1	6	0	6
1E-03	145	26	76	0	75
1E-03	158	32	87	1	85
1E-02	1331	221	851	12	836
1E-02	1311	232	828	18	805
1E-01	13544	2442	8438	162	8244
1E-01	13508	2403	8508	140	8338
1E+00	133726	23721	84750	1307	83010
1E+00	130954	23576	83294	1357	81594

Brackish water	Phytoplankton	PE-phyto	Phyto-select	Phyto 10-50 µm	Phyto < 10 µm
1E+00	77059	16024	28395	408	24011
1E+00	80401	16756	29314	413	24352
1E-01	7167	1510	2625	37	2224
1E-01	7485	1549	2907	28	2441
1E-02	1161	253	446	5	377
1E-02	1166	237	423	5	355
1E-03	198	39	69	1	62
1E-03	181	30	74	1	59
1E-04	123	24	40	0	33
1E-04	130	26	46	0	43
1E-05	72	11	28	2	22
1E-05	60	10	18	0	17
1E-06	16	2	5	0	4
1E-06	16	1	6	0	5
1E-07	41	3	10	0	13
1E-07	26	4	4	0	3

Marine water	Phytoplankton	PE-phyto	Phyto-select	Phyto 10-50 µm	Phyto < 10 µm
1E+00	6211	20	2425	65	1150
1E+00	7605	26	2675	104	1206
1E-01	645	3	250	6	131
1E-01	594	1	227	5	103
1E-02	64	0	23	0	15
1E-02	64	0	19	2	6
1E-03	13	1	2	0	1
1E-03	6	0	2	0	1
1E-04	6	0	2	0	2
1E-04	5	0	1	0	2
1E-05	2	0	0	0	0
1E-05	8	0	0	0	0
1E-06	2	0	0	0	0
1E-06	2	1	0	0	0
1E-07	2	0	0	0	0
1E-07	1	0	0	0	0