Rapid and specific quantification of indicator bacteria in biofilms and water concentrates

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Author(s)
Simona Larsson, Linda Mezule and Talis Juhna, RTU

Quality Assurance
Beate Hambsch, TZW

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Summary

Escherichia coli is still used as the principle indicator for drinking water pollution monitoring, however, despite the thorough monitoring the proportion of waterborne disease outbreaks has been increasing over the years. Since it has been established that the probability of pollution detection according to monitoring program of Water Directive is rather low it may be concluded that the traditional methodology for water sampling and analysis is not always able to ensure public safety. The sampling strategy is limited to sampling the water only, whereas most of the bacteria are attached to the inner surfaces of the pipes forming the biofilm and the fate of these (ingress, virulence, detachment) is not clear. A method, DVC-FISH was developed whereby the cells are incubated with an antibiotic preventing the cell division but not growth, then concentrated on filter membranes and hybridized with a fluorescent probe. After this procedure the cells are viewed and quantified under the epifluorescence microscope. For this an accurate quantification procedure was also developed.

This report summarizes the efforts and results obtained using DVC-FISH method, indicates some potential problems and advantages. It also provides some recommendations for the use of the method.

1 Introduction

Escherichia coli is still used as the principle indicator for drinking water pollution monitoring, since enterotoxic and enterohemorrhagic forms are one of the major causes of water-related outbreaks (Percival *et al.* 2000). The proportion of waterborne disease outbreaks associated with the distribution system failures has been increasing over the years (Moe and Rheingans 2006), meanwhile the probability of pollution detection according to monitoring program of Water Directive is rather low (van Lieverloo *et al.* 2007).

Thus the traditional methodology for water sampling and analysis is not always able to ensure public safety. The sampling strategy is limited to sampling the water only, whereas most of the bacteria are attached to the inner surfaces of the pipes forming the biofilm and the fate of these (ingress, virulence, detachment) is not clear. Bacteria in the environment, including drinking water network, are exposed to different stresses through which they may become uncultivable using the common media for these bacteria (Kjelleberg *et al.* 1993) thus the use of the traditional methods may be not the one safeguarding the population. The attachment of organisms to surfaces has been shown to alter their physiology (Backer 1984; LeChevallier *et al.* 1996; Gilbert and Brown 1995; Keevil *et al.* 1990; LeChevallier *et al.* 1988; Saby *et al.* 2005). In addition, recent research shows that *E. coli* may grow in the conditions which persist in the drinking water distribution systems (Vital *et al.* 2008).

In order to bring more information on these questions a number of studies have been undertaken by RTU (e.g. D.3.5.3, D.3.6.8.1, D.3.6.8.2, D. 3.6.8.3 and D. 3.6.8.4, all of them found on website www.techneau.org).

A method, DVC-FISH was developed whereby the cells are incubated with an antibiotic preventing the cell division but not growth, then concentrated on filter membranes and hybridized with a fluorescent probe. After this procedure the cells are viewed and quantified under the epifluorescence microscope. For this an accurate quantification procedure was also developed.

This report summarizes the efforts and results obtained using DVC-FISH method, indicates some potential problems and advantages.

2 Analysis protocol

2.1 Biofilm analyses

The collectors made of stainless steel are placed in the drinking water distribution system for a period of 2 weeks. After that they are removed and processed in the laboratory by sonication in appropriate volume of sterile distilled water. The coupon must be covered in the water thus the volume depends on the size of the coupon and the sonication vessel. Usually the volume is between 16 to 40 ml. The liquid is processed as described below (see DVC-FISH section).

Sample collection and treatment

1. Remove coupon from coupon collector and place in a sterile vessel. Add 16 - 40 ml of sterile distilled water (depending on coupon type). Repeat this for additional 2 coupons.
2. Sonicate both surfaces of each coupon for 2 min at 20 µA and 22 KHz.
3. For further analysis the suspension obtained is used.

The biofilm sonicates are further subjected to microbiological analyses and quantification (see 2.3 and 2.4).

2.2 Ultrafiltration method for concentration of large volumes of water

The method was developed within the TECHNEAU project and is described in publicly available deliverable D. 3.2.4 by KWR Watercycle Research Institute (formerly KIWA). The water concentrates are further subjected to microbiological analyses and quantification (see 2.3 and 2.4).

2.3 Microbiological analyses

2.3.1 Total bacterial number

1. Take 0.001 – 0.01 ml of sonicated sample and filter on 25-mm-diameter 0.2-µm-pore-size filters (Anodisc; Whatman plc).
2. After filtration fix the sample with 3-4% formaldehyde for 15-20 minutes without removing the filter from filtration device.
3. Then wash the filter with sterile distilled water (on filtration device).
4. Stain the sample with 10 µg/ml DAPI (4',6-diamidino-2-phenylindole, Merck) for 10 minutes.
5. Wash the filter with sterile distilled water, remove the filter from filtration device and air-dry.
6. Count at least 20 fields of view for each sample.
7. Express the result as number of cells per cm^2 of coupon surface ((average cell number per volume * fields of view on filter * total sonicated volume)/area of coupon)

2.3.2 DVC-FISH

1. Put 1 ml of suspension in a sterile Eppendorf tube and centrifugate for 2 min at 6,000 rpm (2500g).
2. Carefully remove the supernatant and resuspend the pellet in 1 ml of Tryptone Soya broth and 10 µg/ml Nalidixic acid mix.
3. Incubate the samples in the dark for 6 h at 30°C.

4. After incubation wash the samples 5 times by centrifugation at 6000 rpm (2500g) for 2 minutes. Each time resuspend the pellet in sterile distilled water. Final resuspension should yield 1 ml of sample. Proceed with FISH by filtering 1 ml of the sample through the filter (see below). If water concentrate/sonicated biofilm is used for FISH directly, start with the step No 5.
5. Filter 1 ml of sample on 25-mm-diameter 0,2-µm-pore-size filters and add 3-4% formaldehyde solution.
6. Fix the samples on filtration device for 15 minutes, then wash the filter by filtering large volume (approximately 100 ml) of sterile distilled water.
7. Remove the filter from the filtration device, put on clean glass slide and air-dry.
8. Put 20 – 30 µl of PNA hybridization mix consisting of hybridization buffer and 200 nM of fluorescently labeled ECOLIFILM PNA probe. Cover the filter with cover slide and place into a humidified vessel.
9. Incubate the samples in the dark for 60 minutes at 57°C.
10. After incubation place the filters back on filtration device and wash by filtering through large volume (approximately 100 ml) of sterile distilled water.
11. Apply 10 µg/ml DAPI and stain for 10 minutes.
12. Wash with plenty of sterile distilled water.
13. Remove the filter and air-dry.

14. Visualize the samples by epifluorescence microscopy. For detection of *E. coli* with ECOLIFILM probe use a narrow range Y3 filter (Ex: 545 ± 30; Em. 610 ± 75, dichromatic mirror 565 nm).
15. Count 1/5 of the filter area according to the section below.
16. Calculate number of cells in filtered volume (average cells per field of view * 5).
17. Express the result as number of positive events on 1 cm^2 of coupon surface (number of cells in x ml used for sonication/ area of coupon surface).

2.4 Enumeration of DVC positive cells

Samples were visualized with epifluorescence microscope. For detection of *E. coli* with ECOLIFILM probe, a narrow range Y3 filter (Ex: 340/380 nm;. Em: >425 nm, dichromatic mirror 565 nm) was used. To obtain a detection limit of minimum 5 events per filter a manual scanning technique was developed.

1. First the diameter of the microscope field is estimated.
2. Then number of sectors to be scanned is estimated by the formula:

$N = F` / f$

Where, N – number of sectors to be scanned; F` – $Diameter_{filter}/5$; f – $diameter_{field\ of\ view}$

3. The filter is placed under the microscope and the upper left corner of the filter is regulated in the microscope field of view.
4. By thorough viewing the sample is visualized from the upper part to the bottom and number of positive events is recorded.

5. When the bottom part of the filter is seen in the eyepiece the stage is moved so to reach the next sector. This is performed with the help of microscope ruler. The distance is calculated by the formula:

$D = F/N$

Where D – distance; N – number of sectors to be scanned; F – $Diameter_{filter}$.

6. The next sector is scanned from the bottom to the upper part without loosing the sample view. All positive events are recorded. This is repeated for all of the remaining sectors.
7. Total number of events recorded is as:

Sum of events recorded * 5

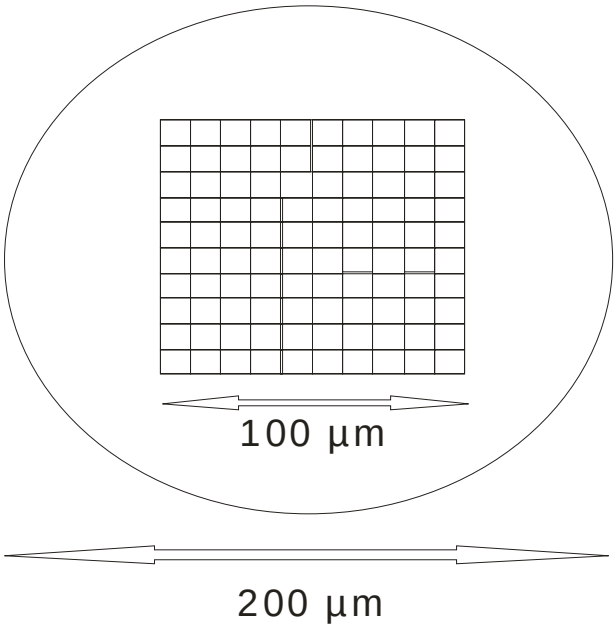

Figure 1. Diameter of the sample counting grid (100 µm) and total diameter of the microscope field of view (200 µm). The circle represents one total field of view on the microscope.

Sample:

1. From microscope data f = 200 µm = 0.02 cm (Fig. 1)
2. N = (2 cm/5)/0.02 cm = 20

5. D = 2 cm / 20 = 0.1 cm (Fig. 2)

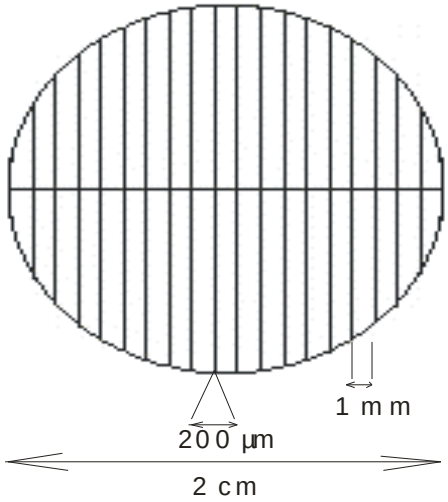

Figure 2. Main parameters for the counting: filter diameter (2 cm), sector width (200 µm) and sector distance (1 mm). The circle represents the filter.

Considering that (i) the recovery of the Hemoflow was about 80% (see TECHNEAU D. 3.2.4 by KWR) and (ii) that the recovery of the applied FISH-DVC method is 80% (see D 3.5.3 by RTU), it is possible to analyze about 60% of the *E. coli* cells present. Furthermore, if 1 cell is found by scanning 1/5th of the filter, through which 1 ml of the sample was filtered thus making the minimum detectable amount 5 cells/ml the overall limit of the detection is 3 cells/ml. Depending on the degree of concentration this comes to 0.008 cells/ml with the lowest degree of concentration used in this study (364) down to $5*10^{-4}$ cells/ml with the highest degree of concentration (5496).

Regarding the detection limit in the biofilm, considering that 80% of the cells are retained after DVC and that the coupon having a surface area of 1.77 or 7.0685 cm^2 was sonicated in 16 - 40 ml of water, and the minimum detectable cell number (scanning 1/5 of a 2 cm^2 filter through which 1 ml of the sonicate has been filtered) again is 5 cells/ml, the overall detection limit of the FISH-DVC method is from 1.105 to 8.83 cells/cm^2.

3 Results and Discussion

3.1 Overview

As indicated in the introduction the opinion that traditional microbiology methods are not able to guarantee the safety of the population has appeared in the press. With interest in rapid microbiological methods raising a variety of new technologies available for the use in quality control could move the traditional microbiology laboratory into the 21st century. Many of the rapid methods are dissimilar to the current methods included in the pharmacopoeia (PCR, flow cytometry, viable stains etc.)

There is a need to separate between the methods which rely on the traditional approach but are automated and the alternative methods since they require two separate validation approaches (Jones 2006). These alternative tests use different principles of measurement and enumerate different targets (as opposed to colonies), such as bioluminescence or, as in this case, fluorescent events.

During the TECHNEAU project the DVC-FISH method was developed, compared to several viability stains (CTC, esterase activity and LIVE/DEAD). Samples, both biofilm and water concentrates were tested, more that 100 of biofilm and about 50 of the water concentrates during the project, both in the experimental stage and in the case study context. The results and comparisons refer only to Riga distribution network and intralaboratory comparison of the different methods. All methods have been performed by several people each and 3 repeats per sample were always performed.

3.2 Comparison with culturable bacteria

Grab sampling, sonicated biofilm and water concentrates

The number of the samples from water distribution network taken failing to meet the Directive (0/100 ml) regarding *E. coli* was none. No CFU forming *E. coli* was found in the drinking water biofilm as well.

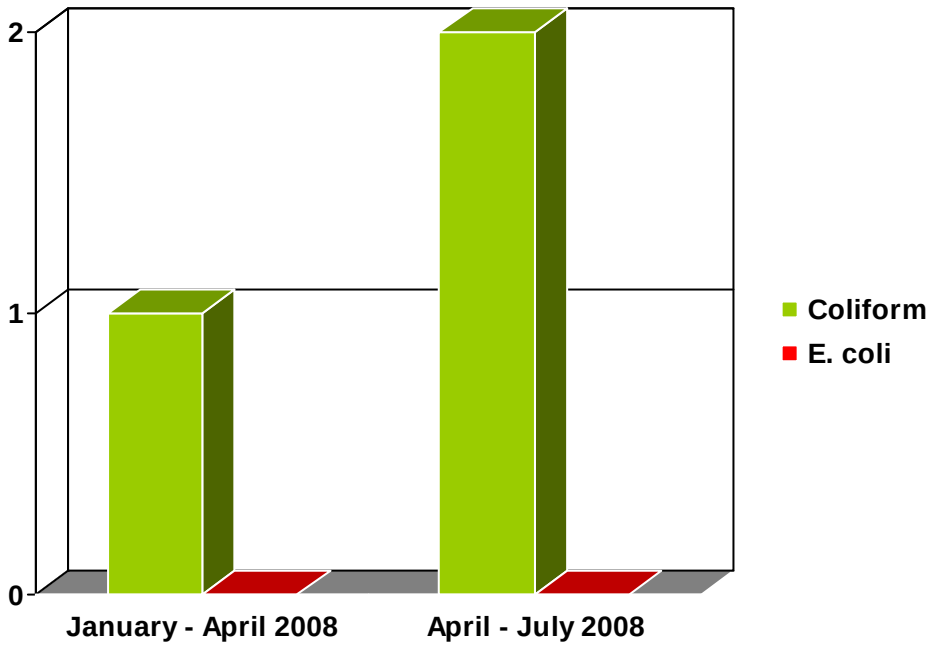

Figure 3. Number of samples from water distribution failing to meat the Directive (0/100 ml). Quarter average values (CFU bacteria/100 ml, LVS EN ISO 9308-1: 2001) are shown (courtesy of Riga Waterworks). In total around 50 samples were analyzed using the standard membrane filtration method**.**

The picture was slightly different when the water concentrates were analyzed using the MPN method since in some cases a minor amount of *E. coli* was detected, however still corresponding to the Directive.

Table 1. The results from a campaign sampling using the ultrafiltration method (Hemoflow device, see D. 3.2.4 by KWR) in May-June 2009. The raw water is not shown.

Site	TBN	Cultivable coliforms/ 100 ml	Cultivable *E. coli*/ 100 ml	DVC-positive *E. coli*/ 100 ml
G-DW 1	**4,57E+03**	**0,0000**	**0,0000**	**0, 10 ± 0, 2**
G-DW 2	5,66E+04	0,0000	0,0000	0, 00
S-DW	9,77E+03	0,0000	0,0000	0, 00
G-Net 2	1,30E+06	**0,0196**	**0,0000**	**1,08 ± 0.2**
G-Net 1	2,16E+05	**0,0064**	**0,0064**	**0,09 ± 0.1**
G-Net 2	1,27E+05	**0,0169**	**0,0127**	**0,93 ± 0.4**
G-Net 1	3,46E+04	**0,0000**	**0,0000**	**0,09 ± 0.2**

Thus, concentration helps to reveal a potential hazard as opposed to the grab sampling.

The FISH-DVC method, however, resulted in about 100 times more of viable *E. coli* indication still *E. coli* in levels still corresponded to the Directive. It could be concluded that the method is more sensitive than the traditional methods. It has been also noted before that the cultivation-independent detection methods detect at least 10 times more cells (Bjergbaek and Roslev 2005). Perhaps it should be mentioned that the probe has been tested against the common drinking water bacteria and no cross-hybridization has occurred (see previous Deliverables by RTU, mentioned above) thus the probe may be regarded as specific. It has been noted before that the main drawback of the classical, growth based viability assays is the possibility of false negative results; false positives can be excluded by correct sterile technique (Davey *et al.* 2004). It has also been shown that DVC-FISH method

gave systematically higher *E. coli* counts than a reference culture-based method (MPN method). Furthermore, the ratio between both counts (DVC-FISH/MPN) increased with decreasing abundance of culturable *E. coli* indicating that the proportion of non-culturable *E. coli* (detectable by the DVC-FISH procedure and not by a culture-based method) was higher in low contaminated environments (Garcia-Armisen and Servais 2004).

3.3 Comparison with other viability methods

The DVC-FISH method has been compared to other viability methods such as the detection of esterase activity, the activity of the electron transport chain (ETC) and the membrane permeability assessments. While each of these methods may be useful by themselves, it is difficult to combine them with the fluorescent identification of *E. coli*. Figure 4 shows an example of ETC activity determination for water bacteria. The assay is very rapid and easy to perform; however, no identification possibilities using a single test with the developed probe are available.

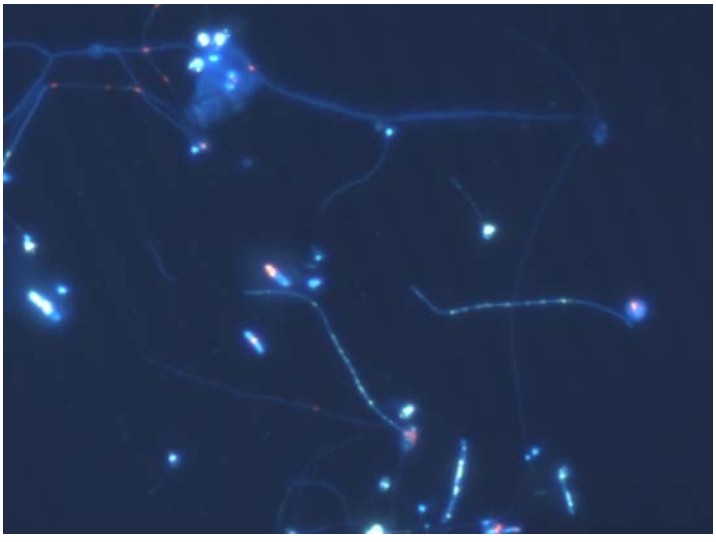

Figure 4. CTC analyses of a sample from a coupon, inserted in water distribution network.

There are differences in the protocol or interaction with the dye which currently gives the best signal (CY 3). It has also been reported that the esterase activity, which may be very useful in different context, do not work with *E. coli* (Hoefel *et al.* 2003), as we have shown as well, for the epifluorescence microscopy. More details on this can be found in D.3.5.3. www.techneau.org.

The LIVE/DEAD approach is disadvantageous technically for the epifluorescence microscopy since the sample drying affects the membrane permeability and when many samples are processed it is difficult to ensure that some of them do not dry out thus providing false negative results (dead cells). It, however, may again be the method of choice for the other kind of approaches.

Examination of field samples from real distribution networks can be very challenging, because such samples can contain a lot of impurities of different origin that can complicate the discrimination of the target cells. The use of the DVC method prior to FISH dramatically improves the detection of *E. coli* cells. In sonicated samples, metabolically active cells were elongated, allowing much better discrimination of the cells, in particular in biofilm samples from cast iron material, which contained many impurities. This could be due to poor penetration of the biofilm by nutrient and antibiotic mixture. The method improved the discrimination of the target cells from the fluorescent particles of different origins and significantly facilitated the enumeration of FISH-positive bacteria (Fig. 4). However when DVC was applied to the undisturbed biofilms (whole coupons, data not shown) only a slight increase in cell length and volume was observed, which did not give enough statistical credibility for the use of DVC method for routine examination of viable *E. coli* cells in undisturbed biofilms. We found that sonication

of the surfaces is necessary. This is one of the drawbacks of the method. Another drawback is the "rapidness" since the elongation still requires 6 hours. The results, however can be obtained in one working day, which is considerably faster than using the conventional methods. Again, the sensitivity issue can be brought up in this connection.

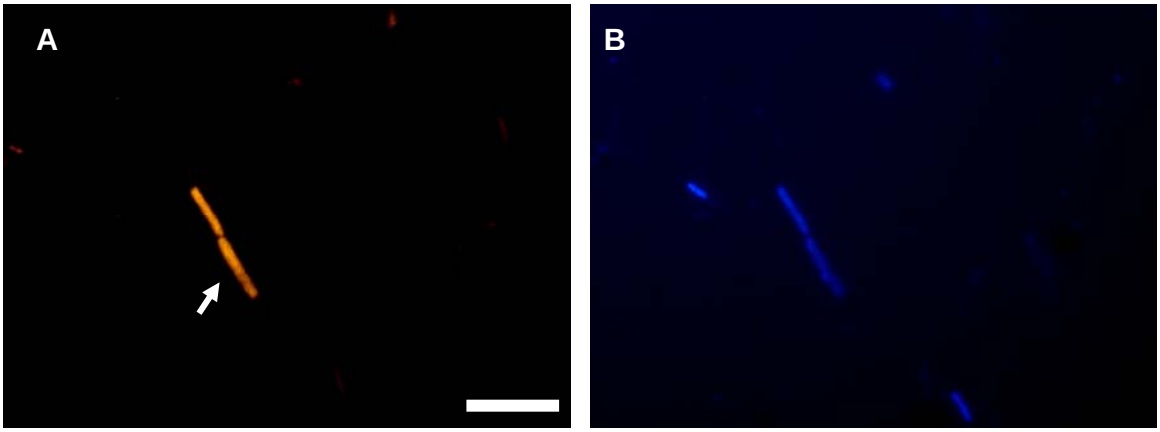

Figure 5. *E. coli* in biofilm sample from distribution networks supplied with groundwater (G-Net 6). Sonication was used prior to DVC-FISH. Elongation was observed suggesting that bacteria are viable (able to divide). A) *E. coli* with FISH probe B) total bacterial number with DAPI. Bar 10 µm.

The traditional method for enumeration is the appearance of the colonies on agar medium where the CFU may arise from one cell or a clump of cells (Janssen *et al.* 2002) even as much as 1000 (Wilson *et al.* 2004). Moreover, it is visible to the naked eye only after sufficient time has passed to generate several millions of cells. This is the fundamental difference of the rapid methods – they are not based on CFU but on different criteria. Thus it is apparent that the same validation principles may not be applicable.

A major concern with acceptance of alternate microbiological methods is uncertainty over validation (Jones 2006). Weighing the gains which could be obtained by faster and more sensitive processing of water samples *vs* traditional approach it is worth considering a clear set of standards for validation of these, non-traditional, emerging methods.

4 Conclusions

This report presents a new rapid method, FISH-DVC, which has been successfully tested in the field conditions, during the case study in Riga. The method is more sensitive than the traditional methods and can be performed within one working day. It has several advantages over other viability tests, such as the ability to identify the fecal indicator and its viability at the same time. Since the validation standards of the rapid methods are not completely clear yet, the method was tested within the laboratory (by different individuals) and compared against several other viability stains as well as the traditional membrane filtration method.

Despite the time needed for the concentration the ultrafiltration method followed by FISH-DVC is applicable for analysis of drinking water whereas the biofilm may be analyzed after 2 weeks of residence time.

Presence of even low levels of *E. coli* in biofilm compromise water quality (e.g. analyses and health), thus more attention to on-line monitoring is needed.

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