Development and validation of a FISH-based method for the detection and quantification of *E. coli* and coliform bacteria in water samples

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**ABSTRACT**

Monitoring of microbiological contaminants in water supplies requires fast and sensitive methods for the specific detection of indicator organisms or pathogens. We developed a protocol for the simultaneous detection of *E. coli* and coliform bacteria based on the Fluorescence in situ Hybridization (FISH) technology. This protocol consists of two approaches. The first 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 subsequent detection of the grown micro-colonies. Both approaches were validated using drinking water samples spiked with pure cultures and naturally contaminated water samples. The effects of heat, chlorine and UV disinfection were also investigated. The micro-colony approach yielded very good results for all 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 cannot be applied for water samples which have been disinfected by UV irradiation. In addition, our results indicated that green fluorescent dyes are not suitable to be used with chlorine disinfected samples.

**Key words** | coliform bacteria, disinfection, drinking water, *E. coli*, FISH

**INTRODUCTION**

Public health protection requires safe drinking water, which means that drinking water must be free of pathogenic microorganisms. The presence of pathogens in water has been associated with faecal contaminations; as a result the microbial safety of drinking water has been determined by testing for the presence of faecal indicator bacteria (Stevens et al. 2005). Widely used microbial indicators for drinking water quality/safety are coliform bacteria (total coliforms) and *Escherichia coli*. While *E. coli* bacteria specifically indicate faecal contamination, the presence of other coliform bacteria can also be a result of natural processes (Stevens et al. 2005). However, drinking water is not a natural environment for coliform bacteria. Therefore their presence in drinking water always indicates water quality deterioration (Rompré et al. 2002). In addition to coliform bacteria and *E. coli*, other indicators of importance are e.g. Enterococci, Clostridia or bacteriophages (Figueras & Borrego 2010).

At the moment, the presence of *E. coli* appears to provide a very good indication of faecal contamination in drinking water as well as in other coastal and freshwater environments (e.g. beaches, springs, surface waters) used for recreational activities (Tallon et al. 2005), despite the fact that alternative indicators (e.g. *Bacteroides*, Bifidobacteria, etc.) have been discussed in the literature for several years now (see Cabral (2010) and Figueras & Borrego (2010) for recent reviews). Consequently the monitoring of *E. coli* is of major importance not only to water supplies, but also for recreational waters and it requires fast and sensitive methods. Standard cultivation methods are time-consuming and modern water safety management requires faster methods. Consequently molecular-based methods without cultivation become attractive.

The Fluorescence in situ Hybridization (FISH) technology has been proven to represent a sensitive and
rapid molecular method for the specific detection of microorganisms (Amann et al. 1990, 1995; Amann & Fuchs 2008). As nearly all microbial parameters in drinking water need to be monitored quantitatively and limit values are fixed in the guidelines, quantification of E. coli or coliform bacterial cells in water samples with the FISH method is an essential premise in order to apply the method for the analysis of drinking water samples. Within the TECHNEAU project, an integrated project funded by the European commission (http://www.techneau.org), Vermicon AG (Munich, Germany) developed a protocol for the simultaneous detection of E. coli and coliform bacteria named ScanVIT-E.coli/coli-forms. The existing slide-based FISH-technology was transferred into a protocol which can be applied to filter membranes allowing the simultaneous detection and quantification of E. coli and coliform bacteria cells on filter membranes after filtration of a defined volume of water. 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 6 h prior to staining of the grown micro-colonies by FISH (Figure 1). E. coli bacteria cells are detected with a green fluorescent dye, coliform bacteria (including E. coli) with a red fluorescent dye.

In this study, the ‘ScanVIT-E.coli/coli-forms’ kit was validated using spiked and natural water samples. The effects of heat treatment, as well as 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.

MATERIALS AND METHODS

Media and cultivation methods

For culture-based detection of E. coli and coliform bacteria, between 10 and 100 mL of water samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore, Schwabach, Germany) and the filters were subsequently incubated on chromocult coliform agar (Merck, Darmstadt, Germany) for 24 h at 36°C.

Strains used for spiking water samples

Escherichia coli (DSMZ 1103) and the common coliform strains 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 nutrient agar (Merck), suspended in 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.

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 (10 to 100 mL) 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.

Fluorescence in situ Hybridization (FISH)

For the detection of E. coli and coliform bacteria by the FISH-method, the ScanVIT-E.coli/coli-forms kit (vermicon AG, Munich, Germany) was used according to the user manual. Two approaches were used, the direct analysis of single cells (approach 1) or the analysis of micro-colonies...
needs to be performed. Therefore, the filter membrane was placed on a lactose-TTC-agar plate (Heipha, Eppelheim, Germany) and incubated for 6–7 h at 37°C, prior to hybridization of the grown micro-colonies. Hybridization and washing were carried out in special reactors (part of the kit) which prevent cell losses and evaporation of solutions. 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/coliforms 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.

**Disinfection procedures**

In order to test the possible impact of 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). Water samples were autoclaved (20 min, 121°C) to achieve heat-inactivation of the cells. For chlorination, chlorine solution (0.5 or 6 mg chlorine per L, regular and maximum allowed concentrations, respectively) was added to the water samples. After 30 min, free chlorine was removed using 0.01 mol/L Na2S2O3 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/m²). After disinfection treatment, the samples were immediately analyzed.

**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. In addition, natural water samples (river water of the Rivers Rhine and Main, wastewater of two wastewater treatment plants) were investigated using the micro-colony approach. 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. The natural microbial bioocoensism from river water was obtained by filtering river 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 (i.e. within the river bioocoenaosis) was determined through cultivation on chromocult coliform agar. The desired number of cells was added to drinking water samples from the obtained suspension. These samples were treated the same way as the samples with the water matrix in order to see the influence of the water matrix of river water.

**RESULTS AND DISCUSSION**

**Validation of the single cell protocol**

For the validation of the single cell protocol the effect of heat, chlorine and UV treatment was tested using drinking water samples spiked with pure cultures of E. coli and C. freundii to a final concentration of approximately 10⁶ cells/mL. After heat or 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 cell counts determined in the untreated sample after FISH-staining. These results are summarized in Table 1.

In the untreated sample, only a fraction of the added cells could be cultured on chromocult coliform agar (37% for E. coli and 10% for C. freundii), thus part of the cells were dead or in a viable but not culturable state. The fluorescent cell counts determined in the untreated sample after FISH-staining were in the same range as the colony counts (Table 1). Hence, the FISH-method is adequate for the detection of viable E. coli/coliform bacterial cells.

**Single cell detection before and after heat treatment**

Drinking water samples spiked with cells from laboratory cultures of E. coli and C. freundii were heat treated by autoclaving (20 min, 121°C). Comparison of the total cell counts (acridine orange staining) before and after heat treatment revealed no significant difference, 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 (Table 1, Figure 2). Thus, the cellular ribosomal RNA (the target molecule for the FISH probes) 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 (see below).

![Figure 2](https://iwaponline.com/wst/article-pdf/64/7/1435/444746/1435.pdf)

**Table 1** | Comparison of total cell counts, colony counts and fluorescent cell counts after FISH (single cell protocol) in drinking water samples spiked with *E. coli* or *C. freundii* (untreated and after disinfection or heat treatment)

<table>
<thead>
<tr>
<th>Spiked samples</th>
<th>Total cell counts (acridine orange) cells/mL Mean values (st.dev.)</th>
<th>Colony counts (chromocult coliform agar) CFU/mL</th>
<th>Fluorescent cell counts (after FISH) Filter N2.1 (red) coliforms cells/mL Mean values (st.dev.)</th>
<th>Filter LS (green) <em>E. coli</em> cells/mL Mean values (st.dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em>, untreated</td>
<td>1.20 × 10⁶ (1.5 × 10⁵)</td>
<td>0.44 × 10⁶</td>
<td>0.35 × 10⁶ (1.3 × 10⁵)</td>
<td>0.35 × 10⁶ (0.8 × 10⁵)</td>
</tr>
<tr>
<td><em>E. coli</em>, after heat</td>
<td>0.51 × 10⁶ (1.7 × 10⁵)</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
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<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em>, after</td>
<td>1.20 × 10⁶ (3.1 × 10⁵)</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
<td>0.50 × 10⁶ a</td>
</tr>
<tr>
<td>chlorination</td>
<td></td>
<td></td>
<td></td>
<td>(1.8 × 10⁵)</td>
</tr>
<tr>
<td><em>E. coli</em>, after UV-</td>
<td>1.10 × 10⁶ (1.6 × 10⁵)</td>
<td>&lt;dl</td>
<td>0.49 × 10⁶ (1.7 × 10⁵)</td>
<td>0.47 × 10⁶ (1.7 × 10⁵)</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. freundii</em>, untreated</td>
<td>4.90 × 10⁶ (3.0 × 10⁵)</td>
<td>0.49 × 10⁶</td>
<td>0.14 × 10⁶ (0.7 × 10⁵)</td>
<td>&lt;dl</td>
</tr>
<tr>
<td><em>C. freundii</em>, after heat</td>
<td>4.20 × 10⁶ (8.2 × 10⁵)</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>C. freundii</em>, after</td>
<td>5.40 × 10⁶ (3.6 × 10⁵)</td>
<td>&lt;dl</td>
<td>&lt;dl</td>
<td>0.79 × 10⁶ a</td>
</tr>
<tr>
<td>chlorination</td>
<td></td>
<td></td>
<td></td>
<td>(3.1 × 10⁵)</td>
</tr>
<tr>
<td><em>C. freundii</em>, after UV-</td>
<td>1.90 × 10⁶ (5.2 × 10⁵)</td>
<td>&lt;dl</td>
<td>0.12 × 10⁶ (0.8 × 10⁵)</td>
<td>&lt;dl</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<dl: below detection limit (<1 CFU/100 mL for colony counts, <5 cells/mL for fluorescent cell counts).

aUnspecific green signals, CFU: colony forming units, st.dev.: standard deviation.


**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 1, Figure 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 1). Hence, the DNA was not destroyed by chlorination. With the FISH method, *E. coli* or *C. freundii* 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. 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).

The reason for this unspecific 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 was used to label the *E. coli* FISH-probe, as not suitable for the detection of living *E. coli* cells in chlorine treated samples (see also below).

**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/m²). The UV treated cells showed no growth on chromocult coliform agar, whereas total cell counts and fluorescent cell counts (after FISH) were nearly the same before and after the application of UV irradiation (Table 1, Figure 2). The 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), although they could not be cultured on chromocult coliform agar anymore. Thus, the cultivability of the cells is impaired, while the DNA and the rRNA seems to be still intact.

As it might take some time until the ribosomal RNA is degraded an additional experiment was performed to further investigate this aspect. A drinking water sample spiked with *E. coli* was treated with UV irradiation and subsequently stored at 4°C. After 1, 5, 24 h and 7 days samples were taken and hybridized with the *E. coli* and coliform probes. The results are shown in Table 2 (Figure 3).

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 coliform agar after this period of time, indicating that they were somehow damaged by the UV irradiation. In contrast, an untreated control sample which was also left at 4°C for 7 days showed cultivability. Thus we can conclude that the single cell FISH protocol is not suitable for water samples which have been disinfected by UV irradiation. Further, more detailed studies are needed in order to analyze the degree of cell damage by UV irradiation.
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 6–7 h prior to FISH staining of the grown micro-colonies. For the validation of the micro-colony approach, drinking water samples spiked with various cell numbers of E. coli and coliform bacteria (C. freundii, E. cloacae and S. marcescens) were analyzed as well as naturally contaminated water samples (river water and wastewater). 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 (Figure 4).

The validation of the micro-colony approach also included the investigation of naturally contaminated water samples, i.e. water samples from the rivers Rhine and Main, as well as wastewater samples (data included in Figure 4). These samples harbour elevated concentrations of E. coli and coliform bacteria. In order to assess the effect of the organic matter present in the water – the river water was either used directly or the mixed natural biooensis of the water was concentrated through filtration methods and resuspended in drinking water. The effects of disinfection methods were investigated by treating the spiked river samples with disinfectants (e.g. chlorine) and monitoring the survival of E. coli and coliform bacteria.

### 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
water samples with chlorine (0.5 or 6 mg/L) or with UV light (Table 3). When river water samples were investigated, the water matrix did not interfere with the method (data not shown). As shown in Table 3, the micro-colony approach also proved useful for the analysis of disinfected samples.

**CONCLUSIONS**

In this study we investigated a novel method for the specific detection of *E. coli* and coliform bacteria in various water samples. This technique is based on the FISH-technology and allows the simultaneous detection of *E. coli* and coliform bacteria cells on filter membranes after filtration of a defined volume of water. The protocol allows two different approaches, the direct FISH staining and subsequent microscopic detection of single cells on the filter membrane, or the detection of micro-colonies after an incubation step on nutrient agar and subsequent hybridization.

The single cell FISH method worked well with untreated and heat-treated water samples. However, the method had drawbacks when disinfection procedures were applied. It did not work for UV treated samples, since non-culturable cells were still detectable. 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 low 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 useful for routine analyses of E. coli/coliform bacteria in water samples, otherwise the detection limit increases to about $10^2$–$10^3$ cells/mL (cf. Eschenhagen et al. 2008).

In contrast, the micro-colony approach 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 in drinking water samples as well as in natural water samples (such as, wastewaters, surface waters, recreational waters, etc.). Due to the incubation step of 6–7 h, the micro-colony approach is more time-consuming than the direct single cell approach and it takes approximately 10 h to get results with this method. However, this is only half the time compared to the fastest solely culture-based methods (e.g. Cowburn et al. 1994; Fricker et al. 1997; Schets et al. 2002; Bernasconi et al. 2006).

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References


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