RIGA TECHNICAL UNIVERSITY

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BIOLOGICAL STABILITY IN CHLORINATED DRINKING WATER DISTRIBUTION NETWORKS

Summary of the Doctoral Thesis

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RTU Press
Riga 2017

THE DOCTORAL THESIS
PROPOSED TO RIGA TECHNICAL UNIVERSITY
FOR THE PROMOTION TO THE SCIENTIFIC DEGREE OF
DOCTOR OF ENGINEERING SCIENCES

To be granted the scientific degree of Doctor of Engineering Sciences, the present Doctoral Thesis will be defended at the open meeting of RTU Promotion Council on June 16th, 2017 at the Faculty of Architecture of Riga Technical University, Ķīpsalas street 6, room 433.

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I hereby declare that the Doctoral Thesis submitted for the review to Riga Technical University for the promotion to the scientific degree of Doctor of Engineering Sciences is my own. I confirm that this Doctoral Thesis had not been submitted to any other university for the promotion to a scientific degree.

Alīna Neščerecka ……………………………. (signature)

Date: ………………………

The Doctoral Thesis has been written in English. It consists of an Introduction; 5 Chapters; Conclusions; 55 figures; 7 tables; the total number of pages is 116. The Bibliography contains 182 titles.

CONTENTS

RELEVANCE OF THE STUDY

The concept of drinking water biological stability implies that biological water quality at the point of consumption should be the same as it was for finished water directly after treatment [1], and it basically reflects one of the main goals of the drinking water supply system. Drinking water biological quality deterioration could lead to a number of problems, including consumer discontent with the water's aesthetic properties and in the worst case bacterial infections, which could cause serious health problems and even deaths. Therefore, finished water (directly after treatment) and water in the distribution network (DN) should not contain pathogenic bacteria, and bacterial growth in DN should be prevented or at least limited. Bacterial growth is particularly undesirable due to potential opportunistic pathogenic bacterial growth. These bacteria represent a part of a natural water microbial composition and in low concentrations usually do not cause diseases in a healthy host. However, they could pose a certain risk for individuals with weakened immune systems, especially in high concentrations.

Biological stability of drinking water could be achieved by two main approaches: (1) drinking water disinfection with residual compounds or/and (2) removal of growth-promoting nutrients from water, which are necessary for bacteria proliferation. Achievement of biologically stable drinking water by nutrient removal and without final disinfection is a common practice in for example Switzerland and The Netherlands [2, 3]. However, less is known about biological stability in the systems with disinfectant residuals. Disinfection is meant to kill microorganisms, thus in high concentrations of disinfection residuals, bacterial growth should not appear. However, the concentration of residual chlorine usually decreases in the DN, and therefore the ability to prevent growth diminishes. Some studies showed an increase of biomass in DN, even though disinfection was applied prior distribution [4, 5]. Moreover, several studies showed increased concentrations of assimilable organic carbon (AOC) after chlorination [6–8], which is the main nutrient source for heterotrophic bacteria. These controversial features led to a question about a role of chlorination in biological stability.

Latvia is a country in Northern Europe, where natural freshwater is available in quantities large enough to fulfill the needs of the country's population, but it has to be treated to meet legislative requirements for drinking water quality. Generally water quality of natural sources improved in the late 90s due to a decrease of agricultural and industrial wastewater discharge. However, a decrease of water consumption caused an increase of water age in large DN, for example in Latvia's capital Riga, and created favorable conditions for bacterial growth in the network [9]. The Riga DN consists of more than 1400 km of pipes, which makes this particular DN advantageous and interesting for biological stability investigations. Water retention time is long enough to provide bacterial growth if growth-promoting conditions are present in the network, which allows evaluation of spatial biological stability. Moreover, all water undergoes chlorination before entering the DN, which gives a possibility to study biological stability in a real full-scale chlorinated DN. Finally, water of different origins and treatments is supplying the same DN (Fig. 1): the DN in Riga is supplied from chlorinated groundwater and artificially recharged groundwater, and treated surface water, where the last two contain high amounts of humic matter and AOC, which can promote

bacterial growth. Therefore, a different growth potential was expected in drinking water originating from different sources, with particular interest in the areas where water from different sources can get mixed.
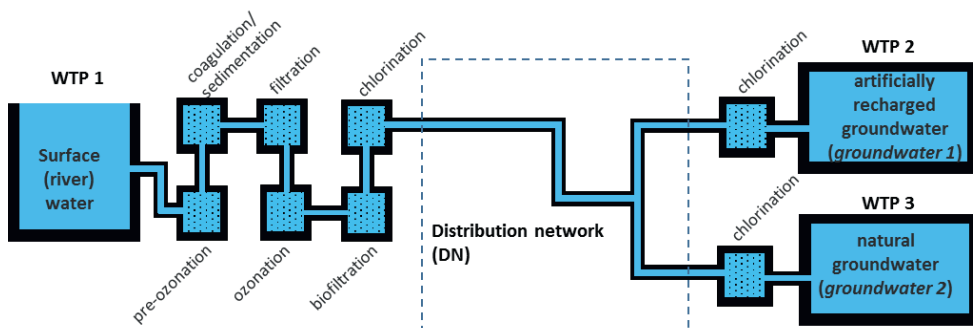

Fig. 1. A principal scheme of the investigated drinking water supply system.
The city was supplied from three main water treatment plants (WTP), which involve full-scale treatment of surface water (WTP 1), and natural and artificially recharged groundwater (WTP 2 & 3). Sampling at different stages of the treatment at three WTPs and more than 50 locations from the DN were included in the present study.

Existing methods for drinking water biological quality and biological stability evaluation have various drawbacks. For example, determination of dissolved and/or assimilable organic carbon provides only indirect evidence of growth risk and could lead to erroneous interpretations, if the water is limited by inorganic compounds. Hence, there was a need to develop a new approach to measure growth potential, based on the quantification of growth limitation caused by various nutrients. Biological instability in the Riga DN was reported in an earlier study, but a correlation between bacterial concentrations and water age in the Riga DN was observed using cultivation-based method for viable bacteria determination [9]. This approach is able to detect only about 1 % of indigenous water bacteria [10] and requires at least 24 h to obtain the results [11]. Accurate and reliable viability determination methods are essential for drinking water systems, where disinfection is applied, and especially for biological stability determination *in situ*. Therefore, a clear need for new methods and approaches existed: cultivation-independent methods for bacterial quantification had to be standardized, and a multiple-nutrient growth potential approach had to be modified and applied for biological stability investigations.

GOAL OF THE STUDY

The main goal of the study was to investigate whether chlorination (as final treatment step) is beneficial or detrimental for bacterial growth in a full-scale chlorinated drinking water distribution system. Various tasks were set in order to accomplish this study:
  • To optimize and standardize a flow cytometric viability method, which is based on SYBR Green I and propidium iodide (SGPI) fluorescent staining, and to test the method on chlorinated water samples.

- To investigate the feasibility of adenosine-triphosphate (ATP) analysis for characterization of chlorination efficacy.
- To apply the mentioned methods in a real full-scale drinking water distribution system with the aim to evaluate water spatial and temporal biological stability.
- To adapt, modify and apply a growth potential method for characterization of bacterial growth limiting factors in the Riga drinking water supply system.

DESCRIPTION OF METHODOLOGY AND THE MAIN CHAPTERS

Testing and optimization of the methods

Flow cytometric measurements of SGPI stained samples and determination of ATP were used for viability assessment as alternatives to the conventional HPC method for drinking water quality characterization and biological stability evaluation in this study. These methods are well-known in water research, but the protocols often differ between different studies. Moreover, while a range of commercial dyes and reagents can be purchased as of-the-shelve kits, they are not necessarily optimized for drinking water analyses. Hence, there is a clear and recognized need for protocol optimization and standardization, as well as for a mechanistic understanding of the applied methods. Increasingly routine applications of these methods require fixed protocols to produce reliable and comparative data across experimental studies.

A pipeline for developing staining protocols for flow cytometry demonstrated with SYBR Green I and propidium iodide viability staining
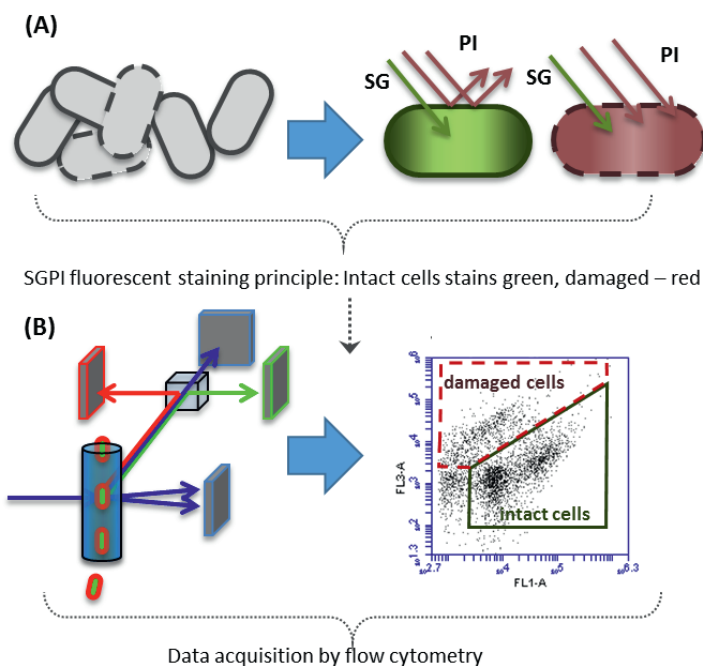
Fig. 2. Principle of viability assessment with SGPI staining and flow cytometric measurements.
When SGPI is added to the sample, cells with intact membranes will be stained green, as only SG could penetrate the cells. PI will penetrate the cells with damaged membranes, quench SG, and stain bacteria red (A). Naturally fluorescent or pre-stained water sample flows through the flow cell, where the cells cross the laser beam one by one. Forward and side scatter (blue), and fluorescence signals (red and green) are recorded electronically (B).

Application of fluorescent dyes combined with flow cytometric measurements (FCM) was often used for total cell count and cell viability determination in water research. It is assumed that bacteria lose vital functions when the cell membrane is damaged, since they cannot maintain electrochemical gradient. The SGPI staining principle is based on ability of SYBR Green I (SG) to penetrate all bacteria in the sample, while propidium iodide (PI) could enter only cells with a disrupted membrane, thus distinguishing between cells with the intact and damaged cell membrane (Fig. 2A). Flow-cytometric analyses represent measurements of light scatter and fluorescence, emitted of fluorescent probes when a linear stream of cells is passing a laser beam at a right angle (Fig. 2B).

A general pipeline for method optimization of staining for FCM in general, and for SGPI fluorescent staining in particular, was developed during this PhD project. River water samples without treatment and heat-treated or chlorinated samples were stained with SGPI, incubated within different conditions and measured with 40–60 s time intervals. The pipeline included testing of various parameters, such as the dye solvent, concentration of dye, staining time and temperature, which could severely affect staining outcome if not used correctly. For example, it was shown that DMSO (commonly used to dissolve FCM dyes) enhanced membrane permeability in autochthonous bacterial communities and resulted in erroneous data interpretation (Fig. 3); TRIS buffer was suggested as an alternative.
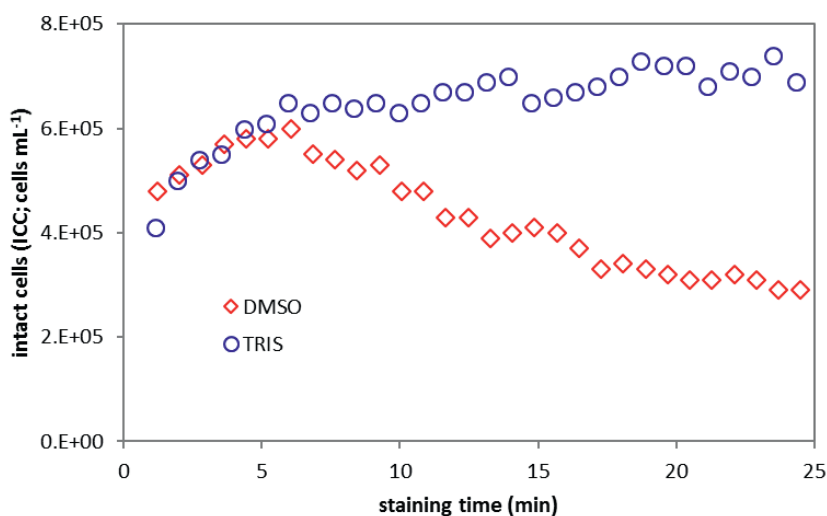

Fig. 3. The impact of TRIS buffer (pH = 8.1) and DMSO as solvents used for preparing dye working solution on SGPI staining.
River water samples were stained 25 min at 37 °C with 3 μM PI in 10,000 x diluted SG (n = 30) [12].

Similar tests demonstrated that PI addition below 3 μM resulted in incomplete staining of damaged cells, while concentrations higher that 12 μM resulted in false PI-positive staining of intact cells. We selected 6 μM as optimal concentration. Low temperatures (25 °C) resulted in a slow reaction and did not stain all bacteria, while 44 °C caused damage to cells and false PI-positive results. Hence, 35 °C was selected as optimal staining temperature. Approximately 12–15 min were needed to stain all cells and achieve stable results with

the selected concentrations and temperatures listed above. The addition of EDTA resulted in 1–39 % more PI-positive results compared to an EDTA-free sample, clearly calling for further research on whether EDTA should be used or not. The final outcome was a standardized protocol supported by scientific data from each step as well as a defined methodological approach to study and optimize other dyes in the future [12].

Assessing ATP analysis for chlorinated samples

ATP are the molecules that serve as the main energy carriers for many metabolic processes in cells together with other vital functions [13]. Thus, the presence of intracellular ATP most likely indicates the presence of viable microorganisms in a sample. However, ATP analysis has some potential pitfalls, notably, interference from extracellular ATP. In a water sample, ATP could be detected as intracellular ATP, which is associated directly with viable bacteria, and in extracellular environment or extracellular ATP (Fig. 4). Although the existence of extracellular ATP was already known, its origin was not completely clear, and sometimes the detection thereof was even attributed to methodological artifacts. Moreover, the method was usually applied during the studies of non-chlorinated water, when used for drinking water analyses, and was not properly tested for chlorinated water specifically. Thus investigation of ATP behavior and its stability during chlorination was necessary.
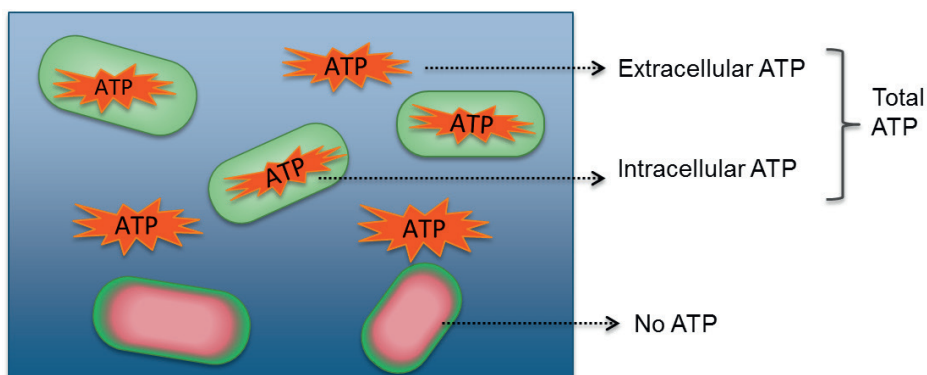

Fig. 4. Schematic demonstration of ATP presence in aquatic samples.
Total ATP consists of intracellular ATP in cells, which is an essential molecule for cell metabolism, and extracellular ATP. Bacteria without ATP are considered dead. Accurate bacterial viability measurements could be obtained by distinguishing intracellular and extracellular ATP.

To investigate the effect of chlorination ATP, an *Escherichia coli* suspension was diluted in filtered river water to a final concentration of approximately 3×10^6 cells mL^{-1}, then sodium hypochlorite was dosed to subdivided samples with initial free chlorine concentrations from 0.04 to 22.4 mg L^{-1} in the samples. Total and extracellular ATP were measured, and the same samples were stained with SGPI, and intact cell counts were obtained with FCM. Changes of ATP and intact cell count are demonstrated in Figure 5.
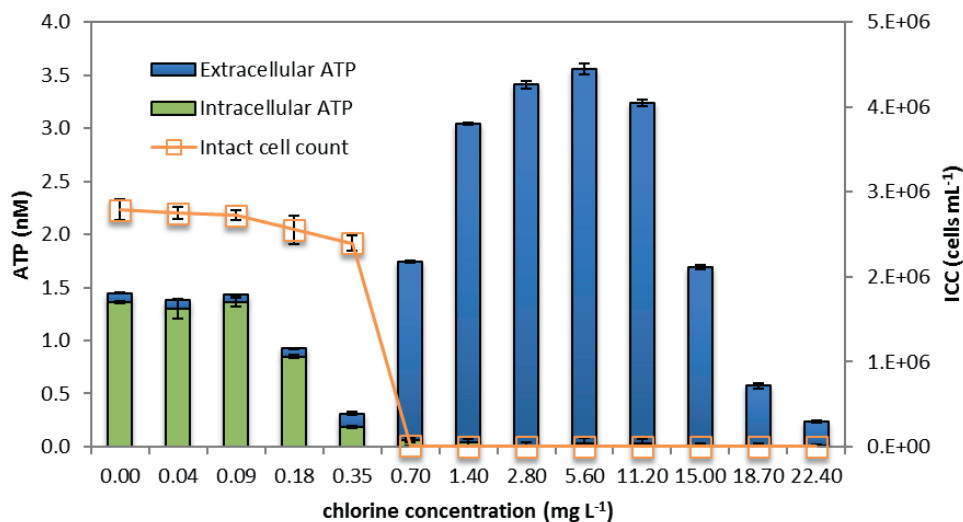
Fig. 5. Chlorine disinfection (5 min, room temperature) of an *E. coli* pure culture suspended in river water with different hypochlorite doses.
Stacked columns show the total ATP as the sum of intracellular ATP (green) and extracellular ATP (blue). Intact cell counts (ICC; circles) were measured by flow cytometry following SGPI staining. Error bars indicate standard deviation from triplicate experiments [14].

Release of ATP from damaged bacterial cells was observed during chlorination, but both the mechanism and extent of ATP release depended on the hypochlorite dosage. Five minutes exposure to high concentrations of chlorine (> 0.35 mg-Cl$_2$ L^{-1}) caused a considerable release of ATP from bacteria, while lower concentrations resulted only in a decrease of intracellular ATP without the release of ATP (Fig. 5). The dramatic increase in extracellular ATP coincided with an equally evident decrease in ICC to < 0.4 % of the initial concentration, indicating comprehensive disruption and damage of the bacterial cell membranes, and with the consequence that about 100 % of all ATP was extracellular. This observation suggests that ATP rapidly leaks out of the cells when the membrane is permeabilized to an extent detectable with SGPI staining and flow cytometric assessment. The same experiment with an indigenous river water bacterial community in river water produced similar results, suggesting that this behavior is universal for bacteria exposed to oxidative disinfection.

Several tests have been performed to study stability of extracellular ATP in the aquatic environment. The results showed that extracellular ATP molecules were stable in sterile environments for at least 20 h, but slowly decreased in filtered river water ($k = 0.051$ h^{-1} at 5 °C ($R^2 = 0.996$) and $k = 0.145$ h^{-1} at 30 °C ($R^2 = 0.999$)) by extracellular enzymes and/or the fraction of filterable (0.1 µm) bacteria. However, extracellular ATP decline was considerably faster ($k = 0.368$ h^{-1}; $R^2 = 0.96$) in the presence of an indigenous bacterial community. Interestingly, 12-h long experiment showed, that decreasing extracellular ATP coincided with increase of intracellular ATP (Fig. 6), which clearly indicated bacterial growth. Therefore, we conclude that a considerable fraction of the extracellular ATP was degraded and/or

taken up by growing bacteria. We tested an ability of drinking water bacterial community to consume or degrade ATP as a source of carbon and phosphorus specifically. The results showed that extracellular ATP was used as a source of phosphorus for bacteria, whereas carbon from ATP molecules was not utilized, suggesting a potential role of extracellular ATP in biological stability of drinking water.
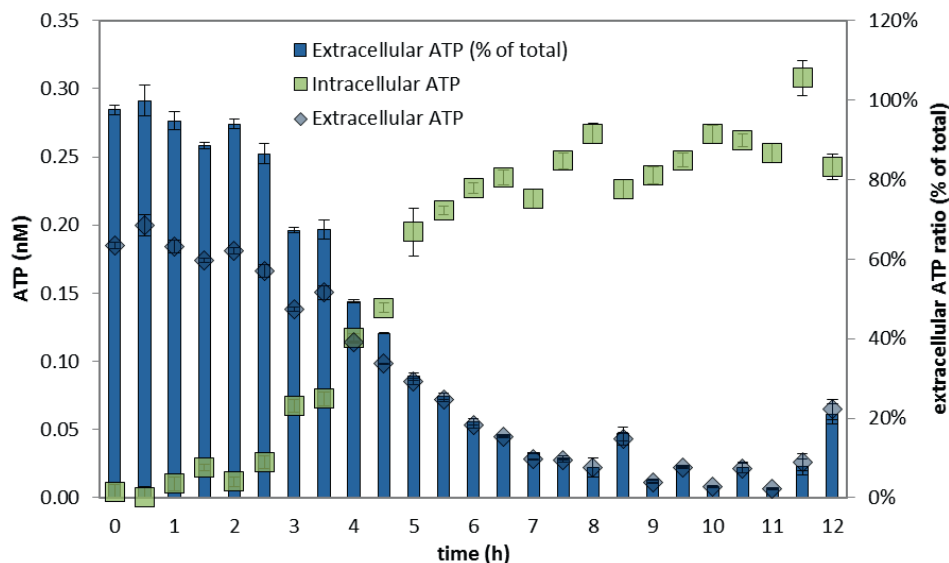

Fig. 6. Changes in intracellular and extracellular ATP during batch growth (12 h, 30 °C) of an indigenous river water bacterial community in filtered river water with natural-occurring extracellular ATP.
Extracellular ATP (diamonds) decreases concurrent with increases in intracellular ATP (squares), thus completely reversing the ratio of extracellular to intracellular ATP (bars) over time [14].

Considering ATP release during chlorination, it is evident that ATP released from bacteria at concentrations above a certain value (c.a. 0.2 g-Cl_2 g-DOC^{-1}), can be a source, albeit minor, of phosphorus for microbial growth and thus a contributing factor towards biological instability in (drinking) water. Evidently, the extent to which this can play a role in drinking water systems depends on the bacterial concentrations in both the biofilm and suspended phases prior to chlorination, as well as the applied chlorine concentration. The overall results underline the necessity to specifically determine intracellular ATP during analyses of the chlorinated water samples [14].

Case studies: Temporal and spatial biological stability assessment in Riga

The above-mentioned methods were applied to study biological stability in chlorinated drinking water DN in Riga. A total of 49 sampling sites were selected across the city to cover the network broadly and to include both proximal and distal zones relative to the treatment plants, in order to investigate spatial biological stability. These samples included three water treatment plants (WTP), which are supplied with natural and artificially recharged groundwater (WTP 2 & 3) and surface water (WTP 1) (Fig. 1, Fig. 7).
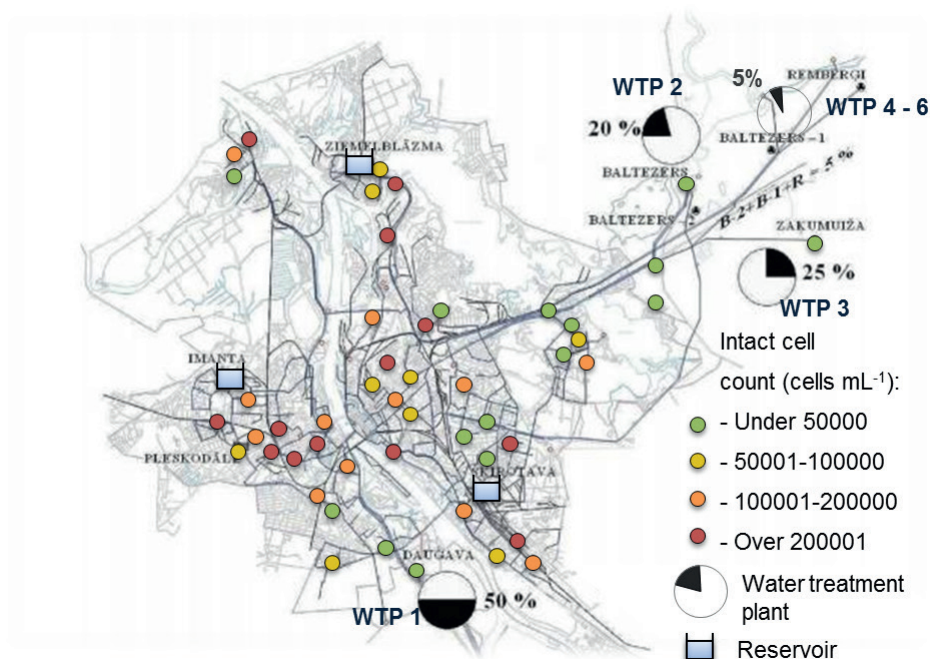

Fig. 7. Actual distribution of the classes of intact cells (colored circles) throughout the drinking water distribution network.
WTP 1, WTP 2, WTP 3 represent location and productivity of the main water treatment plans supplying the city: WTP 1 operates using surface water, WTP 2 – artificially recharged ground water, WTP 3 – natural groundwater. WTP 4 – 6 indicates on other three pump stations with less significance for the city water supply [15].

To examine the spatial distribution of growth/instability in the network, the data was divided into four broad categories based on the extent of growth and visualized on the sampling map (Fig. 7). The sampling points with the lowest intact cell concentration (less than $5{\times}10^4$ cells mL^{-1}) are marked with green bullets. Yellow and orange colored bullets indicate higher concentrations, while the points with the highest values (over $2{\times}10^5$ cells mL^{-1}) are shown as red bullets. Treated water contained between $1.84{\times}10^5 – 5.63{\times}10^5$ total cells mL^{-1} and between $9.7{\times}10^3 – 2.13{\times}10^4$ intact cells mL^{-1} (hence 2–5 % intact cells) depending on WTP. The data confirms effective final disinfection in all treatment plants. The total cell concentration values of the drinking water samples from the distribution network (n = 49) varied from $1.62{\times}10^5$ cells mL^{-1} to $1.07{\times}10^6$ cells mL^{-1} and the range of

the intact cell concentration was from 5.28×10^3 cells mL^{-1} to 4.66×10^5 cells mL^{-1} (3–59 % intact cells). Notably, 50 % of all samples contained more than 1.06×10^5 intact cells mL^{-1} corresponding to an increase of at least one order of magnitude in those samples compared to effluent water, which clearly shows that bacterial growth in the distribution network was not an isolated occurrence.

As could be expected, the map shows that the green colored points are mostly concentrated in areas close to the WTPs. In turn, higher ICC concentrations were mostly observed at the areas, distant from the WTPs, and in so-called "mixing" zones. These observations indicate on biological instability in the DN, and on potential bacterial growth. Relatively low ICC close to the WTPs could be explained by short water retention time, growth inhibition by residual chlorine, low probability of water stagnation due to high flow rate. The prevalence of the samples with higher cell concentrations in more distant areas could be related to the argument that increasing distance and water residence time could lead to chlorine decay and nutrient release due to oxidation; both these events would favor bacterial growth. Moreover, mixing zones are potential hot-spots for bacterial growth, as one water might well contain the nutrients that are growth limiting in the other. However, notably uneven distribution of bacteria, where relatively high and low ICC were measured within a small area, could be also related to temporary variations. For example, the results of the experiment, where the sample in the DN was monitored with ATP and FCM methods continuously during 21 h, showed that biological quality changed dramatically during the day (Fig. 8). The reasons of this could be related to changes in water consumption, which affects daily operation of reservoirs, different water retention time and influence bacteria detachment rate from biofilms.

The results of this study demonstrated biological instability in the DN, which was caused by bacterial growth in the network. Although there was no evidence that chlorination promoted bacterial growth at any degree, it was clear that residual chlorine could not prevent proliferation of microorganisms [15].
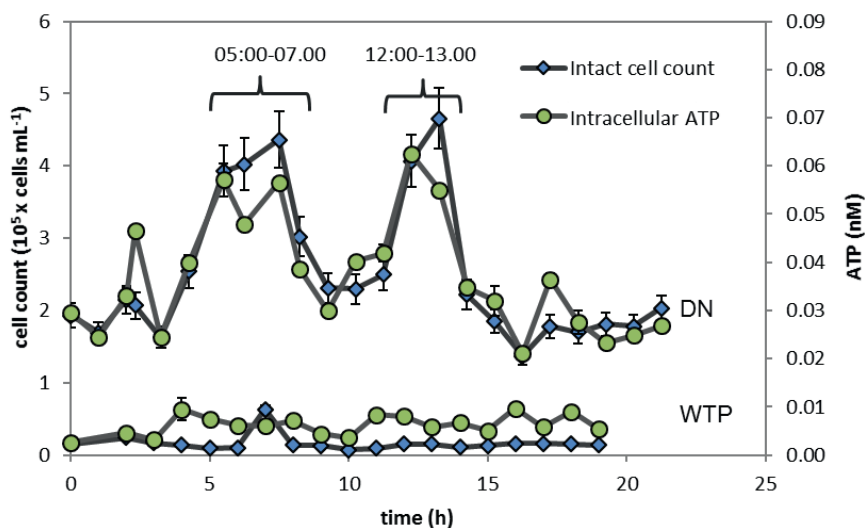

Fig. 8. Diurnal changes in bacterial parameters of WTP and DN points.
Intensive sampling of one WTP (n = 19) and one point in the DN (n = 23) during 21 hours reveals steady cell concentrations at the treatment plant but clear variations in the distribution network [15].

Case studies: Long-term biological stability study

While the first case study was limited to two-weeks sampling and focused on spatial instability, a follow-up investigation took place in the same distribution system in order to understand whether biological instability has a long-term effect, thus focused on temporal instability. TCC and ICC were measured at two locations in the DN, where sampling was performed every week during one year. One location was supplied mostly from WTP 1 (treated surface water), and the second likely had also an influence from groundwater (WTP 2 & 3).
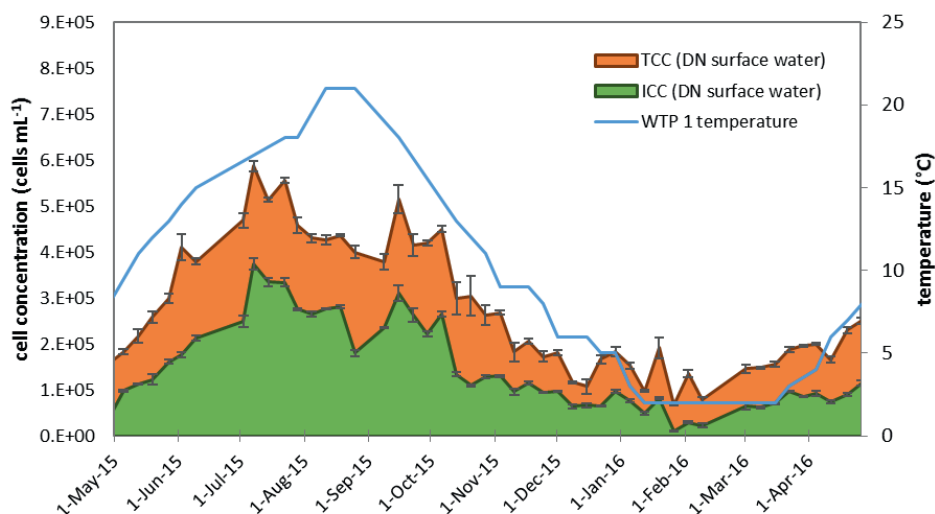

Fig. 9. Temporal bacterial fluctuations in the DN, which was supplied with treated surface water, and temperature of finished water at WTP 1 over long-term monitoring (n = 48)

Clear seasonal variations in TCC and ICC were observed in the drinking water distribution network samples over 12-months (n = 48) (Fig. 9). TCC values of the DN sample that originated from treated surface water varied from 0.67 to 5.87×10^5 cells mL^{-1}, and ICC values were from $0.1–3.75 \times 10^5$ cells mL^{-1}, which means that 1-log different concentrations of bacteria could be expected at different times of the year. One-year monitoring showed that generally higher bacterial concentrations were observed during warmer times of the year: all ICC and TCC values, which were higher than the average, occurred in a time period from May to October, and were lower in the remaining times of the year, following similar trend as the water temperature at the treatment plant. A strong correlation between bacterial concentrations and temperature at WTP 1 was obtained: TCC and temperature resulted in $R^2 = 0.82$, while $R^2 = 0.8$ was found between ICC and finished water temperature (n = 37). The results indicated that water temperature affected various processes, which further influenced drinking water quality in the network. Firstly, raw surface water had lower bacterial counts in winter, when water temperature dropped down. Then, bacterial concentrations at the biofilter effluent, which are the major source of bacteria in finished water, were significantly lower in winter, when water temperature was as low as 1 °C, in comparison with summer months and water temperature 21 °C. Finally, comparison of WTP 1 effluent and

corresponding DN samples showed less growth in the DN during winter in comparison to summer months. In contrast, groundwater was almost not affected by the seasons, and water temperature and bacterial concentrations were similar in summer and winter.

Overall results from this study suggest that drinking water quality and particularly biological stability should be evaluated with respect to different seasons and water origin. Thus seasonal fluctuations should be considered when defining a baseline for bacterial parameters in water, and operating water treatment plants (final disinfection).

Determination of growth-promoting nutrients in Riga DN

In previous chapters, we showed that spatial and temporal instability is present in the DN. Moreover, these observations indicate that the amount of residual chlorine was apparently not enough to prevent bacteria growth. On the other hand, growth could occur only in the presence of microbially available nutrients. While various studies showed that AOC could be formed during chlorination, it was discovered during this PhD project that bacteria release ATP during exposure to chlorine, which could be consumed by bacteria as a source of phosphorus. Therefore, a further goal of the thesis was to test whether chlorination could promote bacterial growth by releasing enough nutrients in the real drinking water distribution system.

Modification of the existing methods to determine microbially available nutrients was needed, because conventional methods were mostly focused on a single parameter (usually carbon), which could give inaccurate results if other nutrients were limiting, and were not able to compare the influence of different growth limiting/promoting compounds due to different concentrations, required for growth. The method, used in this study, was based on the combination of the growth potential method proposed by Prest and colleagues [16], and microbially available phosphorus (MAP) determination methods, proposed by Lehtola and colleagues [17]. Direct incubation of quenched and non-quenched water samples was used as a negative control, and an addition of other required nutrients in excess with respect to 1 mg L^{-1} AOC served as positive control. Exclusion of the target nutrient from the media allowed to determine whether this compound is present in the sample and until which extent it could promote bacterial growth in comparison with controls and other tested nutrients. Bacterial growth was determined after 3-day incubation at 30 °C with TCC measurements. The samples at three WTPs were taken before and after chlorination, and DN samples were chosen, considering water origin – treated groundwater or surface water. All samples were tested for carbon, phosphorus, nitrogen and iron limitation.
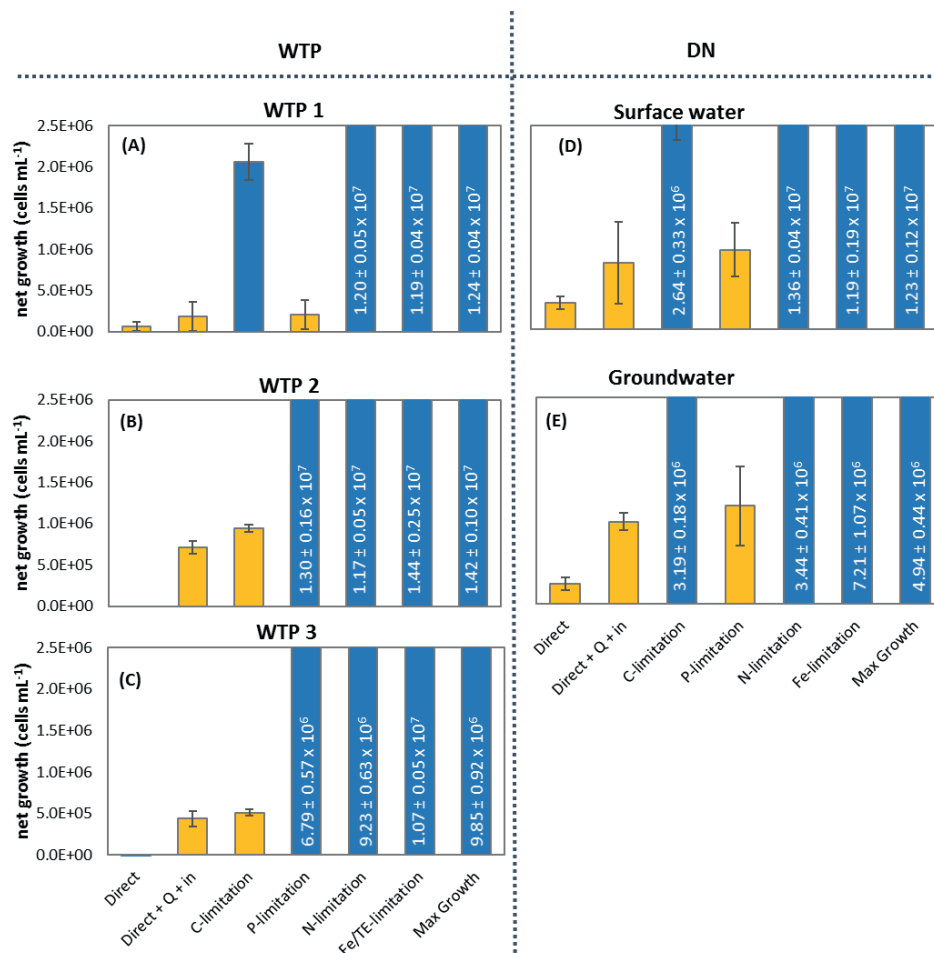
Fig. 10. Bacterial growth represented as changes in total cell count (TCC) after 72 h incubation.

Growth potential approach was tested for effluent water after chlorination at three WTPs originated from: (A) surface water (WTP 1); (B) artificially recharged (WTP 2) and (C) natural groundwater (WTP 3). The same approach was tested on the DN samples, which were supplied principally from (D) treated surface water; (E) treated groundwater. The control samples without addition of nutrients (direct and direct + Q +in) and with combination of nutrients, which resulted in the least growth, are marked yellow.

Direct incubation of the samples showed that treated water at all WTPs effluent did not promote bacterial growth as such, presumably due to the presence of sufficient chlorine residuals at this point. Interestingly, surface water and groundwater after treatment have different growth-promoting/growth-limiting properties. Phosphorus was the primary growth-limiting nutrient in the treated surface water sample (WTP 1) (Fig. 10A). When all other nutrients were in excess, the available phosphorus in the water could promote $2.07 \pm 1.75 \times 10^5$ cells mL^{-1} net growth, which was not statistically different from the result of the quenched sample $(1.83 \pm 1.78 \times 10^5$ cells mL^{-1}, P > 0.05) without additional nutrients (Fig. 10A).

In contrast, organic carbon was the primary growth-limiting nutrient in the treated groundwater samples (WTP 2 & 3) (Fig. 10B, 10C). The amount of carbon in groundwater from WTP 2 could promote $9.42 \pm 0.474 \times 10^5$ cells mL^{-1} net growth (94 µg AOC L^{-1}), and the net growth in WTP 3 without addition of carbon was $5.11 \pm 0.37 \times 10^5$ cells mL^{-1} (ca. 51 µg AOC L^{-1}), which was not statistically different from the quenched sample ($6.4 \pm 0.908 \times 10^5$ cells mL^{-1}, P > 0.05). Bacterial growth, obtained in conditions without additional phosphorus, was not statistically different from the samples with the presence of all nutrients, which shows that phosphorus was not a growth-limiting element in either groundwater.

Growth-promoting nutrients changed during distribution. Phosphorus became the primary growth-limiting nutrient in both surface water and groundwater DN samples (Fig. 10D, 10E). Phosphorus in the groundwater DN sample could promote only $1.19 \pm 0.474 \times 10^6$ cells mL^{-1} growth (10-fold lower than in WTP sample). Although phosphorus remained a growth-limiting nutrient in surface water, it could promote 5-fold higher bacterial growth in water from DN than directly after treatment: net growth without additional phosphorus in surface water DN sample was $9.76 \pm 3.29 \times 10^5$ cells mL^{-1}. Changes in the AOC concentration were observed in the groundwater sample, where more carbon was available after distribution ($3.19 \pm 0.18 \times 10^6$ cells mL^{-1}, or 3-fold higher than in WTP sample). However, the AOC difference between the surface water samples before and after distribution was not significantly different (P > 0.05).
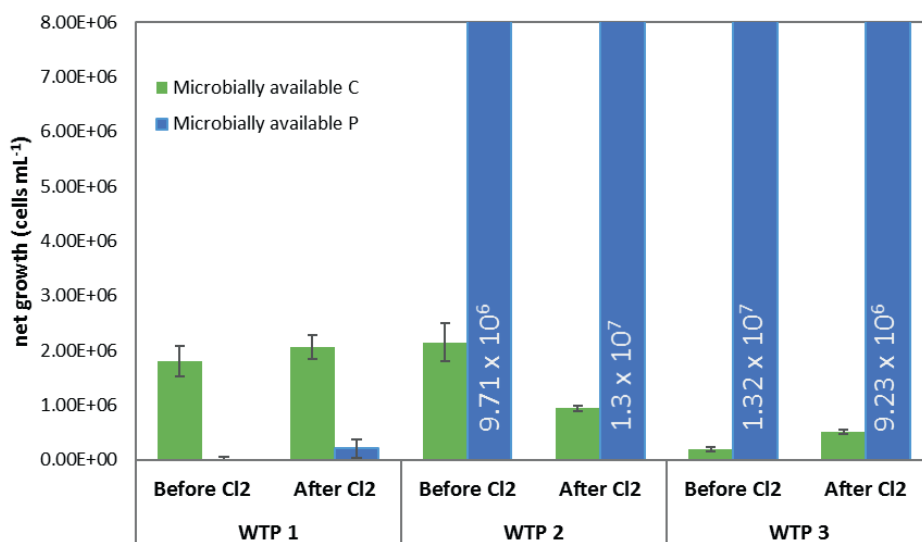
Fig. 11. Changes of growth-limiting nutrients after chlorination.

The effect of chlorination on nutrient release differed depending on the water type and treatment. The growth potential measurements of the samples, taken before and after chlorination, showed significant (P < 0.05) bacterial growth due to increased concentration of microbially available phosphorus at WTP 2, while the increase of microbially available carbon was significant at WTP 3 (Fig. 11), which was approximately 31 µg AOC L^{-1}. Changes of growth-promoting nutrients at WTP 1 due to chlorination were negative

(concentration decreased) or not statistically significant ($P > 0.05$). In terms of biological stability, chlorination posed a risk only at WTP 3, where an increase of growth-limiting carbon was observed. While phosphorus was produced during chlorination at WTP 2, water was not limited by phosphorus, thus we do not consider this as potential instability risk.

This data shows that water from different origin/treatment, distributed in the same network, has different and complementary growth-limiting nutrients. Although an increase of growth-limiting nutrients after chlorination was observed at WTP 3, the overall results indicate that nutrients that are produced during chlorination, are not likely a major cause of biological instability in the studied DN. Changes in biological stability properties suggest that transfer of nutrients is likely to occur in the system, which is supplied from water sources with different initial growth-promoting parameters. Thus potentially increased the risk of bacterial regrowth should be considered in such DN.

SCIENTIFIC NOVELTY AND
THE MAIN RESULTS OF THE STUDY

- Various parameters were tested for SGPI staining optimization, and some of the tests were performed using unique fully-automated flow cytometer. A general pipeline for staining optimization was developed. For this particular method, TRIS buffer was chosen for SGPI working solution preparation, 6 μM of PI was selected as optimal, 35 °C was the most appropriate staining temperature, and approximately 12–15 min were needed to stain all cells and achieve stable results with the selected concentrations and temperatures listed above. It highlighted the need for standardization in all viability protocols.
- The ATP method was tested specifically for chlorinated samples, and the disinfection mechanism was demonstrated in more detail than previously known. Five min exposure to high concentrations of chlorine (> 0.35 mg-Cl_2 L^{-1}) caused a considerable release of ATP from bacteria, while lower concentrations resulted only in a decrease of intracellular ATP without a release of ATP. Hence, disruption of the membrane as a result of chlorination causes the release of ATP, instead of ATP hydrolysis (= decrease of total ATP) within the cell.
- Extracellular ATP was used as a source of phosphorus for bacteria, whereas carbon from ATP molecules was not utilized, suggesting a potential role of extracellular ATP in biological stability of drinking water.
- Biological stability in the chlorinated DN was investigated in detail with novel bacterial viability determination methods:
    - the study included 49 sampling locations in the DN and WTP samples for spatial biological stability assessment;
    - two locations were analyzed weekly during one year for temporal biological stability assessment;
    - 21-h monitoring of WTP effluent sample and corresponding DN sample was performed, considering water retention time.
- Spatial instability was observed in the DN, where higher bacterial concentrations were detected in distant areas from WTPs. Notably, 50 % of all samples contained more than 1.06×10^5 intact cells mL^{-1} corresponding to an increase of at least one order of magnitude

in those samples compared to effluent water, which clearly shows that bacterial growth in the distribution network was not an isolated occurrence.

- Seasonal fluctuations were observed in the DN, with up to 1-log higher bacterial counts in warm months than in winter. This was related to generally lower initial microorganisms counts in raw surface water due to low water temperatures, and lower bacterial activity and growth in the DN and in biofilters at WTPs in winter months in comparison to the warm season. However, groundwater was affected by water temperature to lesser extent.
- A bacterial growth-potential method was modified and used for determination of biological instability in the DN. Finished water did not promote bacterial growth. However, different but complementing growth-promoting nutrients were observed in different types of water, which could increase a risk of drinking water instability in the DN. Moreover, drinking water biological stability and the amount of available nutrients changed during the distribution: treated surface water had up to 5-fold more microbially available phosphorus during distribution, and treated groundwater had 3-fold more assimilable organic carbon during distribution but 10-fold less phosphorus.
- The increase of growth-promoting nutrients was different depending on the WTP. While chlorination at the full-scale surface water WTP did not promote the release of nutrients, phosphorus was produced at WTP 2, and carbon (31 µg AOC L^{-1}) at WTP 3, and only the latter could actually affect biological stability. We believe that bacterial growth risk is the DN was rather related to low residual chlorine concentration and different but complementing growth-promoting nutrients in different water sources, which would create favorable growth conditions when mixed.

PRACTICAL IMPLICATIONS

ATP and flow cytometric SGPI staining methods proved to be fast, reliable and quantitative bacteria viability determination tools and were used for biological stability evaluation in the network and growth potential tests. The methods could be recommended for application in routine drinking water monitoring analysis in addition or, ideally, instead of HPC since in the case of real biological contamination danger, these would provide much faster response and give a possibility to prevent distribution of contaminant. Moreover, the methods could generate a lot of data within short time, which would provide better knowledge about drinking water system performance. Nowadays there is increased interest in modelling and so-called "early warning systems", which could be used for prediction of bacterial behavior and distribution in the drinking water DN, and notification to the responsible institutions in case of drinking water contamination. The collection of large amounts of data is essential for development of such models, and it is an obstacle for model development, based on biological data. Fast and descriptive ATP and FCM methods could help to solve this problem by producing the data, which could be integrated into the models in order to develop, improve and validate the systems. Moreover, automation of fluorescent staining and flow cytometric measurements is possible, which could be used for online drinking water monitoring and data collection in real-time.

Drinking water utilities should consider that FCM and ATP methods are not standardized and regulated by law. At the moment, these methods could be used only as additional methods to ensure drinking water quality by individual water utilities. The reasons for that

are that HPC is the only regulated standardized method for determination of bacteria in the drinking water samples, and only fecal indicator analyses are obligatory for tap drinking water [18, 19]. Interestingly, despite the well-known limitations of the HPC method, Council Directive 98/83/EC, which is the basis of Latvian legislation act, says that "*the methods used to analyse the quality of water intended for human consumption should be such as to ensure that the results obtained are reliable and comparable*" [18], while suggesting time-consuming and inaccurate HPC as bacteria determination method. Ideally, legislation of cultivation-independent methods should be evaluated.

Evidence of biological instability in the studied DN, and generally higher growth potential in the DN in comparison to WTP indicates that drinking water biological stability assessed at the WTP does not necessarily predict potential bacterial regrowth in DN. Thus ideally, growth potential should be evaluated at various sites in the DN in order to get an overall reflection of the situation and to identify the most problematic locations in DN, where concentration of bacteria and/or growth potential are the highest. These areas most likely represent distal places with long water retention time, "mixing" zones if more than one water source is supplying the network, sites, which are often supplied from water reservoirs, and low water consumption areas with long water retention time, for example, former industrial areas. Moreover, the one-year study demonstrates a strong seasonal tendency, however, it also differs depending on the location and water source. Such seasonal effects should be taken into consideration while monitoring DN samples: the same bacterial concentrations could be normal during warm season, but it would indicate contamination if measured in winter. Additionally, WTP performance could be optimized both from a safety and economy perspective. For example, chlorination could be minimized or canceled for surface water treatment in winter, when bacterial concentrations and growth in biofilters are low. Similarly, ozonation intensity could be reduced, when fewer microorganisms are present in raw water and if chemical water quality allows that. However, risk assessment of disinfection efficacy has to be evaluated in order to reduce disinfection. In contrast, with the presence of high amount of bacteria and during hot weather, chlorination could be intensified to prevent bacterial growth in DN.

An interesting finding of different growth limitations in different water sources supplying the same DN emphasizes the need to test several nutrients, which could relate to the biological stability concept. As was observed in the present study, different water can contribute to different growth limitations, which implies the potential additional growth problems in the areas of "mixing" waters, i.e. where water sources could change depending on hydraulic conditions and water consumption. Possible solutions in order to reduce the amount of microbially available nutrients should be considered. This could be achieved by installing additional treatment at groundwater WTPs, for example, biofiltration. In the case of effective removal of microbially available carbon and phosphorus, a possibility to cancel final chlorine disinfection could be evaluated. For example, our results showed that in the Riga DN, chlorination removed/inactivated a large part of bacteria during disinfection, whereas residual chlorine was too low to prevent bacterial growth further in the DN. While it is not likely that nutrients released during chlorination in the studied system could be a reason for overall biological instability in the DN, it is still questionable whether chlorination is necessary and useful in such a system. Moreover, examples of drinking water systems in Amsterdam and Zurich proved that water could be biologically stable without final

chlorination [2, 3]. Before making decisions on this, various actions should be undertaken. The main goal of disinfection is to kill pathogens and the absence of pathogens before distribution has to be checked and ensured. To be sure that stopping chlorination would not lead to pathogen contamination and bacterial growth problems, cancellation of chlorination could be realized by gradual reducing chlorine dose, with enhanced monitoring of carefully selected DN water samples. In contrast, the amount of chlorine could be increased to solve a problem of bacterial growth in the DN. However, it should be understood which type of bacteria proliferates in DN and whether it poses any risk. These considerations should be taken into account for the development of a water safety plan for Riga city.

Increased growth-promoting parameters in the real DN are likely caused by a combination of different factors, and it is clear that interactions between bulk water and pipe surface/biofilm play an important role in water chemical composition alteration too. Thus preventing actions, e.g. flushing of distribution pipes could be undertaken to improve water quality if high regrowth risk exists.

GENERAL CONCLUSIONS

The purpose of this thesis was to investigate biological stability of drinking water in chlorinated distribution networks, with the specific focus on the distribution system of Riga, Latvia. Spatial and temporal instability was observed in the chlorinated DN, which was clearly linked with bacterial growth in the DN.

Chlorination could produce growth-promoting nutrients under certain conditions. The amount of released nutrients is different, depending on the water type and quality. An increase of growth-limiting nutrients after chlorination was observed only at one WTP in the studied DN. Although we generally conclude that chlorination was not the main reason of biological instability in the Riga DN, it created favorable conditions for bacterial growth: chlorination did not result in significant release of nutrients to affect biological stability in this particular DN, however, neither it could prevent bacterial growth. Hence, growth of microorganisms most likely was caused by nutrients from different types of water.

ATP release during chlorination and ability of aquatic bacteria to use extracellular ATP as phosphorus source for growth were demonstrated in the study. It is assumed that at certain conditions, ATP, released during disinfection, could pose a risk of biological instability. While generally we proved that extracellular ATP was used for bacterial growth tests in the current project, future research is needed to understand whether ATP, released due to chlorination, could lead to higher bacterial numbers and change bacterial composition in comparison to non-chlorinated samples. Moreover, while this study was focused on chlorination of suspended bacteria, a possible influence of ATP, released from biofilm bacteria, should be investigated.

The importance of method optimization and proper interpretation of the results was demonstrated. The study on staining pipeline development and testing ATP analysis for chlorinated water allowed a better understanding of the mechanism of cellular death and the mechanism of the methods in general.

Different growth-promoting properties of different water types and changes their changes in the DN highlight an importance of evaluating their role and risks, related to

biological stability in mixed distribution systems. Future research is needed to understand the processes, which could influence nutrients transfer in such distribution networks.

Summarizing observations from our study, we generally conclude that the necessity of chlorination should be evaluated both from disinfection efficiency and biological stability prospective in every individual water supply system. Understanding of growth risks regarding water origin, the design of the DN, growth-promoting nutrients, seasons is mandatory to optimize water treatment, minimize bacterial growth and provide safe water to every customer.

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