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Validation of the FISH-based detection and quantification of *E.coli* and coliform bacteria in water samples

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Colophon

Title
Validation of the FISH-based detection and quantification of *E. coli* and coliform bacteria in water samples

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Summary

Monitoring of microbiological contaminants in water supplies requires fast and sensitive methods for the specific detection of indicator organisms or pathogens. The standard cultivation methods are too time-consuming to match the requirements of modern water safety management, i.e. approaching the equivalent of online measurements. Within the TECHNEAU project we developed a protocol for the simultaneous detection of *E. coli* and coliform bacteria based on the Fluorescence *in situ* Hybridization (FISH) technology, which has been proven to represent a sensitive molecular method for the specific detection of microorganisms. The developed FISH protocol consists of two different approaches. One approach allows the direct detection of single *E. coli* and coliform bacterial cells on the filter membranes. The second approach includes incubation of the filter membranes on a nutrient agar plate and the subsequent detection of the grown micro-colonies.

In this study, both approaches were validated using drinking water samples spiked with pure cultures (both approaches) and naturally contaminated water samples (approach 2). The effects of heat, chlorine and UV disinfection on the FISH based detection of *E. coli* and coliform bacteria were investigated.

The micro-colony approach yielded very good results for all different samples and conditions tested, and thus can be thoroughly recommended for usage as an alternative method to detect *E. coli* and coliform bacteria in water samples. However, during this study some limitations became visible for the single cell approach. The method can not be applied for water samples which have been disinfected by UV irradiation. We found out, that cells inactivated by UV irradiation apparently can still be stained with the FISH probes. In addition, our results indicated that the green fluorescent dyes are not suitable to be used with chlorine disinfected samples. As all cells (FISH stained and not stained, target as well as non-target cells) emitted an unspecific green fluorescence at the excision wavelength of the green fluorescent dyes after chlorine treatment, false-positive results can be obtained. Furthermore, without the implementation of an appropriate automation procedure for cell detection and counting, this approach can become very labour-intensive, especially for samples with low numbers of *E. coli* or coliform bacterial cells.

Contents

	Summary	**1**
	Contents	**2**
1	**Introduction**	**3**
2	**Materials and Methods**	**5**
2.1	Media and cultivation methods	5
2.2	Strains used for spiking water samples	5
2.3	Determination of total cell numbers	5
2.4	Fluorescence in situ hybridisation (FISH)	5
2.5	Disinfection procedures	6
2.6	Natural water samples	6
3	**Results**	**7**
3.1	Validation of the single cell protocol	7
3.1.1	Single cell detection before and after heat inactivation	8
3.1.2	Single cell detection before and after chlorination	8
3.1.3	Single cell detection before and after UV treatment	10
3.1.4	Development of a red fluorescent *E. coli* FISH probe	11
3.2	Validation of the protocol for micro-colonies	12
3.2.1	Determination of detection limits	12
3.2.2	Validation of natural water samples with and without disinfection	13
4	**Discussion**	**16**
4.1	Protocol for single cells	16
4.2	Protocol for micro-colonies	18
5	**References**	**19**

1 Introduction

Public health protection requires safe drinking water, which means that drinking water must be free of pathogenic microorganisms. The presence of pathogens correlates well with the presence of faecal contaminations, so consequently the microbial safety of drinking water has been determined by testing for the presence of microbial indicator organisms (Stevens et al., 2003). Faecal bacteria are indicators of both, faecal contaminations as well as the possible presence of disease-causing microbes. Coliform bacteria including *Escherichia coli* are considered as indicator bacteria for drinking water contaminations. While *E. coli* bacteria specifically indicate faecal contaminations, the presence of other coliform bacteria can also be a result of natural processes (Stevens et al., 2003). However, as drinking water is not a natural environment for coliform bacteria, their presence indicates microbial water deterioration (Rompré et al., 2002).

At the moment, the presence of *E. coli* appears to provide the best indication of faecal contamination in drinking water (Tallon et al., 2005). Consequently the monitoring of *E. coli* is of major importance to water supplies and its monitoring requires fast and sensitive methods. The standard cultivation methods are too time-consuming to match the requirements of modern water safety management, i.e. coming close to online technology. Within the working area 3 of the TECHNEAU project better "online" monitoring systems are to be developed.

The Fluorescence *in situ* Hybridisation (FISH) technology has been proven to represent a sensitive and rapid molecular method for the specific detection of microorganisms (Amann and Fuchs, 2008). To apply this technique for the analysis of drinking water, absolute quantification has to be possible as nearly all microbial parameters need to be monitored by absolute threshold values. Therefore, major modifications and adaptations of the standard FISH-procedures were necessary. The TECHNEAU-partners vermicon and TZW developed a protocol for the simultaneous detection of *E. coli* and coliform bacteria. The existing slide-based FISH-technology was transferred into a protocol, which can be applied to filter membranes leading to quantitative results after filtration of a defined volume of water (for the protocol development see deliverable 3.4.1).

Two different approaches were developed: Either the direct detection of single *E. coli* and coliform bacteria cells on the filter membrane or an approach including an incubation step on a nutrient agar plate for a few hours prior to staining of the grown micro-colonies by FISH. Figure 1.1 gives an overview of both approaches. For the detection of *E. coli*, a green fluorescent dye was used. In parallel for the detection of coliform bacteria, a red dye was applied.

Both approaches were optimized for the analysis of spiked water samples (deliverable 3.4.1). In this study, the optimized protocol was validated using

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spiked and natural water samples. The effects of heat, chlorine and UV disinfection were tested in water samples spiked with pure cultures. The results obtained by the FISH technique were compared to results obtained by culture-based methods and by total cell counts.

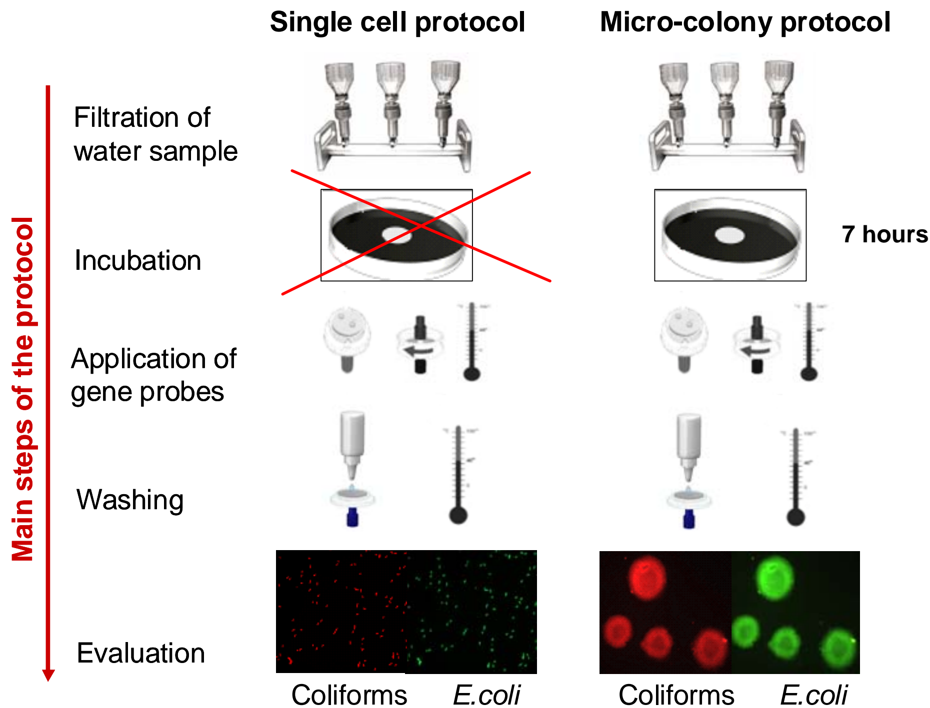

Figure 1.1: Schematic comparison of the FISH based single cell and micro-colony approaches.

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2 Materials and Methods

2.1 Media and cultivation methods

Lactose-TTC-agar with tergitol 7 was obtained from Heipha (Eppelheim, Germany), chromocult coliform agar and DEV agar from Merck (Darmstadt, Germany).

For culture-based detection of *E. coli* and coliform bacteria, spiked or natural water samples were filtered through 0.45 µm nitrocellulose membrane filters (Millipore, Schwalbach, Germany) and the filters subsequently incubated on chromocult coliform agar for 24 h at 36°C. *E. coli* and coliform bacteria form bluish or reddish coloured colonies, respectively.

2.2 Strains used for spiking water samples

Escherichia coli (DSMZ 1103), *Citrobacter freundii* (DSMZ 30039), *Enterobacter cloacae* (DSMZ 30054) and *Serratia marcescens* (laboratory isolate) were used for spiking of water samples. The strains were grown on DEV agar, suspended in approximately 10 mL phosphate buffered saline (PBS) solution and filtered through 0.2 µm polycarbonate filters (Millipore). After a washing step (150 mL PBS), the filtered cells were resuspended in 100 mL PBS. The cell concentration within the solution was quantified by culturing (chromocult coliform agar) after membrane filtration. The solutions were used to spike water samples with the desired cell concentrations of the respective strains.

2.3 Determination of total cell numbers

For the detection of total cell numbers, the acridine orange direct count method (Hobbie et al., 1977) was used. Defined volumes of water samples were filtered through 0.2 µm polycarbonate filters (Millipore), stained with acridine orange solution (Merck), and all cells were counted using a fluorescence microscope (Leica DMRE, Leica, Wetzlar, Germany; λ = 470 nm). This method detects all cells, living as well as dead cells.

2.4 Fluorescence *in situ* hybridisation (FISH)

For the detection of *E. coli* and coliform bacteria by the FISH-method, the within the TECHNEAU project developed ScanVIT-E.coli/Coliform kit (vermicon, Munich, Germany) was used (see deliverable 3.4.1). Two approaches were used, the direct analysis of single cells (approach 1) or the analysis of micro-colonies (approach 2). In advance to FISH for approach 2 an incubation needs to be performed. Therefore, the filter membrane was placed on a lactose-TTC-agar plate and incubated for 6-7 h at 37°C, prior to

hybridization of the grown micro-colonies. For the quantification of *E. coli* and/or coliform bacteria, two probes labelled with two different fluorescent dyes are implemented in the ScanVIT-E. coli/Coliform kit. The coliform probe was labelled with a red-fluorescent dye, the *E. coli* probe with a green-fluorescent dye. The wavelength of the light used to induce fluorescence of the probes was 530 nm (coliforms) or 480 nm (*E. coli*); the microscopic filters used were N2.1 and L5 (Leica, Wetzlar, Germany), respectively. The magnifications used in the microscopic evaluation were 630 for approach 1 and 50 for approach 2.

2.5 Disinfection procedures

In order to test the possible impact of standard drinking water disinfection methods on the FISH protocol, water samples spiked with *E. coli* and/or *C. freundii* cells were treated with different disinfection methods (heat, chlorine, UV).

Spiked water samples were autoclaved (20 min, 121°C) to achieve heat-inactivation of the cells. Chlorine treatment of the samples involved 30 min incubation in chlorine solution (0.5 or 6 mg chlorine per L). After 30 min, free chlorine was neutralized using 0.01 M $Na_2S_2O_3$ solution. To test the effects of UV irradiation, 150 mL of the water sample were poured into sterile Petri dishes (diameter: 14 cm) and exposed to UV irradiation for 15 min (450 J/m^2). After disinfection treatment, the samples were immediately used or stored at 4°C.

2.6 Natural water samples

For the validation of the single-cell approach drinking water samples spiked with *E. coli* or *C. freundii* were used. For the validation of the micro-colony approach, drinking water was also spiked with *E. cloacae* and *S. marcescens* (Table 3.3). To test the influence of the water matrix, a mixture of river water and drinking water was used (end concentration: 6% river water). This water mixture was also used for testing the influence of disinfection procedures with natural water samples (Table 3.4). The natural microbial biocoenosis from river water was obtained by filtering Rhine water through 0.2 µm polycarbonate filters (Millipore). After a washing step (100 mL PBS), the microorganisms on the filter were resuspended in 40 mL PBS. The concentration of *E. coli* and coliforms in the suspension was determined through cultivation on chromocult coliform agar and the desired numbers of cells were added to drinking water samples. These samples (Table 3.5, natural microbial biocoenosis without matrix) were treated the same way as the samples with the water matrix in order to directly see the influence of the water matrix of river water.

3 Results

3.1 Validation of the single cell protocol

For validation of the single cell protocol for the detection of *E. coli* and coliform bacteria by the FISH technique, the effects of heat, chlorine and UV disinfection were tested on drinking water samples spiked with pure cultures of *E. coli* and *C. freundii*. After disinfection treatment the cells were quantified by a culture-based method (colony count after cultivation on chromocult coliform agar), by total cell counts (after staining with acridine orange) and by fluorescent cells counts after FISH. The obtained results are summarized in Table 3.1.

Table 3.1: Comparison of total cell counts, colony counts and fluorescent cell counts (after FISH) in water spiked with *E. coli* or *C. freundii* (untreated and after disinfection treatment).

Spiked samples	**total cell counts (acridine orange)**	**colony counts (chromocult coliform agar)**	**fluorescent cell counts (after FISH)**	
			filter N2.1 (red) coliforms	**filter L5 (green) *E. coli***
	cells / mL	**CFU / mL**	**cells / mL**	
	mean values st.dev.		**mean values** st.dev.	
***E. coli* (untreated)**	**1.20×10^6** 1.5×10^5	**0.44×10^6**	**0.35×10^6** 1.3×10^5	**0.35×10^6** 0.8×10^5
***E. coli* (after autoclaving)**	**0.51×10^6** 1.7×10^5	**0**	**<dl**	**<dl**
***E. coli* (after chlorination)**	**1.20×10^6** 3.1×10^5	**0**	**<dl**	**0.50×10^6 *** 1.8×10^5
***E. coli* (after UV-treatment)**	**1.10×10^6** 1.6×10^5	**0**	**0.49×10^6** 1.7×10^5	**0.47×10^6** 1.7×10^5
***C. freundii* (untreated)**	**4.90×10^6** 3.0×10^5	**0.49×10^6**	**0.14×10^6** 0.7×10^5	**<dl**
***C. freundii* (after autoclaving)**	**4.20×10^6** 8.2×10^5	**0**	**<dl**	**<dl**
***C. freundii* (after chlorination)**	**5.40×10^6** 3.6×10^5	**0**	**<dl**	**0.79×10^6 *** 3.1×10^5
***C. freundii* (after UV-treatment)**	**1.90×10^6** 3.2×10^5	**0**	**0.12×10^6** 0.8×10^5	**<dl**

<dl: below detection limit
* atypical green signals
CFU: colony forming units
st.dev.: standard deviation

For both strains, total cell counts (acridine orange) yielded significantly higher cell numbers than culture-based colony counts. For *E. coli*, 37% of the total cells could also be cultured on chromocult coliform agar. For *C. freundii* , only 10% of the total cells could be detected by the cultivation approach. Fluorescence cell counts obtained after FISH were in the same order of magnitude than colony counts on chromocult coliform agar. For *E. coli*, the fluorescence cell counts after FISH match very well to the culture based

counts, whereas in the case of *C. freundii* they were lower than colony counts (see Table 3.1, untreated cells).

3.1.1 Single cell detection before and after heat inactivation

Drinking water samples spiked with cells from laboratory cultures of *E. coli* and *C. freundii* were heat inactivated by autoclaving (20 min, 121°C). Subsequently, the cells were FISH-labelled and counted in the microscope. The results were compared with untreated cells. While most heat inactivated *E. coli* cells could still be detected after staining with acridine orange, no cells could be detected with FISH, neither with filter N2.1 for the red labelled coliform probe, nor with filter L5 for the green labelled *E. coli* probe (see Table 3.1, Figure 3.1). This is in accordance with the colony counts which implicated no living cells after the autoclaving step. The same results were obtained with *C. freundii* cells.

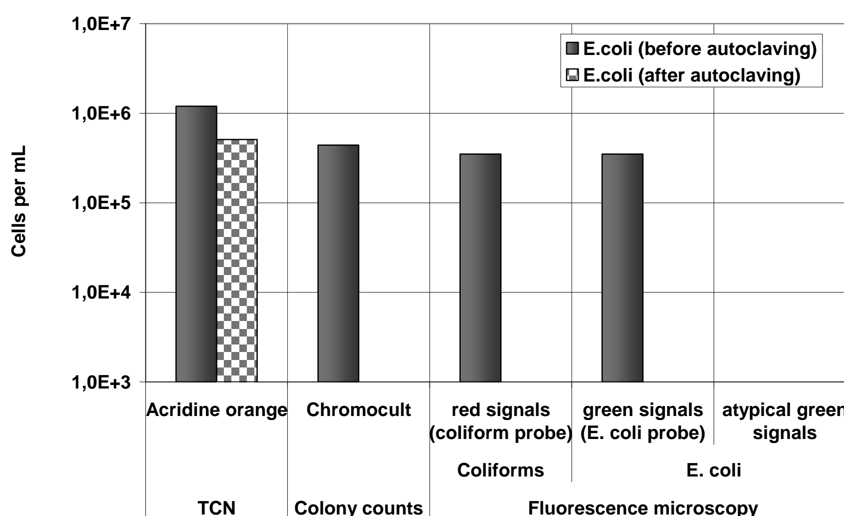

Figure 3.1: Quantification of *E. coli* cells before and after heat inactivation.

3.1.2 Single cell detection before and after chlorination

In order to investigate the effects of chlorine disinfection, drinking water samples spiked with *E. coli* and *C. freundii* cells were treated with chlorine solution (6 mg/L, 30 min). The chlorine treated cells could not be cultured on chromocult coliform agar (Table 3.1, Figure 3.2). However, chlorine treated cell suspensions could still be stained with acridine orange and total cell counts did not show a decrease in cell numbers before and after the treatment (Table 3.1). With the FISH method, neither *E. coli*, nor *C. freundii* could be detected in fluorescence microscopy using filter N2.1 for the red labelled coliform probe. However, cells of both strains showed green fluorescent emissions when filter L5 was used (Figure 3.3). These "atypical" green fluorescence signals could be seen, regardless whether a fluorescence dye was present or not.

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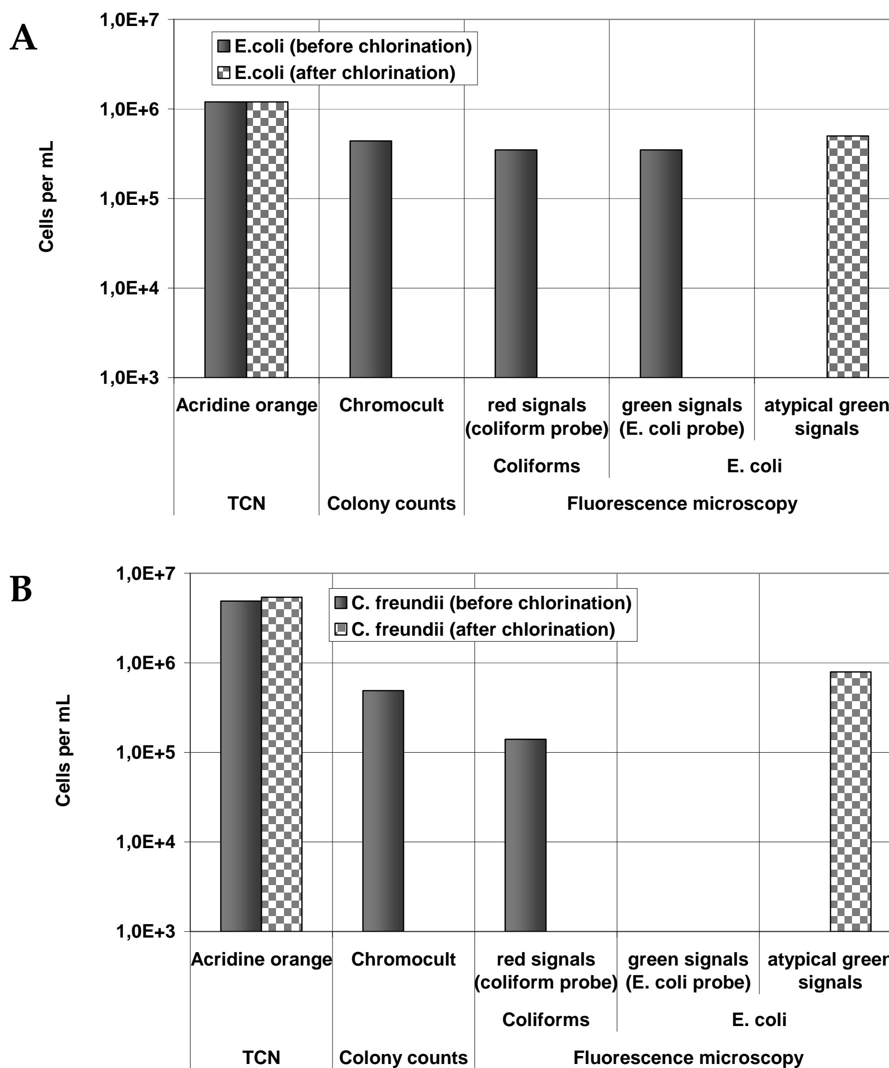

Figure 3.2: Quantification of *E. coli* (A) and *C. freundii* (B) cells before and after chlorination.

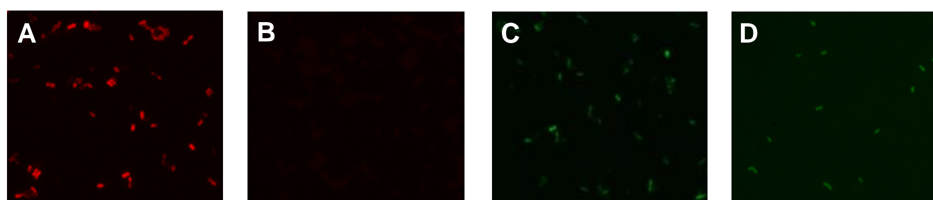

Figure 3.3: Microscopic pictures of *E. coli* cells labelled with the *E. coli* and coliform probe. (A) Filter N2.1 for red labelled coliform probe before chlorination, (B) filter N2.1 after chlorination, (C) filter L5 for green labelled *E. coli* probe before chlorination, (D) filter L5 after chlorination.

3.1.3 Single cell detection before and after UV treatment

The effect of disinfection via UV irradiation was investigated by exposing drinking water samples spiked with *E. coli* or *C. freundii* to UV light (15 min, 450 J/m^2). The UV treated cells showed no growth on chromocult coliform agar (Table 3.1). Before and after the application of UV irradiation, nearly the same total cell counts were obtained after acridine orange staining (Figure 3.4). A similar result was obtained for UV treated cells after FISH. The UV treated cells showed the same fluorescence signal as untreated cells (Figure 3.5), regardless which probe was used. Thus UV treated *E. coli* cells showed clearly visible red and green fluorescence signals, and UV treated *C. freundii* cells could still be hybridized with the red labelled coliform probe.

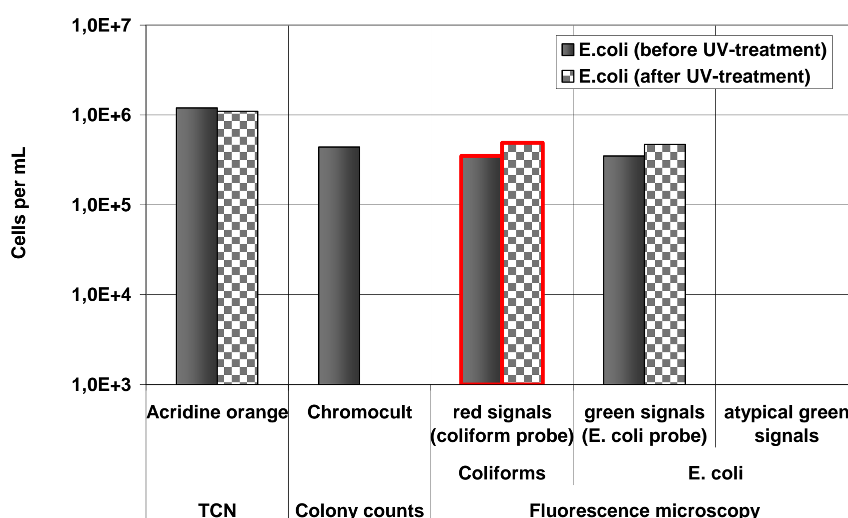

Figure 3.4: Quantification of *E. coli* cells before and after UV treatment.

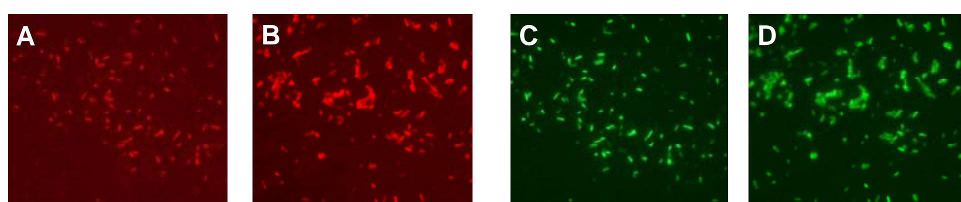

Figure 3.5: Microscopic pictures of *E. coli* cells labelled with the *E. coli* and coliform probe. (A) Filter N2.1 for red labelled coliform probe before UV treatment, (B) filter N2.1 after UV treatment, (C) filter L5 for green labelled *E. coli* probe before UV treatment, (D) filter L5 after UV treatment.

UV treated *E. coli* or *C. freundii* cells could still be stained with the FISH probes, although they could not be cultured on chromocult coliform agar anymore. This could mean that UV irradiation somehow damaged the cells without damaging the ribosomal RNA (the target molecule for the FISH probes).

In the described experiments, the FISH staining was performed immediately after the UV treatment. As it might take some time until the ribosomal RNA is degraded an additional experiment was set up to further investigate this aspect. After UV treatment (15 min, 450 J/m^2) of a drinking water sample spiked with *E. coli*, the sample was kept for 1 h, 5 h, 24 h and 7 days at 4°C and subsequently hybridized with the *E. coli* and coliform probes. The results are shown in Table 3.2. Interestingly, the number of FISH stained cells did not decrease significantly, even after 7 days storage at 4°C. The *E. coli* cells could still not be cultured on chromocult agar after this period of time, indicating that they were seriously damaged by the UV irradiation. In contrast, an untreated control sample which was also left at 4°C for 7 days showed cultivability.

Table 3.2: Comparison of total cell counts, colony counts and fluorescent cell counts after FISH in water spiked with *E. coli* cells (untreated and after UV treatment).

Spiked samples	total cell counts (acridine orange)	colony counts (chromocult coliform agar)	fluorescent cell counts (after FISH)	
			filter N2.1 (red) coliforms	filter L5 (green) *E. coli*
	cells / mL	CFU / mL	cells / mL	
	mean values st.dev.		mean values st.dev.	
E. coli (untreated)	**2.8×10^6** 4.0×10^5	**1.6×10^6**	**0.53×10^6** 3.6×10^5	**0.52×10^6** 2.9×10^5
E. coli (after UV, 1h at 4°C)	**2.8×10^6** 3.6×10^5	**0**	**0.64×10^6** 6.6×10^5	**0.69×10^6** 4.5×10^5
E. coli (after UV, 5h at 4°C)	**2.9×10^6** 4.1×10^5	**0**	**0.48×10^6** 2.8×10^5	**0.47×10^6** 2.8×10^5
E. coli (after UV, 24h at 4°C)	**2.4×10^6** 3.4×10^5	**0**	**0.72×10^6** 1.3×10^5	**0.69×10^6** 1.2×10^5
E. coli (after UV, 7 days at 4°C)	**2.3×10^6** 7.4×10^5	**0**	**0.72×10^6** 4.6×10^5	**0.72×10^6** 4.5×10^5
E. coli (untreated, 7 days at 4°C)	**3.5×10^6** 3.9×10^5	**0.88×10^6**	**0.53×10^6** 4.7×10^5	**0.51×10^6** 4.2×10^5

CFU: colony forming units
st.dev.: standard deviation

3.1.4 Development of a red fluorescent *E. coli* FISH probe

Chlorine treated bacterial cells showed an "atypical" green fluorescence signal, regardless whether a green fluorescent dye was applied via the labelled *E. coli* probe or not. Consequently, the green labelled *E. coli* FISH probe was not suitable for the detection of *E. coli* cells in chlorine treated water samples. Since no atypical red fluorescence was observed, a new red labelled *E. coli* probe was developed and used to overcome this drawback. Using this probe, no false positive *E. coli* cells occurred in chlorination experiments any more. An experiment with untreated *E. coli* cells showed, that fluorescence cell counts of *E. coli* cells stained with either green labelled or red labelled *E. coli* FISH probes yielded the same cell numbers (data not shown).

3.2 Validation of the protocol for micro-colonies

The second approach of the FISH protocol for the detection of *E. coli* and coliform bacteria in drinking water samples includes a short incubation step on a nutrient agar plate (TTC agar) for 6 to 7 hours prior to FISH staining of the grown micro-colonies. For the validation of the micro-colony approach, drinking water samples and natural water samples were used. In order to investigate the lower and upper detection limit of this approach, the samples were spiked with different numbers of *E. coli*, *C. freundii*, *E. cloacae* or *S. marcescens* cells from pure cultures. Mixed cultures of *E. coli* and coliform bacteria were also investigated. The effects of disinfection methods (heat, chlorine, UV) were tested using natural water samples spiked with *E. coli*. The results obtained with the FISH method were compared to results obtained by a culture-based method (cultivation on chromocult coliform agar).

3.2.1 Determination of detection limits

In order to determine the detection limits and standard deviations, drinking water samples were spiked with different concentrations of *E. coli* and coliform cells. The used concentration range was between 1 and 10^4 cells per 100 mL. The micro-colonies were manually scanned and counted and the numbers were correlated with colony counts obtained through the culture-based method. For each experiment three parallels were counted. The results are summarized in Table 3.3. Both methods show congruent cell numbers, in the low as well as in the high cell concentration range.

Table 3.3: Comparison of total colony counts and fluorescent micro-colony counts after FISH in drinking water samples spiked with *E. coli* and coliform cells.

Bacteria used for spiking of water samples	sample number	selection criteria (filter used for FISH)	colony counts	fluorescent micro-colony counts (after FISH)
			CFU / 100mL	micro-colonies / 100 mL
			mean values st.dev.	mean values st.dev.
drinking water + *E. coli*	1.1	coliforms (N2.1)	**9** 4.5	**8** 3.4
		E.coli (L5)	**9** 4.5	**8** 3.4
	1.2	coliforms (N2.1)	**9900** 450	**12600** 500
		E.coli (L5)	**9900** 450	**12500** 600
drinking water + *C. freundii*	2.1	coliforms (N2.1)	**8** 1.2	**11** 1.5
		E.coli (L5)	**0**	**0**
	2.2	coliforms (N2.1)	**13400** 1800	**17300** 760
		E.coli (L5)	**0**	**0**
drinking water + *E. cloacae*	3.1	coliforms (N2.1)	**14** 5.2	**14** 4.6
		E.coli (L5)	**0**	**0**
	3.2	coliforms (N2.1)	**14500** 1400	**18000** 800
		E.coli (L5)	**0**	**0**
drinking water + *E. coli* + *C. freundii* + *E. cloacae*	4.1	coliforms (N2.1)	**32** 5	**33** 2.9
		E.coli (L5)	**7** 3.2	**12** 1.1
	4.2	coliforms (N2.1)	**14600** 1250	**16500** 850
		E.coli (L5)	**4370** 250	**4290** 250
drinking water + *E. coli* + *S. marcescens*	5.1	coliforms (N2.1)	**14** 3	**16** 1
		E.coli (L5)	**8** 0.6	**6** 3.3
	5.2	coliforms (N2.1)	**12300** 1150	**12500** 720
		E.coli (L5)	**5830** 720	**6530** 290

CFU: colony forming units
st.dev.: standard deviation

3.2.2 Validation of natural water samples with and without disinfection

The validation of the micro-colony approach also included the investigation of naturally contaminated water samples. Therefore, water samples from the river Rhine were used since the river water harbours elevated concentrations of *E. coli* and coliform bacteria. In order to assess the effect of the water matrix – i.e. the effect of the organic matter present in the water – the water was either used directly (Table 3.4) or the mixed natural biocoenosis of the water was concentrated through filtration methods and resuspended in drinking water (Table 3.5). The effects of disinfection methods were investigated by treating the spiked natural water samples with chlorine (0.5 or 6 mg/L) or

with UV light. The results are shown in Table 3.4 (water matrix present) and Table 3.5 (without water matrix). Again the results from fluorescence microscopy after FISH were compared to colony counts after culturing on chromocult coliform agar. The results of both methods were comparable. We could not detect any difference between samples with and samples without water matrix. No growth of *E. coli* could be observed in the disinfected samples, neither with the micro-colony approach, nor with the direct culturing method.

An atypical green fluorescence was not disturbing in this approach, as the FISH-counts are done by smaller magnification in the microscope, so only micro-colonies can be detected, no single cells. Inactivated (dead) *E. coli* cells showing such an atypical green fluorescence would not form micro-colonies and therefore were not seen in the microscope.

Table 3.4: Comparison of total colony counts and fluorescent micro-colony counts after FISH in natural water samples (untreated and after disinfection treatment).

water sample and bacteria used for spiking of water sample	sample number	selection criteria (filter used for FISH)	colony counts	fluorescent micro-colony counts (after FISH)
			CFU / 100mL	micro-colonies / 100 mL
			mean values st.dev.	mean values st.dev.
natural water sample*	6.1	coliforms (N2.1)	**364** 40	**366** 48
		E.coli (L5)	**11** 2.7	**11** 1.5
natural water sample* + *E. coli*	6.2	coliforms (N2.1)	**374** 16	**300** 30
		E.coli (L5)	**24** 6.5	**20** 2.6
natural water sample* + *E. coli* chlorine treated (0.5 mg/L)	6.2.1	coliforms (N2.1)	**1**	**0**
		E.coli (L5)	**0**	**0**
natural water sample* + *E. coli* chlorine treated (6 mg/L)	6.2.2	coliforms (N2.1)	**0**	**0**
		E.coli (L5)	**0**	**0**
natural water sample* + *E. coli* UV treated	6.2.3	coliforms (N2.1)	**0**	**0**
		E.coli (L5)	**0**	**0**

CFU: colony forming units
st.dev.: standard deviation
* 6 mL river water + 94 mL drinking water

Table 3.5: Comparison of total colony counts and fluorescent micro-colony counts after FISH in drinking water samples spiked with a mixed biocoenosis (untreated and after disinfection treatment).

water sample and bacteria used for spiking of water sample	sample number	selection criteria (filter used for FISH)	colony counts	fluorescent micro-colony counts (after FISH)
			CFU / 100mL	**micro-colonies / 100 mL**
			mean values st.dev.	mean values st.dev.
drinking water + mixed biocoenosis*	7.1	coliforms (N2.1)	**488** 80	**492** 96
		E.coli (L5)	**11** 1.5	**11** 2
drinking water + mixed biocoenosis* + *E. coli*	7.2	coliforms (N2.1)	**652** 125	**508** 40
		E.coli (L5)	**18** 3.5	**25** 8.7
drinking water + mixed biocoenosis* + *E. coli* chlorine treated (0.5 mg/L)	7.2.1	coliforms (N2.1)	**0**	**0**
		E.coli (L5)	**0**	**0**
drinking water + mixed biocoenosis* + *E. coli* chlorine treated (6 mg/L)	7.2.2	coliforms (N2.1)	**0**	**0**
		E.coli (L5)	**0**	**0**
drinking water + mixed biocoenosis* + *E. coli* UV treated	7.2.3	coliforms (N2.1)	**0**	**0**
		E.coli (L5)	**0**	**0**

CFU: colony forming units
st.dev.: standard deviation
* from river water

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4 Discussion

The monitoring of *E. coli* and coliform bacteria as indicator organisms for drinking water contaminations requires fast and sensitive methods. Within the working area 3 of the TECHNEAU project, a novel method for the specific detection of *E. coli* and coliform bacteria based on the FISH-technology has been developed (see deliverable 3.4.1). This technique allows the simultaneous detection of *E. coli* and coliform bacteria cells on filter membranes after filtration of a defined volume of water. The protocol comprises two different approaches, the direct FISH staining and subsequent microscopic detection of single cells on the filter membrane, or the detection of micro-colonies. The second approach requires an incubation step on nutrient agar prior to the hybridization.

The purpose of this study was the detailed validation of the developed protocol. Both approaches were used to investigate natural water samples and drinking water samples spiked with *E. coli* and/or coliform bacteria. The results were compared with already approved methods, namely total cell numbers (after staining with acridine orange) and colony count on chromocult coliform agar. Furthermore the effects of different disinfection methods (heat, chlorine, UV) on the FISH based detection method were investigated.

4.1 Protocol for single cells

For validation of the single cell protocol, water was spiked with *E. coli* or *C. freundii* cells to a final cell number of approx. 10^6 cells/mL. Only a fraction of these cells could be cultured on chromocult coliform agar (37% for *E. coli* and 10% for *C. freundii*), thus most of the cells were dead or viable but not culturable (VBNC). The cell numbers determined by fluorescence microscopy after FISH-staining within untreated samples were in the same range than the numbers obtained by colony counts (Table 3.1). Hence, the FISH-method is an adequate method for the detection of living *E. coli*/coliform bacterial cells.

When the cells were inactivated by heat treatment (autoclaved), the total cell number (acridine orange staining) before and after heat treatment did not change, since also dead cells are detected with the acridine orange method., However, heat treated cells could neither be seen in the fluorescence microscope after FISH, nor be cultured on agar. Thus, the cellular rRNA was destroyed during the autoclaving step and the probes could not hybridize. Interestingly, different results were obtained using chlorine or UV irradiation as disinfecting agent.

When chlorine as disinfecting agent was used, *E. coli*/coliform cells could not be detected by fluorescence microscopy after applying the coliform probe labelled with a red fluorescent dye. These results indicate that the rRNA molecules were destroyed by the chlorine treatment and the probes could not bind to them anymore. In consistence, the cells could also not be cultured on

chromocult coliform agar after the chlorine treatment. However, *E. coli* as well as *C. freundii* cells showed green fluorescing signals when filter L5 was used, regardless whether a fluorescent dye was present or not (Figure 3.2). The reason for this unspecific (or "atypical") fluorescence of the chlorine treated cells is unknown. Since we could not clearly distinguish between the unspecific signals and the specific green *E. coli* signals, we must consider the green fluorescent dye, which is used to label the *E. coli* FISH-probe, as not suitable for the detection of living *E. coli* cells in chlorine treated samples.

As a consequence of these results the *E. coli* probe was labelled with an alternative dye. After investigating a series of different fluorescent dyes, only a red dye proved useful. However, since both the *E. coli* and the coliform probes were coupled to a red fluorescent dye now, the simultaneous detection of *E. coli* and coliform cells in a sample was no longer possible and thus each sample needs to be analyzed separately for *E. coli* and coliform bacterial cells.

After disinfection by UV irradiation, the treated cells could still be stained with the FISH probes and were still visible in the fluorescence microscope (Figure 3.4, Table 3.2). In contrast, the treated cells could not be cultivated on chromocult coliform agar anymore, even after 7 days storage at 4°C. This indicates that the cells were permanently damaged by UV irradiation and could not recover from this damage. However, the rRNA seems to be still intact even after 7 days storage at 4°C, as the FISH probes could still bind to the target rRNA molecules. No decrease in the number of counted cells was observed over that time period (Table 3.2). Thus we can conclude that the single cell FISH protocol is not suitable to differentiate between dead and living *E. coli*/coliform bacteria in water samples which have been disinfected by UV irradiation.

In a nutshell, the single cell FISH-method works well with untreated and heat-treated water samples. However, the method has drawbacks when other disinfection procedures are applied. It does not work for UV treated samples, since apparently dead cells were still detectable with the method. Chlorine treated samples need separate analyses for *E. coli* and coliform bacteria, as due to unspecific fluorescence the green labelled *E. coli* probe can not be used in parallel to the red coliform probe.

Furthermore, for small cell numbers, the method becomes extremely labour-intensive, since the whole membrane filter must be scanned manually in the microscope with a high magnification to detect single fluorescent *E. coli* and/or coliform cells. Hence, there is a need for automation of the cell detection and counting in order to make the single cell approach practicable for routine analyses of *E. coli*/coliform bacteria in water samples.

4.2 Protocol for micro-colonies

For the validation of the micro-colony approach, drinking water samples spiked with different cell numbers of *E. coli* and coliform bacteria and natural water samples were analyzed. The results of colony counts of the FISH-stained micro-colonies were compared to the colony counts obtained by cultivation on chromocult coliform agar. The results of both, FISH micro-colony counts and colony counts on chromocult coliform agar match up very well (see Tables 3.3, 3.4, and 3.5). The micro-colony approach of the FISH method works well for low (less than 10 cells/100mL) and high numbers (up to 10^5 cells/100 mL) of *E. coli*/coliform cells. When water samples from the river Rhine were investigated, the water matrix did not interfere with the method. Unlike the single cell approach, the micro-colony approach is also suitable for the analysis of disinfected samples, as by this approach only micro-colonies are counted and consequently the cells need to grow before they are detected with this method.

Overall, the micro-colony approach of the FISH-method is a suitable method to detect even low numbers of *E. coli*/coliform bacteria in water samples and it can be used as an alternative to conventional methods. Due to the incubation step of approx. 7 h, the micro-colony approach is more time-consuming than the direct single cell approach (Figure 1.1.). All in all, it takes approx. 10 h to get to results with this method. However, this is only half the time compared to the up to now fastest solely culture-based method (Colilert-18/Quantitray).

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