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Effect of Labile Organic Carbon on Growth of Indigenous *Escherichia coli* in Drinking Water Biofilm

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The finding that *Escherichia coli* can grow in water with low carbon concentration and compete with natural microbiota has challenged the applicability of this bacterium as an indicator for recent fecal contamination. With molecular tests *E. coli* can be frequently detected in drinking water biofilm but not in the water. At the same time the water usually complies with all quality standards. These observations enhance the questions about true fate of these bacteria in drinking water supply systems. The aim of this work was to determine if indigenous (naturally found) *E. coli* are able to colonize drinking water biofilm and show response to favourable growth conditions. Therefore, no inoculation of culture-isolated *E. coli* was performed throughout the studies. 5 week long monitoring on concentration dynamics was performed in full scale drinking water distribution system (artificially recharged groundwater, 28 hours of residence time, < 0.2 mg/L free available chlorine) and showed that even if no cultivable *E. coli* are found in the biofilm, as many as 240 *E. coli* per cm^2 can be detected with Fluorescent *in situ* hybridization. Subsequent increase in the nutrient availability (AOC ~ 500 µg/L), increased *E. coli* concentration in the biofilm for more than 30 times. The results showed a trend of *E. coli* accumulation in the biofilm of a water supply system and that the addition of nutrients (often accounted in biologically unstable water) significantly increase *E. coli* concentration.

1. Introduction

The finding that *Escherichia coli* can grow in low-nutrient water (Vital et al., 2008) and compete with natural microbiota (Vital et al., 2012) questions the reliability of this bacteria as an indicator of recent fecal contamination and puts under the risk water safety issues due to the possibility of its survival and multiplication. Moreover, it has been shown that in oligotrophic conditions *E. coli* is able to attain a state of non-cultivability but still retain its metabolic activity (viable but not culturable, VBNC) (Oliver, 2010) where its detection with traditional assays become impossible. To analyze the occurrence of such bacteria numerous molecular based tools, like, quantitative PCR, flow cytometry or whole cell *in situ* hybridization (Fluorescent *in situ* hybridization, FISH) have been employed over many years (Tora et al., 2014; Juhna et al., 2007; Rompre et al., 2002). High detection limits, inability to analyze specific target organisms, no information on cell viability, high costs and labour-consuming protocols have often been the cause for inability to evaluate environmental systems with respect to low concentration target organisms. Generally FISH analyses are limited to the volume of sample analyzed and subsequent microscopy counting. The use of high-binding probes, like PNA and LNA, combination of FISH with a viability method and improved quantification techniques have been suggested (Mezule et al., 2013; Wagner et al., 2012). Nevertheless, a lot of the studies on pathogen survival and behaviour in environmental systems are mostly limited to analyses with laboratory cultures (van der Kooij, 2003). Previous research have described the presence of non-culturable *E. coli* in both drinking water and biofilm (Juhna et al., 2007), however, the true fate of these bacteria into the drinking water distribution systems has not been assessed. The aim of this work was to determine if indigenous (naturally found) *E. coli* are able to colonize drinking water biofilm and show response to favourable growth conditions.

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2. Materials and Methods

2.1 Reactor installation and site location

To monitor *Escherichia coli* concentration dynamics Propella^{TM} (2.23 L) reactors equipped with stainless-steel coupons (1.77 cm^2 each) were used. The preparation of the reactors for installation involved a complete disassembling followed by mechanical cleaning, disinfection and sterilization (121 °C, 15 min). On the day of the installation the reactor was filled with *E. coli* free ultra-pure water, connected to the motor and operated to ensure complete mixing. After 15 min, ATP of the water samples was measured according to a modified method described by Vital et al. (2008). Cleaning was repeated, if ATP values for circulated and control water differed for more than 20 %.
One or two identical reactors were installed in Riga (Latvia) drinking water supply (in parallel with the drinking water source) at a point in the system which is supplied by artificially recharged groundwater after final chlorination. Water residence time at the point was estimated around 28 h, water quality parameters are given in Table 1. The average flow rate estimated into the reactor was 9.12 L/h. The complete mixing of the water was performed with a motor (Heidolph, Type: RZR1) at around 1500 rpm. The discharged water was directly connected to the wastewater system.

Table 1: Chemical and biological characteristics of the inlet drinking water (artificially recharged groundwater after final chlorination, 28 h residence time in the system) for the reactors.

Parameter	Mean	STD	No. of determinations
Total organic carbon (mg/L)	2.83	0.67	19
Dissolved organic carbon (mg/L)	1.86	0.42	19
Turbidity (NTU)	1.25	1.59	19
Conductivity (µS/cm)	568	1	3
Total chlorine concentration (mg/L)	<0.1	-	13
Temperature (°C)	12	1.5	16
AOC (µgC/L)	113	67	5
ATP (relative light units)	1372	1489	18
Heterotrophic plate counts on day 7 (cfu/L)	$1.17*10^6$	$6.01*10^5$	5
Total bacterial counts (cells/L)	$1.77*10^8$	$9.68*10^7$	18
Cultivable *Escherichia coli* (cfu/L)	0.20	0.54	23

2.2 Sampling

The first coupons were removed before reactor installation and after two h of circulating *E. coli* free sterile distilled water to ensure that the reactor does not contain *E. coli* initially. After installation the samples from the reactors were collected weekly over a period of five weeks. Longer sampling was avoided due to too extensive maturation of biofilm. Each time three coupons were withdrawn. The biofilm from the coupons was removed by gentle sonication (ColeParmer, USA for two min at 20 µA and 22 KHz) in 15 mL of ultrapure - *E. coli* free water. Processor probe was placed 1 cm above coupon. For water samples 2 L each time were collected from the inlet and outlet water of the reactor. All samples were taken to the laboratory and analyzed within two h.

2.3 Addition of labile organic carbon

The system of two identical Propella^{TM} reactors was set up as described previously and run for 3 weeks during which water and biofilm samples were collected. To simulate continuous pollution of the system with wastewater, one of the reactors was continuously supplied with ultra-filtrated (<100 cells/L; *E. coli* free) wastewater (final AOC concentration in the inlet water 500 µgC/L) for 14 days. Biofilm, inlet and outlet water samples were collected in triple on days 2, 7 and 14. The water temperature inside the supplied reactor was increased (max. 22 °C) by heating the inner cylinder of the reactor. Subsequently, another study was performed where one of the reactors was fed with glucose-salt solution (1.25 g/L glucose; 4.55 g/L $(NH_4)_2SO_4$; 0.2 g/L KH_2PO_4; 0.1 g/L $MgSO_4$ *$7H_2O$; 0.1 g/L $CaCl_2$ *$2H_2O$; 0.2 g/L NaCl, distilled water, pH 7 ± 0.5; final AOC ~ 500 µg/L, max. 22 °C) for 8 days. Biofilm, inlet and outlet water samples were collected in triple on days 1, 3, 6 and 8.

2.4 Sample analyses

Total Cell Count (TCC). To monitor overall population dynamics cell staining with DAPI (4',6-diamidino-2-phenylindole, Applichem) was performed. In brief, samples were filtered onto 25-mm-diameter 0.2-µm-pore-size filters (Anodisc; Whatman plc), fixed with 3 – 4% (v/v) formaldehyde and stained with 10 µg/mL DAPI for 15 min. Cell numbers were 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). The estimated detection limits were $1.57*10^5$ cells/L and $6.6*10^3$ cells/cm^2 respectively.

Cultivation analyses. To obtain heterotrophic plate counts (HPC) samples were serially diluted in sterile distilled water and then immediately inoculated onto R2A agar plates by spread plate technique. All plates were incubated in dark at 22 °C for 7 days. The results were expressed as colony forming units (cfu) per cm^2 (biofilm) or mL (water) of sample. For cultivable *E. coli* samples were analyzed by membrane filtration method in a certified reference laboratory according to EN ISO 9308-1:2000 (ISO, 2000).

Assimilable organic carbon. AOC measurements were performed according to the method described by Hammes and Egli (2007). In brief, water sample under analysis was filtered with 0.1 µm syringe filter, inoculated with a pre-cultured natural microbial community ($1*10^4$ cells/mL), incubated at 30 °C until the onset of stationary growth phase. Then the samples were stained with SYBR Green I (Invitrogen, USA), counted with a flow cytometer (488 nm blue laser, CyFlow, Partec, Germany) and converted to carbon concentration.

DVC-FISH analyses. *E. coli* potential to divide and total counts were determined by a modified Direct Viable Count method combined with FISH according to the procedure described by Mezule *et al*. (2013). After incubation of the samples with double diluted Tryptone Soya broth and 10 µg/mL nalidixic acid for 6 hours at 30 °C, the samples were fixed with 3-4 % (v/v) formaldehyde for 20 min and immobilized onto 25-mm-diameter 0.2-µm-pore-size filters (Anodisc; Whatman plc), washed with sterile distilled water and air-dried. Then, 20 µL of PNA hybridization mix containing 200 nM CY3 (Ex: 550, Em: 570) labeled PNA probe (5`-TCA ATG AGC AAA GGT-3`), was applied to the filters. The samples were incubated at 57 °C for 60 min in a tight vessel in dark. After hybridization the filters were washed with plenty of sterile distilled water, air-dried and visualized with epifluorescence microscopy (Ex. 535 ± 25 nm; Em. 610 ± 37 nm, dichromatic mirror 565 nm, Leica DM LB). The detection limits were calculated based on the volume of the analyzed sample, repetitions made and microscope fields of view examined and were below 5 *E. coli* cells/cm^2 of the biofilm analyzed. The detection limits for water samples were > 300 *E. coli*/L. The detection limit for *E. coli* in ultra-filtrated wastewater was < 1 cfu/L and < 5 cells/L according to FISH.

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. Pearson product-moment correlation coefficient (r) was used to estimate the linear correlation between two data sets.

3. Results and discussion

Four times the reactors were connected to a water supply system that complied with microbiological quality standards for *E. coli*. The results of five week monitoring showed no culturable *E. coli* in grab samples of 100 mL of water. Occasionally trace amounts of cultivable *E. coli* were found in both inlet and outlet waters of the reactors when higher volumes of water samples were used; however, the concentration did not increase to more than 2 cfu/L in the inlet water and 1.3 cfu/L in the outlet water but produced high data variability. At the same time FISH analyses for both inlet and outlet water showed that on average 1 % of total microbial population account for *E. coli* (2 – 3 cells/mL) where the able to divide (DVC positive) cells contributed to a maximum of 0.2 % from total population (0.7 - 2 cells/mL).

The comparison of total and able to divide *E. coli* concentration in the inlet water during all sampling periods did not accounted for a statistical significance ($p > 0.05$), thus, excluding any unreported or minor pollution events of the system. The same trend was observed for the outlet water. The comparison of *E. coli* concentration between the inlet and outlet water did not accounted for a significant increase ($p > 0.05$), however, it was observed that higher *E. coli* concentration accounted for the water leaving the reactors (39 % increase for total *E. coli* and 59 % increase for able to divide *E. coli*), indicating on potential accumulation or growth processes into the reactor. Only on week 5 in one of the reactors the amount of DVC positive *E. coli* in the inlet exceeded the outlet concentration, however, still not being statistically different ($p \geq 0.05$). Despite the slight increase in the concentration of *E. coli* leaving the reactor, no cultivable *E. coli* was found in the biofilm at all times.

The analyses of total and DVC positive *E. coli* in the biofilm showed that at low concentrations *E. coli* can be found in each of the analyzed biofilm samples (Figure 1) and can reach as many as 240 cells per cm^2. Similarly as observed in other studies (Camper et al., 1996; Robinson et al., 1995) indigeneous *E. coli* had the tendency to accumulate into the biofilm within the first 20 days what could be explained by simultaneous growth and formation of the biofilm itself. Thus, both total and able to divide *E. coli* counts increased, then rapidly decreased to only 13 % from maximum. Then again the concentration increased more than two times on week four to reach 33 % from the maximum. The irregular changes in the *E. coli* concentrations could be linked to the biofilm formation and establishment on the surface of the reactor or competition with indigenous

microflora (Vital et al., 2012). No presence of *E. coli* bacteriophages was observed in any of the samples. On average 58 ± 23 % of the *E. coli* found in the biofilm had the potential for dividing. The highest percentage of viable from total *E. coli* was on day 22, where it reached more than 95 %. At the same time the amount of *E. coli* in the biofilm never increased 0.0047 % (viable *E. coli* – 0.0043 %) from total bacterial numbers. The difference from previous studies where *E. coli* contributed to as much as 0.001 – 0.1 % from total population (Juhna et al., 2007) could be explained by different detection limits of microscopy methods and different types of biofilm samples analyzed.

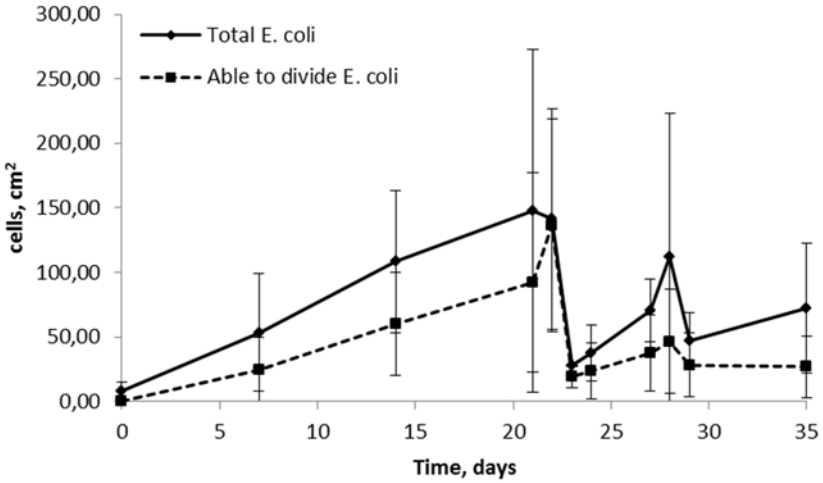

Figure 1: Total (black) and able to divide (viable) (dotted) Escherichia coli in the biofilm of Propella^{TM} reactor attached to drinking water supply. Standard deviation represents the dispersion of the results of five separate reactor installations separated in time.

Till now the research on ability of *E. coli* to grow and proliferate in nutrient poor environments with natural microbiota are mostly limited to laboratory studies (Vital et al., 2008; Vital et al., 2012), however, nutrient increase and elevated environment temperatures are still regarded as survival enhancing factors for enteric pathogens (Donlan, 2002; Torvinen et al., 2007). To analyze the influence of these parameters on *E. coli* naturally occurring in drinking water, the system of two identical reactors was run as described previously and again weekly monitoring of population dynamics and *E. coli* concentration was performed ($p = 0.16$ between the two reactors). To fully simulate the pollution of the drinking water distribution system, low amount of natural wastewater (ultra-filtered and *E. coli* free) was supplied for two weeks. The concentration of the wastewater was regulated to such an extent that the AOC level did not increased 500 µgC/L and no specific smell or discoloration of the water occurred. The observed increase in total bacterial counts in the biofilm and outlet water was only two times and only slight increase ($p \geq 0.05$) in viable *E. coli* in the biofilm was observed after the continuous supply of wastewater (Figure 2). After 2 weeks a mere 60 % increase in TCC was observed in the supplied reactor when compared to the control. The increase in total *E. coli* was 40 % from which nearly all cells were DVC positive, however, the microscopic analyses revealed no formation of extra-elongated cells. In the control reactor no increase in total *E. coli* counts was observed with a 20 % increase in DVC positive cells which corresponded to the monitoring data observed previously. Moreover, inlet and outlet water analyses showed no significant growth of *E. coli* in the reactor.

During the whole period of sampling no culturable *E. coli* from the biofilm was obtained, indicating on a low potential of *in situ* resuscitation of non-culturable, drinking water harbored *E. coli* when supplied with wastewater. Thus, the traditionally occurring increase in TCC and fecal indicators such as *E. coli* in the polluted systems could be explained by their origination from the wastewater itself where the naturally occurring biofilm in the drinking water simply increase the colonization opportunity for the pollutant microflora (Donlan 2002). Since no extensive chemical analyses of the wastewater were performed, the occurrence of resuscitation blocking factors like the elevated concentrations of nutrients producing superoxide and free radicals (Arana *et al*., 2007) or heavy metals (Grey and Steck, 2001) could also explain the failure to resuscitate.

To further investigate the growth potential of *E. coli* found in the drinking water supply, another study was performed where similarly two identical reactors were set up and run for 3 weeks. During this period the estimated difference between DVC positive *E. coli* in both reactors was not significant ($p = 0.34$). After the

viable *E. coli* reached previously observed concentration maximum on week 3, one of the reactors was supplied and continuously fed with glucose-salt solution for 8 days. Glucose concentration used in the study was low enough to exclude potential growth inhibition by acetate formation (Luli and Strohl, 1990).

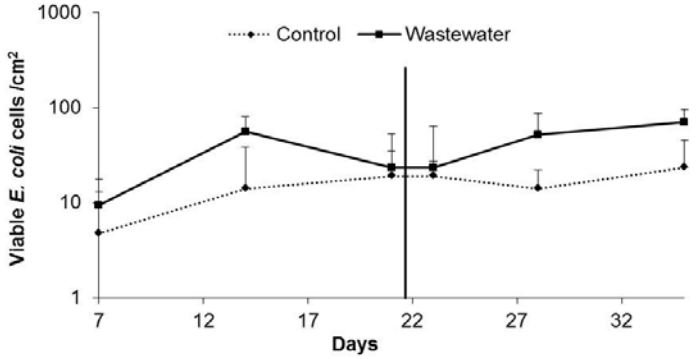

Figure 2: Concentration of viable (DVC positive) E. coli in the biofilm of two identical Propella™ reactors. Control reactor represents the unsupplied (dotted) and the supplied reactor is represented as wastewater (black). Vertical black line represents the moment of the initiation of wastewater supply to one of the reactors. The standard deviation represents the dispersion of the results of three repetitions.

After initiation of nutrient supply to one of the reactors an increase in TCC in the biofilm and outlet water was observed. After 8 days TCC concentration increased for more than 1 log and more than 30 times higher total *E. coli* concentration was observed. At the same time no significant increase in *E. coli* concentration in the control reactor was observed. Moreover, the cells from the reactor supplied with glucose-salt solution showed increased metabolic activity (higher rRNA content and increased potential for dividing) resulting in more fluorescent and elongated cells. The fluorescence intensity of the *E. coli* was at least double relative light units (RLU) as for control samples – from non-supplied reactor. In total the amount of viable *E. coli* in the biofilm increased 6 times and showed a distinct trend of increasing potential for dividing (Figure 3). The estimated difference between the two reactors showed to be significantly different ($p < 0.05$).

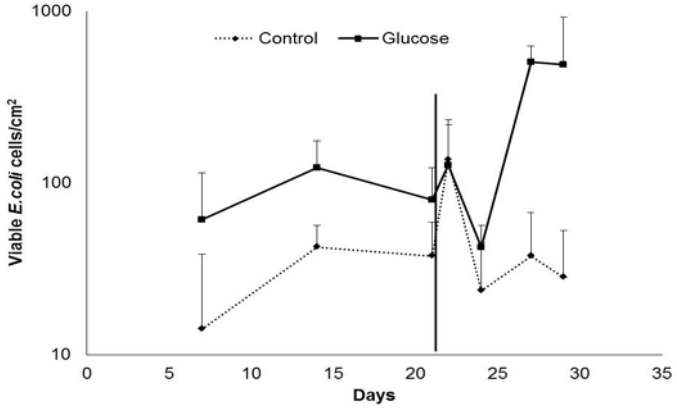

Figure 3: Concentration of viable (DVC positive) E. coli in the biofilm of two identical Propella™ reactors. Control reactor represents the unsupplied (dotted) and the supplied reactor is represented as glucose (black). Vertical black line represents the moment of the initiation of nutrient supply to one of the reactors. The standard deviation represents the dispersion of the results of three repetitions.

This study shows a trend of *E. coli* accumulation in water supply system biofilm and provides insight into previously unexplained sporadic occurrence of these bacteria in drinking water. *E. coli,* dominantly in non-culturable state, is accumulated onto surfaces of pipe material within the biofilm. Then their concentration is reduced by protozoan grazing or outcompeted by faster growing oligotrophic bacteria. The fact that bacteria

pass through water treatment and can resident on the surfaces of pipes provides new knowledge on water safety which should be implemented by water companies. Moreover, the addition of nutrients increases *E. coli* concentration in a real water supply system. Insofar biological stability of water was mostly linked to the overall microbiological quality, e.g., HPC of the water (van der Kooij, 2003), however, our results stress the need to control biological stability also to avoid regrowth of enteric bacteria such as *E. coli*, where high concentration of AOC may pose the risk of pathogen regrowth even in oligotrophic systems such as drinking water.

4. Conclusions

The following conclusions can be drawn from the present study: *1)* indigeneous *E. coli* accumulate in the biofilm of drinking water supply networks, where their concentration is controlled by various biological processes such as antagonism; 2) The distinct increase in able to divide *E. coli* found in drinking water biofilm after nutrient enrichment could pose a serious threat for the consumer.

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