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# Disinfection effect of electrochemically generated chlorine on surface associated *Escherichia coli* in a drinking water system

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ABSTRACT

For many years, electrochemical treatment has been proposed as a potential alternative to conventional drinking water chlorination due to its simplicity, ease of use and ability to generate active disinfectant from ions naturally found in the drinking water The aim of this study was to evaluate the survival of *Escherichia coli* on the surfaces of water distribution system after exposure to *in situ* electrochemically generated chlorine. To analyse the effect of chlorine and its reaction intermediates, completely mixed reactor with or without ingenuous biofilm was supplied with natural drinking water containing low amount of chloride ions (<10 mg/L) and treated with non-stoichiometric titanium oxide electrodes ($TiO_{2-x}$) at low current density (4.1–8 mA/cm$^2$) which generate predominantly chlorine species. Various cell viability markers (cultivability, ability to divide as such and respiratory activity) were assessed in this study. The results showed that electrochemical disinfection was very effective to neutralize the suspended *E. coli* (>5 log decrease in cultivability and 2 log decrease in respiratory activity was obtained after 1 h of treatment). However, surface and biofilm analyses showed significantly lower inactivation rates (1.49–1.79 log after 1 h of treatment). Moreover, after 24 h, biofilm still contained 16% ability to divide *E. coli*. The study clearly showed that surface- or biofilm-attached *E. coli* is more resistant towards electrochemically generated chlorine than the suspended ones, and this should be taken into account when choosing optimal doses for electrochemical disinfection.

*Keywords: Escherichia coli*; Biofilm; Electrochemical disinfection

## 1. Introduction

Chlorination is the most frequently used disinfection technique for drinking water due to its low cost, high efficiency and residual disinfectant effect. Dosage of chemicals in the form of liquid or gas to generate active chlorine in the water is the most commonly applied chlorination approach. Active chorine can also be generated directly in the water with electrolysis from chloride ions naturally present in raw waters [1]. This, so called, electrochemical water disinfection has been considered as potentially better alternative to the conventional disinfection technologies due to several advantages, like the fact that no reagents are needed,

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less by-products are formed and the process is simple and easily controllable [2]. Moreover, the constant supply of chlorine ensures long-term disinfection process which cannot be performed with point chlorination. During electrolysis not only active chlorine but also other oxidative species such as $^{\bullet}OH$, $O_3$, $H_2O_2$ and $^{\bullet}O_2^-$ can be generated [3], and they may provide additional disinfecting effect [4]. Since chlorine is generated *in situ* smaller intermediates of the reactions may penetrate deeper into the biofilm and enhance its dispersal which otherwise protects pathogens from chlorine. However, due to the complexity of the reactions, the influence of electrochemical water disinfection on bacteria is still not completely understood and there are no comprehensive studies about the effect of this type of disinfection on survival of pathogens and faecal indicator *Escherichia coli* in the biofilm. The aim of this study was to evaluate the survival of *E. coli* on the surfaces of water distribution system after subjection to electrochemical disinfection.

Under the stress, such as low nutrient concentration and presence of oxidants (e.g. chlorine) in the water, micro-organisms can lose their ability to form colonies on routine microbiological media, however, retain their metabolic activity (become viable but non-culturable) [5], thus, will not be detected after disinfection and could create false impression of having the system under control. Therefore, in addition to culture-based assays, molecular techniques, namely cell ability to divide and respiratory activity were employed in this study to measure metabolic activity of bacteria. These two techniques were chosen among the other available (membrane integrity, membrane potential, enzymatic activity, etc.) due to their robustness during the sample preparation for the analyses and previously reported applicability in environmental studies [6,7]. Cell ability to divide was detected according to direct viable count DVC assay [8] and combined with fluorescent *in situ* hybridization which has shown to have potential for species targeted analyses of non-cultivable but able to divide bacteria, especially *E. coli* [6,7]. Respiratory activity was determined by incubation of cells with a redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride) which in the presence of functioning electron transport chain act as an artificial electron acceptor, resulting in the formation of fluorescent insoluble formazan crystals inside metabolically active cells [9]. The study was carried out in laboratory-scale biofilm reactor in batch supplied with a natural drinking water.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*E. coli* ATCC 25922 grown on TBX agar (Oxoid, UK) was inoculated into pre-filtered liquid Tryptone soya broth (Oxoid, UK) and incubated with constant shaking (150 rpm) overnight at 37°C. Then, the culture of *E. coli* was centrifuged at 6,000 rpm (2000 × g) for 2 min (MinispinPlus, Eppendorf, USA) and the pellet was washed twice with sterile phosphate-buffered saline (PBS, 7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$ and 130 mM NaCl, pH 7.2) and re-suspended in sterile PBS. In order to determine the number of cells in the suspension, a small amount of sample was filtered through a 25 mm diameter, 0.2 μm pore size filter (Anodisc, Whatmann), without removing from the filtration device fixed with 3–4% formaldehyde solution for 10 min, washed with sterile distilled water and stained with DAPI (10 μg/mL) for 5 min, washed with sterile distilled water and air dried. Cell concentration in the suspension was determined by epifluorescence microscopy by counting 20 random fields of view (Ex: 340/380 nm; Em: >425 nm, dichromatic mirror 565 nm, Leica DM, LB).

### 2.2. Reactor setup

For the disinfection studies, a Propella™ reactor (4.2 L) equipped with 20 stainless steel coupons (1.77 $cm^2$ each) was used (Fig. 1). The preparation of the reactor for the study involved a complete disassembling followed by mechanical cleaning, disinfection and sterilization (121°C, 15 min). To obtain indigenous biofilm, the reactor after assembling was connected to the drinking water system (water treatment plant Daugava, Riga, Latvia; after biologically active carbon filtration, TOC ~5 mg/L; 0.3 NTU; pH 7.2; 0 mg/L total and free chlorine) and operated for 2–3 weeks to obtain detectable cell count ($1.8–2 \times 10^6$ cells/$cm^2$). On the day of disinfection the reactor was disconnected and transported to the laboratory. In experiments not involving the biofilm, the reactor was used directly after sterilization. Before all disinfection experiments, a pre-cleaned, pure $TiO_{2-x}$ ceramic electrode consisting of one anode (12.1 $cm^2$) and two cathodes (24.2 $cm^2$) was fit into the lower part of the reactor and connected to HQ Power, PS5005 (0–50 V DC, 0–5 A direct current rectifier). Then, the reactor was filled with pre-filtered (<0.01 CFU/mL) biologically stabile water (EVIAN, 6.8 mg/L $Cl^-$, AOC < 100 μgC/L), connected to the motor (Heidolph, Type: RZR1) and operated for 5 min to ensure complete

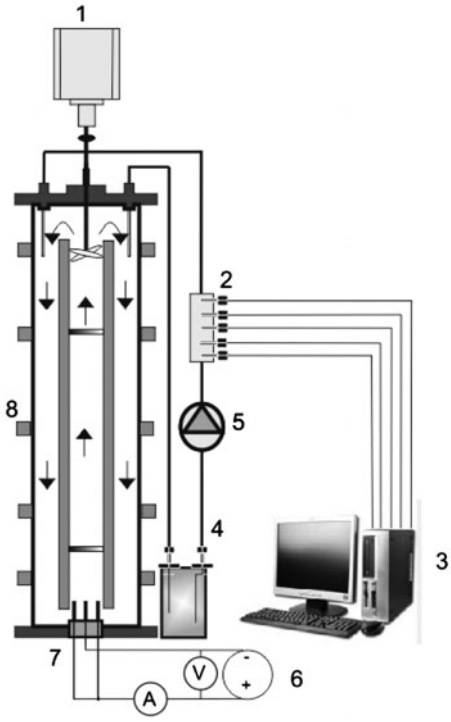


Fig. 1. Schematic overview of the electrochemical disinfection unit. (1) electric motor; (2) sensor cell; (3) data logger connected to PC; (4) extra water sample supply (1 L); (5) peristaltic pump; (6) direct current source; (7) $TiO_{2-x}$ ceramic electrodes; and (8) Propella$^{TM}$ reactor with 20 biofilm sampling devices.

mixing (0.25 m/s, $t^{\circ} < 25$°C). Online measurements of pH, $t^{\circ}$, ORP, $O_2$ and μS were performed with multiparameter data logger (Ahlborn, Germany).

### 2.3. Disinfection studies

Before reactor spiking, biofilm and water samples were collected to determine total microbial counts (reactors with biofilm) or absence of bacteria (clean reactor) and chlorine content. Additionally, potential presence of *E. coli* was determined in each sample.

Initially, the reactor was spiked with *E. coli* (~$1 \times 10^6$ cells/mL) and operated for 1 h to obtain attached cells. Biofilm and water samples were collected for total, *E. coli* and chlorine measurements. Then, the system was connected to 0.2 A current intensity to begin chlorine generation. Biofilm and water samples were collected after 0, 15, 30, 60, 120, 240 and 1440 min. Total and free chlorine concentration was determined by the N,N-diethyl-p-phenylenediamine colorimetric method [10]. For microbiological analyses, 0.02 M of sodium thiosulfate ($Na_2S_2O_3$) was immediately quenched. Biofilm and surface attached *E. coli* were removed by ultrasonication for 2 min at 20 μA and 22 kHz. A total of 16–40 mL of suspension in sterile distilled water was obtained and analysed further.

To exclude excessive chlorine formation after 120 min of operation, the current intensity was reduced from 0.2 to 0.05 A. Each study involved three separate reactor runs and at each sampling 100 mL of water and two coupons were collected.

### 2.4. Microbiological analyses

Total bacterial count was estimated with DAPI described in section 2.1.

#### 2.4.1. Cultivable counts

To obtain heterotrophic plate counts (HPC) samples were decimally diluted in sterile distilled water or membrane filtered (sterile, 0.22 μm pore size cellulose membrane, Millipore) and inoculated onto R2A agar (Oxoid, UK). All plates were incubated in dark at 22°C for 7 d. Results were expressed as colony forming units (CFU) per $cm^2$ (biofilm) or mL (water) of sample. For cultivable *E. coli* samples were decimally diluted or membrane filtered and inoculated into TBX media. After 24 and 48 h of incubation at 37°C, all blue/green colonies were enumerated and expressed as *E. coli* CFU per $cm^2$ or mL.

#### 2.4.2. *Direct* E. coli *measurements*

Total and able to divide *E. coli* concentration was determined by DVC-FISH technique according to the protocol described by Mezule et al. [7]. In brief, samples were incubated in double diluted Tryptone soya broth containing 10 μg/mL nalidixic acid for 6 h. Then, the samples were fixed with 3–4% formaldehyde for 10 min, immobilized onto 25 mm, 0.2 μm pore size membranes (Anodisc, Whatman plc) and incubated in a hybridization buffer with CY3-labelled PNA probe targeting *E. coli* (5′-TCA ATG AGC AAA GGT-3′) for 1 h at 57°C. After hybridization, the samples were washed with sterile, distilled water, stained with 10 μg/mL DAPI and visualized with epifluorescence microscopy (dichromatic mirror 565 nm, Leica DM LB. For DAPI filter sets with Ex: 340/380 nm and Em: >425 nm were used and for *E. coli*—Ex. 535 ± 25 nm; Em. 610 ± 37 nm). For data analyses on cell ability to divide at least 300 randomly chosen cells were captured (Leica, DFC) and measured with Image Pro Plus 4.5.1 software. Cells were recorded as viable if they were ≥1.5 times longer than their original length (no DVC treatment).

#### 2.4.3. Respiratory activity measurements

Cell respiratory activity was measured by a modified CTC assay described by Rodriguez et al. [9]. In brief, the samples were incubated in equal amount of Luria-Bertrani (LB) broth (tryptone—10 g/L, yeast extract—5 g/L, NaCl—10 g/L) and 4 mM final concentration of CTC (Fluka, BioChemika) for 2 h in the dark at room temperature on an orbital shaker. Then, the samples were filtered through a 25 mm diameter, 0.2 μm pore size filter, fixed with 3–4% formaldehyde, rinsed with sterile distilled water and stained with 10 μg/mL DAPI for 15 min. Actively respiring and non-respiring cell numbers were determined with epifluorescence microscopy for DAPI (Ex: 340/380; Em: >425) and for fluorescence the red CTC-formazan crystals (Ex: 545 ± 30 nm; Em: 610 ± 75 nm). Metabolically active cells were determined by counting 60 random fields of view giving a detection limit of 174 cells/mL.

#### 2.4.4. Statistical analyses

MS Excel 2007 ANOVA single parameter tool (significance level ≤0.05) was used for analysis of variance on data from multiple reactor installations (monitoring of concentration dynamics). To determine if the data-sets are significantly different or not, *t*-test analyses (MS Excel 2007) were performed for two-tailed distributions. Probabilities of ≤0.05 were considered as significant.

## 3. Results and discussion

Up to date numerous types of electrodes for electrolytical disinfection have been developed and tested. The most popular materials include Pt, graphite, $PbO_2$, $SnO_2$, $IrO_2$, Pt–Ir, $RuO_2$, $TiO_2$ and boron-doped diamond [2,11]. Here we used non-stoichiometric titanium oxide electrodes ($TiO_{2-x}$) [12], since this type of electrodes generates predominantly chlorine species, whereas production of other oxidants ($OH^-$ radicals) is negligible. In this way, the study was focused only on the influence of active chlorine species and their reaction intermediates but not on other oxidants formed [3]. Controlled system parameters were obtained in a completely mixed reactor with or without natural biofilm and inoculated with pure culture of *E. coli*.

The results showed that when natural drinking water with low chloride ion concentration (below 10 mg/L) was subjected to low current intensity (0.1 A with 8 mA/cm$^2$ on the anode), the formation of active chlorine began within the first minutes of the experiments irrespective of the microbial load in the system (Table 1). In a sterile system, more than 6 mg/L of active chlorine was generated within 2 h, indicating on effective conversion of chloride ions to chlorine. As it was expected, suspended micro-organisms (*E. coli* and the biofilm) consumed chlorine and accounted for relatively lower free chlorine concentrations in the systems ($p > 0.05$). To decrease the free available chlorine concentration to the recommended values of <0.5 mg/L [13], current intensity after 2 h of operation was decreased to 0.05 A (4.1 mA/cm$^2$). As a result chlorine concentration began to decrease to reach the detection limit below 0.2 mg/L after 4 h. Simultaneous monitoring of other parameters did not show any extensive changes in water pH (7.31 ± 0.16) or temperature (20–24 ℃) during the treatment period; thus, maintaining the system as acceptable drinking water source.

First *E. coli* inactivation was tested in a system not containing any natural biofilm. Prior to disinfection experiments *E. coli* attachment to the surface was obtained by constant circulation of the water in the reactor for 1 h reaching an average concentration of $1.44 \pm 0.22 \times 10^4$ cells/cm$^2$. Subsequent circulation for 4 h did not account for a significant increase in *E. coli* attachment ($p > 0.05$) and cell concentration did not exceed $4.12 \times 10^4$ cells/cm$^2$ in any of the experiments.

The disinfection results showed that for the suspended *E. coli* more than 5 log decrease in the

Table 1
Total and free chlorine (mg/L) formation kinetics in various reactor systems at 8 mA/cm$^2$ current density and low chloride ion concentration (<10 mg/L). (Mean ± SD from 3 replicates)

| | Without microorganisms | | With *E. coli* | | With *E. coli* and biofilm | |
|---|---|---|---|---|---|---|
| Time, min | Total $Cl_2$ | Free $Cl_2$ | Total $Cl_2$ | Free $Cl_2$ | Total $Cl_2$ | Free $Cl_2$ |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 1.13 ± 0.70 | 0.20 ± 0.34 | 0.93 ± 0.30 | 0 | 2.38 ± 0.0 | 0 |
| 30 | 2.98 ± 0.40 | 1.65 ± 1.52 | 2.58 ± 0.72 | 0 | 2.68 ± 0.42 | 0.89 ± 0.42 |
| 60 | 5.23 ± 0.46 | 4.57 ± 0.91 | 4.04 ± 0.50 | 2.51 ± 0.23 | 3.77 ± 0.28 | 2.28 ± 1.26 |
| 120 | 8.07 ± 0.75 | 6.75 ± 0.75 | 6.82 ± 0.30 | 4.57 ± 0.20 | 4.57 ± 0.84 | 3.67 ± 1.54 |

cultivable counts were achieved within 1 h of treatment (2.5 mg/L free chlorine), which can be considered as very high removal efficiency (Fig. 2). Longer treatment times did not indicate any cultivable *E. coli* in the water (>6 log inactivation, below the detection limit). Simultaneously, only two log removal ($p < 0.05$ between the groups, after 1 h of treatment) in respiring *E. coli* was observed. Extended treatment (over 4 h) did not show a significant decrease ($p > 0.05$) in respiratory activity anymore, however, microscopic examination showed a tendency to decrease in fluorescent crystal size with increasing treatment time what could be linked to decrease in overall cell metabolic activity. Analyses on the attached *E. coli* showed that after 1 h of electrochemical treatment cultivable *E. coli* concentration decreased for only 1.47 log (Fig. 2). Whereas only 0.84 log removal in the attached respiring *E. coli* was observed. Subsequent treatment did not show a significant effect on the attached *E. coli* cultivability and respiratory activity. As it was expected, more cells showed respiratory activity than were cultivable on conventional culture media. Analyses on the cell potential to divide showed that initially 99% of all *E. coli* (both suspended and attached) were able to divide, however, after 60 min of treatment, the concentration decreased to 0.8 log. Further treatment did not have any significant effect on decrease in *E. coli's* ability to divide. This could be connected to relatively low detection limits of the DVC method used where it was impossible to observe more than 2 log decrease.

For further studies, natural (2–3 weeks old) drinking water biofilm was used (~$2 \times 10^6$ cells/cm$^2$; less than 10% cultivable). Again prior electrolysis, *E. coli* attachment with intense water circulation was performed. Presence of the biofilm did not facilitate *E. coli* attachment when compared to the system with no biofilm ($p > 0.05$) what could be linked to similarities in the environment, roughness of the surface itself or insufficient biofilm matrix [14]. The concentration of *E. coli* in the biofilm did not vary significantly throughout the study—from $9.42 \times 10^3$ cells/cm$^2$ after 1 h of circulation (without electrochemical treatment) to $1.20 \times 10^4$ cells/cm$^2$ after 24 h of treatment (average $1.39 \pm 0.4 \times 10^4$ *E. coli* cells/cm$^2$) indicating on no apparent attachment of suspended dead *E. coli*.

As previously, electrochemical treatment again showed to be very effective for the inactivation of suspended *E. coli*. Less than 30 min were required to obtain 2 log inactivation of suspended cultivable *E. coli*. After 1 h, the amount of cultivable cells decreased below the detection limits (more than 6 log inactivation) of the method (Fig. 3). Slightly lower ($p > 0.05$) inactivation rates were observed for cultivable counts of all bacteria in the system (*E. coli* and biofilm-associated-microorganisms). After 24 h of treatment when no cultivable *E. coli* were detected in the water anymore, $2 \times 10^3$ cfu/mL were still detected, indicating on minor detachment of more chlorine resistant biofilm bacteria. Biofilm analyses again demonstrated different inactivation rates of attached *E. coli* when compared to the suspended ones ($p < 0.05$). Only 1.79 log inactivation of biofilm-attached cultivable *E. coli* was obtained after 1 h (Fig. 3) and after 24 h of electrochemical treatment, the amount of cultivable *E. coli* decreased for only 3.24 log. The assessment of the biofilm effect in survival of cultivable *E. coli* did not account for a significant effect ($p > 0.05$) when

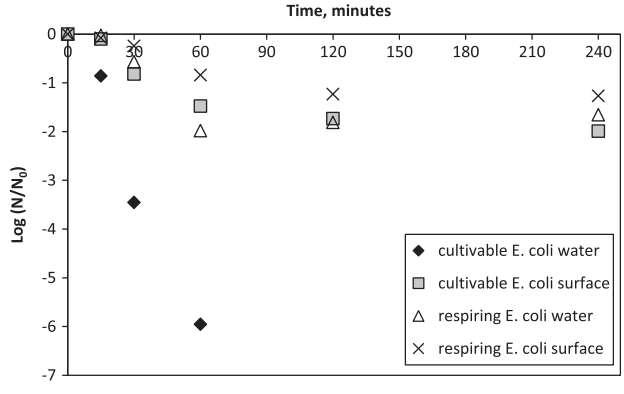


Fig. 2. Log reduction of respiring *E. coli* (cells) and cultivable *E. coli* (cfu) in water and on the surface of the reactor without natural biofilm after chlorine inactivation for 240 min. Data represent the average from three separate runs. 6 log reduction for cultivable counts and 3 log reduction for metabolic activity were estimated as detection limits.

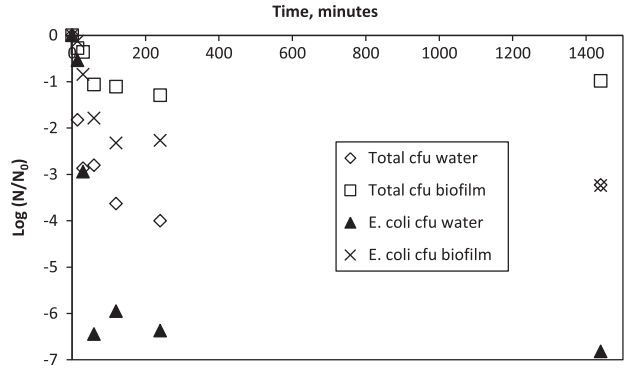


Fig. 3. Log reduction of cultivable microorganisms (as HPC) and cultivable *E. coli* in water and biofilm after chlorine inactivation for 24 h. The data represent the average from three separate runs. The detection limits for both methods were estimated as 6 log.

compared to surface attached *E. coli* in pure culture system (1.47 log against 1.79 log after 1 h).

Apparent biofilm-based cell detachment was observed in the system after 24 h when no cultivable *E. coli* was detected in the water; however, as many as $2 \times 10^3$ cfu/mL of HPC was obtained. This also supports the observation that as high as 4 log inactivation in HPC was observed in the water after 4 h of treatment but only 3.2 log after 24 h indicating on increased cultivable cell concentration in the system. Since no growth facilitating factors have been introduced in the system under mild electrochemical treatment, cell detachment from the biofilm [15] could explain the observation. Moreover, more than four times higher HPC were obtained in biofilm samples after 24 h of treatment when compared to 4 h.

Respiratory activity measurements showed high initial (before electrochemical treatment) metabolic activity (>80%) of suspended cells, what was linked to the inoculated *E. coli*. At the same time only 30% metabolic (respiratory) activity was observed for the biofilm population. This was linked to both occurrence of dead cells in the biofilm and relatively small crystals formed inside the cells what made the detection difficult. Disinfection studies again showed a more rapid decrease in the respiratory activity of suspended cells (2.2 log in 4 h) than for the biofilm associated ones (<1 log in 4 h) ($p < 0.05$ between the groups) and similarly as for cultivable counts an increase in metabolically active cell proportion between 4 and 24 h of treatment was observed. Since the assay was not species specific, no discrimination between respiring *E. coli* and other bacteria was possible.

The analyses on *E. coli* ability to divide further confirmed the extended survival potential for sessile *E. coli* —after 2 h of treatment the concentration of able to divide *E. coli* fell below detection limits of the assay; however, even after 24 h of treatment, biofilm contained as many as 16% of able to divide *E. coli* (Fig. 4).

Survival of micro-organisms (including pathogens) in the biofilm has been shown for various systems in the past [6,15]. Biofilm associated bacterial resistance to various chemicals has now been recognized as a common feature [16,17]. Despite drinking water, chlorination is recognized as the most ofteny used disinfection technique, short periods of chlorine depletion can have a positive effect on bacterial growth and survival capacity [18]. Maintaining of constant chlorine levels throughout the water supply system is often impossible due to their complexity. Over the years, various physical and chemical disinfection techniques have been proposed as alternatives or supplements to chlorination. Often electrochemical disinfection has been regarded as superior to

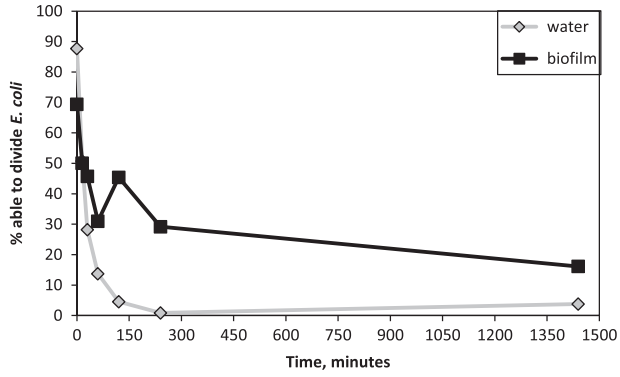


Fig. 4. Percent of able to divide *E. coli* in the water (light) and biofilm (dark) after 24 h of electrochemical disinfection. Data represent the average from three separate runs.

traditional chemical disinfection and point-limited physical assays, since the technique allows adjustment of disinfecting effect on-site and no reagents are required [11]. Moreover, the technique enables the production of the active disinfectant over a long period of time unlike point chlorination in traditional addition of the reagent. The high efficiency for electrochemical disinfection of suspended bacteria was shown in this study where more than 5 log inactivation of cultivable and 2 log inactivation in actively respiring cells was obtained after 1 h of treatment at low current density (below 10 mA/cm$^2$) in natural water containing low chloride ion concentration. However, the disinfection properties of sessile (surface or biofilm attached) *E. coli* showed much slower inactivation rates for all viability parameters tested and allowed to obtain cultivable *E. coli* even after 24 h of treatment; thus, indicating on apparent insufficiency of electrochemical disinfection alone. Yet, cultivable *E. coli* represented only about 0.1% of total *E. coli* cells in the biofilm; however, as many as 16% still possessed the ability to divide, which agrees with previous observations that cells lose their ability to form colonies on agar media much faster than other markers of viability [5]. The presence of indigenous biofilm did not facilitate the survival of attached *E. coli* when compared to the system with no biofilm. The observation contradicts previous observations on biofilm ability to protect pathogens and indicator organisms [14,15]; however, this could be linked to relatively short period of attachment (1 h) prior disinfection. To determine sole biofilm potential in protecting specific pathogens or indicators during electrochemical disinfection, additional studies should be performed.

The study showed that electrochemical treatment of water, when formation of other oxidative species

except chorine is minimal, is an effective method for disinfection of water; however, it has no advantages in comparison to conventional chlorination for killing micro-organisms (including indicators and pathogens) that are attached to surfaces or harboured in the biofilm. The study also highlights shortages of commonly used theory (Ct law) for designing of chlorination units and this law does not take into account bacteria which are associated with surfaces.

## 4. Conclusions

- Prolonged chlorination during electrochemical treatment of drinking water (<10 mg/L chloride ions) was highly efficient for removal of cultivable *E. coli* (more than 5 log/h) and less effective for metabolically active (respiring) population (2 log/h) in the water phase.
- Research on electrochemical inactivation of *E. coli* showed significantly different removal rates for surface- and biofilm-attached *E. coli*, where after 24 h only 3 log decrease in cultivable cells was observed and 30% of all biofilm associated *E. coli* still retained their ability to divide.
- Attached *E. coli*, both with or without biofilm, is significantly more resistant towards electrochemically generated chlorine than water-based *E. coli*, and this should be taken into account when choosing optimal doses for electrochemical disinfection.

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