Applicability of biofilm sampling for detection of pathogens in drinking water distribution networks

Data from coupons and concentration methods

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

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Summary

This report describes evaluation of two sampling strategies for microorganisms, namely the concentration of large volumes of water and sampling of biofilm using coupons. The results were compared to those from the grab sampling technique.

Bacteria in the environment are exposed to different stresses through which they may become unculturable using the common media for these bacteria. Such bacteria, often called VBNC (viable but not culturable) or ABNC (active but not culturable) have developed recalcitrance to culture. Thus molecular methods may reveal the bacterial concentration more precisely, compared to the traditional methods. It is well known that biofilm harbors bacteria thus it is important to include biofilm in the sampling as well.

Previous studies in TECHNEAU project involved the development of a Hemoflow filter for concentration of large volumes of water (see D.3.2.4. by KWRWater), which was used in this study. In addition, biofilm collecting technique, described earlier (see e.g. D.3.6.8.2. by RTU) was used. The grab sampling data were obtained from the city A water supplier and the State Health Agency.

Using the grab sampling and culture-based detection methods virtually no *E. coli* was found in the routine monitoring samples and in the samples collected in this study. However, both large volume concentration and biofilm analyses using DVC-FISH method revealed the presence of viable *E. coli* in the drinking water.

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1 Introduction

If properly applied, current protocols in municipal water treatment should be effective at eliminating pathogens from water. However, inadequate, interrupted, or intermittent treatment has repeatedly been associated with waterborne disease outbreaks (Reynolds et al. 2008).
The proportion of waterborne disease outbreaks associated with the distribution system failures has been increasing over the years (Moe and Rheingans 2006). A total of 86 waterborne outbreaks have been reported during 1990-2004 in 10 out of 25 EU countries (Risebro et al. 2006). Both surface and groundwater supplies were implicated in these outbreaks, however groundwater supply outbreaks reported almost by half more cases of illness compared to the surface water supplies. The predominant agents were *Cryptosporidium*, *Campylobacter*, Norovirus, *Giardia* (Risebro et al. 2006) and *E. coli* O157 (Mannix et al. 2007). The common causes of outbreaks were surface runoff in groundwater, which is only chlorinated and cross-connections. Maintenance work and negative pressure has also been implicated to aid pathogen intrusion in the drinking water supply system (Nygard et al. 2007). In US documented waterborne disease outbreaks are primarily the result of technological failures or failure to treat the water (Craun et al. 2006).
Most water supplies monitor their drinking water for the absence of indicator organisms in a small volume of water (100-500 ml). However, the probability of detection fecal contamination according to monitoring program of Water Directive is rather low (van Lieverloo et al. 2007). The absence of an indicator in 100 ml does not guarantee the safety of the drinking water due to the high detection limit. This is illustrated by the fact that some of the outbreaks have occured through water which met the *E. coli* standard of absence in 100 ml (Anderson and Bohan, 2001). Methods of large volume sampling have become available in recent years (Hijnen et al. 2000, Smeets et al. 2007) and it has been shown that increase of the sample volume to 100 liters lowers the detection limit to 0.01 CFU/L or less (Hijnen et al. 2000).
Hence, traditional methodology for water sampling and analysis is not always able to ensure public safety regarding both (i) the strategy of sampling and (ii) the choice of the detection method. Not only the sampling strategy is limited to sampling a relatively small amount of water, but actually most of the bacteria are attached to the inner surfaces of the pipes forming the biofilm. The phenomenon of biofilm formation, or the attachment of microorganisms to the inner surfaces of the drinking water distribution system, has been well documented (see reviews in references Keevil 2002; O'Toole et al. 2000; Parsek and Singh 2003). The attachment of organisms to surfaces has been shown to alter their physiology. Attached organisms were found to be generally more active in absorbing nutrients, as well as more resistant to environmental stress such as starvation, heavy metals, and chlorine (Backer 1984; LeChevallier et al. 1996). It has also been shown that bacteria attached to surfaces show greater resistance to disinfection (Gilbert and Brown 1995; Keevil et al. 1990; LeChevallier et al. 1988; Saby et al. 2005). Biofilms in distribution systems may provide a favorable condition for some bacteria, such as opportunistic pathogens (e.g., *Legionella* spp., *Pseudomonas aeruginosa*, and *Mycobacterium avium*), to colonize it and may harbor pathogens, such as *Salmonella*

enterica serovar *Typhimurium*, which have entered the distribution system (Armon et al. 1997; Berry et al. 2006; Keevil 2002; Parsek and Singh 2003).
In addition the absolute majority of cells in the drinking water are not culturable at all (even though they may be viable) meaning that they will not grow in the culture media (Colwell and Grimes, 2000). The cells which are otherwise culturable (e.g. *E. coli*) under certain conditions may enter a state of unculturability and these are called VBNC (viable but not culturable) (Oliver, 2005) or ABNC (active but not culturable) cells. The latter term is often preferred (Kell et al. 1998).
Bacteria in the environment are exposed to different stresses through which they may become unculturable using the common media for these bacteria. Such bacteria have acquired stress resistance by active mechanisms, which, in turn are genetically programmed but have developed recalcitrance to culture (Kjelleberg et al. 1993). The formation of VBNC or ABNC cells has been proposed as a survival strategy as a response to mild environmental stress, such as nutrient deprivation/starvation (Ganesan et al. 2007; Yamamoto et al. 1996) temperature, notably cold (Weichart et al. 1997), osmotic shock (Xu et al. 1982, Asakura et al. 2005) sunlight radiation (Besnard et al. 2002; Pommepuy et al. 1996), low pH (Chaveerach et al. 2003) and presence of certain metals (Grey and Steck 2001). Such VBNC bacteria may retain their virulence (Baffone et al. 2003).

It has been shown in lab-scale experiments that *E. coli* can survive in biofilters (Li et al. 2006) and even multiply in the biofilm (Camper et al. 1991; Fass et al. 1996; Keevil 2001; Robinson et al. 1995; Williams and Braun-Howland 2003). *E. coli* may survive and even exibit metabolic activity in distribution networks. During UV–low chlorine disinfection, *E. coli* was found to persist at low levels, suggesting that the UV treatment had caused an adaptive mutation. During UV–chlorine-dioxide treatment, the *E. coli* that was initially below the detection limit reappeared during a low level of disinfection (0.2 mg/L) in the cast iron systems (Murphy et al. 2008). Moreover, although *E. coli* is often detected in the drinking water, the source of the contamination is not clear. Since culture methods will most likely, not detect all active bacteria, alternatives must be sought. The advantages of such alternative activity measurements are not only detection of non-culturable organisms but also their rapidity as no lengthy incubation is needed. FISH (fluorescence in situ hybridization) was selected as the method of choice since this method is quick for identification on the species level which can be made within a couple of hours and cheap enough to be used on a routine basis. The method can be used for the detection of ABNC cells and it is possible to combine the FISH method with some viability or activity assays, such as CTC (5-cyano-2,3-ditolyl tetrazolium chloride) and/or DVC (direct viable count). PNA probes are preferred since these have been found useful when investigating drinking water biofilms due to their ability to penetrate even the thick biofilm layers (Wilks and Keevil 2006).

The aim of this study was to analyze *E. coli* in the drinking water using a method for concentration of large volumes by ultrafiltration (further referred to as Hemoflow) and biofilm sampling. The results were compared to those of grab sampling of 100 ml of drinking water. In addition, the analysis was performed using the traditional culture method for coliforms and *E. coli*, performed both *in situ* and in a certified laboratory and FISH, including the viability indicating DVC test.

The sampling sites were source water, drinking water treatment plants and drinking water distribution network in the city A. A river flows through the city and the left bank is supplied with surface water from the river (uptake located about 20 km upstream) and the right bank is supplied mostly by groundwater. The surface water is treated by a multi-step treatment train (coagulation, filtration, ozonation, final chlorination) while the groundwater is only chlorinated. The sites S-DW 1 and 2 were within the surface water treatment station (after biofilters) and finished water, S-Net 3 and 4 are two locations in the network, supplied by surface water, G-DW 5 was located in the groundwater treatment plant after chlorination while the groundwater is only chlorinated. The sites G-Net 6 and 7 were supplied with the groundwater. S-Net 4 and G-Net 6 were located closer to their corresponding treatment plants, compared to S-Net 3 and G-Net 7, respectively.

2 Materials and methods

2.1 Ultrafiltration method

The method was developed within the TECHNEAU project and is described in publicly available deliverable D. 3.2.4. The findings of the study (D.3.2.4.) were:

− The concentration of maximum volumes of 2000 litres produces high recovery rates (> 65%) for all organisms except *Campylobacter*,
− The results of the several experiments have a standard deviation between 7-33%,
− The efficiency of the detection of *Cryptosporidium* and *Giardia* is much higher and more reproducible than with the existing Envirochek concentration method,
− Concentrates obtained with the Hemoflow-installation can be postconcentrated without a significant reduction of the recovery rate,
− Post-concentration of phages must take place by centrifugation with Centricon®,
− Post-concentration of bacteria must take place by centrifugation (5-10 min, 900 g) with complete examination of the pellet,
− For the assessment of F-specific and somatic phages, the examinable volume has been increased from 10 ml to 2000 liters,
− The Hemoflow concentration method makes it possible to simultaneously concentrate the organisms that are to be examined.

The recovery data is summarized in Table 1.

Table 1: Mean recovery rate (in % ± SD) for various micro-organisms in different water types, determined directly in the Hemoflow-concentrate. Figures in brackets show the numbers of experiments.

Organism	Drinking water (20-1146 l)	Water from purification plant (600 l)	Surface water (50 l)
E.coli	81 ± 33 (3)	93 ± 8 (3)	93 ± 22 (3)
Enterococci	82 ± 25 (3)	74 ± 23 (3)	81 ± 9 (3)
Clostridium	90 ± 24 (3)	89 ± 18 (3)	91 ± 22 (3)
F-specific phages	82 ± 25 (8)	115 ± 7 (3)	79 ± 21 (3)
Somatic phages	65 ± 28 (3)	93 ± 21 (3)	111 ± 13 (3)
Campylobacter	52 ± 9 (3)	96 ± 66 (3)	35 ± 7 (3)
Cryptosporidium	87 ± 14 (8)	101 ± 29 (3)	67 ± 29 (7)
Giardia	93 ± 14 (8)	85 ± 13 (3)	86 ± 13 (7)

2.2 Biofilm collectors

The collectors made of stainless steel (Fig. 1) were placed in the drinking water distribution system for a period of 2 weeks. After that they were removed and processed in the laboratory. The coupons were sonicated and the liquid was subjected to FISH and DVC-FISH analyses.

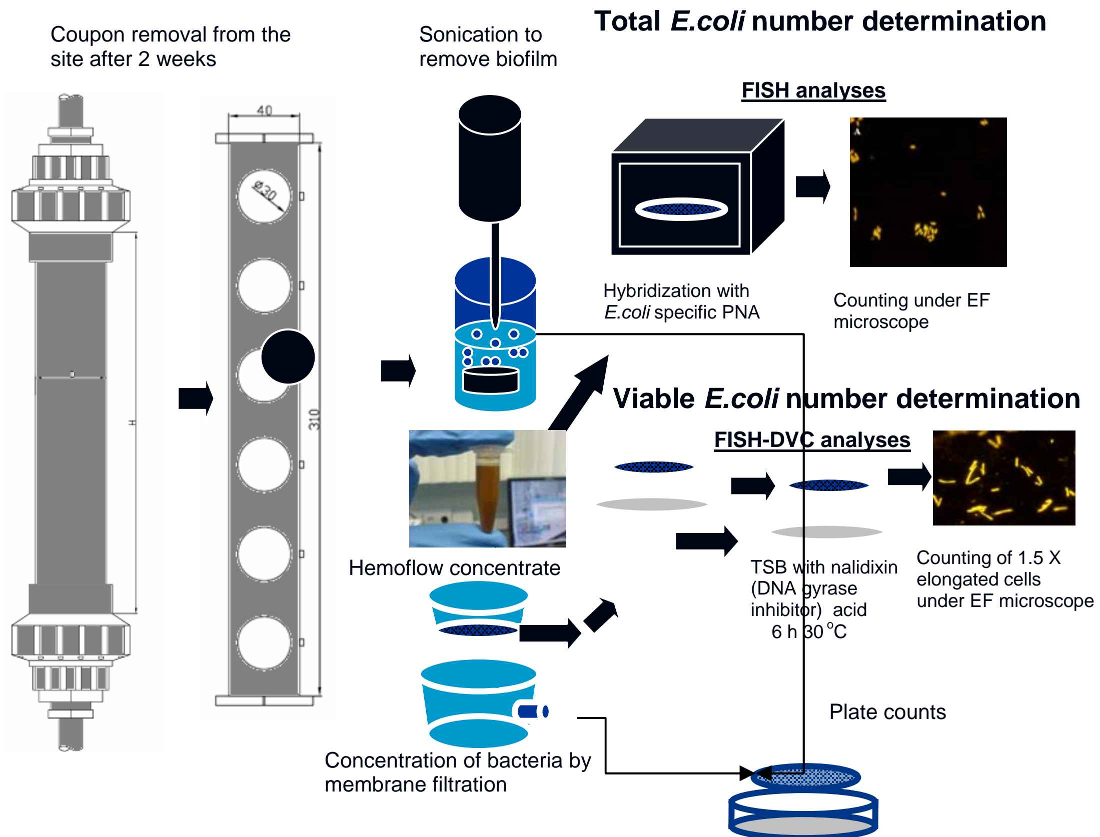

Figure 1. Biofilm collectors and the treatment of the samples for total and viable/active cell analyses.

2.3 Direct Viable Count

The method was developed by Kogure et al. (1979) and later improved by Joux and Lebaron (Joux and Lebaron 1997) and it is based on the incubation of bacteria with an antibiotic (nalidixic acid), which prevents cell division but not the biosynthesis processes. As a result, the active cells become elongated whereas inactive cells retain their appearance. Later improvements of the technique concerned the application of a cocktail of antibiotics and thus the technique became applicable for bacterial community analyses including bacteria which are resistant to nalidixic acid. According to the critical review by Kell et al. (1998) the exact mechanism of DVC is not clear, although the cell elongation is assumed to be growth potential related. The technique has gained quite a lot of popularity (Baudart et al. 2002; Kalmbach et al. 1997; Lisle et al. 1998; Pommepuy et al. 1996; Regnault et al. 2000), also together with FISH as the elongated cells build up 16S rRNA and are marked by the probe even more strongly. It has been shown before that DVC counts are ususally higher than the plate counts (Hoefel et al. 2003; Joux and Lebaron 1997) perhaps, because the bacteria which have been exposed to stress only have an ability to divide certain number of times (Button et al. 1993) and it is not sufficient to produce visible colonies.

2.4 Protocol for Biofilm Analysis

2.4.1 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.

2.4.2 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)
8. Stain the sample with 10 µg/ml DAPI (4',6-diamidino-2-phenylindole, Merck) for 10 minutes.
9. Wash the filter with sterile distilled water, remove the filter from filtration device and air-dry.
10. Count at least 20 fields of view for each sample.
11. 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)

2.5 FISH/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 positive events for 20 fields (if 350 or more cells are present) or 60 (if less than 350 cells are present) fields of view.
16. Calculate number of cells in filtered volume (average cells per field of view * number of fields of view on filter).
17. Express the result as number of positive events on cm^2 of coupon surface (number of cells in 40 ml / area of coupon surface).

2.6 Cultivation methods

Cultivable *E. coli* from both water and biofilm samples were detected by the plate count technique. The membrane filters were incubated on TBX medium (Oxoid Ltd, UK) for 24 hours at 37°C. Typical blue/green colonies were counted and results expressed as CFU per ml (water samples) or per cm^2 (biofilm samples). All samples were analyzed in triple.
The heterotrophic plate count was performed using R2A medium with incubation at 35°C for 48 - 72 hours.

2.7 Analysis of the parasitic protozoa

A commercially available kit Aqua-Glo™ G/C which is EPA - approved for use in Methods 1622 and 1623 was used according to manufacturer's instructions (Waterborne Inc, New Orleans, LA, USA).

3 Results and Discussion

3.1 Limits of detection

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), 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.

3.2 Culturable bacteria

The number of culturable coliform bacteria in the source water was relatively high in the month of January, after which it decreased, reaching the lowest level in the month of April. After that, the number of culturable coliforms increased again. Faecal coliforms and *E. coli* followed the same trend (Fig. 2)
The number of the samples from water distribution network taken either in 1st or the 2nd quarter of the year and failing to meet the Directive (0/100 ml) regarding *E. coli* was none. Regarding the total coliforms, two samples failing to meet the Directive were observed in the 2nd quarter of the year as opposed to one sample in the 1st quarter.

The data from State Health Agency indicate that there are a rather high number of drinking water samples which do not conform to standard. However, the data must be interpreted in the light of the fact that the monitoring results are not shown separately for the city A (where the samples for biofilm analyses and concentration were taken).

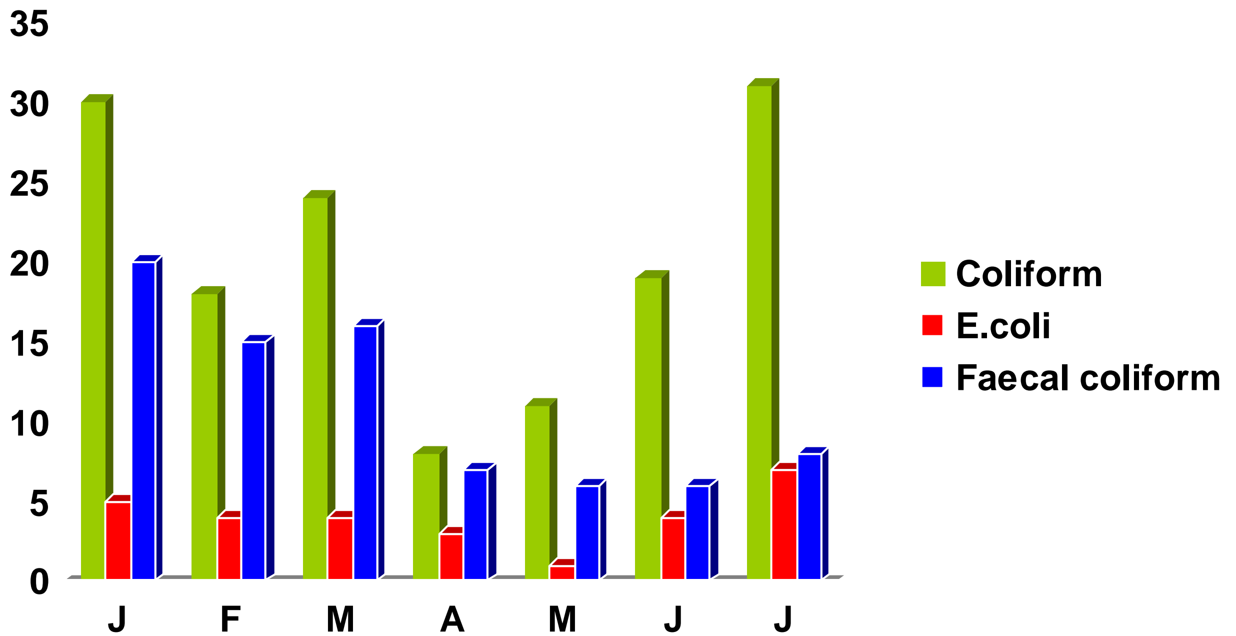

Figure 2. Results from routine sampling analyses of raw water source (courtesy of the water supplier). Month average values (CFU/100 ml) are shown. The cell number was analyzed using the membrane filtration method LVS EN ISO 9308-1: 2001.

Table 2. The results of audit monitoring (State Health Agency)

Year	Water supply systems	Microbiological parameters		Chemical parameters	
		Total	Non-conformity	Total	Non-conformity
2005	32	50	1	50	19
2006	21	39	2	39	17
2007	41	59	9	59	24

Table 3. The results of yearly monitoring (State Health Agency, SHA)

Year	Water supply systems	Microbiological parameters		Chemical parameters	
		Total	Non-conformity	Total	Non-conformity
2005	68	694	12	694	42
2006	57	680	6	680	63
2007	96	631	1	631	44

The audit monitoring (collection of a smaller amount of samples but unexpected by the water supplier) data indicate that the concentration of coliforms increased from 2005 till 2007 however, the increase is connected to the changes of territorial division of SHA where some smaller cities about 60 km from city A were audited together with the city A. The Table 3 shows the yearly monitoring results (processing of larger amount of samples taken in accordance with an agreement between SHA and the water supplier) and gives a more balanced view on the water

quality whereupon the microbiological quality is increasing. In the year 2008 there was one instance where coliforms (but no *E. coli*) were found in a water sample in the city A.

Most of the studies that have examined the presence of *E. coli* in biofilms have used culture-based methods (Wingender and Flemming 2004). These methods have limitations, including duration of incubation, antagonistic organism interference, lack of specificity, and poor detection of slow-growing or non-dividing microorganisms (Rompre et al. 2002). Plate count methods also result in some inaccuracy since the cells can be clumped together and intertwined with other biofilm components (Li et al. 2006). It must be emphasized that methods using microbial growth will be unable to detect non-dividing cells at all. Therefore, the number of *E. coli* in the drinking water distribution network could be, and likely is, underestimated. The presence of *E. coli* was inadequately indicated by the traditional culture-based methods in the our previous studies (see e.g. D.3.6.8.2.), a finding in agreement with previous findings showing that cultivation-independent detection methods detect at least 10 times more cells (Bjergbaek and Roslev 2005).

Table 4. Summary of total cell, heterotrophic bacteria and cultivable *E. coli* data from 5 independent concentration experiments performed in the same spot within the distribution network. The duration of the concentration was 10-12 hours and the amount of water concentrated was 400-600 liters. The presented results were recalculated according to the corresponding degree of concentration and shown as cells or CFU/ml in the water.

Site	**G-NET-7.1**		**G-NET-7.2**	**G-NET-7.3**	**G-NET-7.4**	**G-NET-7.5**
Time		01.12.08.	02.12.08.	03.12.08.	08.12.08.	09.12.08.
Degree of concentration		745	1291	480	453	457
Total bacteria, ml water	average	5,32E+04	3,11E+04	1,08E+04	3,09E+04	3,61E+04
	STD	5,51E+03	2,17E+03	9,25E+01	5,39E+02	2,48E+03
Heterotrophic bacteria, ml	average	1,20E+03	2,94E+02	1,13E+02	5,78E+02	8,27E+02
	STD	2,94E-01	6,54E-02	1,47E-01	3,54E-01	2,86E-01
CFU *E.coli*, ml	average	0	0,017	0	0	0
	STD	0	0	0	0	0
CFU coliforms*, ml	average	0	0,017	0,015	0,47	n.a.
CFU *E.coli**, ml	average	0	0	0	0	n.a.

*analysis performed by a certified lab

In this study a method for concentration of a large water volume (500-3000 liters) was applied. Using this ultrafiltration concentration technique a small number of cultivable *E. coli* was found in one instance within the drinking water network

(Table 4), in the raw water source and in one instance – after the biofilters in the surface drinking water treatment plant (Table 5.).
An independent commercial laboratory found coliforms (but not *E. coli*) in 3 out of 5 concentrates.

Table 5. Summary of total cell, heterotrophic bacteria and cultivable *E. coli* data from 4 independent concentration experiments performed in the same spot within the treatment train (after biofilters). The duration of the concentration was 40-90 hours and the amount of water concentrated was 1.5 – 3.8 m^3. In the raw water source only 400 liters were concentrated. The presented results were recalculated according to the corresponding degree of concentration and shown as cells/ml in the water.

Site	**Raw water source***	**After biofilters**	**After biofilters**	**After biofilters**
Time	09.05.08.	07.05.08.	16.04.08.	04.04.08.
Degree of concentration	364	2395	2422	5946
Total bacteria, ml	2,83E+06	2,55E+06	3,46E+04	1,00E+06
Heterotrophic bacteria, ml	5,16E+04	1,86E+04	7,45E+02	6,17E+03
CFU *E.coli*, ml	1,83	0	0	0,6

* 400 liters were concentrated due to the plugging of the filter.

Biofilm analysis of 72 coupons did not reveal any cultivable *E. coli* cells. The average concentration of total bacteria/cm^2 ranged from about $1.8*10^3$ till $5.5*10^7$ and the concentration of heterotrophic bacteria ranged from 17 till $1.5*10^6$ depending on the sampling site and time.
Some culturable *E. coli* cells (see Table 5.) were detected using the concentration method, but not the biofilm collectors. The highest concentration (1.83 CFU/ml) was found in the raw water source. Comparing to the routine sampling of 100 ml in this study 10 times more cells were found : 183 CFU/100 ml *vs.* of average of about 2 (see Fig. 2). In one instance *E. coli* was found in the drinking water as well (1.7 CFU/100 ml). According to the data obtained in this study the concentration of large volumes of drinking water analyzed using the cultivation of *E. coli* indicates that the drinking water meets the standard in practically all cases. This finding is similar to what has been shown previously (Hambsch et al. 2007).

3.3 FISH and DVC-FISH data

In contrast to the culture method (Table 4 and 5), the FISH method indicated the presence of viable *E. coli* in all the concentrated samples (Table 6). *E. coli* cells were found in the raw water source and in the sample with the highest degree of concentration, as it can be expected. However, the *E. coli* cells were also present and DVC positive (see Fig 3.) in the drinking water.

Table 6. Summary of *E. coli* concentration from 5 independent concentration experiments performed in the same spot within the distribution network. The duration of the concentration was 40-90 hours and the amount of water concentrated was 400-600 liters. The presented results were recalculated according to the corresponding degree of concentration and shown as cells/ml in the water.

Site		**G-NET-7.1**	**G-NET-7.2**	**G-NET-7.3**	**G-NET-7.4**	**G-NET-7.5**
Time, date (started)		01.12.08.	02.12.08.	03.12.08.	08.12.08.	09.12.08.
Degree of concentration		745	1291	480	453	457
FISH (cells/ml)	average	0,05	0,03	0,02	0,04	0,09
DVC-FISH (cells/ml)	average	0,013	0,004	0,008	0,017	0,077

E. coli was found in 90% of the 72 coupons and the concentration ranged from 1 till 65 cells/cm^2 if biofilm. The numbers found in biofilm lie in the range of what has been found previously in biofilm of *e.g.* drinking water networks in Europe (Juhna et al., 2007). Rough calculations taking in account the surface area of the city A drinking water distribution system and the amount of water, which passes through daily, indicate that the concentration of *E. coli* in biofilm is higher than in water.

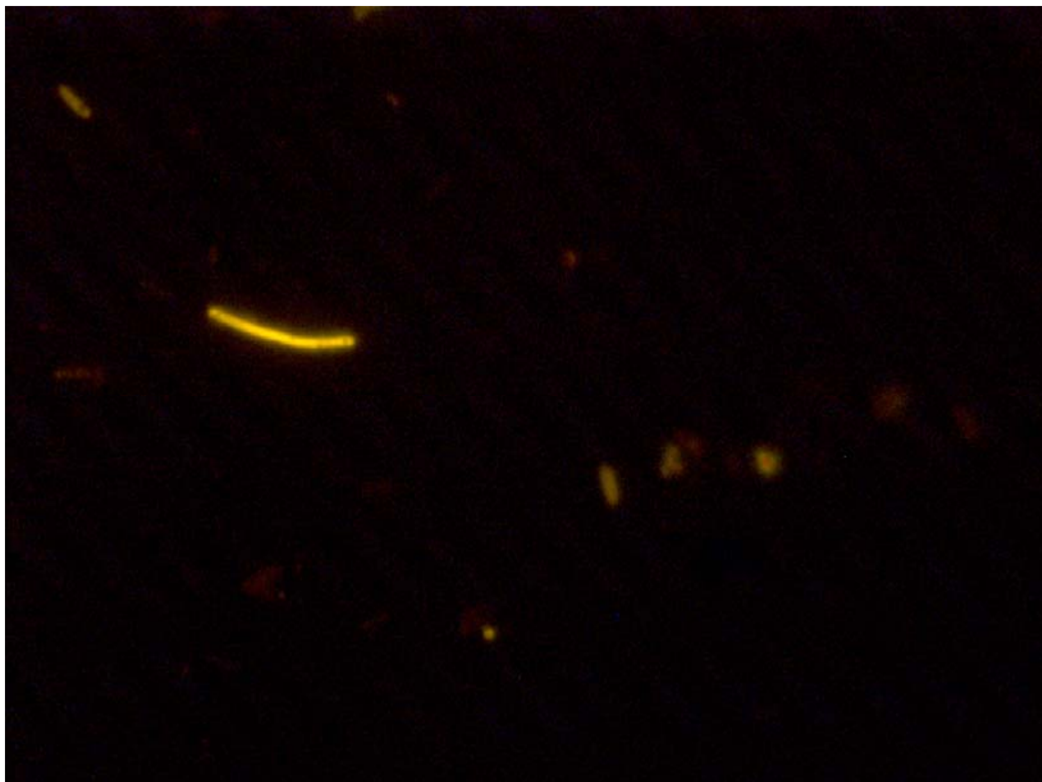

Figure 3. A DVC-FISH positive *E. coli* cell from the drinking water network.

In summary, it can be proposed that *E. coli* cells are often not detected by the grab sampling method because, routinely, only small volumes of water are analyzed, and this is done using culture or enzymatic methods which do not detect active but non-culturable bacteria. The FISH-DVC number in all the samples was lower than FISH number, which indicates that some of the cells are not viable. It must also be noted that the number of viable *E. coli* in the raw water source obtained by the culture method (1.83 CFU/ml) was very close to the number obtained after DVC analyses (1.9 cells/ml), which indicates that the DVC method might be considered as a complement to the existing methods..

Table 7. Summary of *E. coli* data from 4 independent concentration experiments performed in the same spot within the treatment train (after biofilters). The duration of the concentration was 40-90 hours and the amount of water concentrated was 1.5 – 3.8 m^3. In the raw water source only 400 liters were concentrated. The presented results were recalculated according to the corresponding degree of concentration and shown as cells/ml in the water.

Site	**Raw water source***	**After biofilters**	**After biofilters**	**After biofilters**
Time, date (started)	09.05.08.	07.05.08.	16.04.08.	04.04.08.
Degree of concentration	364	2395	2422	5946
FISH (cells/ml)	5,28	0	0	0,59
DVC-FISH (cells/ml)	1,92	0	0	0,29

* 400 liters were concentrated due to the plugging of the filter.

Biofilm analysis showed that comparing the S-Net 3 and 4 the lowest concentration of *E. coli* was observed in the latter. S-4 is located just a few kilometers from the surface water treatment plant. Similarly G-Net 7 displayed higher concentration of *E. coli* compared with G-Net 6 and the highest concentration in total. This site is the furthest within the network from the treatment plants. Thus the data indicate that the farther from the treatment plant, the longer the residence time of the drinking water, the higher is the concentration of *E. coli*.

3.4 Pathogenic protozoa

Pathogenic protozoa were analyzed in the water concentrates and in some samples *Cryptosporidium parvum* was found (Table 8 and 9, Figure 4). *Giardia lamblia* was not found in any of the samples.
The presence of *C. parvum* was confirmed in the surface water treatment station, after the biofilters. That particular sample was the most concentrated sample in the series. No parasitic protozoa were found in the biofilm, except one case of a possible *C. parvum* and there the identification could not be 100% confirmed due to low fluorescence signal.

Table 8. Protozoa in drinking water network

Sample	G-NET-7.1	G-NET-7.2	G-NET-7.3	G-NET-7.4	G-NET-7.5
Cryptosporidium	+/-	-	-	-	+/-
Giardia	-	-	-	-	-

Table 9. Protozoa in the treatment train

Sample	After biofilters	After biofilters	After biofilters	Raw water source
Cryptosporidium	-	-	+	+/-
Giardia	-	-	-	+/-

The +/- indicate that an absolutely conclusive positive could not be affirmed due to low signal or strange shape.

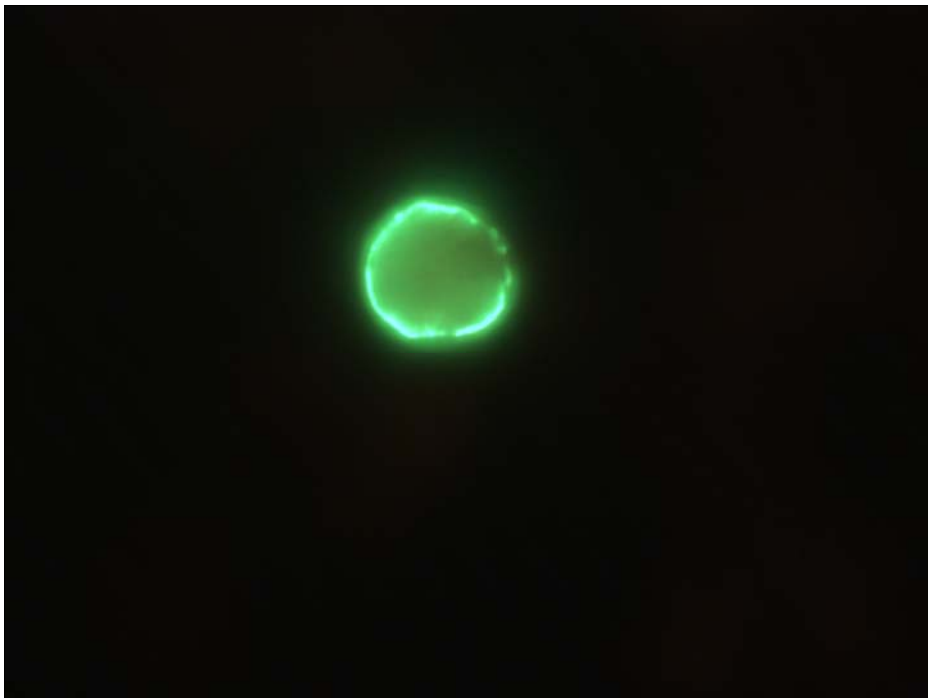

Figure 4. *C. parvum* after treatment in biofilters.

4 Conclusions

The sample concentration technique is a very useful tool for drinking water analyses as it allows the concentration of large water volumes and the analysis data obtained are thus more representative. The biofilm analyses complement the concentration experiments data and are also less costly compared to the concentration method so both sampling techniques are useful and advisable for the general use. The concentration technique allows for detection of pathogens, such as parasitic protozoa's.

E. coli was present in a water distribution network even if water most of the time met EU water quality standards, as checked by plate counting.

Viable *E. coli* was detected in biofilm of water distribution networks supplied with groundwater and surface water. The surfaces (and biofilm) of pipes in water distribution networks act as reservoirs of *E. coli* which has entered water distribution networks. It is possible that a small number of *E. coli* cells accumulate over a long period of time – either through malfunction of the treatment train or other intrusion and then the cells are washed out together with the rest of biofilm into water in higher amount as a result of changes in the pressure.

The concentration of *E. coli* level tended to increase with water residence time in distribution networks supplied with chlorinated groundwater and treated surface water.

Presence of even low levels of *E. coli* in biofilm compromises the water quality, thus more attention to on-line monitoring and probabilistic risk assessment is needed. The DVC method might in future be considered as an alternative to the culture methods.

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