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The Effect of Chlorination on *Escherichia Coli* Viability in Drinking Water

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***Abstract* – The assessment of drinking water disinfection efficiency traditionally involves the estimation of faecal indicator inactivation rates in the form of reduction of cultivable counts. Widely described, viable but noncultivable (VBNC) state as a form of bacterial survival strategy in oligotrophic conditions is not considered in these estimations. The aim of the present research is to assess the effect of free chlorine disinfection on faecal indicator *Escherichia coli* in order to study the succession of cellular alterations in response to chlorine exposure. To determine the inactivation rates, cell ability to form colonies, ability to divide as such and metabolic activity have studied. 0.064 mg/L min^{-1} free chlorine has been needed to obtain 99 % inactivation of cultivable counts; however, 5 till 200 times higher disinfectant dose x contact time has been required to reduce cell ability to divide and metabolic activity, respectively. The results have shown that to determine correct drinking water disinfectant doses multiple cell viability parameters should be analyzed.**

***Keywords* – Chlorination, *Escherichia coli*, inactivation kinetic, viability.**

I. Introduction

In drinking water treatment, disinfectant doses and subsequent disinfection kinetics are determined by using the concept of residual disinfectant concentration multiplied by contact time (CT) [1]. The kinetics of *E. coli* inactivation is usually described by Chick-Watson first order equation, and it uses the data obtained from plate counts, where accurate enumeration is of high importance to assess microbiological water quality [2]. However, many pathogens like *Giardia* [3] and *Cryptosporidium* [4] are resistant to the disinfection doses currently applied for drinking water. Moreover, an additional protection to microorganisms can be attained by their attachment to the biofilms [5].

It is accepted that the detection of *E. coli* in the water indicates the presence of possibility of waterborne pathogens with a subsequent risk to public health. For preparation of drinking water, including disinfection processes, at the moment all engineering calculations are based only on microbial ability to form colonies. However, many bacteria, including pathogenic *E. coli*, when subjected to stress may be present in the drinking water in a noncultivable state often referred to as viable but noncultivable (VBNC) state. It is assumed that bacteria in the VBNC state fail to grow on routine bacteriological media, on which they would normally grow and develop into colonies, but are alive and capable of renewed metabolic activity [6]. Nevertheless, it has been shown that in this state, bacteria are able to retain their pathogenicity [7] and even resuscitate [8]. Therefore, to assess the disinfection kinetics of waterborne microorganisms not only cultivable but all viable cells should be evaluated. Since the VBNC concept still requires some clarification, viability markers (membrane integrity, enzymatic activity etc.) are the most appropriate [9], the detection of at least two would be recommendable.

In this study, with traditional culture based assays, two viability techniques – cell ability to divide (direct viable count, DVC) [10] and respiratory activity (CTC assay) [11] were used to assess the effect of chlorine disinfection to neutralize *E. coli* in the drinking water. The DVC technique involving the incubation of the samples with nutrients and an antibiotic (nalidixic acid) allows detecting all cells having any potential for dividing, but not exclusively to form colonies on a solid culture medium. Limited ability to divide can be linked to the exposure to stress [12], however, is not a restricting potential to form toxic substrates [6]. Cell respiratory activity measurements in the form of introduction of an artificial electron acceptor (CTC) were selected due to their robustness (formation of insoluble crystals visible by fluorescence microscopy) and simultaneous toxicity of formed formazan crystals [13], thus, excluding cell division during sample treatment. Moreover, the method was chosen as a marker for a metabolic activity with no connection to cell multiplication.

To reflect the chlorine inactivation kinetics on *E. coli*, two reagent based disinfection techniques were used in this study – chemical chlorination (addition of hypochlorite) as active disinfectant and electrochemical disinfection, where active disinfectant was generated from salt ions naturally found in water. Electrochemical approach was chosen because of its ability to constantly generate low amounts of active disinfectant over a period of time, thus, providing longer contact time.

II. Materials and Methods

A. Bacterial Strains and Culture Conditions

E. coli ATCC®25922 was inoculated in sterile tryptone soya broth (TSB, Oxoid Ltd, UK) and cultivated at 37 °C with constant shaking at 200 rpm. Then *E. coli* cells were centrifuged in a microtube at 2000 g for 2 minutes (Minispin, Eppendorf) and rinsed with sterile distilled water. The centrifuging and rinsing processes were conducted thrice to completely remove the culture medium. The final bacterial pellet was re-suspended in sterile phosphate buffered saline (PBS, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl, pH 7.2) to obtain a stock solution of *E. coli* (~ 10^7 CFU mL^{-1}).

In order to determine the exact cell concentration 0.005 mL of the stock suspension was filtered through a sterilized 25-mm-diameter 0.2-µm-pore-size filter (Sartorius, Polycarbonate

Track- Etch Membrane, Germany) and fixed with 3 % – 4 % formaldehyde for 10 minutes, washed with sterile distilled water and stained with 10 μg/mL DAPI (4',6-diamidino-2-phenylindole, Merck, Germany) for 5–10 minutes. Cell concentration was determined with epifluorescence microscopy (Ex: 340/380; Em: > 425, dichromatic mirror 565 nm, Leica DMLB, Germany) by counting of 20 random fields of view.

B. Disinfection Experiments

1. Chlorination

Batch scale experiments were performed in well-mixed 250 mL Erlenmeyer glass flasks containing 100 mL sterile, pre-filtered phosphate buffer solution (0.039 M NaH_2PO_4, 0.061 M Na_2HPO_4, pH 7.0). The flasks were mixed with constant shaking (Biosan, Latvia) at 150 rpm at room temperature 25 ± 2 °C. First, different sodium hypochlorite chlorine doses were added (0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L and 5 mg/L as free chlorine), which were prepared by diluting commercially available sodium hypochlorite (NaClO, VWR International Ltd., Poole, BH15 1TD, England) in sterile distilled water, and the free chlorine concentrations were determined using the N, N-diethyl-p-phenylenediamine (DPD) colorimetric method, according to the procedure described by LVS EN ISO 7393-1:2001 [14]. In this method, the DPD was oxidized by free chlorine, resulting in a solution with color intensity proportional to the free chlorine concentration. Then a known concentration of *E. coli* cells was added to 100 mL sterile, pre-filtered 0.2 M phosphate buffer solution containing a known concentration of chlorine. After inoculation (final concentration 1×10^6 *E. coli* cells mL^{-1}), inactivation kinetics was studied for 1 h (0.5 min., 1 min., 5 min., 10 min., 30 min., 60 minutes). Immediately after the sampling, residual chlorine was neutralized with equal or excess volume of 0.02 M sodium thiosulfate ($Na_2S_2O_3$). Each experiment was performed in triplicate. All the results were expressed in milligrams of chlorine as Cl_2 per liter.

2. Electrochemical Disinfection

To 0.5 L of sterile synthetic *E. coli* free water (0.039 M NaH_2PO_4, 0.061 M Na_2HPO_4, 1 mM KCl, pH 7.0) *E. coli* with a final concentration of 1×10^6 /mL was added. Electrochemical treatment of the samples was performed in a specially made electrolytic cell consisting of pure TiO_{2-x} ceramic anode [15] with an area of 12.1 cm^2 and a cathode made of stainless steel (AISI 304), with a total surface area of 18 cm^2. The cathode consisted of two identical plates placed in parallel on either side of the anode, 5 mm away from it [16]. Power to the electrochemical reactor was supplied by HQ Power, PS5005 (0 – 50 V DC, 0 – 5 A, direct current rectifier). Standard electrolysis process was carried out at current intensity of 0.1 A, 23 °C ± 2 °C with intensive stirring. Samples were collected before electrolysis (0 h) and after 5 min, 15 min, 30 min and 120 minutes of treatment. Immediately after the sampling, residual chlorine was neutralized with equal or excess volume of 0.02 M sodium thiosulfate ($Na_2S_2O_3$). Each experiment was performed in triplicate.

C. Cultivable Cell Counts

Cultivable *E. coli* was estimated with the plate count technique: 0.1 mL aliquot of the sample or its decimal dilution was inoculated on TBX agar (Oxoid Ltd., UK) and incubated for 24 hours at 37 °C. Each sample was plated in triplicate. The results were expressed as colony forming units (CFU) per milliliter.

D. Direct Viable Count

Cell potential for dividing was determined by modified direct viable count (DVC) method [10] and combined with DAPI staining. In brief, samples were incubated in double diluted tryptone soya broth (Oxoid Ltd) and 10 μg/mL nalidixic acid for 6 hours at 30 °C on an orbital shaker at 200 rpm (Biosan, Latvia). After incubation the samples were fixed with 3 % – 4 % (final concentration) formaldehyde solution for 10 minutes. Then the samples were filtered through a 25-mm-diameter 0.2-μm-pore-size filter (Anodisc, Whatman International Ltd., Germany), rinsed with 100 mL of sterile distilled water and stained with 10 μg/mL DAPI for 15 minutes. After staining, the samples were rinsed with sterile distilled water and air-dried.

For data analyses at least 20 microscope images were made (Ex: 340/380; Em: > 425, dichromatic mirror 565 nm, Leica DMLB, Germany) and 300 randomly chosen cells lengths (μm) were measured with Image Pro Plus 4.5.1 software. Cells were set as elongated if they were ≥ 1.5 times longer than their original length (no DVC treatment).

E. Metabolic Activity Measurements

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 (5-cyano-2,3-ditolyl tetrazolium chloride, Fluka, BioChemika) for 2 hours in the dark at room temperature on an orbital shaker (200 rpm). Then the samples were filtered through a 25-mm-diameter 0.2-μm-pore-size filter (Anodisc, Whatman International Ltd., Germany), fixed with 3 % – 4 % formaldehyde, rinsed with sterile distilled water and stained with 10 μg/mL DAPI for 15 minutes. Actively respiring and non-respiring cell numbers were determined with epifluorescence microscopy (Leica DM LB, Germany) for DAPI (Ex: 340/380; Em: > 425) and for red fluorescent CTC-formazan crystals (Ex: 545 nm ± 30 nm; Em: 610 nm ± 75 nm). Metabolically active cells were determined by counting 60 random fields of view, giving a detection limit of 174 cells per mL.

F. Disinfection Kinetics

The effectiveness of disinfection process is obtained using the concept of residual disinfectant concentration multiplied by contact time or CT [17]. The kinetics of *E. coli* inactivation is usually described by Chick-Watson first order equation:

$$log\ (N/N_0) = -kC^nT \qquad (1)$$

where N/N_0 is the ratio of survived *E. coli* at time *t*, *N* is the concentration of viable *E. coli* exposure to a disinfectant, N_0 is the initial concentration of viable *E. coli*, *C* is the residual disinfectant concentration in mg/L, and *k* and *n* are empirical

constants (n = 1), T is the contact time in minutes when residual disinfectant is in contact with the water [18]. CT concept is used by public supply systems as one tool for ensuring adequate inactivation of organisms during disinfection [19]. Basically, log inactivation is expressed as the number or percent of microorganisms inactivated (killed or unable to replicate) through the disinfection process. It means that 1-log inactivation value is equivalent to 90 % of microorganisms reduction, 2-log is 99 % reduction, 3-log is 99.9 % reduction, 4-log is 99.99 % reduction, and so on [20].

G. Statistical Analysis

Statistical analyses were performed in MS Excel using Data analysis ANOVA tool or T-test to compare data sets. The level of significance was set at $P < 0.05$ for all comparisons.

III. Results

The inactivation effect of electrochemical disinfection and chlorination using sodium hypochlorite solution (NaOCl) on *E. coli* was investigated. Three different methods (CFU count, the number of DVC-elongated cells, the number of CTC-positive cells) were used for *E. coli* detection in the drinking water after chlorination and electrochemical disinfection.

A. Chlorination

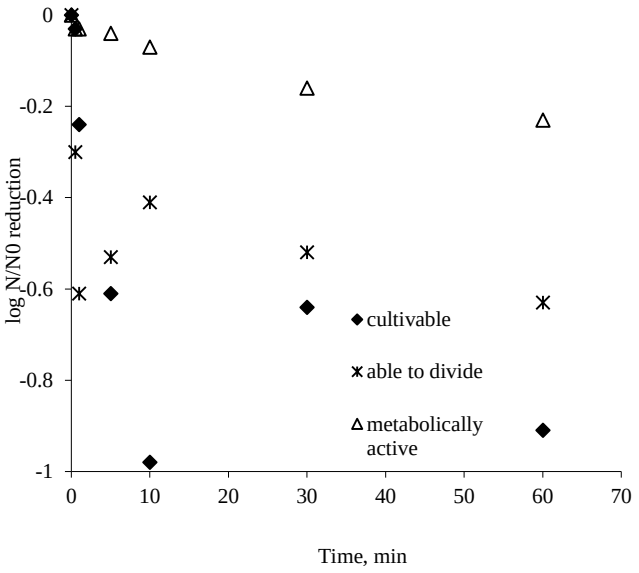

Fig. 1. Effect of 0.05 mg/L chlorine concentration on inactivation of cultivable (♦), able to divide (×) and metabolically active (Δ) *E. coli*. The data are the mean of three separate experiments.

The results showed (Fig. 1) that at a low initial chlorine concentration (0.05 mg/L as Cl_2), the number of able to divide *E. coli* cells decreased by 0.63-log after 1 hour. At the same time, a higher decrease was observed for cultivable counts (0.91-log after 1 hour). However, the respiratory activity of *E. coli* cells decreased less, showing only a decline of 0.23-log after 1 hour. During the first 10 minutes of treatment able to divide and metabolically active *E. coli* cells concentration rapidly decreased and the prolonged low chlorine treatment resulted in an increase. This can be explained as an onset of chlorine stress, but since the chlorine concentration was too low, *E. coli* could recover.

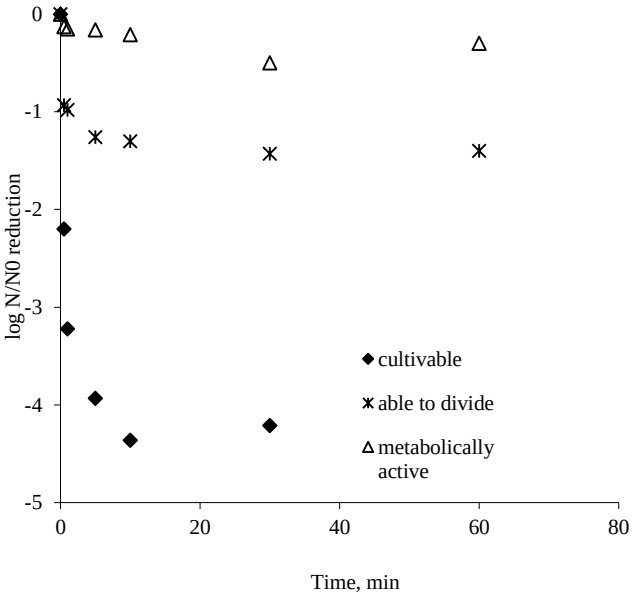

Fig. 2. Effect of 0.1 mg/L chlorine concentration on inactivation of cultivable (♦), able to divide (×) and metabolically active (Δ) *E. coli*. The data are the mean of three separate experiments.

For the disinfection at initial chlorine concentration of 0.1 mg/L Cl_2 (Fig. 2), the highest viable cell counts after 1 hour were observed with the CTC method (only 0.30 log reduction). At the same time, a 2.20-log reduction was observed for cultivable counts within 30 seconds and almost a 5-log reduction after 1 hour. The DVC method showed a 1.40-log reduction in able to divide counts after 1 hour, indicating a rapid decrease in the ability to form colonies but still retaining the capacity for dividing. Similar findings have been described for *E. coli* cells in chlorinated drinking water [21] and biofilm [22].

Higher initial chlorine concentrations (0.2 mg/L, 0.5 mg/L and 5 mg/L Cl_2) reduced the cultivability of *E. coli* cells for at least 5-log units in 30 seconds. In other words, after 30 seconds of treatment no *E. coli* cells formed colonies on a solid medium. This observation of cultivability loss is in agreement with what has been previously reported for *Helicobacter pylori* [23] and *E. coli* [24]. At the same time, only a 0.42-log reduction after 1 hour (0.2 mg/L Cl_2) of treatment was observed for a metabolic activity. To rapidly reduce metabolically active *E. coli* cell concentration, high chlorine concentration (5 mg/L Cl_2) was necessary. Only then a 5-log reduction within 1 minute was observed.

At high initial chlorine concentrations (0.5 mg/L and 5 mg/L Cl_2), the number of able to divide *E. coli* cells decreased to 90 % after 30 seconds. Increasing the contact time to 1 hour did not further increase the inactivation rates, suggesting that DVC method was not efficient in the enumeration of viable (non-cultivable) *E.coli* after chlorination. However, similar observations [22] have been previously explained as a rapid onset of death for cells ongoing division. Thus, the increase in the detection limits of the DVC method to determine low concentration of able to divide cells and use of stationary phase

cultures could be suggested. Furthermore, the combination of the DVC method with *in situ* hybridization could be a useful method to simultaneously detect and specifically identify low concentration of viable (able to divide) cells in water and biofilm samples after chlorination [23], [25].

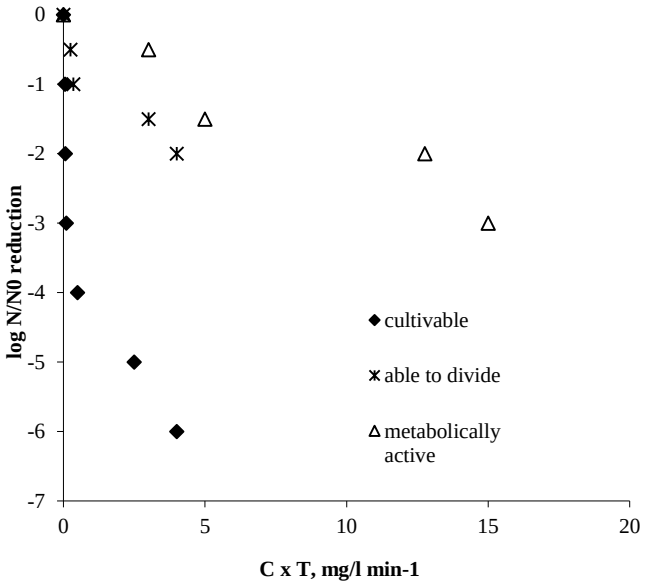

Fig. 3. Relationship between log survival and CT. Chlorine concentration (mg/L) and contact time (minutes) required to reduce cultivable (♦), able to divide (×) and metabolically active (Δ) *E. coli.* The data are the mean of three separate experiments.

The results (Fig. 3) showed that, when chlorinated, *E. coli* first lost its cultivability giving a low CT value (0.064 mg/L min^{-1}, 99 % inactivation) which was similar to earlier reported for coliforms (0.075 mg/L min^{-1} at pH 7.0 and 20 °C) [26] and in the ranges (0.04 mg/L min^{-1} – 0.92 mg/L min^{-1}) of previously reported values for *E. coli* at different temperatures, pH values and chlorine compounds used [18]. Moreover, the recommended doses (0.08 mg/L min^{-1} at pH 7.0 and 1 °C – 2 °C) for bacterial inactivation were not exceeded [2]. However, to decrease the *E. coli* ability to divide for 90 %, much higher CT values were needed (0.35 mg/L min^{-1}). Finally, the highest inactivation doses were necessary to neutralize cell metabolic activity (CT = 12.76 mg/L min^{-1}, 99 % inactivation), indicating higher *E. coli* tolerance limits to chlorine disinfection than considered before.

B. Electrochemical Disinfection

The application of electrochemical disinfection at chloride ion concentrations similar to drinking water (~ 35.5 mg/L Cl^-) gave no significant decrease ($p > 0.3$) in cultivable counts after 5 minutes of treatment; however, after 15 minutes a significant decrease ($p < 0.05$) was observed and accounted for more than a 5-log decrease (Fig. 4). This can be explained by a rapid onset of active chlorine formation after 5 minutes of electrolysis. Similar results were reported previously [16] when low chloride ion concentrations (below 7 mg/L Cl^-) were used.

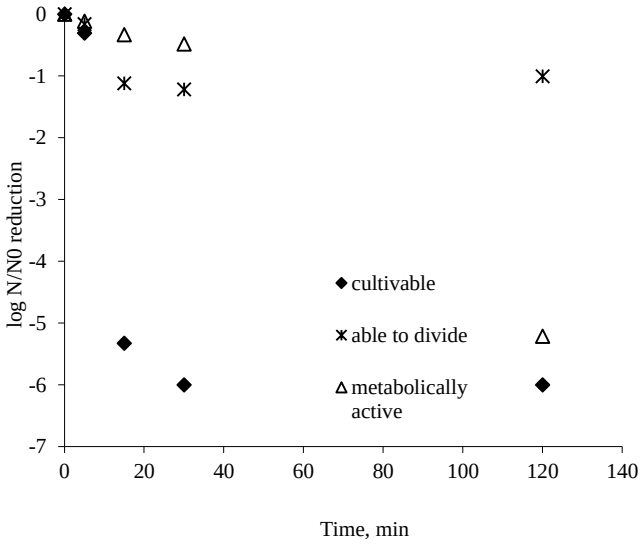

Fig. 4. The effect of electrochemical disinfection (0,1 A, 35.5 mg/L Cl^{-1}, RT) to reduce cultivable (♦), able to divide (×) and metabolically active (Δ) *E. coli* with respect to mg/L of free available chlorine (black line) generated. The data are the mean of three separate experiments.

No significant decrease ($p > 0.3$) in able to divide cell counts was observed within 5 minutes of treatment with electrolysis, after 15 minutes, similarly as for chlorination studies, only 90 % decrease was obtained ($p < 0.05$). This again could be explained by the limits of the DVC method applied.

As previously reported [16], the decrease in metabolically active cell concentration was the slowest. No significant ($p > 0.05$) decrease in metabolically active cell counts were observed after 1 hour of treatment. However, after 2 hours the amount of metabolically active cells fell below the detection limit, accounted for a significant decrease. Thus, it was possible to demonstrate a full neutralization (no metabolic activity) of *E. coli* when electrochemical disinfection with no active disinfectant added was used.

IV. Discussion

The choice of the viability parameters in this study was based on the review of previous studies [21], [25], [27], [28] which described that the respiratory activity (CTC), substrate responsiveness (DVC assay) and cultivability were more direct methods for cell viability.

The results of the study showed that traditional faecal indicator – *Escherichia coli* which is regarded as very susceptible to chlorination – could survive for much longer periods than it was assumed. The CT values for chlorination and electrochemical treatment time necessary to inactivate metabolically active and able to divide cells were much higher than for the cultivable cells. However, it must be emphasized that the use of elevated chlorine concentrations would be impossible due to the fact that drinking water must be not only microbiologically but also chemically safe and should not exhibit any inappropriate taste and odor.

Although studies have shown that the presence of *E. coli* in drinking water may represent either a true contamination event or can be released in the drinking water from the biofilm [29],

the application of culture based methods for the estimation of the disinfection efficiency is somewhat overestimated [30]. This observation was supported also in this study, where cultivability showed 5 to 200 times lower CT value than DVC and CTC assays, respectively, which would support the observation that other viability methods should be used in drinking water quality monitoring together with the cultivability.

Finally, although chlorination still remains as one of the most widely used disinfection techniques worldwide, it might be advisable that additional point-of-use water treatment techniques like electrochemical disinfection should be reevaluated and applied in places, where remaining disinfectant concentrations are below critical limits.

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Viktorija Deņisova, Linda Mežule, Tālis Juhna. Hlorēšanas ietekme uz *Escherichia coli* dzīvotspēju dzeramā ūdenī.
Dzeramā ūdens hlorēšana ir viena no visbiežāk pielietotajām dezinfekcijas metodēm attīstītajās valstīs. Dzeramā ūdens kvalitātes un dezinfekcijas efektivitātes novērtēšanai nosaka fekālā piesārņojuma indikatororganismu, galvenokārt, *Escherichia coli*, klātbūtni sagatavotajā ūdenī. Dezinfekcijas efektivitātes aprēķināšanai izmanto mikroorganismu spēju vairoties mikrobioloģiskās barotnēs (kultivēšanas metode), tomēr zināms, ka oligotrofā vidē daudzi mikroorganismi, t.sk. *E. coli* var būt sastopami dzīvotspējīgā, bet nekultivējamā stāvoklī. Pētījuma mērķis bija noteikt dezinfekcijas efektivitāti ne tikai ar tradicionāli pielietotu metodi, bet arī ar citām *E. coli* fizioloģiskā stāvokļa novērtēšanas molekulārajām metodēm. Darba ietvaros tika analizēta *E. coli* šūnu spēja veidot kolonijas, dalīšanas potenciāls, kā arī *E. coli* metaboliskā aktivitāte. Eksperimentu norises laikā tika novērots, ka vispirms samazinās *E. coli* kultivējamība, pēc tam dalīšanās potenciāls un tikai tad samazinās *E. coli* šūnu metaboliskā aktivitāte, tāpēc kultivēšanas metodes pielietojums hlorēšanas efektivitātes novērtēšanā netiek uzskatāms par pietiekoši reprezentatīvu. Pētījumu rezultātā tika noskaidrots, ka, lai neitralizētu 99 % kultivējamo *E. coli*, nepieciešami 0,064 mg/L min^{-1} hlora. Taču, lai samazinātu dalīties spējīgo un metaboliski aktīvo *E. coli* šūnu koncentrāciju, bija nepieciešama attiecīgi 5 līdz 200 reizes lielāka dezinfekcijas deva reizkontaktlaiks. Rezultāti parādīja, ka pareizai dzeramā ūdens dezinfekcijas devas noteikšanai nepieciešams izvērtēt vairākus šūnu dzīvotspējas rādītājus.

Виктория Денисова, Линда Межуле, Талис Юхна. Влияние хлорирования на жизнедеятельность *Escherichia coli* в питьевой воде.
Хлорирование воды – наиболее распространенный способ обеззараживания питьевой воды. Индикатором качества питьевой воды и эффективности процесса дезинфекции является наличие фекального загрязнения, в основном *Escherichia coli*, в воде. Традиционно для оценки эффективности дезинфекции используется поверхностный метод для культивирования микроорганизмов. Однако известно, что многие микроорганизмы, в том числе и *E. coli*, в олиготрофных условиях могут быть жизнеспособными, но некультивированными, т.е. не образующими колонии на твёрдых питательных средах. Цель исследования заключалась в определении эффективности дезинфекции не только традиционным методом, но и другими молекулярными методами для изучения физиологических свойств *E. coli*. В ходе работы были проанализированы колониеобразующие свойства клеток *E. coli*, их способность делиться, а также их метаболическая активность. В результате нашего исследования эффективность хлорирования воды для удаления 99 % культивированных клеток *E. coli* составила 0.064 mg/L min^{-1}. Однако, чтобы уменьшить способность *E. coli* клеток к делению и метаболическую активность, было необходимо увеличить отношение дозы дезинфектанта ко времени (доза х время) от 5 до 200 раз соответственно. Другими словами, сначала уменьшаются колониеобразующие свойства клеток *E. coli*, потом их способность делиться, и только затем уменьшается их метаболическая активность. Результаты показали, что для определения правильной дозы дезинфектанта для обеззараживания питьевой воды необходимо анализировать несколько параметров жизнеспособности микроорганизмов.