Feasibility-report of a quantitative method for rapid assessment of microbial population composition in drinking water using flow-cytometry combined with FISH

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Feasibility-report of a quantitative method for rapid assessment of microbial population composition in drinking water using flow-cytometry combined with FISH

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## Contents

### 1 Introduction

1.1 Goal of this work  

### 2 Results and Discussion

2.1 Overview  
2.2 Cell loss during the FISH procedure  
2.3 Challenges of omitting the washing steps  
2.4 Omission of washing and resuspension after incubation with FISH-probes  
2.5 Alternative fixation procedures  
2.6 Performance of the FISH protocol on a filter followed by resuspension  

### 3 General discussion  

### 4 References
1 Introduction

Fluorescent in situ hybridization (FISH) is a method which uses rRNA-targeted oligonucleotide probes to visualize and identify specific bacteria or bacterial groups in natural environments. The method is not cultivation based, but the presence of RNA in cells suggests that they are likely to be viable (Amann et al., 1990). Visualization of the targeted bacteria is done through fluorescent labelling of the oligonucleotide probes, and is normally accomplished with epi-fluorescence microscopy or with confocal laser scanning microscopy (CLSM). The method has been developed long ago, and has since become one of the major techniques in environmental microbiology, specifically with regards to detection of cells which are not culturable on conventional media (Wagner et al., 2003).

Flow cytometry is a method with which fluorescently labelled microbial cells in solution can be quantified rapidly. This method can be used for direct enumeration of the total cell concentrations in water (Hammes et al., 2007; Hammes and Egli, 2005; Lebaron et al., 1998), staining and detection of specific cellular features such as viability (Hoefel et al., 2003; Phe et al., 2005; Berney et al., 2006), or staining and enumeration of specifically targeted cells with antibodies (Vital et al., 2007). A lucrative feature of flow cytometry is that it is fast, accurate and quantitative. The advantage of combining flow cytometry with FISH is that it has the potential to provide rapidly information the the population composition in water samples. This has been recognized previously, and several groups have tested the use of FCM for this purpose (Baudant et al., 2002; Wallner et al., 1993).

The primary challenge of using FCM combined with FISH with drinking water bacteria is that the original FISH procedure involves 2 washing steps and is usually done with the sample fixed on a solid surface. For FCM, the sample needs to be in solution, which means that either centrifugation-resuspension steps or a filtration-resuspension step is required. In addition, the FISH method is based on the presence of ribosomal RNA in cells, and drinking water bacteria growing in oligotrophic environments often are small cells with low concentrations of rRNA, thus resulting in weak signals. This can be compensated for with signal enhancing (CARD-FISH) (Pernthaler et al., 2002).

1.1 Goal of this work

The aim of this study was to investigate experimentally the combination for FISH and flow cytometry (FCM-FISH) as a tool for rapid and quantitative assessment of the microbial population composition in drinking water. Since FCM-FISH have been demonstrated before, the main target was to have a truly quantitative method. As a model organism, E. coli has been used.
2 Results and Discussion

2.1 Overview

The goal of this feasibility study was to enable quantitative measurements of FISH tagged bacteria on a flow cytometer. *E. coli* was used as a model organism. The method of Wallner and coworkers (Wallner et al., 1993) was the basis of this study. The original protocol consist of the following basic steps:

1. Incubation of the sample in fixation and permeabilization buffer
2. Washing step (to get rid of the fixation buffer)
3. Incubation with FISH probes solution
4. Washing and resuspension in PBS (to get rid of unbound FISH probes)

In this protocol, the washing step is done with centrifugation. The main issue for making the FCM-FISH procedure quantitative is to omit or replace washing steps, where samples either have to be centrifuged and re-suspended or filtered and re-suspended because of the inevitable loss of cells during these procedures, which was demonstrated previously in our group (see Section 2.2 of this report).

2.2 Cell loss during the FISH procedure

Table 2.2.1. shows the significant cell loss during the performance of the FISH procedure which is described above, in which centrifugation and re-suspension is used during the washing steps. A total of 65 % of the initial cell concentration is lost this way. These experiments were done with relatively large *E. coli* cells which are easy to centrifuge, and it may well be expected that the recovery of small natural bacteria would be even lower.

<table>
<thead>
<tr>
<th>procedure step</th>
<th>counts/mL by flow cytometry</th>
<th>percentage of initial concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial concentration</td>
<td>$2.04 \times 10^8 \pm 3 %$</td>
<td>100 %</td>
</tr>
<tr>
<td>after first washing step</td>
<td>$1.17 \times 10^8 \pm 2 %$</td>
<td>57 %</td>
</tr>
<tr>
<td>after first washing step</td>
<td>$7.16 \times 10^7 \pm 11 %$</td>
<td>35 %</td>
</tr>
</tbody>
</table>

2.3 Challenges of omitting the washing steps

- The fixation/permeabilisation step is typically done using paraformaldehyde (PF). However, PF causes interference with fluorescence signals. Therefore, unless a washing step is possible to remove PF from the samples, paraformaldehyde should not be used. As an alternative fixation procedure we tested UVA
irradiation, which is known to permeabilise bacterial cell walls (Berney et al., 2006).

- After hybridization, cells have to be washed in order to remove unbound FISH probes. The unbound probe causes unspecific background fluorescent signals which would render measurement impossible. In order to avoid this washing step, we have used a second stain (DAPI), coupled with a specific feature of flow cytometry - multiparameter analysis with two different lasers - to discriminate cells from the background signal. Essentially this approach allows the user to discriminate with the 1st laser between background signals and bacteria (based on DAPI staining), and with the second laser to discriminate between FISH-labelled bacteria and un-labelled bacteria in the DAPI population.

2.4 Omission of washing and resuspension after incubation with FISH-probes

The approach of using multiparameter flow cytometry with two different lasers to account for the problem of background fluorescence of unbound probes was successful. After incubation with the FISH probes *E. coli* samples were directly stained with DAPI and analysed on a flow cytometer with double laser setup (UV Laser (355nm) and Blue Laser (488nm)). In Figure 1 three different treatments are compared: A. Addition of washing buffer, centrifugation and resuspension. B. Addition of washing buffer. C. No washing. The procedure was applied to an *E. coli* culture, which was treated either with EUB probes, non-EUB probes (negative control) or no probes (control). All three versions lead to a clear discrimination of EUB tagged bacteria (R2) from the background signal (R1). If no washing is applied (C) the unspecific staining (non-EUB) is more prominent but still well distinguishable from the background. Hence, omission of washing can already reduce time and consumables needed for this step.

However, note that for these experiments we have used *E. coli* pre-cultured under optimal conditions. The result was large cells which gave strong fluorescent signals with both DAPI and the FISH probes. With drinking water bacteria which grow in an oligotrophic environment, one can expect significantly smaller cells with weaker fluorescent signals, which might complicate the measurements. Also, the use of dual lasers is not common for every flow cytometer, and thus adds additional costs to the instrumentation needed.
Figure 1: Influence of washing on the performance of the FISH-method on an E. coli culture. Before analysis the samples were stained with DAPI and measured with a dual laser setting (UV laser for DAPI, Blue laser for FITC). A: Washing and resuspension, B: addition of washing buffer, C: direct measurement without washing. On all FCM dot-plots, the x-axis represent DAPI fluorescence intensity, and the y-axis represent FITC (FISH probes) fluorescence intensity. Each dot indicates one particle which was detected based on its DAPI fluorescence, and the corresponding FITC fluorescence of this particle.
2.5 Alternative fixation procedures

This step is much more difficult to replace or omit. Bacteria have to be fixed and permeabilized before they can be tagged with FISH-probes and the use of paraformaldehyde has proven most efficient in this respect. This compound, though, has to be washed of the samples after permeabilization because it provokes clustering of cells and it interferes with the fluorescence signals during FCM. We tried several strategies to fix and permeabilize the cells (e.g. using UVA irradiation to permeabilize the membrane lowering paraformaldehyde concentration or) but none of these strategies have proved successful.

2.6 Performance of the FISH protocol on a filter followed by resuspension for FCM analysis

As an alternative to avoid centrifugation steps during the FISH protocol, the performance was done using E. coli cells fixed on a filter as previously described (Pernthaler et al., 2002). Different resuspension procedures have been applied in order to resuspend FISH labelled bacteria for FCM analysis.

Table 2.6.1. Resuspension procedures after FISH labelling of E. coli cells fixed onto a filter.

<table>
<thead>
<tr>
<th>treatment</th>
<th>cell recovery in percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Cut filter into pieces, put into 1 mL 1 x PBS, 5 min vortexing</td>
<td>26.8 % ± 5 %</td>
</tr>
<tr>
<td>II. Give filter in sterile Evian containing 1 % lysis buffer, 10 min vortexing</td>
<td>18.7 % ± 6.75 %</td>
</tr>
<tr>
<td>III. Put filter in 1 mL 1 x PBS, sonication for 1 min , 10 min vortexing</td>
<td>cells broke</td>
</tr>
</tbody>
</table>

No resuspension step was successful in order to enable quantitative analysis.
3 General discussion

It has been concluded previously that the combination of FISH and flow cytometry is a viable option for qualitative analysis of population structures in drinking water (Baudant et al., 2002; Wallner et al., 1993; Rompre et al., 2002; Fuchs et al., 2001; Tang et al., 2005). However, the washing steps which are used in standard FISH protocols results in inevitable loss of bacterial cells. Unless the FISH-FCM method can be developed to an extend where it is truly quantitative, it holds little value over the conventional FISH-microscopy method on filtered samples, where quantitative analysis is possible.

The use of a dual laser approach with FCM was a significant step to avoid the second washing step in the FISH protocol (removal of unbound probes). Through this approach, background problems caused by remaining probes in the sample could be avoided completely. Nevertheless, for quantitative analysis the permeabilization step so far poses an insurmountable hurdle. Hence, the well-established FISH method based on epi-fluorescence microscopy will prevail as the best option for quantitative assessment of bacterial population distributions in drinking water. Recent advances in laser-scanning microscopy improved this approach to make it faster and more convenient by automation of counting. This approach is now called solid phase cytometry (Lisle et al., 2004; Mignon-Godefroy et al., 1997).

The aim of this work package is the development of methods for drinking water analysis, which can be applied by drinking water facilities in order to facilitate the monitoring of water quality. In our opinion the approach of using flow cytometry for the enumeration of FISH labelled cells is not practical at this stage.
4 References


