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BIOLOGICAL STABILITY IN CHLORINATED DRINKING WATER DISTRIBUTION NETWORKS

Doctoral Thesis

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Contents

Annotation

The thesis “Biological stability in chlorinated drinking water distribution networks” investigates the factors and processes in the drinking water distribution network (DN), which could promote bacterial growth. The thesis is particularly focused on bacterial growth in chlorinated DN, since in previous works biological stability was studied mostly in non-chlorinated DN. While generally disinfection is meant to reduce bacterial concentrations, there is a hypothesis that by killing a significant fraction of bacteria and oxidizing organic matter, chlorination could create an environment favorable to bacterial growth. Riga drinking water supply system was suitable for such investigation, based on the fact that chlorination is common practice in Riga. Moreover, large and complex Riga drinking water DN is supplied from chlorinated surface water, artificially recharged and natural groundwater, which gave a possibility to study an influence of chlorination and different water sources on bacterial growth in the network.

The thesis consists of two main parts – testing and optimization of the methods for quantification of the viable biomass specifically for chlorinated samples, and two case studies, which investigated Riga DN to evaluate biological stability and to identify the reasons of bacterial growth. A general pipeline for SYBR Green I and propidium iodide (SGPI) staining method’s optimization for flow cytometric (FCM) measurements has been developed during the present work. Adenosine triphosphate (ATP) measurements were tested specifically for chlorinated samples, and used for biomass characterization of the distribution network samples. It was discovered that ATP was released from bacteria with high sodium hypochlorite dose, and extracellular ATP could be consumed by bacteria as a phosphorus source, thus contributing to biological instability of water. Application of these methods in the real chlorinated drinking water supply system showed temporal and spatial biological instability in the network. A modified microorganism growth potential approach allowed to determine growth-limitation during drinking water treatment and distribution, caused by various nutrients. It was demonstrated that carbon, released during chlorination of natural groundwater, could lead to increased bacterial growth in the DN. Moreover, we found that the main growth-limiting nutrients were different for the different types of water: treated surface water was phosphorus-limited, while carbon was the main growth-limiting nutrient in groundwater that contained phosphorus in excess. We overall conclude that nutrients released during chlorination is not the main reason of instability in the DN, but it creates favorable conditions for bacterial growth, whereas different water types with different growth-limiting compounds can pose a high risk of biological instability in the DN.

The thesis is written in English and consists of 116 pages, 55 figures, 7 tables and 182 literature sources were used for development of the thesis.

Anotācija

Promocijas darbs "Bioloģiskā stabilitāte hlorētos dzeramā ūdens sadales tīklos" aptver pētījumu par faktoriem un procesiem dzeramā ūdens sadales tīklā, kuri var veicināt baktēriju augšanu (vairošanos). Promocijas darba pētījuma objekts ir tieši hlorēta ūdens sadales tīkls, jo iepriekšējos pētījumos bioloģiskā stabilitāte pārsvarā tika pētīta nehlorētos tīklos. Kaut gan hlors iznīcina mikroorganismus, tiek uzskatīts, ka nogalinot lielāku daļu baktēriju un oksidējot organiskās vielas, hlorēšana var radīt baktēriju augšanai labvēlīgu vidi. Hlorēšanu izmantoto ūdens sagatavošanas procesā Rīgā, kas padara šo lielu un sarežģītu ūdensapgādes sistēmu par īpaši piemērotu pētījumam. Turklāt, ūdens, kas tiek padots uz Rīgas sadales tīklu, nāk no hlorēta virszemes ūdens, mākslīgi papildināta gruntsūdens un dabīga pazemes ūdens. Tādā veidā hlorēšanas ietekmi uz bioloģisko stabilitāti sadales tīklā var apskatīt atkarībā no ūdens avota veida.

Promocijas darba pētījumu var sadalīt divās galvenās daļās – dzīvotspējīgas biomasas novērtēšanas metožu pārbaude un optimizēšana, paredzēta tieši hlorētiem ūdens paraugiem, un Rīgas ūdens sadales tīkla izpēte ar mērķi novērtēt bioloģisko stabilitāti un noskaidrot baktēriju augšanas iemeslus. Promocijas darba izstrādes laikā tika izstrādāts vispārīgs SYBR Green I un propīdija jodīda (SGPI) krāsošanas metodes optimizēšanas protokols. Šī metode ļauj izšķirt dzīvas baktērijas no baktērijām ar bojātu membrānu, mērot ar plūsmas citometru. Adenozīntrifosforskābes (ATP) mērījumi tika pārbaudīti uz hlorētiem ūdens paraugiem, un tika izmantoti biomasas raksturošanai ūdens sadales tīklā. Tika novērots, ka augstas nātrija hipohlorīda devas rezultātā ATP tiek izdalīts no baktērijām, savukārt baktērijas var izmantot ārpus šūnas ATP kā fosfora avotu, tādā veidā apdraudot bioloģisko stabilitāti. Abas metodes tika pielietotas Rīgas ūdens sadales tīkla paraugiem, kas parādīja ka ūdens nav bioloģiski stabils, un baktēriju daudzums svārstās laikā un atšķīrās starp dažādām paraugu ņemšanas vietām. Ar uzlabotu baktēriju augšanas potenciāla metodi bija iespējams noteikt dažādas baktēriju augšanu limitējošas barības vielas. Tika noskaidrots, ka no dabīga gruntsūdens hlorēšanas rezultāta izdalītais ogleklis var veicināt baktēriju augšanu. Turklāt, galvenie baktēriju augšanu limitējoši parametri bija dažādi starp dažādiem ūdens avotiem: attīrītā virszemes ūdenī bija fosfora trūkums, bet ogleklis bija limitējošais abos pazemes ūdeņos, kur fosfors bija pārākumā. Mēs secinājām, ka hlorēšana nav galvenais iemesls bioloģiskās stabilitātes samazināšanai apskatītājā ūdensapgādes sistēmā, kā arī nenovērš baktēriju vairošanos tīklā. Savukārt ūdens no dažādiem avotiem ar atšķirīgām barības vielām var ievērojami paaugstināt baktēriju augšanas risku ūdens sadales tīklā.

Promocijas darbs ir uzrakstīts angļu valodā un satur 116 lappuses, 55 attēlus, 7 tabulas, un 182 literatūras avoti tika izmantoti promocijas darba izstrādē.

INTRODUCTION

Waterborne diseases' outbreaks, associated with drinking water distribution systems, are still common in 21st century even in developed countries. Biological drinking water quality deterioration in DN could be caused by external contamination and bacterial growth in the network. Bacterial proliferation in the DN and associated processes are attributed to biological stability concept. Biological stability is a quantification of the microbial quality of drinking water that implies no changes occurring in the concentrations, composition and viability of the microbial community in the water during distribution [1, 2]. From an operational perspective, this relates directly to drinking water treatment and distribution practices, of which a key point worldwide is the presence or absence of disinfection residuals (chlorine) in the distributed water. While a considerable amount of work has been done on biological stability in the absence of chlorine residuals, much less is known about the impact of disinfection practices on biological stability. Whereas chlorination is meant to kill microorganisms, at the same time, oxidative properties of the disinfection agent could lead to increase of nutrients, essential for bacterial growth.

This study was focused particularly on the chlorinated drinking water DN, which is supplied with water from different water sources, and different treatment is applied before distribution. Its ability to maintain sufficient microbiological quality was tested with cultivation-independent methods, which were specifically tested and optimized for chlorinated drinking water samples. Short-term, long-term and spatial changes in drinking water microbiological quality were monitored. A new bacterial growth potential approach was applied to test growth-limiting nutrients in different types of water and get more insight into the reasons of water quality deterioration. The thesis outline is following:

Chapter I represents a theoretical background of the studied matter, namely, drinking water quality, various challenges related to achievement of microbially safe drinking water, and relevance of the study in Latvia. Interaction between drinking water biological stability and disinfection is discussed in detail, and a goal and objectives of the thesis are defined.

Chapter II describes the problem of biological instability in a full-scale chlorinated drinking water distribution network. Biological stability was evaluated using cultivation independent FCM and ATP methods and conventional HPC method. Sampling was performed at different locations in Riga distribution network and at WTPs' effluent, thus spatial biological instability was observed. 24 h drinking water monitoring of DN and WTP effluent samples was performed in order to assess short-term biological instability. Moreover, applied bacteria determination methods are compared, and correlations between them, advantages and potential pitfalls are discussed.

In **Chapter III**, a need of accurate staining protocols is described, and an optimization pipeline of SGPI staining of natural treated and untreated water samples for flow cytometric measurements is defined. Detailed SGPI staining tests show influence of different dyes solvents, dye concentration, staining time and temperature on staining outcome. Moreover, addition of chelating agents, as well as various specific cases, where the method should be used with prudence, are discussed.

Chapter IV is focused specifically on ATP measurements of chlorinated natural water samples. Changes of intracellular and extracellular ATP with increasing chlorine concentration reveal a new chlorine disinfection mechanism. Stability of ATP in extracellular environment within different scenarios is described.

Chapter V investigates possible reasons of biological instability in the Riga water supply system. A one-year drinking water monitoring shows seasonal influence on drinking water quality. A modified bacterial growth potential method reveals important differences in growth-

promoting nutrient in different water sources, which supply the same network and might contribute to biological instability.

General discussion and recommendations summarize the results, obtained during the study, and discusse practical implementation of the presented approaches. The role of chlorination is discussed generally, and particularly in Riga drinking water supply system. The reasons of biological instability in Riga distribution network and potential solutions to improve water quality are discussed.

1. CHAPTER I: THEORETICAL BACKGROUND AND RELEVANCE OF THE STUDY

1.1. Drinking water quality

Drinking water quality could be evaluated by aesthetic, physically-chemical and microbiological parameters. Aesthetic properties include odor, taste, color and turbidity, which are extremely important from the customer point of view, as they are the measures of water acceptability. Any variations from normal aesthetic parameters are usually associated with poor water quality. Although these changes indeed are caused by chemical or biological processes, this does not obligatory indicate on health risks. And vice versa – contaminated and unsafe water not always could be detected by sense organs [3–5]. Temperature, pH and conductivity are classified to physical parameters. However, physical properties of water are mostly relevant for acceleration or inhibition of chemical and biological processes rather than posing a real health risk. In turn, chemical water quality requires more rigorous attention and regulation. Numerous hazardous organic and inorganic chemicals could be present in water, including heavy metals, nitrates, pesticides, disinfection byproducts. They differ by the type and origin, as well as their harmful effect on human organism [6, 7].

Nevertheless, biological contamination has higher impact on health in comparison with chemical water quality deterioration [7–9]. Although various distribution network's faults could lead to both chemical and biological contamination, most of the reported waterborne outbreaks were caused by agents of biological nature (Fig. 1.1). Moreover, outbreaks, which resulted in hospitalization and deaths, occurred also mostly because of microbiological contamination. Water-borne bacterial infections could target human gastrointestinal tract, but for example, *Legionella* species are spread by water droplets in the air and could cause lung diseases when inhaling. Thus occurrence and proliferation of such microorganisms is undesirable and should be prevented.

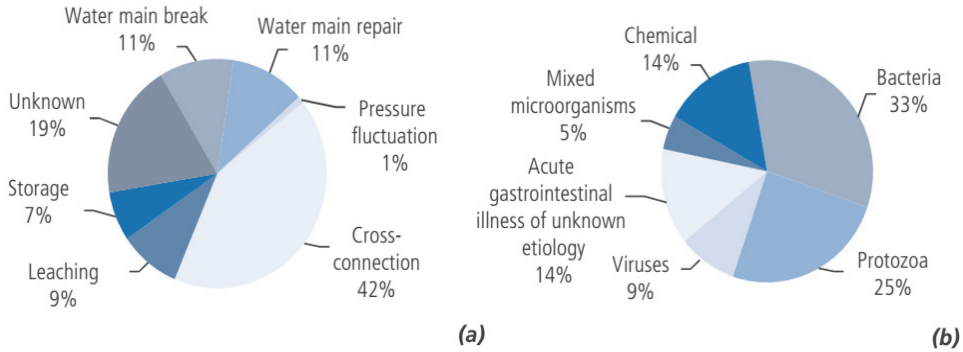


Fig. 1.1. Waterborne outbreaks associated with distribution systems in the USA, 1981–2010, by (a) system fault and (b) causative agent [10]

1.2. Challenges in drinking water distribution system

Although drinking water quality has to meet legislative requirements, and therefore often is treated before distribution, it can deteriorate on the way to the consumer, i.e. in a distribution network (DN). It is difficult to control processes in DN due to its size and complexity: DN consists of pipes, which could be hundreds and thousands kilometers long within the same DN, as well as storage tanks, pumps, reservoirs, valves, fittings, hydrants and other hydraulic elements [7]. Water requires many hours and sometimes several days in the DN to overcome the distance until it reaches customer [11]. Interactions between water bulk phase, pipe surface

and biofilms occur in the DN, and amongst other processes often are accompanied by an increase of water temperature, disinfection residual decay, corrosion, deposition of sediments and bacterial regrowth [1, 7, 11–13]. Deterioration of microbiological water quality particularly is associated with bacterial regrowth in DN or bacteria detachment from biofilms, if not caused by external contamination, and the extent of bacterial growth in both bulk water and biofilms depends on influent water quality and the previously mentioned processes in the DN. Although it is difficult to maintain even drinking water quality during distribution, it is important to study and understand potential risks in order to prevent or minimize its deterioration.

1.3. Drinking water biological stability

Meanwhile, drinking water should never be considered as sterile – numerous types of non-pathogen microorganisms are normally present in water and cause no harm to human organism. However, these communities could contain opportunistic bacteria species, which could be harmful in high concentrations to people with weakened immune system [14, 15]. Thus it is important to prevent bacteria proliferation and to aim drinking water biological stability in the network.

A strict definition of biologically stable water implies no changes in bacterial concentration and composition during drinking water distribution [1, 2]. Practically, drastic changes in bacterial concentration should not be allowed as this could pose a risk of proliferation of pathogen and opportunistic bacteria too. Biological stability could be evaluated directly by comparing microbiological parameters before and after distribution and could be defined as spatial (in)stability [1, 16–18]. Differences in bacterial concentration within the same location are characterized by temporal biological instability. These changes could have long-term effect, for example, seasonal changes, and short-term variations, which are mostly related to changing hydraulic conditions, which result in water stagnation or bacteria detachment from biofilms [18–20] (Table 1.1). Prediction of biological stability is obtainable by various growth potential tests, which goal is to assess whether water could promote bacterial growth. These tests vary from simply direct incubation of water sample and measuring bacterial growth, to determination of specific growth-promoting nutrients or conditions for particular organisms [21–25]. Understanding of factors and processes, influencing biological stability, allows to prevent bacterial growth in the network and improve drinking water safety.

Table 1.1.

Biological stability assessment principles and influencing factors in drinking water distribution systems

Biological stability in the DN			
Types	**Spatial**	**Temporal**	
		Short-term	**Long-term**
Assessment principle	Bacterial changes between: – WTP and DN – Different locations	Changes within short time period: – hours - a few weeks – High sampling frequency	Changes within long time period: – months - years – High or low sampling frequency
Primary influencing factors	– Distance – Water age	– Hydraulic conditions – Water consumption	– Seasons – Climate change

1.4. Production of biologically stable drinking water

Biological stability could be achieved by either bacteria inactivation prior and during distribution, or elimination of nutrients, necessary for microbial metabolism. However, this is not entirely clear how disinfection influences availability of nutrients in water. While some studies showed increased concentrations of assimilable organic carbon (AOC) due to chlorination [26–28], others did not see significant effect of chlorine or results were controversial [29, 30]. Moreover, drinking water with organic carbon as a growth limiting compound was investigated the most [27, 31, 32], while other nutrients and indirect factors were often ignored.

1.4.1. Disinfection

Disinfection is often applied as a part of the drinking water treatment or the only treatment during drinking water production process. Its main task is inactivation of pathogens in water and prevention of bacterial regrowth during distribution. Drinking water chlorination is the most common practice, applied in order to achieve these goals [33]. In terms of disinfection, chlorine refers to active compounds, which are formed in water from chlorine gas or hypochlorite salts, and it is highly effective against vegetative bacteria already in low doses, while higher concentrations can affect fungi, viruses, algae and bacterial spores [34].

Prevention of bacterial regrowth in distribution system is achieved by residual chlorine, which can be in free and/or combined form of chlorine. Free chlorine consists of hypochlorous acid and hypochlorite ions and is more active than combined chlorine in form of chloramines, thus often residual chlorine is described either by free chlorine or total chlorine, which is sum of free and combined forms of chlorine. Although effectiveness of chlorine against microorganisms is undoubted, it has certain limitations when used in drinking water distribution systems. It can cause corrosion of the metal pipes, and reactions with organic matter could lead to formation of disinfection by-products, which could be potential carcinogens, such as trihalomethanes and haloacetic acids [34, 35]. Additionally, odor and taste of chlorine deteriorate aesthetic water quality. Despite the fact, that chlorine concentration guideline value is as high as 5 mg L^{-1} [35], it should be minimized to reduce possible negative outcome. However, relatively low chlorine dose could lead to chlorine decay during distribution under recommended threshold of 0.2 – 0.5 mg L^{-1}, where its ability to kill bacteria and inhibit bacterial growth decreases [35–37]. Together with a risk of increased AOC concentration due to chlorination, as was reported in various studies [27, 28], it could reduce drinking water biological stability (Fig. 1.2). In this case alternative approach should be considered to ensure biological stability.

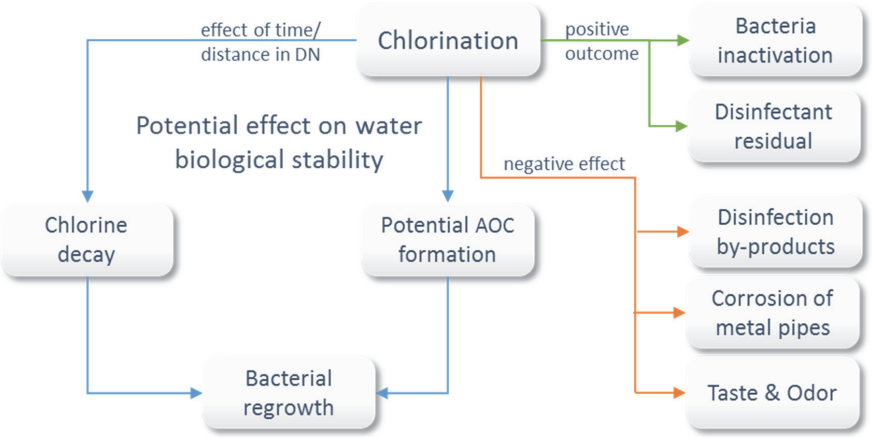


Fig. 1.2. Influence of chlorine disinfection on drinking water quality in distribution network

While main advantages and disadvantages are well known, effect on biological stability is still not clear

1.4.2. Assessment and control of growth-promoting nutrients

Elimination of nutrients in finished water is another solution to achieve biological stability. An obligatory requirement for bacterial growth is availability of energy source. Bacteria are classified into two main groups by the way they access carbon as an energy source. Heterotrophic bacteria consume readily available organic carbon, while autotrophic bacteria can produce organic carbon from other sources, including inorganic compounds. Most of human pathogens belong to heterotrophic bacteria, which is likely to be a reason why heterotrophic bacteria are controlled the most, and organic carbon in water is considered as a major growth-promoting nutrient for drinking water bacteria [32, 38, 39]. Moreover, bacterial elemental composition or nutrient demand corresponds to approximate 100:10:1 carbon:nitrogen:phosphorus (C:N:P) atomic ratio, where carbon is obviously predominant [2, 23].

Organic matter in water is described firstly by total organic carbon (TOC) and dissolved organic carbon (DOC), which represents a fraction of TOC without solid particles. However, only a fraction of DOC, which could be utilized by bacteria, or biodegradable organic matter (BOM), is important in terms of biological stability. Depending on the determination approach, biodegradable dissolved organic carbon (BDOC) and AOC are distinguished [23, 28, 40, 41]. Various modifications of the methods exist, however, the main principles are the following: BDOC is measured as a difference between DOC concentrations in water after contact with biomass, while changes in biomass are not considered. In contrast, AOC concentration is derived from bacterial growth yield after incubation. Both parameters are used for biological stability characterization, however, AOC approach has lower detection limit and generally better correlates with bacterial regrowth [28, 40, 42, 43].

Different guidelines were proposed to achieve biological stability. BDOC under 0.15 mg L^{-1} should not promote bacterial growth in drinking water without disinfectant residuals, and in chlorinated water this value is 0.25 mg L^{-1} [17, 44]. AOC thresholds are significantly lower: 10 µg L^{-1} AOC in drinking water systems without chlorination, and 50 – 100 µg L^{-1} AOC in the systems with disinfection residuals [23, 28, 32]. The most common and effective treatment for AOC and BDOC elimination in distributed water is biological filtration, where up to 90 % of BOM removal could be achieved [45, 46]. High efficacy of BDOC removal (97 %) could be also achieved by nanofiltration, however, AOC was not affected by this technology [40].

Although conventional growth potential assays, based on organic carbon, were thoroughly studied and applied for drinking water characterization, these methods could lead to erroneous conclusions if other than organic carbon compounds are growth-limiting in water [22, 47]. It is particularly important for drinking water supply systems, where organic carbon is present in excess, while it is limited by phosphorus, as was reported for drinking water in Finland, Japan and Latvia [47–49]. For example, phosphorus limitation can occur in the systems, where coagulation is used in drinking water treatment. The microbially available phosphorus (MAP) approach was developed to control phosphates, which could lead to bacterial growth in the network [21, 50]. Although there are no guidelines suggested for MAP concentration, it was shown that linear correlation with bacterial growth was observed starting with 0.05 µg of PO_4-P, in turn 1 µg of PO_4-P leads to 180 times higher bacterial count than 1 µg AOC, indicating that MAP should be maintained as low as possible [21].

Although organic carbon and phosphates are likely the most influencing compounds in drinking water, regarding biological stability, other inorganic matter could also promote growth. Thus it is important to evaluate biological stability, considering multiple nutrients. Prest et al. presented an approach to describe growth potential, which allows to determine whether organic, inorganic or multiple limitation is in the sample [22]. Undoubtedly it provides better insight of biological stability, however, it could be advantageous to modify the method in order to determine various specific growth-limiting compounds.

1.5. Bacteria enumeration for biological stability assessment and drinking water microbiological monitoring

Accurate, reliable and fast bacteria enumeration methods are essential for drinking water monitoring and for biological stability evaluation. However, conventional cultivation-based methods, or heterotrophic plate count (HPC), are often the only legislated methods for drinking water monitoring in many countries including Latvia [51, 52]. The method is based on bacteria cultivation on the artificial media, and subsequent counting formed colonies, which represent viable microorganisms. Cultivation can take from 1 day up to 2 weeks to obtain the results [38], which is too long time to undertake actions in case of microbiological contamination. Another disadvantage of the method is related to the fact that only small fraction (< 1 %) of water indigenous bacteria could be cultivated on plates [31, 53]. Moreover, bacteria could enter viable but nonculturable (VBNC) state, when undergo stress, including a presence of disinfection agent [54, 55]. Hence using alternative to HPC methods should be considered for drinking water monitoring, and chlorinated drinking water monitoring particularly.

1.5.1. Fluorescent staining and flow cytometry

Application of fluorescent dyes combined with flow cytometric measurements (FCM) was often used for total cell count and cell viability determination in water research [53, 56–58]. Depending on the analysis, preparation of the sample takes only 10 – 30 min, while measurement time depends on the instrument and normally it takes less than 5 min. Flow-cytometric analyses are based on the measurement of light scatter and fluorescence, emitted of fluorescent probes, when a linear stream of cells is passing a laser beam at a right angle [59] (Fig. 1.3), thus counting all cells in the sample. Another advantage of the method is a possibility to use it remotely, as far as fully automated online flow cytometry systems have been already successfully approved for drinking water monitoring [19, 60].

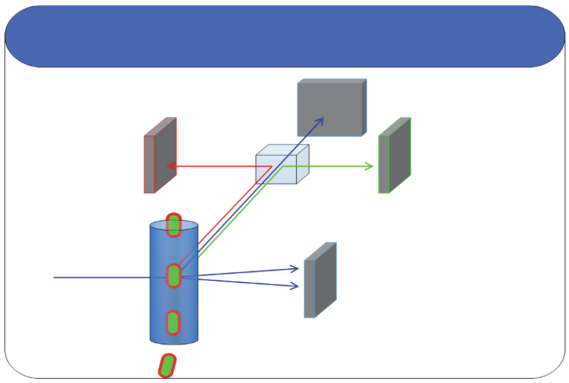


Fig. 1.3. A principle of two-color flow cytometric measurement

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 scatters (blue), and fluorescence signals (red and green) are recorded electronically.

While untreated drinking water could be well described by total bacteria concentration, viability assessment is essential for disinfected water to control treatment efficacy. SYBR Green I® (SG) is fluorescent membrane permeable dye used for determination of total cell count (TCC). SG binds to cell DNA, and emits green fluorescence when excited by blue laser (λ = 488 mm). In turn, propidium iodide (PI) is membrane impermeable dye, which could be used alone or in combination with counterstains. PI could bind to DNA only when the cell membrane is damaged, resulting in emission of red fluorescent light after excitation. The cells with damaged membrane are considered not viable, since they cannot maintain electrochemical gradient [61]. In contrast, PI-negative bacteria indicate on intact membrane, and on potentially viable cells accordingly.

SG and PI dyes together (SGPI) in combination with FCM were often used for intact and damaged cells determination [56, 57, 67–63]. Nevertheless, SGPI staining methods differ in PI concentrations, staining time, and temperature is often not defined. Optimization of viability method is particularly important, because improper staining parameters could lead not only to incomplete staining, but result in membrane damage and staining artifacts. Previous studies demonstrated testing of PI concentrations and staining time, but to our knowledge no literature was available on working solution solvents test, and only one work showed testing staining temperature [61, 63–65]. Although by no means a strict method should be applied to any sample, these observations show a clear need to develop a pipeline for staining protocol optimization.

1.5.2. Adenosine triphosphate (ATP) measurement

ATP is a molecule, which is present in all living cells. Besides other functions, ATP molecules are the main energy carriers for many metabolic processes in cells [66]. Thus the presence of intracellular ATP most likely indicates the presence of viable bacteria in a sample. ATP analysis was argued to be a useful and prospective tool for bacterial viability determination in both chlorinated and non-chlorinated drinking water environments [18, 67–69].

ATP is measured as luminescence, produced as a result of a special enzyme (luciferase) and ATP reaction. Important advantage of the method is that analysis takes only about 5 minutes per sample with the preparation steps. However, one should be aware that ATP could be detected in a free form in extracellular environment, especially when water samples are measured (Fig. 1.4). While in untreated water with relatively low extracellular ATP, it could be acceptable to measure only total ATP in the sample, in many cases extracellular ATP (or

ignoring this) could have potential pitfalls [67, 70–72]. For these reasons, intracellular and extracellular ATP are often measured separately. In the method, used in the current work, intracellular ATP was obtained as the difference between total ATP and extracellular ATP, separated by 0.1 μm filtration. [67].

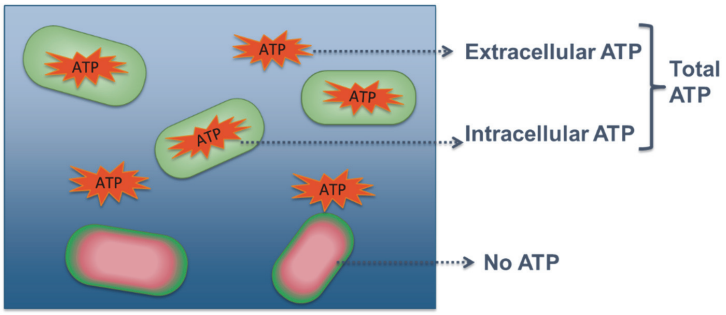


Fig. 1.4. Schematic demonstration of ATP in drinking water sample
Total ATP consists of intracellular ATP in cells, which is essential molecule for cell metabolism, and extracellular ATP. Bacteria without ATP are considered dead. Accurate bacterial viability measurements could be obtained by distinguishing intracellular and intracellular ATP.

Although the existence of extracellular ATP was observed and described already more than three decades ago in both seawater and freshwater environments [70, 73], its origin and stability are still unclear. Some studies have argued that extracellular ATP originates from lysed and stressed cells, or that ATP is released and excreted by higher organisms, or secreted by some types of bacteria during growth [70, 73–75]. Moreover, previous studies suggested that drinking water treatment processes such as oxidative disinfection resulted in considerable increase in extracellular ATP [76, 77]. This raises question regarding ATP method's feasibility to monitor disinfection efficiency as alternative to cultivation based methods, the origin and stability of extracellular ATP, and the impact thereof on drinking water quality.

1.6. Relevance of biological stability study in Latvia

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. Although water quality of natural sources generally improved in late 90-s due to decrease of agricultural and industrial wastewater discharge, water age in a large distribution network on the example of Latvia's capital Riga increased as a result of decrease of water consumption, and caused bacterial growth in the network [78]. Moreover, the distribution network in Riga is supplied from groundwater, artificially recharged groundwater and surface water, where the last two contain high amount of humic matter and assimilable organic carbon (AOC) [24, 48, 78], which can cause biological instability. However, organic carbon provides only indirect evidence of regrowth risk, and different growth potential is expected in drinking water, originated from different sources, with particular interest in the DN, where water can get mixed. Correlation between bacteria concentration and water age in the Riga DN was observed using cultivation-based method for viable bacteria determination [78], which has certain limitations as was discussed before. Finally, chlorination is applied as a final disinfection at all WTPs. Hence, evaluation of biological stability in the Riga DN is a well-suitable site to investigate influence of chlorination, different water sources and treatment on bacterial regrowth in the network, and to test cultivation independent bacteria determination methods in the real full-scale chlorinated drinking water supply system.

1.7. Objectives of the thesis

The main goal of the study was to investigate whether chlorination is beneficial or detrimental for bacterial growth in chlorinated drinking water distribution network. Various tasks were set to accomplish this study:

- To optimize flow cytometric viability method, which is based on SYBR Green I and propidium iodide (SGPI) fluorescent staining, and to test them on chlorinated samples.
- To investigate the feasibility of adenosine-triphosphate (ATP) method for chlorination efficacy characterization.
- To apply the mentioned methods in a real full-scale drinking water distribution system with the aim to evaluate water biological stability.
- To adapt, modify and apply microorganisms' growth potential method for characterization of bacterial growth limiting factors in Riga drinking water supply system.

2. CHAPTER II: BIOLOGICAL INSTABILITY IN A CHLORINATED DRINKING WATER DISTRIBUTION NETWORK

2.1. Introduction

The goal of public drinking water supply systems is to produce water of acceptable aesthetic and hygienic quality and to maintain that quality throughout distribution until the point of consumption. From a microbiological perspective, the quality of treated water can deteriorate as a result of excessive bacterial growth, which can lead to problems such as a sensory deterioration of water quality (e.g. taste, odor, turbidity, discoloration) as well as pathogen proliferation [24, 25, 31, 38, 76, 79–83]. To avoid this, biological stability during distribution can be achieved by maintaining sufficient residual disinfectants in the water, and/or through nutrient limitations [17, 31, 32, 82]. However, drinking water systems should not be viewed as sterile; complex indigenous bacterial communities have been shown to inhabit both chlorinated and non-chlorinated drinking water distribution systems [1, 27, 80, 84–86].

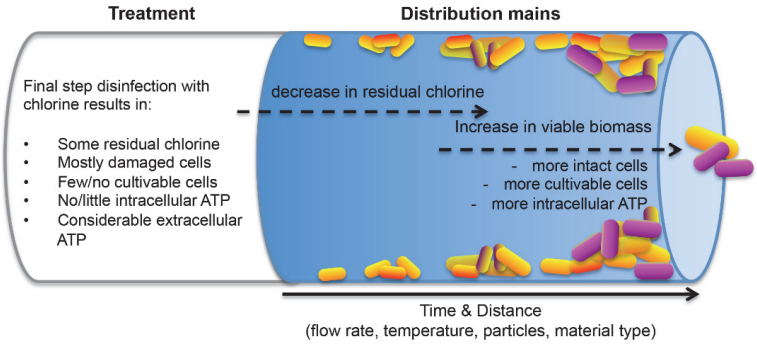


Fig. 2.1. A worst-case-scenario in an unstable, chlorinated distribution network
Prediction of changes in the microbiological state of the water due to the depletion of residual chlorine and the concomitant growth of bacteria, potentially resulting in hygienic and sensory deterioration of the water quality.

The concept of biological stability and its impact on a system's microbiology has been discussed extensively in the framework of non-chlorinated drinking water distribution systems [1, 18, 31, 69, 82, 87]. However, many treatment plants worldwide employ a final disinfection step to ensure that no viable bacteria enter the distribution system. The latter is often achieved by oxidative disinfection, usually by chlorination [88]. Disinfection has a number of implications for a biological system. During chlorination, one can expect that a considerable fraction of bacteria in the water are killed or damaged, while some residual chlorine may remain in the water (Fig. 2.1). This could be visible through numerous microbial monitoring methods. For example, the number of cultivable bacteria, measured with heterotrophic plate counts, would reduce dramatically [89, 90]. Secondly, bacteria cells are likely to display measurable membrane damage irrespective of their cultivability [91], though the rate and extent of damage may differ between different communities. This would be detectable with several staining techniques coupled with epifluorescence microscopy or flow cytometry (FCM). Also ATP, often used as an alternative cultivation-independent viability method [18, 67, 89], will be severely affected. Based on data from Hammes and co-workers [64] one may reasonably expect increased levels of extracellular ATP (so-called free ATP) and decreased concentrations of intracellular ATP (bacterial ATP) following oxidative disinfection. Irrespective of the detection method, the overall consequence of disinfection is a considerable decrease in the viable biomass, potentially opening a niche for microorganisms to occupy downstream of the

treatment process. Following initial disinfection, residual chlorine might provoke undesirable changes during drinking water distribution. Disinfectants target not only bacteria, but it also react with natural organic matter, pipe surfaces and particles in the network, thus potentially forming/releasing assimilable organic carbon (AOC) [26, 28, 30, 92, 93]. AOC can easily be consumed by bacteria, and is therefore seen as a main contributor to biological instability. Moreover, chlorine decay within the network negatively affects its ability to inhibit microbial growth at the far ends of the network [17]. If all factors were considered, the presence of nutrients, a reduction in the number of competing bacteria, and the lack of residual disinfectant would potentially lead to biological instability in the distribution network, manifesting in a subsequent bacterial growth (Fig. 2.1). Besides the importance of nutrients, the extent of bacterial growth will be influenced by a number of factors. For example, increased water temperature can accelerate chlorine decay and favor bacteria growth [18, 94], while changes in hydraulic conditions can alter nutrient supply for microorganisms in biofilms and/or bacteria detachment from the pipe surfaces [95, 96]. Finally, the quality of materials in contact with drinking water, as well as the presence of sediments and loose deposits, can both affect the general microbial quality of the water [81, 97, 98].

In the present study we examined some of the above-discussed concepts in a full-scale, chlorinated distribution system in the city of Riga (Latvia) with a number of microbiological methods. The purpose was a detailed investigation of the entire city's distribution network, asking the basic question whether evidence of spatial and/or temporal biological instability exists, and if so, to which degree. Additional goals were to evaluate the use of fluorescent staining coupled with FCM, as well as ATP analysis, for the assessment of chlorinated drinking water in a distribution network with disinfectant residuals.

2.2. Materials and Methods

2.2.1. Description of study site

Sampling was performed in the full-scale distribution network of Riga (Latvia) with a total length of about 1400 km. The city is supplied with drinking water from six water treatment plants (WTP) produced from both surface and groundwater (150 000 m^3 d^{-1}). Only the three major WTP, which are continuously operated, were included in the sampling campaign. Average WTP effluent water quality parameters for each treatment plant are shown in Table 2.1. The distribution network mainly consists of cast iron (80 %) and unlined iron (15 %) pipes as old as 50 years. The diameters of pipes ranged from 100 to 1200 mm. Three reservoirs are operated in the network to compensate for fluctuations in the daily water demand, while four high-pressure zones are maintained in some distal areas of the network. The high-pressure zones were excluded from the present study. 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. The sampling sites were selected according to the approximate water retention times obtained from a validated hydraulic model made in EPANET 2.0 [99, 100] based on a total length of 538 km (39 % of the total length of the network). Apart from the effluents of the three treatment plants, the sampling sites were in all cases fire hydrants in order to attain some degree of reproducibility between sampling and to avoid localized effects (e.g. household growth). The exact locations of sampled fire hydrants can be obtained from the authors after agreement from the local water utility.

Table 2.1.

Average water quality parameters for the final effluents of the three main treatment plants of Riga (Latvia) [77]

	WTP 1	WTP 2	WTP 3
Source water	surface water	artificially recharged groundwater	groundwater
Final treatment step[a]	Cl_2 (0.5–3 mg L^{-1})	Cl_2 (ca. 1.5 mg L^{-1})	N.A.
Residual chlorine (mg L^{-1})[b]	0.44±0.11	0.51±0.01	0.42±0.26
Total organic carbon (TOC) (mg L^{-1})[a]	6±1	9±3	3
Assimilable organic carbon (AOC) (μg L^{-1})[a]	213±37	209±59	N.A.
Total cell concentration (cells mL^{-1})[b]	$5.31\pm0.97\times10^5$	$5.45\pm0.47\times10^5$	$1.69\pm0.18\times10^5$
Intact cell concentration (cells mL^{-1})[b]	$1.83\pm1.18\times10^4$	$1.4\pm0.86\times10^4$	$1.03\pm0.68\times10^4$
Total ATP (nM)[b]	0.015±0.005	0.029±0.004	0.011±0.002
Intracellular ATP (nM)[b]	0.007±0.003	0.000±0.004	0.001±0.002
HPC 22°C (CFU mL^{-1})[b]	23±24	4±2	4
HPC 36°C (CFU mL^{-1})[b]	16±16	4±2	1
Conductivity (μS cm^{-1}), 25°C[a]	468±101	625±4	272±25
pH[a]	6.63±0.18	7.41±0.04	7.5±0.05

[a]Data supplied by the water utility or measured in previous sampling campaigns.
[b]Data from present study.
doi:10.1371/journal.pone.0096354.t001

2.2.2. Sampling protocol

A specific sampling protocol was designed and followed in order to avoid artifacts due to water stagnation in unused fire hydrants. Each hydrant was pre-flushed at a high velocity (never exceeding 1.6 m s^{-1}) for no more than 60 s, then immediately adjusted to a low velocity of 0.015 – 0.25 m s^{-1} and connected to an online system for monitoring pH, temperature, redox potential, electroconductivity and turbidity. The low sampling velocity was specifically used to ensure a minimal possible impact of cell wall erosion and detachment from biofilms on the samples and measurements. Readings of all parameters were taken at 5 – 10 minute intervals, and water was only sampled for microbiological analysis once all of the parameters stabilized. The impact of this hydrant flushing is demonstrated in an example in Fig. 2.2, Table 2.2 and discussed in detail in the results section. Samples were kept in cold storage (c.a. 5 °C) and analyzed within four hours of sampling.

Table 2.2.

Physical and chemical parameters of water measured on-line during flushing of newly-opened fire hydrant. Some measurements were omitted during the first 20 minutes of flushing due to the high fluctuation in measuring tools readings

Flushing time (min)	pH	Redox (mV H2)	EC (µS/cm)	Turbidity (FTU eq.)	Temperature (°C)
5	-	-	-	128.84	-
10	7.73	7	484	18.613	15.5
15	-	-	-	142.499	-
20	-	-	-	9.714	-
25	7.88	94	525	8.547	15.1
30	7.81	85	524	6.349	15
35	7.77	87	528	4.234	14.9
40	7.77	82	530	2.764	14.9
45	7.76	78	533	1.923	14.9
50	7.77	73	535	1.772	14.9
55	7.76	77	530	-	14.8
60	7.75	78	530	-	14.9

2.2.3. Chemical analysis

Determination of free chlorine was performed according to standard method EN ISO 7393-1, based on the direct reaction with N,N-diethyl-1,4-phenylenediamine (DPD) and subsequent formation of a red compound at pH 6.2 – 6.5. Afterwards titration by means of a standard solution of ammonium iron (III) sulfate until disappearance of the red color was performed. Determination of total chlorine was performed according to EN ISO 7393-1, based on the reaction with DPD in the presence of an excess of potassium iodide, and then titration as described above.

2.2.4. Fluorescent staining and flow cytometry (FCM) of water samples

Staining and FCM analysis was done as described previously [76, 101]. In short, for a working solution, SYBR® Green I (SG) stock (Invitrogen AG, Basel, Switzerland) was diluted 100x in anhydrous dimethylsulfoxide (DMSO) and propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution to a final PI concentration of 0.6 mM. This working solution was stored at -20 ºC until use. From every water sample, 1 mL was stained with SGPI at 10 µL mL^{-1}. Before analysis, samples were incubated in the dark for 15 minutes. Prior to FCM analysis, the water samples were diluted with 0.22 µm filtered bottled water (Evian, France) to 10 % v/v of the initial concentration. FCM was performed using a Partec CyFlow SL instrument (Partec GmbH, Münster, Germany), equipped with a blue 25 mW solid state laser emitting light at a fixed wavelength of 488 nm. Green fluorescence was collected at 520 ± 10 nm, red fluorescence above 630 nm, and high angle sideward scatter (SSC) at 488 nm. The trigger was set on the green fluorescence channel and data were acquired on two-parameter density plots while no compensation was used for any of the measurements. The CyFlow SL instrument is equipped with volumetric counting hardware and has an experimentally determined quantification limit of 1000 cells mL^{-1} [76].

2.2.5. Adenosine triphosphate (ATP) analysis

Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA, USA) as described elsewhere [67]. A water sample (500 µl) and the ATP reagent (50 µl) were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were then combined and then the luminescence was measured after 20 sec reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). For extracellular ATP analysis, each sample was filtered through a 0.1 µm sterile syringe filter (Millex®-GP, Millipore, Billerica, MA, USA), followed by analysis as described above. The intracellular ATP was calculated by subtracting the extracellular ATP from the total ATP for each individual sample. ATP was measured in duplicate for all samples.

2.2.6. Heterotrophic plate counts

To obtain heterotrophic plate counts (HPC), samples were serially diluted in sterile distilled water and then inoculated onto nutrient yeast agar plates using the spread plate technique. All plates were incubated in dark at 22 ºC or 36 ºC for 3 and 7 days, respectively. Results were expressed as colony forming units (CFU) per ml of water sample.

2.2.7. Statistical analysis

Statistical data evaluation was performed with the MS Excel Data Analysis tool (Descriptive statistics, Regression). The reproducibility for indirect/calculated data (e.g., intracellular ATP) was calculated by a propagation-of-uncertainty method. FCM data was not always measured in duplicate, due to practical constraints. In these cases, a 9 % error (average coefficient of variation (CV) (n = 39)) was applied for representing FCM data. The residual chlorine concentration distribution box plot was created using on-line calculator on http://www.physics.csbsju.edu/stats/.

2.3. Results and Discussion

2.3.1. The importance of correct sampling

Sample collection during this study elucidated some of the problems specific for this network and highlighted the broader importance of correct sampling procedures. Fire hydrants were selected as sampling points to enable direct access to the distribution network and avoid potential household effects [102]. We opted for a low velocity water flow in combination with online monitoring to achieve comparable samples. In some cases, the water initially emerging from the fire hydrants were visibly turbid and/or discolored (data not shown). Turbid water is clearly unwanted and serves as a first visual confirmation of some form of system failure. In this regard, a recent study in the Netherlands has established an important link between suspended solids and microbial growth and biological instability [81]. Hence in some instances continuous low velocity flushing of up to 60 minutes was required before stable values for chemical and physical parameters as well as microbiological parameters were obtained (Fig. 2.2; Table 2.2). The data in Fig. 2.2A demonstrate clearly the need for a carefully planned sampling protocol when assessing full-scale systems. It should be noted that Fig. 2.2A represents an example of some of the worst sampling points in the system. Data from other hydrants often showed less fluctuation during flushing (Fig. 2.2B). One potential problem during the sampling procedure is the resuspension of sediments/particles and sloughing of biofilms from the pipes, causing artifacts in the measurements. In this respect, we specifically employed a low velocity (0.015 – 0.25 m s^{-1}) presampling flushing procedure. The latter differs

from extreme flushing applied for network cleaning, which is operated with high velocities of 1.5 – 1.8 m s^{-1} [103, 104]. According to Antoun and co-workers [103] low-velocity flushing (below 0.3 m s^{-1}) does not cause any scouring actions. However, it should be considered that part of the samples, especially during the first minutes of the flushing, can contain biofilm bacteria detached in a result of pre-flushing [95].

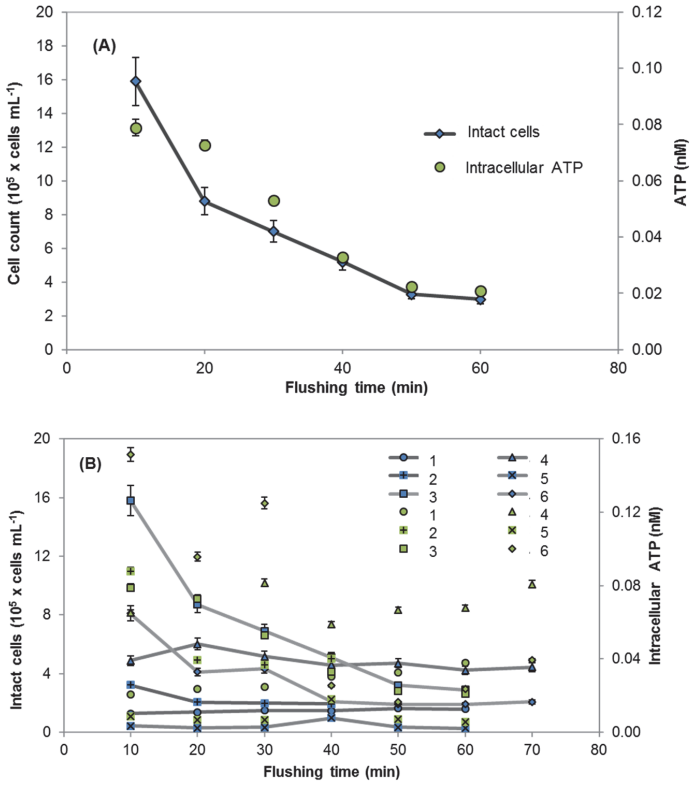
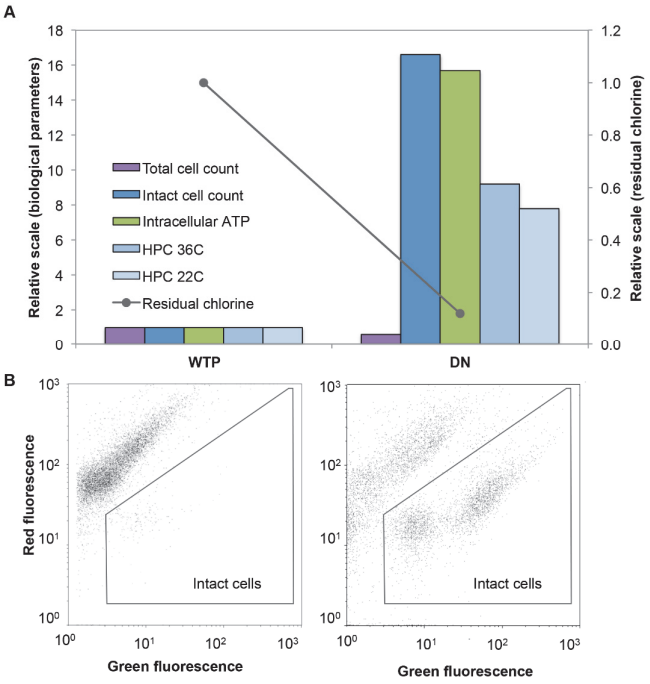


Fig. 2.2. Changes in intact cell concentration and intracellular ATP during flushing in newly-opened fire hydrants

(A) The impact of low velocity flushing on water quality on the example of the in one of the worst sampling point. (B) Changes of biomass during flushing in all 6 newly-opened fire hydrants. Intracellular adenosine tri-phosphate (ATP) data points were derived from duplicate measurements of extracellular and total ATP concentrations. FCM intact cells (after SYBR Green I and propidium iodide staining) were single measurements, with a relative standard deviation of 9 % calculated from all data in the present study. Intact cell concentration values are shown as solid lines, whereas intracellular ATP results displayed as single bullets.

2.3.2. The concept of detecting instability: a single point in the distribution network

In the introduction we proposed the straightforward hypothesis that biological parameters would show an increase between the point of treatment and a point during distribution in case of biological instability (Fig. 2.1). Before the relation between different parameters and the impact on the entire network are discussed in detail below, a single sampling point is compared to its source water as an example to illustrate the concept (Fig. 2.3A). The point was selected on the basis of (1) hydraulic data linking it with a specific WTP, (2) its medial distance from WTP (neither too close and nor too far from the WTP) and, (3) the fact that all microbiological parameters (FCM, ATP and HPC) as well as residual chlorine measurements were performed on this sample. For the purpose of clarity, the data was normalized to the values of the treated water and expressed as the relative change (the raw data are shown in Fig. 2.3C). Evidently the data from Fig. 2.3A and 2.3C supports the basic hypothesis. The microbial parameters such as intact cell concentration, ATP and colony forming units all show a considerable increase in their values. Simultaneously, only 12 % (0.06 mg L^{-1}) of the initial residual chlorine concentration (0.5 mg L^{-1}) was left in the water sample. The data suggests that the residual chlorine in the network was not sufficient to inhibit microbial growth, concurring with earlier report from Prévost and colleagues [12] showing increased HPC, total direct and direct viable bacterial counts in a distribution network coinciding with chlorine depletion. Other studies also showed the presence of viable bacteria in water with chlorine concentration lower than 0.1 mg L^{-1} [90] and that residual chlorine levels below 0.07 mg L^{-1} allows bacterial growth [17]. Data of residual chlorine concentrations in the drinking water network is summarized in Fig. 2.4. Evidently a considerable fraction of samples (18 %) had residual chlorine concentrations below 0.1 mg L^{-1}.



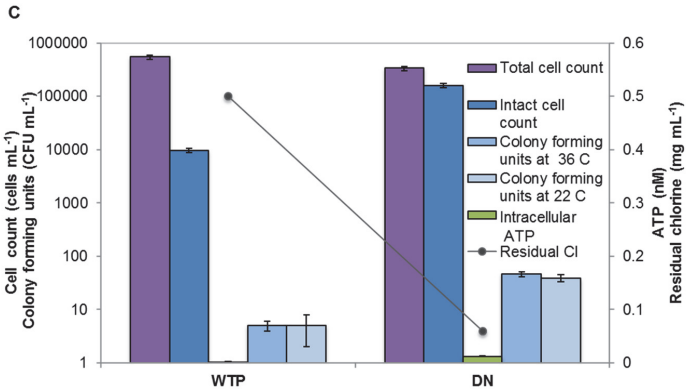


Fig. 2.3. Changes in bacterial parameters between water treatment plant (WTP) and distribution network (DN) sampling points

(A) For comparison, all values at the WTP were set to 1, and values in the DN were expressed relative to their values at the WTP. Data points are average values for duplicate measurements; (B) Flow cytometric density plots of samples stained with SYBR Green I and propidium iodide, showing the intact cell concentration at the plant and in the specific network point; (C) Changes in various bacterial parameters between one water treatment plant and a randomly selected point in the distribution network (actual values for Fig. 2.3A).

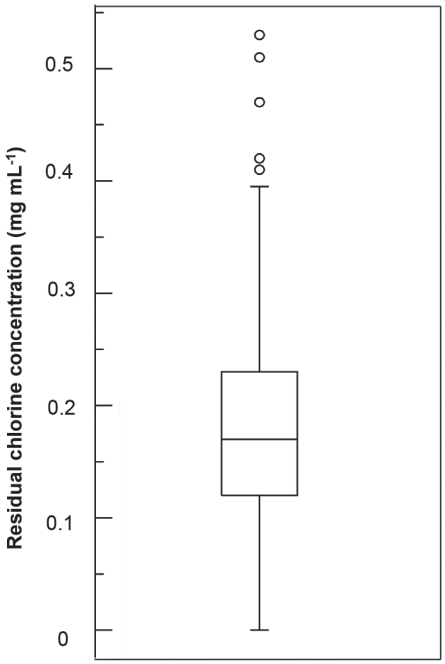


Fig. 2.4. Residual chlorine concentration in the distribution network

50 % of residual chlorine concentration in the network was between 0.12 (first quartile) and 0.23 (third quartile) mg mL^{-1}, with a mean value of 0.17 mg mL^{-1} (n = 27). The whiskers indicate on minimum and maximum values, whereas bullets show outliers of the population.

Staining of bacteria with fluorescent dyes was previously suggested as a way to distinguish between viable and damaged bacteria in real water samples [56, 57], and the application of this approach has been successfully demonstrated in laboratory scale chlorination studies [91, 105]. One focus point of the present study was to determine whether FCM combined with viability staining can be used for a fast and meaningful assessment of viable bacteria in chlorinated drinking water systems. The same samples from Fig. 2.3A, stained with SYBR Green I and propidium iodide (SGPI), are shown as density plots obtained with FCM (Fig. 2.3B). The theory behind the staining method and the interpretation of such data are discussed in detail elsewhere [56, 64, 69, 91, 101]. In the treatment plant sample, where the water was recently exposed to chlorine, 98 % of all cells were measured as membrane compromised, seen by absence of events inside the gated area of the plot (Fig. 2.3B). In the distribution network (DN) sample, a high concentration of intact cells appeared (Fig. 2.3B). Since these intact cells were clearly not present in the influent, the plausible conclusion is that the bacterial growth occurred during distribution.

2.3.3. Detailed assessment of dynamic changes in a single point

High frequency monitoring of a single sampling point revealed temporal instability in the distribution network. We monitored the effluent of one treatment plant and one point in the network with 1-hour intervals during a day (ca. 21 h). The sampling was arranged in such a way that the network sampling started 15 hours after the treatment plant sampling, which corresponded with the estimated water residence time (WRT) for this location. Fig. 2.5 displays the changes of intracellular ATP and intact cell concentrations in the network and the water treatment plant. Values for both parameters were low in the water samples from the treatment plant (n = 19): intracellular ATP varied from 0.0025 nM to 0.0096 nM (mean = 0.0061 ± 0.002 nM) and the intact cell concentration amongst 19 samples varied from 7.5×10^3 to 6.3×10^4 cells mL^{-1} (mean = $1.6\times10^4 \pm 1.2\times10^4$ cells mL^{-1} in average). In turn, the values from the distribution network point (n = 23) were significantly higher: intact cell concentrations ranged from 1.37×10^5 to 4.66×10^5 cells mL^{-1} (mean = $2.5\times10^5 \pm 9.9\times10^4$ cells mL^{-1}), and the ATP concentrations from 0.021 to 0.063 nM (mean = 0.038 ± 0.012 nM). Moreover, a distinct pattern was apparent in the distribution network data, with values peaking at about 05:00 – 07:00 and again at 12:00 – 13:00. During both these events, the intracellular ATP data followed a similar pattern as the intact cell concentration data in the network ($R^2 = 0.81$, $p < 0.005$). Although it is not evident exactly why the bacterial concentrations peaked at these specific time periods, a plausible explanation is a change in the flow velocity due to diurnal changes in water consumption by both industrial and domestic consumers. It was previously shown in laboratory scale experiments that increased flow velocity could lead to increased bacterial detachment from biofilms and a re-suspension of loose deposits, thus leading to an increase in suspended cell concentrations [95, 96, 106]. In addition, it is possible that lower water consumption overnight resulted in considerably reduced flow rates, and consequently a faster decay of chlorine and increased bacterial growth [12, 107].

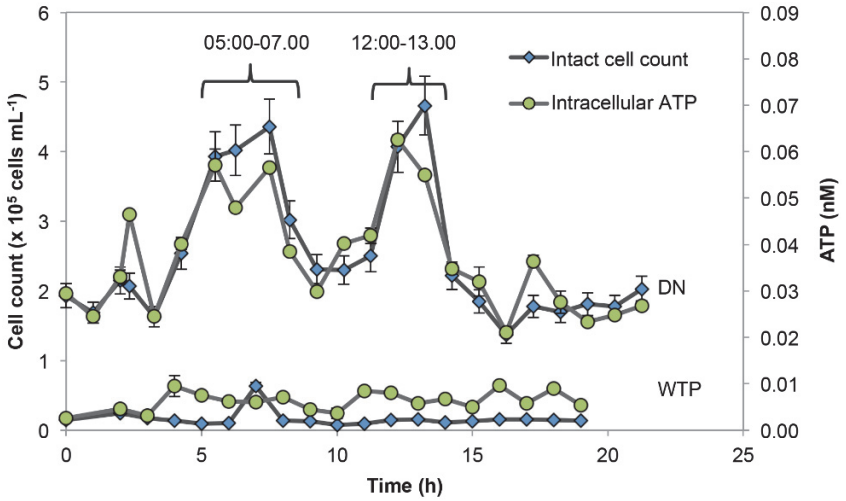


Fig. 2.5. Diurnal changes in bacterial parameters of WTP and DN points

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. Intracellular adenosine triphosphate (ATP) data points were derived from duplicate measurements of extracellular and total ATP concentrations. FCM intact cells (after SYBR Green I and propidium iodide staining) were single measurements, with a relative standard deviation of 9 % calculated from all data in the present study.

Detailed data sets of diurnal changes in the microbial quality of water mains, such as Fig. 2.5, are particularly scarce in literature. Importantly, this clearly demonstrated temporal instability in the network for which the exact cause remains uncertain. Moreover, it shows that the absolute cell concentrations at any sampling point may be influenced by the time of sampling.

2.3.4. Instability data for the entire network

Full-scale distribution networks are complicated systems, not restricted to a single source or a straight distribution line [1]. The Riga distribution network is supplied with drinking water from several separate treatment plants (Table 2.1). One plant treats surface water from the Daugava River (WTP 1) and the others supply natural groundwater (WTP 2) and artificially recharged groundwater (WTP 3). Chlorination is applied as the final disinfection step at all plants, resulting in low concentrations of intact cells, intracellular ATP and cultivable bacteria in the effluents (Table 2.1). A large fraction of the active chorine is rapidly consumed due to relatively high levels of organic matter. Despite the fact that the purpose of chlorination and residual chlorine is to limit microbial growth, a considerable increase in the concentration of intact cells was detected throughout the distribution network. Fig. 2.6A shows the range of intact cell concentrations arranged in ascending order. Treated water contained between 1.84×10^5 – 5.63×10^5 total cells mL^{-1} and between 9.7×10^3 – 2.13×10^4 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 1.62×10^5 cells mL^{-1} to 1.07×10^6 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. The observed increase in intact cell concentration is likely related to the

presence of assimilable organic carbon (AOC) in the distributed water. While AOC was not measured in the present study, previous data for two of the treatment plants were high (in the range of 200 μg L^{-1}; Table 2.1), and nutrient availability in the water is generally regarded as a key factor that promotes microbial growth [39, 92]. It cannot be excluded that some variability in the data resulted from bacteria detached from biofilms or re-suspended from sediments during the fire hydrant sampling procedure. However, the potential adverse impact of this was minimized by the low velocity sampling protocol (see above), while the systematic increase in cell concentrations in the network clearly suggests the occurrence of biological instability rather than sampling artifacts. In contrast to these findings, several studies analyzing drinking water distribution systems without any additional residual disinfectants showed no (or only minute) changes in bacterial parameters during distribution [1, 18, 87]. These distributions systems rely on nutrient limitation to achieve biological stability, and while intact cell concentrations are often relatively high (ca. 1×10^5 cells mL^{-1}) [1, 69], changes during distribution tend to be negligible.

To examine the spatial distribution of the growth/instability in the network, the data was divided into four broad categories based on the extent of growth (Fig. 2.6A). These were visualized on the sampling map (Fig. 2.6B). The sampling points with the lowest intact cell concentration (less than 5×10^4 cells mL^{-1}) are marked with green bullets. Yellow and orange colored bullets indicate higher concentrations, while the points with the highest values (over 2×10^5 cells mL^{-1}) are shown as red bullets. As it could be expected, the map shows that the green colored points are mostly concentrated in areas close to the water treatment plants. Low intact cell concentrations in those areas could be ascribed to (1) disinfection during treatment and (2) growth inhibition from sufficient residual chlorine. Also the flow rate in the outgoing pipes closest to the treatment plants is high, which likely prevents water stagnation, sedimentation and cell adhesion on the pipe surface, and, consequently, biofilm formation and further bacterial growth. A different situation is observed in the distant areas from the water treatment plants and particularly in the so-called mixing zones, where the water from three different water treatment plants potentially mix. The map displays different color points spread in these zones without any visible order. The prevalence of the samples with higher cell concentrations there compared to the areas close to WTPs also corroborates the argument that increasing distance and water residence time could lead to chlorine decay with concomitant oxidation of dissolved organic matter; 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.

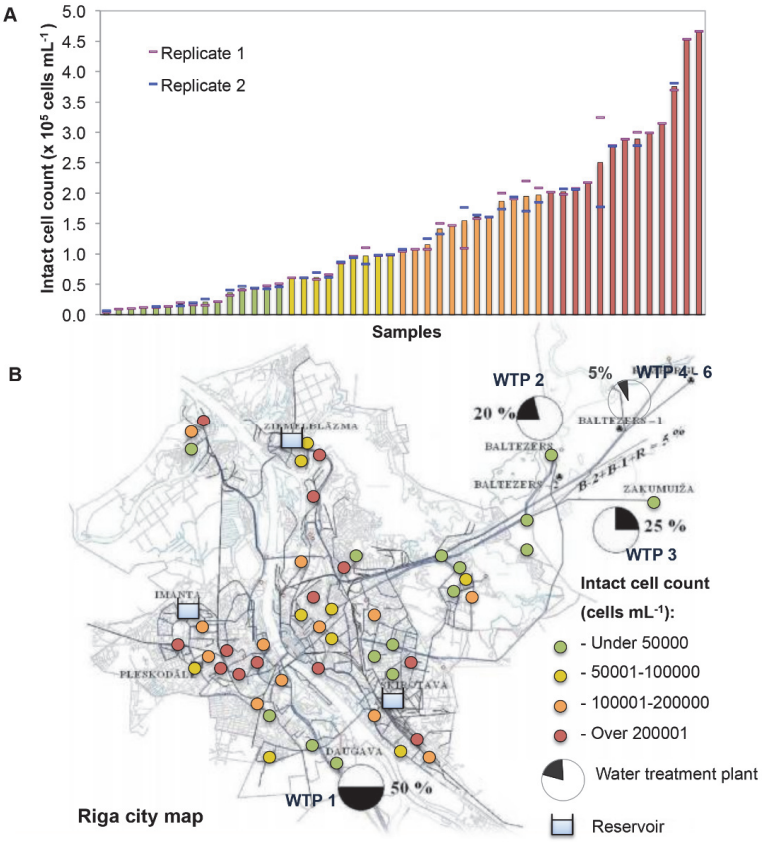


Fig. 2.6. Intact cell concentrations of all samples measured from the distribution network (n = 49)

(A) Intact cell concentrations arranged in ascending order and categorized into four main classes (colored bars) according to increasing concentrations. Data points are average values of duplicate measurements. Blue and purple stripes above and below data bars show the measured values. (B) 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.

The uneven spatial distribution of the samples with different intact cell concentrations is noteworthy, highlighted for example by the three points in upper-left corner of the map. Based on the long distance from the WTPs, high intact cell concentrations were expected, but the samples taken from the hydrants located in this small area rather show variability (respectively 1.82×10^4, 1.87×10^5, 2.51×10^5 intact cell mL^{-1}). Such different intact cell concentrations could be due to several reasons: the time the samples were taken, which is linked to water consumption and the potential impact of which is shown in Fig. 2.5, the condition of the pipes in this specific area (unknown), the way water flows from the treatment plant, and/or the relative proximity of these sample points to one of the reservoirs (Fig. 2.6B), etc. Other authors showed a decrease in AOC [27] and ATP [18] in the some distal points of the distribution networks. Decrease of AOC concentration was explained by its consumption by bacteria within the network. These authors argued that an insufficient amount of nutrients led to starvation and a

decrease in bacterial parameters at the end of the pipelines. However, it is an unlikely reason in the present study, because this phenomenon seems more occasional than systematic.

The combined data demonstrates clearly biological instability throughout the distribution network. However, despite the relative simplicity of the concept (Fig. 2.1; Fig. 2.3A), a complex interplay of chemical, physical and biological parameters and hydraulic conditions should be taken into account for characterization of each particular case of instability.

2.3.5. Comparison of different microbiological parameters

FCM and ATP data showed clear correlations, but these data did not correlate well with conventional HPC data. A total amount of 49 different samples was measured in duplicate with ATP (total and extracellular) and FCM (total and intact cell concentration) analyses, while 38 of those samples were further analyzed with HPC. The significant linear correlation ($R^2 = 0.77$; n = 49) between intracellular ATP and FCM intact cell concentration is shown in Fig. 2.7A. This corroborates previous studies that showed good results comparing total ATP with total cell concentration [18, 108] and intracellular ATP with intact cell count as well [67, 69]. The strong correlation is encouraging, since FCM and ATP analysis are independent viability parameters – integrity of the cell membrane (FCM) and cellular energy (ATP). A correlation between these parameters during disinfection is not necessarily a given fact. The membrane integrity based PI staining method implies that PI positive cells are damaged and thus considered as inactive, yet extreme examples where living cells became permeable for propidium iodide have been described [64]. In turn, [37] showed that after UV-C exposure cells became inactivated, while their membranes remained essentially intact. Discrepancies between intracellular ATP and intact cell concentration can also result from cell morphology, bacterial species and physiological state, that was discussed in detail previously [67]. The results provided by FCM provide information on single cell level, whereas during ATP analyses the values are evaluated per volume. Hence, intracellular ATP-per-cell was calculated for characterization of biomass activity. In the present study, intracellular ATP-per-cell ranges from zero (no cell-bound ATP observed) to 5.92×10^{-10} nM cell^{-1} (= 3×10^{-17} g cell^{-1}) with the average value of 1.68×10^{-10} nM cell^{-1} (= 8.52×10^{-18} g cell^{-1}) (stdev = 9.58×10^{-11} nM cell^{-1}, n = 49). The result is in the same range as ATP-per-cell values obtained from various water sources, which were analyzed with the same methods [67, 69]. This suggests that bacterial activity (ATP values) in the intact cells was not affected by any remaining chlorine residuals, and that membrane damage (SGPI values) was in this case reflective of viability in the sample. The good correlation between these two independent parameters is an optimistic prospect for applying these methods for chlorinated water analyses in future studies.

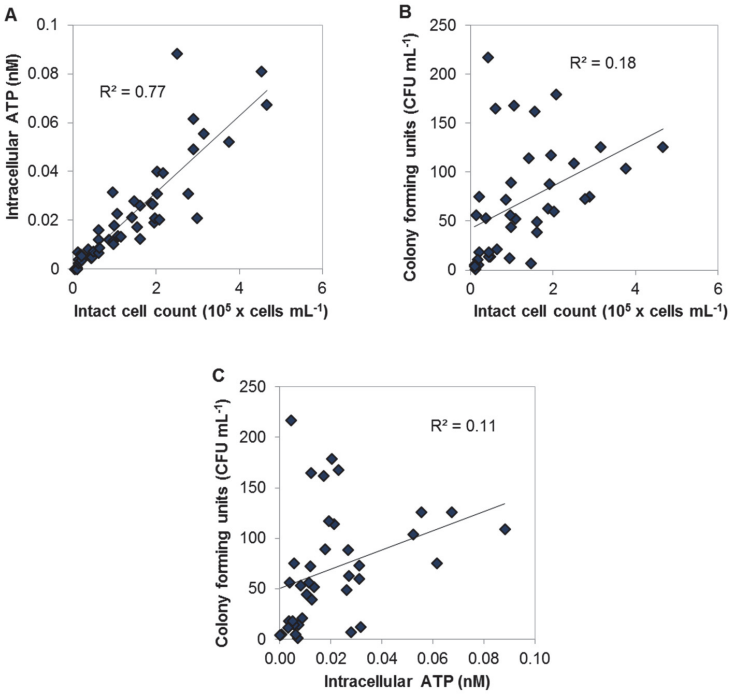


Fig. 2.7. Comparison of the various microbiological parameters

Clear correlations were observed between intact cells and intracellular ATP (n = 49) (A), but no obvious correlations between these two parameters and heterotrophic plate counts at 22 °C (n = 38) (B) (C).

The conventional HPC results were compared with the FCM intact cell concentration values. A weak correlation ($R^2 = 0.18$, n = 38) was observed between HPC (at 22 °C) and intact cell concentrations (Fig. 2.7B), similar to reports in previous studies [101, 108]. It could be explained by the often described phenomenon, that less than 1 % of drinking water bacteria are cultivable on conventional agar plates [67, 108, 109]. In addition, Mezule and co-workers [110] demonstrated evidence of the presence of so called viable but not-culturable (VBNC) bacterial state, in both drinking water and biofilms for the network investigated here, thus indicating further limitations in the HPC method. Since intracellular ATP showed a good correlation with intact cell concentration, but intact cell count correlated weakly with HPC, it was expected that intracellular ATP and HPC would not correlate well (Fig. 2.7C; $R^2 = 0.11$, n = 38). Various studies were performed to compare ATP and HPC parameters from water samples, but good correlations were never observed e.g., $R^2 = 0.20$ [18], $R^2 = 0.36$ [89] and $R^2 = 0.31$ [108]. Our results combined with those from previous studies cast further doubts on the value of using the HPC method for general microbiological drinking water quality control. In our opinion, the clear correlation between two methodologically independent viability parameters (intracellular ATP and FCM intact cell counts), and the absence of any correlations with two different HPC methods, renders the former methods more meaningful for assessing and understanding biological instability, particularly in chlorinated environments.

2.3.6. Importance of measuring extracellular ATP

Arguments for and against the concept and importance of measuring extracellular ATP have been made [18, 67, 69, 71, 72, 76]. To understand this better, we arranged our data according to increasing intracellular ATP concentrations, after which the measured extracellular ATP values were added to each corresponding sample (Fig. 2.8). It is evident that extracellular ATP constitutes a considerable fraction of the total ATP amount in some samples – varying from 3 % up to 100 % – with an average contribution of 36 % (n = 49). Moreover, 33 % of the samples contain more that 50 % of extracellular ATP. This data supports other studies, where analyses showed high extracellular ATP in drinking water samples from the distribution networks [67, 69]. Interestingly, the highest extracellular ATP ratio is mostly observed in the samples with relatively low intracellular ATP, in this case samples with close proximity to the treatment plant. In the case of chlorinated water, this could potentially be explained by the oxidative effect of chlorine on bacterial cells. Previous studies have shown extensive damage to bacterial membranes during chlorination [91, 105], after which a release of extracellular ATP from the damaged bacteria can occur. This membrane damage was also clearly detected in the present study (e.g., Fig. 2.3B). Although, there is lack of detailed data considering the release of extracellular ATP in water samples affected by chlorination, strong evidence of ATP release during oxidation was presented in previous studies [69, 76]. Both these works showed a significant decrease in cell concentrations and intracellular ATP after ozonation, whereas extracellular ATP comprised 83 – 100 % of the total ATP. Moreover, Fig. 2.8 shows that samples with increased intracellular ATP concentrations, which we linked to bacterial growth during distribution, often had considerably less extracellular ATP in relation to total ATP. This could be due to the fact that extracellular ATP can be biodegraded by bacteria or extracellular enzymes in the network [70, 71, 73, 75]. However, it cannot be excluded that a decrease in extracellular ATP during distribution occurs due to oxidation by residual chlorine present in the network.

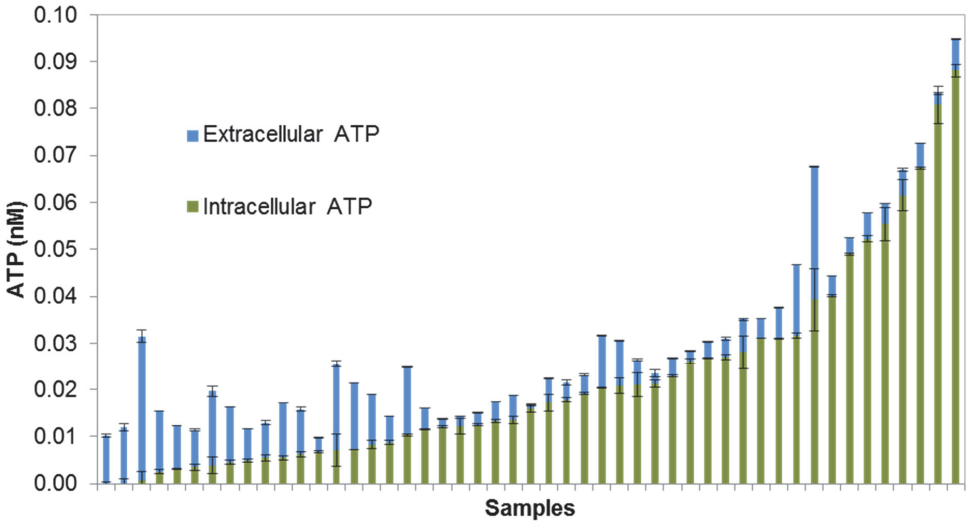


Fig. 2.8. Distribution of intracellular and extracellular ATP in the water samples
In general, higher concentrations and relative percentages of extracellular ATP were measured in samples that exhibited lower intracellular ATP concentrations (n = 49).

2.3.7. Conclusions of Chapter II

- An investigation of a full-scale chlorinated drinking water distribution network with various microbiological methods clearly demonstrated both spatial and temporal biological instability in the network.
- Fluorescent staining with SGPI in combination with ATP measurements provided reliable and descriptive information about bacterial density and viability in chlorinated drinking water samples.
- A good correlation was observed between intracellular ATP and intact cell counts ($R^2 = 0.77$), whereas HPC showed poor correlations with both parameters ($R^2 = 0.18$ with intact cell concentration and $R^2 = 0.11$ with intracellular ATP).
- Extracellular ATP constituted on average 36 % of total ATP in the present study, which confirms the necessity of extracellular ATP subtraction from total ATP measurements during chlorinated drinking water analyses.
- Overall the results raise questions with respect to the offset between increased biological safety gained from disinfection opposed to increased risk from instability (uncontrolled bacterial growth). While an improvement of the chlorination procedure could be a solution, the data suggests looking beyond only disinfection for achieving biological stability of drinking water.

3. CHAPTER III: A PIPELINE FOR DEVELOPING STAINING PROTOCOLS FOR FLOW CYTOMETRY, DEMONSTRATED WITH SYBR GREEN I AND PROPIDIUM IODIDE VIABILITY STAINING

3.1. Introduction

Fluorescent staining coupled with flow cytometry (FCM) is often used for quantification and characterization of bacteria in both natural and engineered environmental water samples [53, 56–58]. Previous studies have clearly demonstrated that staining results obtained from commonly used dyes such as SYBR Green I (SG) and propidium iodide (PI), and particularly their combination (SGPI), are severely influenced by variations in staining protocols [57, 61–64, 111]. For example, Prest and colleagues [111] demonstrated changes in cell concentrations and fluorescence fingerprints at different SG staining temperatures, while other authors showed changes in the percentage of "dead" cells detected at different concentrations of PI [63–65]. While a range of commercial dyes can be purchased as of-the-shelve kits, there is a clear and recognized need for protocol optimization and standardization, as well as for a mechanistic understanding of the staining method. Increasingly routine applications of these methods require fixed protocols to produce reliable and comparative data across experimental studies [111–113]. Hence, the aims of the study were (1) to establish a general methodological pipeline for testing and optimization of staining protocols for flow cytometric analysis of bacteria in environmental water samples and (2) to demonstrate this approach and some related challenges for the SGPI viability staining method.

Viability staining with SGPI was specifically selected as a good example of the case in point. Based on the LIVE/DEAD®, first proposed by Boulos and co-workers as an alternative to plate counts [114], nucleic acid double-staining using PI combined with membrane-permeable SG as a counter stain is frequently used to distinguish between membrane compromised (damaged) cells, which are considered to be dead, from intact cells, which are often described as potentially viable cells [56, 57, 61–63]. At least five separate previous studies have worked towards some degree of testing/optimization of the SGPI staining protocols [57, 61–64], but a comparison of these and a review of currently available literature highlights numerous discrepancies in staining methods (Table 3.1). For example, reported PI concentrations varied 20-fold from 1.5 to 30 µM when used in combination with SG, and 50-fold (1 to 50 µM) when used alone or with other dyes. Moreover, staining temperatures are often not reported, or reported vaguely as "room temperature", and staining times varied between 2 and 60 min. In addition, a few authors investigated the use of chelating agents (EDTA, EGTA) for gram-negative bacteria, when PI-based viability determination methods are applied [56, 101, 115], but without in-depth assessment of potential artifacts arising from this.

Table 3.1.

Variations in protocols used for propidium iodide viability staining [116]

Counter-stain	PI solvent	T (°C)	Time (min)	PI conc. (µM)	EDTA/ EGTA	Sample type	Reference
cFDA	distilled water	37 °C	15	5	-	Pure cultures	(Amor et al., 2002)
SG	distilled water	37 °C	15	15	-	Pure cultures	(Barbesti et al., 2000)
SYTO9	DMSO	20 °C	20	30	-	Pure cultures	(Berney et al., 2006)
SYTO9 SG	DMSO	RT	20	3 - 30	EDTA 5 mM	Pure cultures, freshwater bacteria	(Berney et al., 2007b)
SG	DMSO	20 °C	25	6	EDTA 5 mM	Drinking water	(Berney et al., 2008)
SYTO9	0,085% NaCl	RT	20	30	-	Pure cultures, environ. isolates	(Boulos et al., 1999)
PI alone	distilled water	30 °C	10	30	-	Pure cultures	(Bunthof et al., 2001)
SG	n.a.	RT	20	15	-	Seawater	(Falcioni et al., 2008)
SG, SG II	n.a.	n.a.	30	1.5 - 15	-	Freshwater, seawater	(Grégori et al., 2001)
SG	TRIS buffer	40 °C	5	6	-	Tap water, pure cultures	(Hammes et al., 2012)
BOX; BOX/EB	DBS	n.a.	n.a.	7.5	EDTA 4 mM	Pure cultures	(Hewitt et al., 1999)
PI alone	n.a.	RT	60	50	-	Pure cultures	(Khan et al., 2010)
SG	DMSO	30 °C	10	30	-	Pure cultures	(Kong et al., 2016)
SG	DMSO	n.a.	15	6	-	Drinking water	(Lautenschlager et al., 2013)
GFP, SYTO9	DMSO	n.a.	15	30	-	Pure cultures	(Lehtinen et al., 2004)
Rhodamine Oxonol	water/ Ethanol	RT	2	15	EGTA 1 mM	Pure cultures	(López-Amorós et al., 1995)
SG	n.a.	n.a.	20	30	-	Marine sediments	(Manini and Danovaro, 2006)
PI alone	n.a.	RT	30	15	-	Chlorinated water from WTP	(Phe et al., 2005)
SYTO9	n.a.	RT	15	30	-	Bottled water	(Ramalho et al., 2001) microscopy
SG	DMSO	RT	14-18	6	-	Drinking water, treated water	(Maaike K Ramseier et al., 2011)
Hoechst 33342/alone	PBS	n.a.	10	1	-	Pure cultures	(Shi et al., 2007)
SG	DMSO	37 °C	10	3	-	Pure cultures, natural water	(Van Nevel et al., 2013)
SG	DMSO	n.a.	15	6	-	Drinking water	(Vital et al., 2012)
DAPI	distilled water	n.a.	30	7.5	-	Pure cultures	(Williams et al., 1998)
SYTO9, DAPI, cFDA	DMSO	RT	30	20 - 48	-	Pure cultures	(Zotta et al., 2012)

Accurate staining protocols are critical for all dyes, but particularly so for a viability double-staining approach (e.g., SGPI). Firstly, temperature, time and dye concentrations could influence reaction rates and the efficacy of staining. Secondly, a usage of two fluorescent dyes in combination means potential interaction between their excitation/emission spectra (i.e. energy transfer). For example, it was shown that emission spectrum of SYTO9, which is similar to SG spectrum, causes PI excitation with an increase of the signal intensity and quenching of the green fluorescence consequently [117]. Additionally, a displacement of SYTO9 molecule by PI was demonstrated as a possible mechanism occurring when staining with the two dyes. Appearance of "double positive" cells was described in another study, when inadequate PI concentrations resulted in the incomplete energy transfer between SG and PI [62]. Finally, incorrect staining conditions might cause damage to bacteria, thus creating artifact results. For example, it was demonstrated that high temperatures permeabilize cell membranes, while PI could erroneously penetrate cells when used in too high concentrations [57, 65]. Thus variations of the staining method could severely influence the outcome. For this reason it is imperative to assess the dyes within the context of understanding their viability targets and their mechanism of action.

In the present study we focused specifically on actual environmental samples, using river water as the bacterial source for most experiments. The specific experimental goals of the study were: (i) to assess the influence of the solvents used for dye preparation on the staining result; (ii) to identify an optimal dye concentration, (III) staining time and (iv) staining temperature. Moreover, we use this to propose a pipeline for testing fluorescent dyes for flow cytometry in general.

3.2. Materials and Methods

3.2.1. Sampling

River water (Chriesbach, Dübendorf, Switzerland) was chosen as a water source to provide untreated indigenous bacterial communities for SGPI staining optimization. Nine additional environmental/drinking water samples were taken to test the optimized protocol and the effect of EDTA, namely, river water (Daugava River, Riga, Latvia), river water (Lielupe River, Jurmala, Latvia), bottled natural mineral water (Mangali, Latvia), bottled natural mineral water (Evian, France), sea water (Baltic sea, Gulf of Riga, Jurmala, Latvia), well water (Babite, Latvia) and three chlorinated tap water samples from different locations (Riga, Latvia). Water was collected in sterile 50 mL tubes, and tests were performed within 24 h.

3.2.2. Composition of dyes solutions

The original SG and propidium iodide (PI) staining solution (SGPI) was prepared using SG stock solution diluted in 100× in anhydrous dimethyl sulfoxide (DMSO) and a final PI concentration of 0.3 mM [113]. For optimization purposes various PI concentrations were tested. For a working solution, SYBR® Green I (SG) stock (Invitrogen AG, Basel, Switzerland) was diluted 100x in 10 mM Tris buffer (pH = 8.1). Working SGPI solutions were prepared using this SG working solution and addition of PI with a concentrations ranging from 37.5 μM to 2.4 mM (equivalent to 0.375 μM to 24 μM final PI concentration) (details in 2.5.).

3.2.3. Bacteria inactivation

Two types of inactivation treatments were used to produce real water samples with different amounts of intact and damaged bacteria. Heat treatment was used to ensure substantial bacterial damage. For that, 150 µL of the sample was transferred into an Eppendorf tube and incubated at 80 °C for 3 min [56]. An addition of a moderate chlorine dose (0.35 mg L^{-1} free chlorine as a final concentration) was applied to inactivate a part of total bacterial community. The contact time was 5 min and chlorine was quenched with addition of 10 mM $Na_2S_2O_3$ (1 % (v/v)) before staining. Determination of free chlorine was measured using commercially available chlorine test kits (LCK 310; Hach-Lange) and a photometer (CADAS 30, Hach-Lange).

Additional experiments with different types of bacterial inactivation agents were performed to test bacterial damage mechanism, where SGPI staining might not represent actual cell damage. River water samples were exposed to formaldehyde (38 %, Sigma-Aldrich) with final concentrations 0.3 – 0.4 % and 3 – 4 %. Sodium azide NaN_3 concentrations in the sample were 0.01 % and 0.05 % (w/v). Staining and measurements were performed after 1, 5 and 30 min contact time. Disinfection with heavy metals was performed using $CuCl_2$, in concentrations 0.4, 4 and 40 mg L^{-1}, and $AgNO_3$ with final concentrations of 0.25 and 2.5 mg L^{-1} and contact time 5 and 30 minutes. SGPI staining (15 min, 35 °C, 6 µM PI) for the samples exposed to heavy metals was performed with addition of EDTA (500 mM) with a final concentration of 5 mM and without EDTA.

3.2.4. Fluorescent staining and flow cytometry (FCM) of water samples

Measurements were performed using a BD Accuri® C6 flow cytometer (BD Accuri® cytometers, Belgium), or a Partec CyFlow® SL instrument (Partec GmbH, Münster, Germany). The BD Accuri® C6 instrument is equipped with a 50 mW solid state laser emitting light at a fixed wavelength of 488 nm. Green fluorescence intensity was collected 533 ± 30 nm, red fluorescence at > 670 nm, while sideward and forward scattered light intensities were collected as well. Measurements were performed at pre-set flow rate of 66 µL min^{-1}. The CyFlow® SL instrument is equipped with a blue 25 mW solid state laser emitting light at a fixed wavelength of 488 nm. Green fluorescence was collected at 520 ± 10 nm, red fluorescence above 630 nm, and high angle sideward scatter (SSC) at 488 nm. The trigger/threshold for both instruments was set on the green fluorescence channel and data were acquired on two-parameter density plots, while no compensation was used for any of the measurements. All data were processed with the FCM propriety software, and electronic gating was used to separate positive signals from instrument and water sample background [111].

Three different approaches for fluorescent staining and FCM measurements were applied depending on the purpose (see optimization setup section 3.2.5.): (i) standard measurement (with BD Accuri® C6 or Partec CyFlow® SL); (ii) automated 96-well multi-plate measurements (with BD Accuri® C6); (iii) high frequency time and temperature controlled measurements (with the BD Accuri C6® partially connected to an online FCM device [60]). Standard measurements were performed as described before [113]. If not stated otherwise, the samples were preheated at 37 °C (3 min), then stained with either SG or SGPI at 10 µL mL^{-1} (10,000× final dilution of SG; 3 µM final concentration of PI) followed by 10 min incubation in the dark at 37 °C. When necessary, the water samples were diluted immediately prior to FCM analysis with 0.22 µm filtered commercially available bottled water (Evian, France) to 10 % v/v of the initial concentration.

For temperature-controlled 96-well multi-plate based analyses, the BD Accuri® C6 instrument equipped with autoloader was placed in a walk-in incubator at 37 °C. 200 µL of the sample were transferred to separate wells (n = 32), pre-heated (3 min), then stained simultaneously with a multi-channel pipette, and subsequently 25 µL of each sample was measured [113]. For high frequency measurements, an online FCM system was used with some modifications from the one described before [60]. In short, SG or SGPI (10 µL mL^{-1}) was added into a FCM sampling tube with 2 mL of a water sample, which was pre-heated to the necessary temperature. Directly after addition of the dye, the tube was placed into the FCM sampling port in a temperature-controlled environment and the automated measurement cycle was started. A control unit was programmed to inject sample every 1 min, thus analyzing 19 µL per sampling.

3.2.5. Staining optimization approach

The tests for the optimization approach were performed in the specific order that is schematically demonstrated on Fig. 3.1. The tests, included in this general pipeline, were conducted as follows:

Dye solvents (Step 2): SGPI working solutions were prepared in either Tris buffer (10 mM, pH = 8.1) or DMSO solvent as described in section 3.2.2 to assess impact of the dye solvents on staining results (Fig. 3.1, Step 2). The dyes were added to the pre-heated (37 °C) river water samples at 3 µM PI final concentration, and measurements were performed using 96-well multi-plate approach. FCM sampling and measurements were done at approximately 50 s intervals, and incubation temperature was constant at 37 °C.

Stain concentrations (Step 3): Untreated and heat-treated water samples (Section 3.2.3) were stained with 0.375, 0.75, 1.5, 3, 6, 12 and 24 µM final PI concentrations to assess the impact of dye concentration on SGPI staining. The samples were stained for 10 min at 37 °C prior measurements. All the samples were prepared in triplicate. The test was done using the standard FCM measurement procedure (described above).

Incubation temperature (Step 4): SGPI was added to untreated water samples with 3, 6 or 12 µM final PI concentration, which were measured with the online system approach at 25 °C, 35 °C and 44 °C pre-set temperature to make a decision about optimal staining temperature (Step 4).

Stain concentration and incubation time (Step 3 & 5): Untreated and chlorine-treated (Section 3.2.3) water samples were stained with SGPI dyes with 3, 6 or 12 µM PI concentration, and incubated at 35 °C. Sampling and measurements were done continuously for 25 min with the online system in order to estimate a concentration and time required for stable and optimal staining (Step 5).

Additives and different samples (Step 6 & 8): A necessity of EDTA addition was tested. EDTA (5 mM final concentration) was added to different water samples (Section 3.2.1) together with SG or SGPI dyes, and incubated 15 min at 35 °C. The same samples were stained without EDTA.

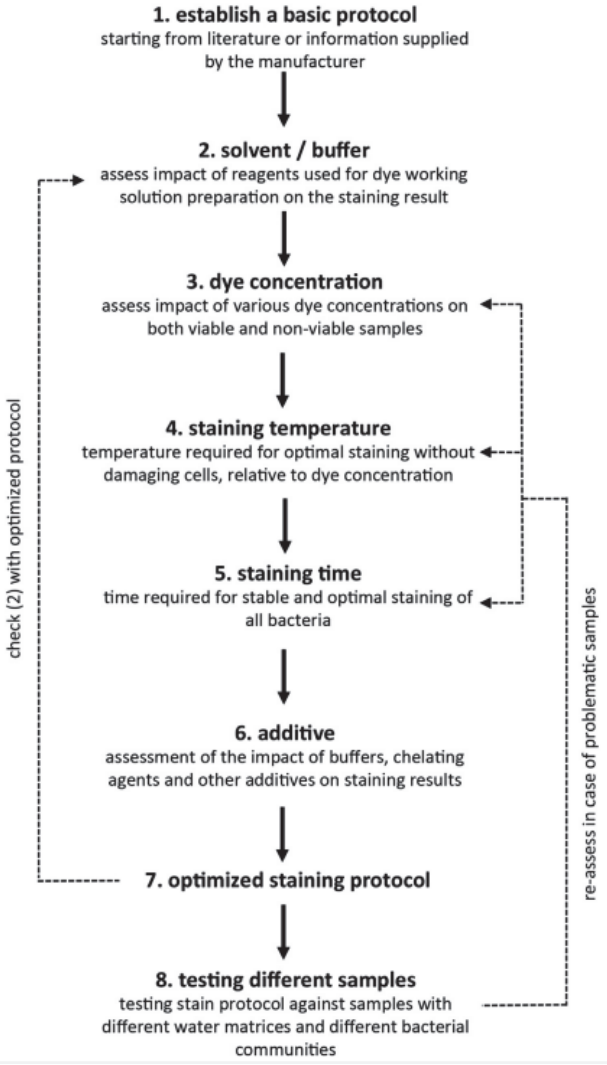


Fig. 3.1. A general pipeline for developing staining protocols for flow cytometric analysis of environmental samples

Steps 3–5 are done either in parallel (this study) or with several iterations to establish optimal conditions. Step 2 is ideally re-done with an optimized protocol if sufficiently different from the original protocol, while adverse/problematic data in Step 8 may well necessitate re-assessment of Steps 3–5.

3.2.6. Statistical analysis

Statistical analysis was made using MS Excel ANOVA Single factor data analysis tool.

3.3. Results and Discussion

3.3.1. Solvents can damage cells during staining

When SGPI was dissolved and diluted in DMSO prior to use, it appeared that the DMSO progressively caused unwanted cell damage during the staining, and this was not observed when the dye was prepared in TRIS buffer (Fig. 3.2). With DMSO as solvent for the dye, intact cell counts (ICC) of an otherwise untreated river water sample reached a maximum after 6 min of staining (6×10^5 cells mL^{-1}), but then gradually decreased with increased staining time (Fig. 3.2A). After 25 minutes, the ICC were only 2.9×10^5 cells mL^{-1}, which is less than 50 % compared to the maximum ICC and only 29 % of the average total cell count (TCC) at the same time point (Fig. 3.2B). When the dye was prepared in TRIS buffer, the ICC was 6.5×10^5 cells mL^{-1} after 6 minutes (similar to the DMSO result) and continued to increase slightly, indicating that staining is time dependent. Notably, TCC values were unaffected by the solvents and were not statistically different between 6 and 25 min of staining, namely $9.88 \pm 0.32\times10^5$ cells mL^{-1} with DMSO, and $9.51 \pm 0.38\times10^5$ cells mL^{-1} with TRIS buffer ($n = 24$) (Fig. 3.2B). Corresponding FCM density plots demonstrated a clear difference between staining with TRIS buffer and DMSO as respective dye solvents after 10 min of staining (Fig. 3.2C). There was a notable shift from the green fluorescence region (“intact cells”) to the red fluorescence region (“damaged cells”) in the DMSO plot, indicating that membrane impermeable PI molecules penetrated the cells. In turn, the FCM plot, obtained within the same conditions with TRIS buffer as solvent, showed clearly separated intact and damaged cells. The TCC data with TRIS buffer showed the same slight increase as the ICC data (Fig. 3.2A, 3.2B), which suggest that this is caused by time-dependent increased staining of cells with SG. This is discussed in detail below (Section 3.3.4) where we suggest staining untreated samples at least for 10 min to obtain stable results.

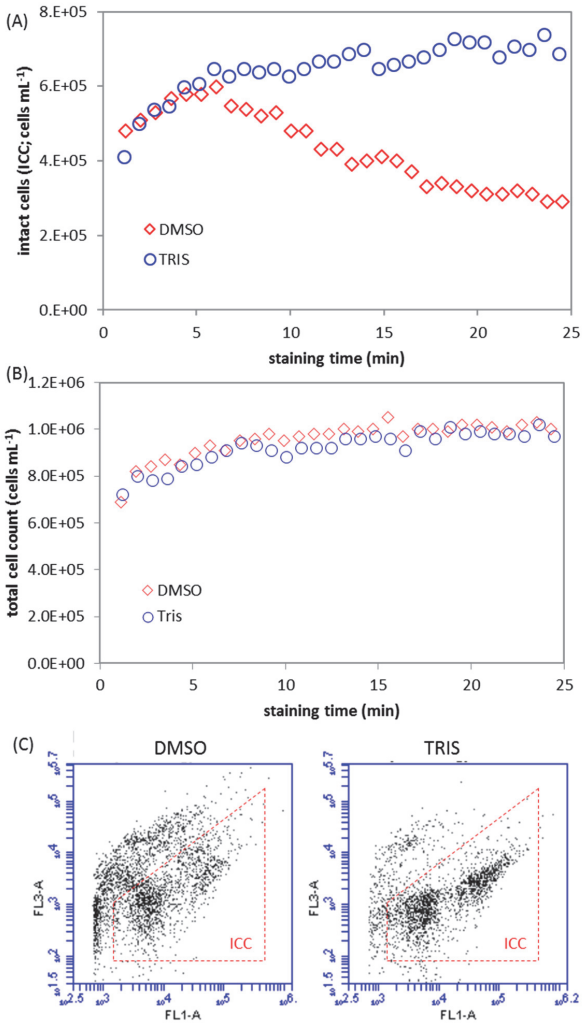


Fig. 3.2. The impact of TRIS buffer (pH = 8.1) and DMSO as solvents used for preparing dye working solution on SGPI staining

River water samples were stained 25 min at 37 °C with 3 µM PI in 10,000 × diluted SG (n = 30). (A) Changes of ICC of the samples, stained with SGPI, prepared in DMSO and TRIS buffer; (B) An impact of TRIS buffer (pH = 8.1) and DMSO reagents used for dye working solution on the SG staining. Samples, stained with SG dissolved either in DMSO or TRIS, shows similar trend over time. Hence, dye's solvent does not play an important role, when total cell count is determined. (C) FCM density plots of untreated river water sample stained 10 min at 37 °C with SGPI (3 µM PI), prepared in DMSO (1 % final concentration) (left) and TRIS (right) (Fig. 3.2A).

DMSO is often used as solvent for fluorescent PI based viability dyes [69, 101, 118–120]. However, DMSO has properties that facilitate high cell permeability [121], apparently also at the low concentrations used in our study (i.e., 100× dilution). Evidence of the ability of DMSO to make cell membranes thinner and enhance pore formation was observed with eukaryotic cells and demonstrated with molecular dynamics simulations [122–125]. A similar effect was also observed with bacterial cells, showing 1 % DMSO enhanced cell permeability [126], which is the same final concentration as was used in the present study. In a series of supporting

experiments we furthermore demonstrated that the decrease in ICC was clearly dependent on the DMSO concentration and exposure time (Fig. 3.3). These concentration and time dependent DMSO reaction data strongly support the hypothesis that DMSO damage of cells is causing a substantial decrease in ICC, and clearly shows the critical impact that a dye solvent can have in the outcome of a viability assay. We conclude that ideally DMSO should not be used to dissolve SGPI working solutions, while other viability dye that targets membrane permeability (e.g., SYTOX Green, ethidium homodimer 2) should be tested on compatibility with DMSO.

Some previous studies showed that TRIS buffer could also alter bacterial membranes [127, 128]. However, the concentrations used in those studies were higher than what was used in the present work. Since no obvious drawbacks were observed with TRIS buffer, it was chosen as a solvent for SGPI staining for all further tests in the current study.

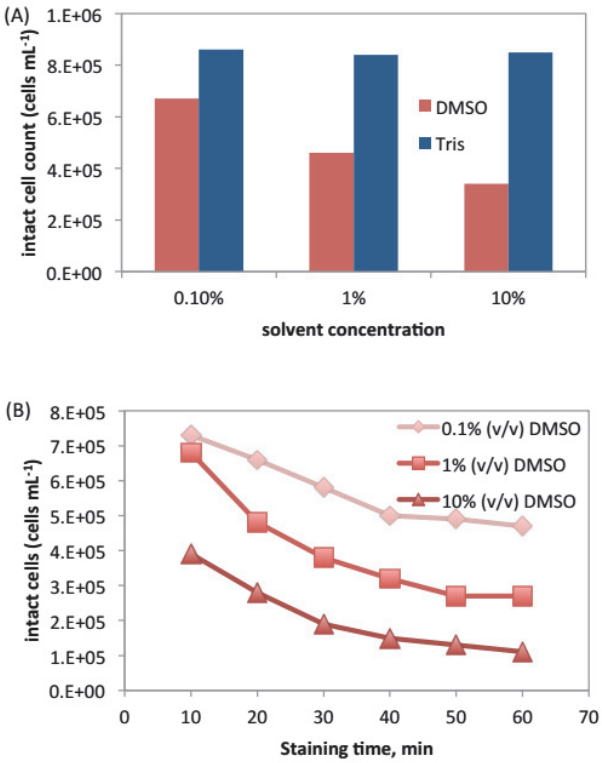


Fig. 3.3. Effect of DMSO and TRIS buffer concentrations on SGPI staining outcome (A) Effect of 10-fold different DMSO and TRIS concentrations (v/v) on SGPI staining of the river water samples; (B) changes of ICC with different DMSO concentrations versus time. Apparently, different concentrations of TRIS do not cause changes in ICC, however, increased DMSO concentration progressively cause decrease in ICC: 50 % damage with ×100 different concentration. DMSO caused damage is time dependent.

3.3.2. Appropriate dye concentrations for viable and non-viable cells

The dye concentration can affect the interpretation of the experimental data, since a too high concentration may lead to false-positive staining, while too low concentrations will inevitably lead to ineffective or false-negative cell staining [65]. Hence, in the case of a viability dye such as PI, the impact of concentration should be tested with both viable and non-viable bacteria, with the latter treated (killed) in a manner that would actually be detected with the specific dye. Here the test was performed with untreated and heat-inactivated river water, with the latter expected to comprise mostly cells with damaged membranes. We selected a broad range of PI concentrations based on peer-reviewed literature (Table 3.1). While the PI concentrations varied, SG concentration had a fixed value (10,000× final dilution from the stock) throughout all tests to ensure the same SG emitted energy and thus direct comparability between data. The results showed that the lowest PI concentration (0.375 µM) already sufficed to detect a 1-log ICC decrease (91 %) in the heat treated sample, resulting in $2.83 \pm 0.289 \times 10^5$ cells mL^{-1} of ICC (Fig. 3.4). Higher PI concentrations (3 – 24 µM) showed considerably more ICC decrease (0.5 – 1.17×10^5 cells mL^{-1}). Notably the ICC values of the untreated samples varied from $2.15 \pm 0.05 \times 10^6$ to $2.58 \pm 0.16 \times 10^6$ cells mL^{-1} within the entire tested PI concentration range. Moreover, the results were not statistically different with PI concentrations in a range between 0.75 and 12 µM. While doubling the PI concentration caused up to 50 % different ICC in heat-treated samples, differences between doubled PI concentrations never exceed 5 % in the untreated samples. This indicates that while the dye concentration was less critical for viability evaluation of the untreated samples, it considerably affected non-viable (dead) cell quantification, and thus underlines the need to assess viability dyes specifically with both viable and non-viable target cells.

Similarly, the effects of staining caused by different PI concentrations were also observed on FCM density plots from the experiment above. Fig. 3.5 shows FCM density plots after 10 min of SGPI staining with identical SG concentrations and respectively low (0.75 µM), middle (3 µM) and high (24 µM) PI concentrations. While low PI concentrations stained a considerable part of heat-damaged cells, the shift from the "intact" to "damaged" regions was clearly incomplete (Fig. 3.4B; Fig. 3.5A). Although the ICC of the untreated sample (predominantly intact cells) were not statistically different when stained with 0.75 µM and 3 µM PI ($P > 0.05$), the low concentrations of PI resulted in poor separation between the clusters for intact and damaged cells, which might be an obstacle for correct data gating and interpretation (Fig. 3.5B; Fig. 3.5D). In turn, at high PI concentrations (24 µM) a clear separation of damaged cells in the heat-treated sample was observed (Fig. 3.5E). However, in the un treated samples all events (including the intact cells as well as the background) displayed higher red fluorescence intensities than in less PI-saturated samples: a 42 % higher median red fluorescence value of the ICC gated events in the sample stained with 24 µM in comparison to 3 µM PI. As a consequence, a fraction of presumably intact cells shifted across the fixed gate into the area allocated to damaged cells, which also explains the 9 % ICC decrease in the untreated sample stained with 24 µM PI (Fig. 3.4A).

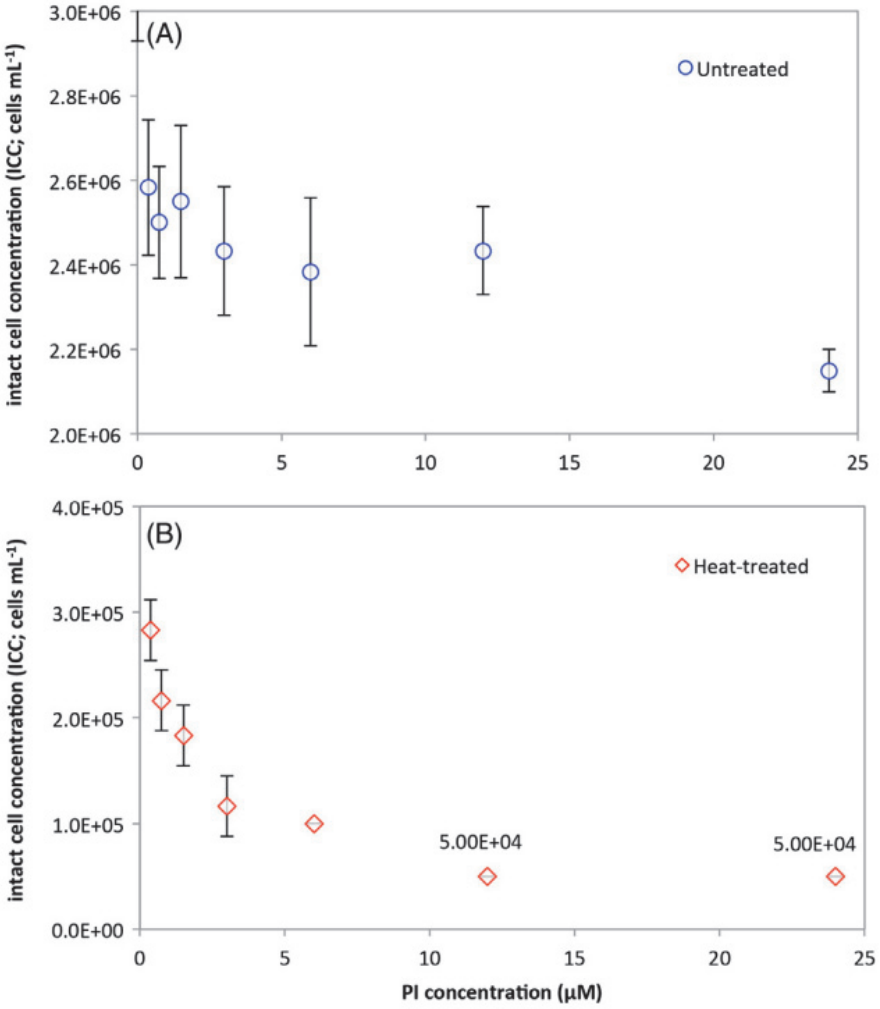


Fig. 3.4. The effect of different PI concentrations on SGPI staining results

Untreated (A, circles) and heat-treated (80 °C, 3 min; B, diamonds) river water samples were stained 10 min with doubling PI concentrations (0.375 – 24 µM final concentrations), while the SG concentration remained constant (10,000× diluted SG).

The optimal PI concentration has to provide complete staining of damaged cells without causing false PI-positive results (staining of intact cells). The mechanism of PI viability staining is based on labeling DNA of the cells with damaged membranes: large negatively charged PI molecules are normally unable to penetrate cells with intact membranes [114, 129]. In our case, staining was considered incomplete if the PI concentration was less than 3 µM. We argue here that the membranes of the cells in the heat-treated samples were definitely damaged, but insufficient PI penetrated the cells. Moreover, there also has to be sufficient PI in solution to achieve maximum intercalation with all target DNA of damaged cells together with SG. The latter will ensure complete energy transfer from SG to PI (known as fluorescence resonance energy transfer (FRET) or "quenching"), which is responsible for a critical shift in the PI signal intensity, and hence for the ability to differentiate between SG-positive and PI-labeled cells [56, 117]. This explanation was also proposed in the study of Barbesti and co-authors [62], where

inactivated cells emitted both green and red fluorescence, when insufficient PI was used. However, we also argue that there is no reason to use higher than 12 µM PI concentrations, since this could pose a risk of false-positive results and/or artifact fluorescence shifts in the FCM plots. The data from earlier studies, obtained with epifluorescence microscopy, showed increased amounts of red fluorescing cells in SGPI and 4′-6′-diamidino-2-phenylindole (DAPI)-PI stained samples, when the PI concentration was higher than 30 µM and 15 µM, respectively [63, 65]. This phenomenon was explained as a staining artifact in both studies, in agreement with our observations above. As a consequence, we conclude that the PI concentration should not be lower than 3 µM and should not exceed 12 µM to obtain reliable results. Hence, three PI concentrations – 3 µM, 6 µM and 12 µM – were selected for further testing in all subsequent experiments.

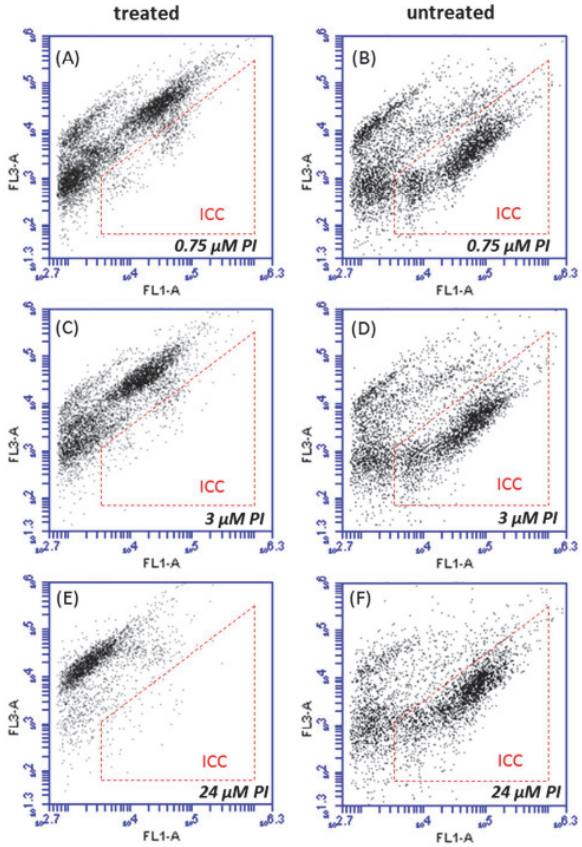


Fig. 3.5. FCM density plots of river water samples stained with different concentrations of PI (selected from Fig. 3.4)

Samples were exposed to different PI concentrations for 10 min: heat treated (80 °C, 3 min) river water samples stained with 0.75 µM (A), 3 µM (C), 24 µM (E) and untreated samples with 0.75 µM (B), 3 µM (D) and 24 µM (F) PI, while the SG concentration remained constant in all (10,000× diluted SG). The X and Y axis represent green (FL1-A) and red (FL3-A) fluorescence intensity respectively. The gated area (dotted red line) indicates the region where intact cells (ICC) are located on the plots.

As a consequence, we conclude that the PI concentration should not be lower than 3 µM and should not exceed 12 µM to obtain reliable results. Hence, three PI concentrations – 3 µM, 6 µM and 12 µM – were selected for further testing in all subsequent experiments.

3.3.3. Temperature influence the staining reaction

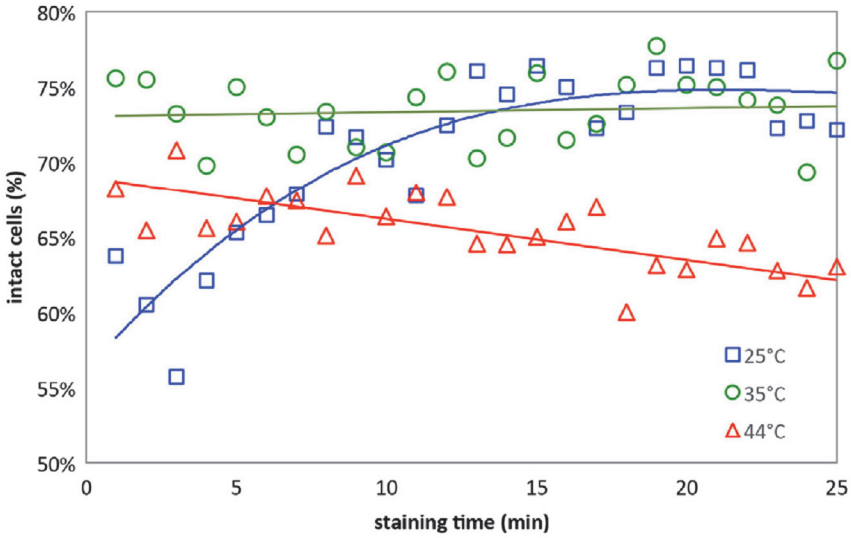


Fig. 3.6. The influence of three different incubation temperatures on the percentage of intact cells (calculated as ICC/TCC × 100) in a river water sample stained with SGPI with 3 µM PI (final concentration)

The actual TCC and ICC concentrations are shown in Fig. 3.7. The measurements were done at 1-min intervals for each sample (n = 25).

The main criteria for selecting a SGPI staining temperature were (a) achieving rapid and complete staining, and (b) avoiding temperature-induced membrane damage. Samples were stained with the three selected SGPI concentrations at 25 °C, 35 °C and 44 °C and measured at 1-min intervals from the moment of staining onwards. Warmer temperatures (35 °C) improved SGPI staining over the 25 °C sample, but too high temperatures (44 °C) permeabilized bacterial cell walls leading to potentially erroneous results (Fig. 3.6). At 25 °C the staining reaction was slow; it took nearly 11 min to reach a stable average of 74 ± 2 % ICC (as a percentage of TCC (% ICC) (n = 14)). Additionally, both ICC and TCC showed proportionally lower values at 25 °C in comparison with the results obtained at the higher temperatures (Fig. 3.7). This corresponds with previous studies where warmer staining temperatures resulted in higher TCC values [111, 130]. At 35 °C the % ICC was stable during all 25 min (73 % ± 3 %; n = 25), suggesting uniform staining with both dyes without apparent cell damage. However, it should be noted that while the % ICC remained constant at 35 °C, the actual cell concentrations still required about 8 min to reach steady state (see Fig. 3.2), similar to previous studies on SG staining [111]. In contrast, staining at 44 °C resulted in significantly less % ICC than the lower staining temperatures, and the % ICC was furthermore progressively decreasing over time, reaching as low as 63 % (25 min).

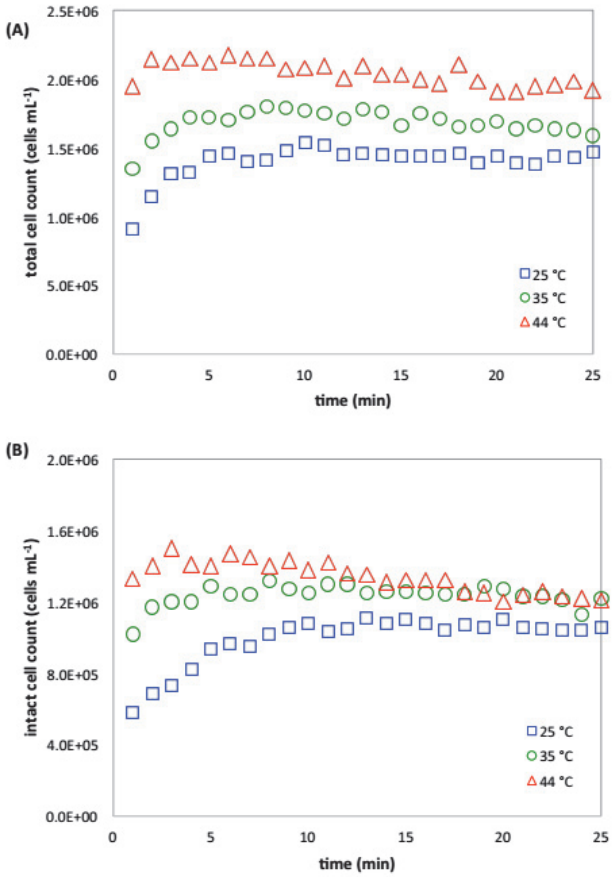


Fig. 3.7. Actual TCC and ICC concentrations of the samples, stained with SG (A) and SGPI with 3 µM PI (B), measured with flow cytometry

The same effects at the different temperatures were observed with the samples stained with SGPI containing 6 µM and 12 µM PI concentrations (Fig. 3.8). These data indicate that a part of the bacteria became membrane-compromised due to the high temperature. This was not entirely unexpected; it was already demonstrated that heat stress caused by 60 - 80 °C temperatures resulted in cell membrane disruption [56, 61, 65, 120], while a recent study demonstrated an increase of PI-positive cells even at 40 °C [130]. The data above emphasizes the importance of establishing an appropriate staining temperature and the absolute necessity of using a fixed value for all measurements. Interestingly, temperature was either not mentioned, or only vaguely described as “room temperature”, in the methods descriptions of several studies (Table 3.1). Our results suggest that 35 °C is the most suitable temperature, which ensures rapid and balanced staining and does not damage the cell membranes of the investigated bacterial populations. However, a broad temperature range (10 °C differences) was tested in the present study, and minor variations from the selected temperature may well produce satisfactory results. For example, the SGPI (dissolved in TRIS buffer) data from Fig. 3.2, recalculated as % ICC, showed that 37 °C also provides stable results without damaging cells: % ICC was 70 ± 2% during the all 25 min of staining (n = 30) (Fig. 3.9). This is important because 37 °C is often more advantageous as incubation temperature due to its

multi-purpose applications, and it was also used previously for SG and SGPI staining [62, 113]. Moreover, the SG staining method for total cell concentration measurements was standardized for this temperature (37 ± 2 °C) [112]. In the broader context, this data clearly emphasize the need to consider staining temperature as a critical parameter when testing dyes and staining protocols for quantitative flow cytometry.

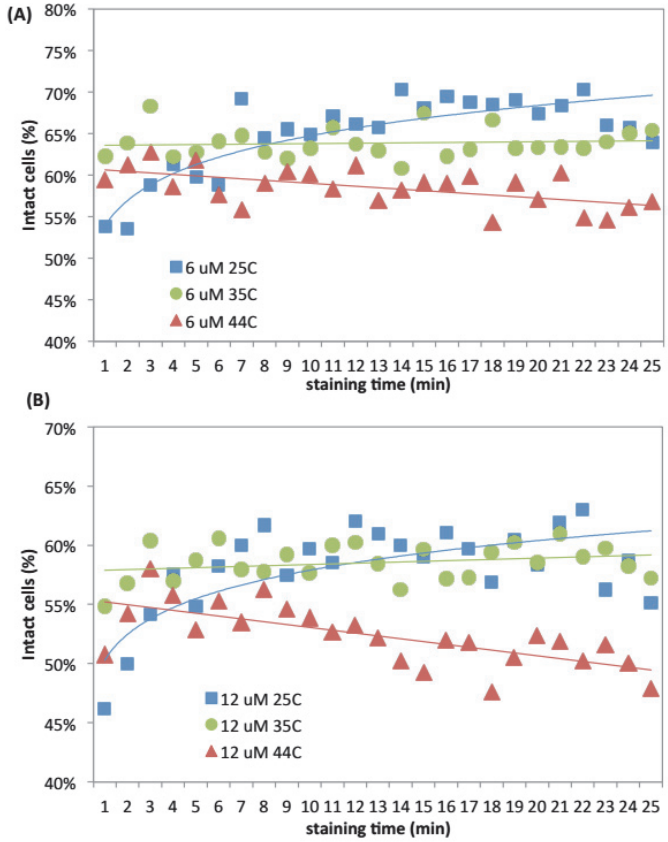


Fig. 3.8. The influence of three different incubation temperatures on the percentage of intact cells

A river water sample stained with SGPI with 6 µM (A) and 12 µM (B) PI concentration. Percentage of intact cells was calculated as ICC/TCC × 100. The measurements were done at 1-minute intervals for each sample (n = 25).

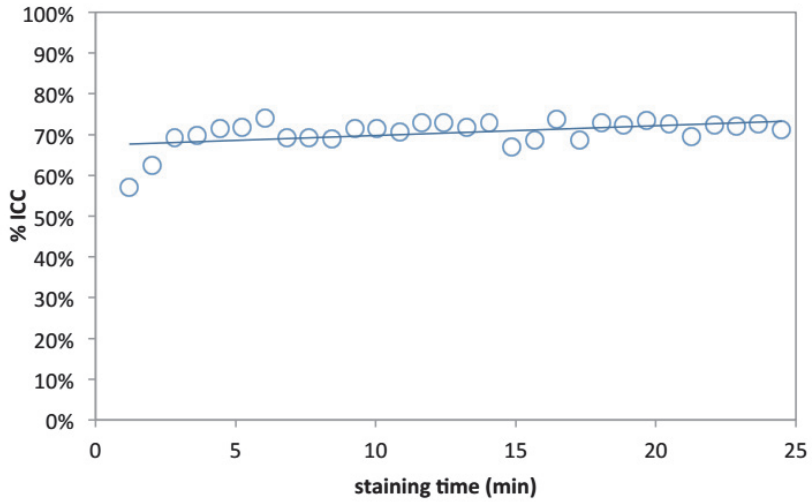


Fig. 3.9. Changes of the percentage of intact cells (calculated as ICC/TCC × 100) in a river water sample stained with SGPI diluted in TRIS buffer and 3 µM PI (final concentration) when incubated at 37 ^{0}C

The actual TCC and ICC concentrations are shown in Fig. 3.2A and Fig. 3.2B (TRIS buffer values). The measurements were done with automated 96-well multi-plate FCM approach (n = 30).

3.3.4. Determination of suitable staining time and final PI concentration

Our entire dye-testing approach by default already included staining time as a variable in the tests with respect to PI concentrations and staining temperatures, and evidently the staining time had the most significant effect on ICC during first 10 min (Fig. 3.2, 3.6 - 3.8). A closer look at staining time and PI concentrations specifically showed that maximum ICC values in an untreated river water sample were obtained after 8 min (1.4×10^{6} cells mL^{-1}), 7 min (1.29×10^{6} cells mL^{-1}) and 11 min (1.21×10^{6} cells mL^{-1}) as a result of the SGPI staining at 35 °C with 3 µM, 6 µM and 12 µM PI concentration respectively (Fig. 3.10A). The staining was stable between 10 and 25 minutes, and the average ICC were $1.33 \pm 0.043\times10^{6}$ cells mL^{-1} with 3 µM PI, $1.20 \pm 0.037\times10^{6}$ cells mL^{-1} with 6 µM PI and $1.13 \pm 0.042\times10^{6}$ cells mL^{-1} stained with 12 µM PI concentration. Thus 10 min was deemed sufficient to distinguish between intact and damaged cells if no additional treatment was applied.

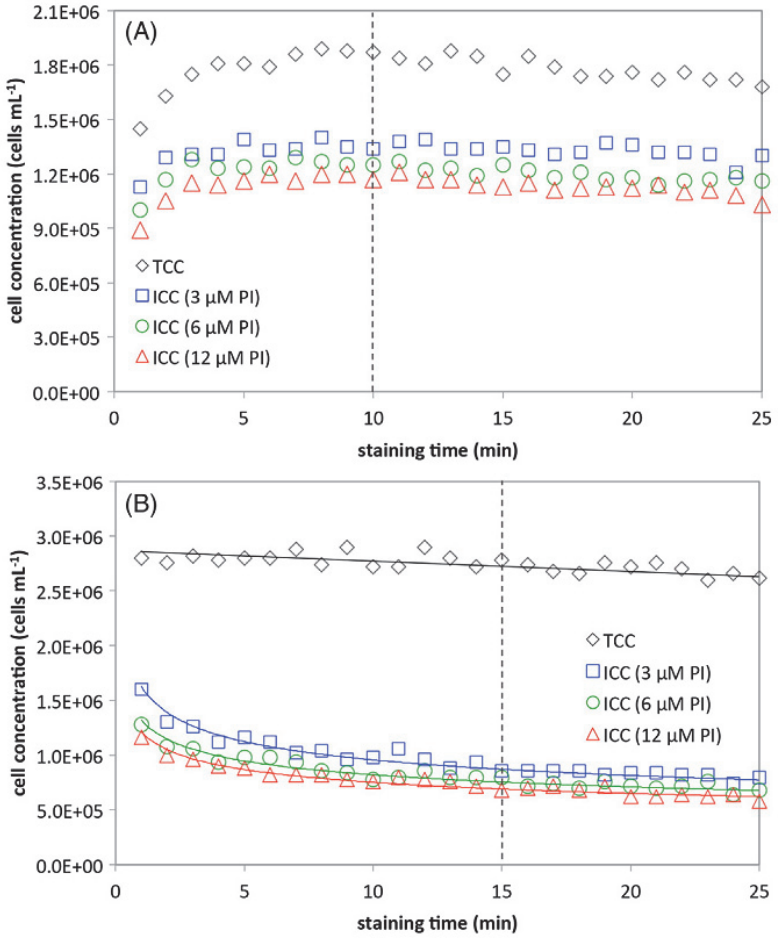


Fig. 3.10. Determining the optimal PI concentration and staining time by sequential measurements of an untreated river water (A) and chlorinated river water sample (B)
Samples were stained and measured at 35 °C with SG for determination of TCC (diamonds) and SGPI with different PI concentrations for ICC: 3 µM (squares), 6 µM (circles) and 12 µM (triangles). A minimum of 15 min staining time and 6 µM PI (final concentration) were selected as the optimal conditions. A dotted vertical line marks time when staining became stable.

In order to test staining time and concentrations on damaged cells, a slightly chlorinated river water sample (0.35 mg-Cl_2 L^{-1}, 5 min) was used. TCC values were stable throughout staining of the chlorinated sample, namely, $2.75 \pm 0.078 \times 10^6$ cells mL^{-1} (n = 25, CV = 3 %) (Fig. 3.10B). In turn, the ICC decreased logarithmically with an increase of time in chlorinated samples. Comparing to the untreated sample, in chlorinated sample the results were more stable (smaller CV values) within 15 and 25 min (n = 11) rather than between 10 and 25 min. ICC were $8.26 \pm 0.34 \times 10^5$ cells mL^{-1} (30 % of TCC, CV = 4 %), $7.14 \pm 0.37 \times 10^5$ cells mL^{-1} (26 % of average TCC, CV = 5 %), $6.54 \pm 0.48 \times 10^5$ cells mL^{-1} (24 %, CV = 7 %) for the 3 µM, 6 µM and 12 µM PI concentrations, respectively. The more stable results obtained after 15 min led to a conclusion that 15 min is optimal time for SGPI staining for both intact and damaged cells.

The importance of an appropriate PI concentration and its selection criteria were already discussed in detail in Section 3.3.2. PI concentrations tested/described in this section (3 – 12 μM) met our requirements, and therefore selection of the appropriate stain concentration was based on statistical difference between the measurements. ICC in the treated samples stained with the dyes containing 6 μM and 12 μM of PI were not statistically different ($P > 0.05$), in other words, doubling the concentration of PI did not result in significant change of ICC. In turn, ICC obtained with 3 μM and 6 μM PI were statistically different ($P < 0.05$). In untreated samples, all three PI concentrations were statistically different, however, 6 μM and 12 μM were less different than other combinations (P value in first case was higher than the rest). Additionally, % ICC difference between 6 μM and 12 μM was only 4% after defined staining time (15 min, $n = 10$), which is not critical for viability assessment as discussed in Section 3.3.2. Thus 6 μM PI was chosen as an optimal concentration for SGPI dye.

The same concentration and staining time were used in some of the studies mentioned before [1, 69, 119]. However, selection of staining time was not explained. Other studies, which showed dependences between time and stained bacteria, usually made the conclusion based on stability of the results. For instance, some researches compared "live"/total bacteria ratios [114] or PI-stained cells ratio [64] considering hypothesized physical state of bacteria. In turn, Williams and co-authors were selecting optimal staining time with an aim to avoid cell leakage and false PI-labelled cells, which might appear due to too long exposure to the stain [65]. Different time values were chosen as optimal in these works: 10 min [64], 20 min [114] and 30 min [65]. Although we can't compare actual time value between different studies unless other parameters are not the same, to our knowledge, staining was not applied for more than 30 min if PI was used with a counterstain (Table 3.1). With that we conclude that selected 15 min staining time is sufficient time to achieve optimal staining results and generally do not contradict with other studies.

3.3.5. Additives: EDTA addition affects SGPI staining

EDTA addition was previously shown to be essential for achieving optimal staining results with pure cultures of gram-negative bacteria [56, 130, 131]. But EDTA addition presents an enigma to viability staining, as it is known to alter properties of cellular membranes [128, 132, 133], hence potentially creating results rather than measuring a result. A broad set of environmental samples measured with and without 5 mM EDTA (final concentration) showed that EDTA addition unevenly increases cell membrane permeability between 1 and 39 % (Fig. 3.11A). The largest ICC decrease was observed in a seawater sample (39 %), and the least in bottled mineral water samples (1 – 8 %) (the actual TCC and ICC values are shown on Fig. 3.11B). Moreover, SGPI staining outcome depended on EDTA concentration: higher EDTA concentrations resulted in lower ICC values (Fig. 3.12). In the river water sample, 0.5 mM EDTA (final concentration) caused almost no changes in ICC ($P > 0.05$), however, ICC decreased from $2.22 \pm 0.035 \times 10^6$ cells mL^{-1} (without EDTA) to $1.37 \pm 0.06 \times 10^6$ cells mL^{-1} with 5 mM EDTA, which represents 38 % ICC decrease in comparison to 0.5 mM EDTA. A further 10-fold increase of the final EDTA concentration resulted in $1.07 \pm 0.104 \times 10^6$ cells mL^{-1} ICC, which was only 48 %, when compared to ICC obtained without EDTA addition. It is important to highlight that SGPI staining with 50 mM EDTA resulted in only 14% less ICC in comparison to 5 mM EDTA containing sample (relatively to ICC of EDTA-free sample). This indicates that further increase of the EDTA concentration above 5 mM had only a limited effect on membrane permeability and a fraction of the bacterial population remained completely unaffected by EDTA and remained PI-impermeable. What is inconclusive from the data is whether EDTA addition enabled correct observation of damaged cells, or whether EDTA addition caused artifact false-positive staining of intact cells.

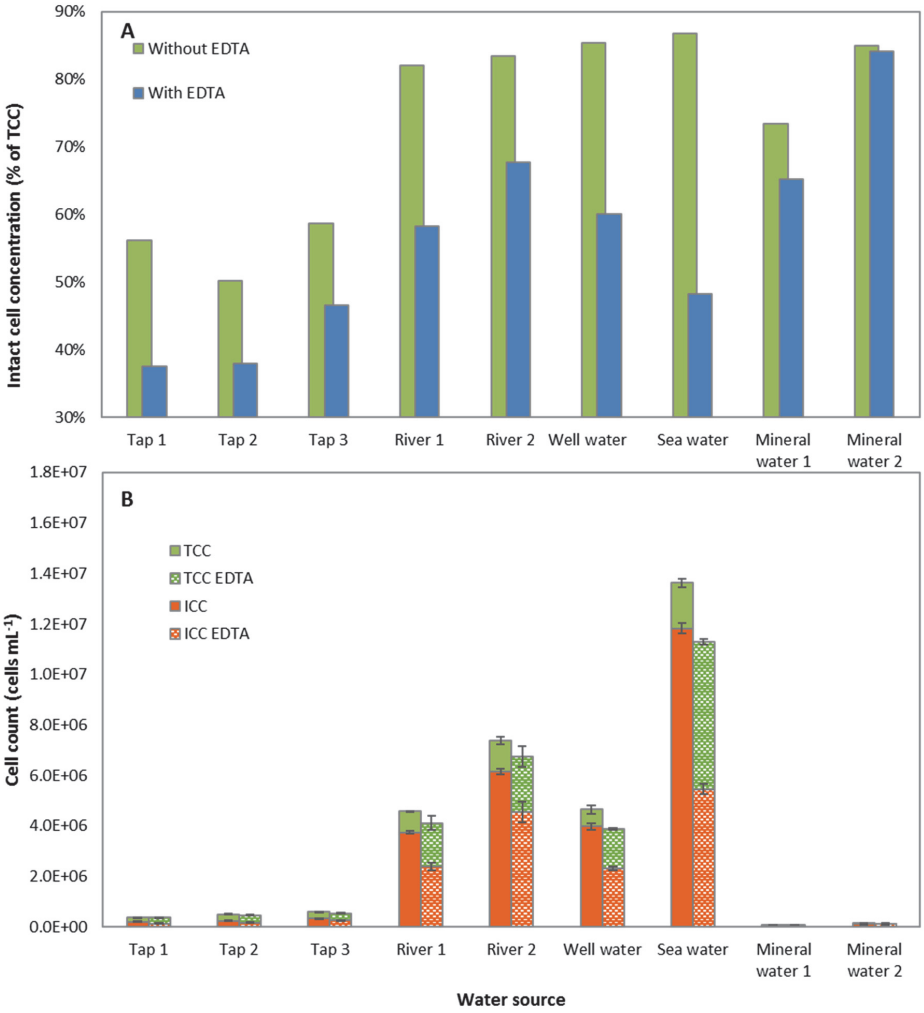


Fig. 3.11. Effect of EDTA addition on ICC and TCC in the samples of different origin and with different chemical and bacterial composition in a variety of water samples with different chemical and bacterial compositions

(A) The effect of EDTA addition on the percentage intact cells (ICC; calculated as ICC/TCC × 100); (B) Actual data of SGPI stained samples from (A). Tap water samples were chlorinated, but the source and treatment differed; the remaining samples were untreated. The percentage ICC was calculated from mean values of TCC and ICC (n = 3).

In earlier studies, the effect of EDTA on membrane permeability was mostly investigated in terms of bacteria susceptibility to various antimicrobial agents, and it was generally concluded that EDTA increases permeability of gram-negative bacteria by chelating metal ions on the outer membrane [132, 134–136]. Much less research have been conducted specifically for gram-positive bacteria. Available sources showed that gram-positive bacteria growth was inhibited in presence of EDTA [137, 138], and increased permeability was also reported [135], however, the mechanism is not clear. These contradicting results suggest that the impact of EDTA addition on bacteria is not completely clear and additional research is needed. However, because of the obvious risk of inducing membrane damage and creating false-positive data, it was decided not to use EDTA for routine analysis of environmental samples until more conclusive data becomes available.

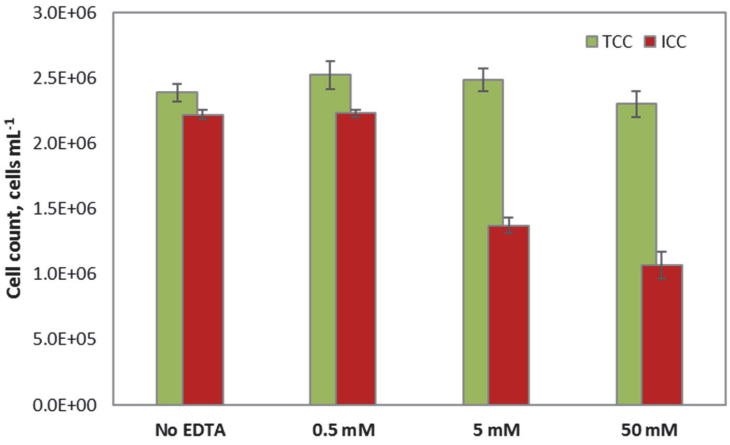


Fig. 3.12. Changes in total (TCC) and intact (ICC) cells with the addition of 10-fold increasing EDTA concentrations

A river water sample was stained at 35 °C for 15 min with respectively SG and SGPI (10,000× diluted SG and 6 μM PI) with or without EDTA. Error bars indicate standard deviation for triplicate experiments.

3.3.6. Considering the mechanism of cell death when assessing viability dyes

Bacteria, like other organisms, die from different causes and it is important to understand that currently no viability dye has been identified that universally distinguishes between life and death [139]. Membrane integrity, determined with SGPI, is a measurement of viability in samples treated with sunlight [56, 140], chlorine [119, 141], ozone [119], heat [101] (Fig. 3.4B), ethanol, soap (data not shown). However, SGPI led to erroneous viability interpretation in samples treated with 0.3 – 4 % formaldehyde or 0.01 – 0.05 % sodium azide (Fig. 3.13). Both of these compounds are toxic to bacteria at these concentrations [34, 142], but did not cause significant membrane permeabilization. Another example is UV-C light exposure, where bacteria become inactivated (DNA damage) long before the membrane gets damaged [143].

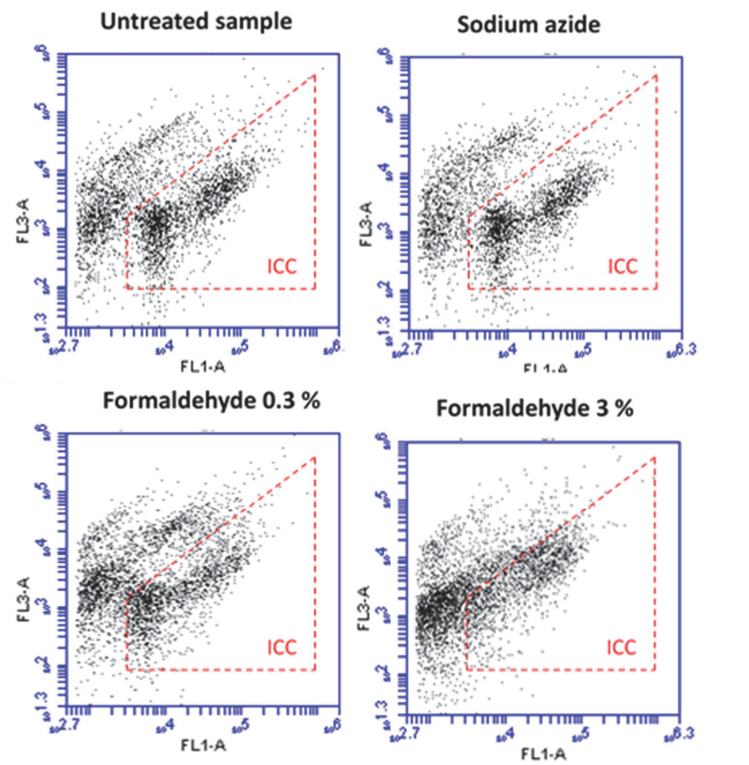


Fig. 3.13. FCM density plots of the untreated sample and samples treated with sodium azide (0.05 % w/v) and formaldehyde (0.3-0.4 % and 3-4 % v/v)

Despite the fact that both fixation solutions are very toxic to bacteria, most of the bacteria are observed in intact cell area, which indicates that the membrane integrity was hardly affected.

A final more complicated example is disinfection with heavy metals. Addition of 4 mg L^{-1} $CuCl_2$ (equivalent to 1.9 mg-Cu^{2+} L^{-1}) to river water samples resulted in merely 7 % ICC decrease, when the samples were stained with the normal SGPI protocol (Fig. 3.14A, 3.14B), but reached 53 % with EDTA addition. A similar situation was observed in samples where silver ($AgNO_3$) was added to inactivate bacteria (Fig. 3.14C). We argue that EDTA addition chelates the membrane-bound metal ions that resulted in a PI-impermeable layer, and thus EDTA treatment is necessary to observe cell damage in this specific case. These results highlight potential risks while using SGPI dye specifically, and underline the fact that all staining protocols should be developed and tested according to the type of samples and applied treatment.

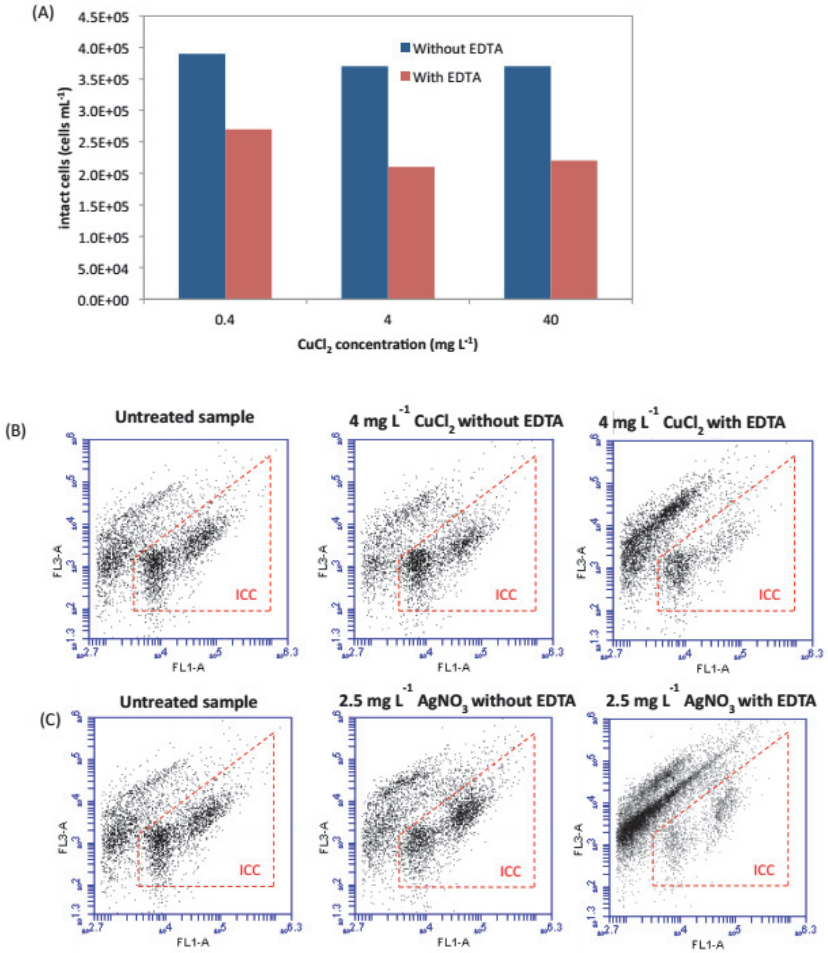


Fig. 3.14: EDTA effect on the SGPI staining of river water treated with heavy metals

ICC obtained after river water treatment with different $CuCl_2$ concentrations (A); FCM density plots of river water sample, stained with SGPI: without any treatment, incubated with 4 mg L^{-1} of $CuCl_2$ for 30 min, incubated with 4 mg L^{-1} of $CuCl_2$ for 30 min and stained with addition of EDTA (B); FCM density plots of river water sample, stained with SGPI: without any treatment, incubated with 2.5 mg L^{-1} of $AgNO_3$ for 30 min, incubated with 2.5 mg L^{-1} of $AgNO_3$ for 30 min and stained with addition of EDTA (C). On the FCM density plots, X and y axis represent green and red fluorescence intensity respectively.

3.4. Conclusions of Chapter III

A pipeline for developing and optimizing staining protocols for flow cytometry was demonstrated in the study with the viability stain combination of SGPI. We specifically showed that:

- *Solvent*: DMSO enhanced membrane permeability in autochthonous bacterial communities and resulted in erroneous data interpretation; TRIS buffer was suggested as alternative.
- *Concentration*: 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.
- *Temperature*: 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.
- *Time*: Approximately 12 – 15 minutes were needed to stain all cells and achieve stable results with the selected concentrations and temperatures listed above.
- *Additives*: 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.

Altogether, the data clearly shows the need to be careful, precise and reproducible when staining cells for flow cytometric analysis.

4. CHAPTER IV: BEHAVIOR AND STABILITY OF ADENOSINE TRIPHOSPHATE (ATP) DURING CHLORINE DISINFECTION

4.1. Introduction

Disinfection is often applied as a final step in drinking water treatment plants to inactivate microbial contaminants, and for this purpose, various chlorine-based oxidants are used worldwide [88]. Heterotrophic plate counts (HPC) and specific plating for indicator organisms are commonly used to control the microbiological quality of water in general and disinfection efficacy in particular [38, 88]. Despite well-described shortcomings [38, 101, 108], HPC remains an accepted method for drinking water analysis in most countries. During the last decade, a number of cultivation-independent methods have been proposed as alternatives to HPC for general microbial water quality assessment. Among those, adenosine triphosphate (ATP) analysis was argued to be a useful and prospective tool for bacterial viability determination in both chlorinated and non-chlorinated drinking water environments [18, 67–69].

Besides other functions, ATP molecules are the main energy carriers for many metabolic processes in cells [66]. 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 [67, 70–72]. Although the existence of extracellular ATP was observed and described already more than three decades ago in both seawater and freshwater environments [70, 73], its origin and stability remain unclear. Some studies have argued that extracellular ATP originates from lysed and stressed cells, or that ATP is released and excreted by higher organisms, or secreted by some types of bacteria during growth [70, 73–75]. With respect to the former, previous studies suggested that oxidative disinfection of drinking water resulted in considerable increase in extracellular ATP [76, 77]. A plausible explanation for this phenomenon is that extracellular ATP is released from damaged/disrupted bacteria. Chlorination damages bacterial membranes [91] and excessive chlorination could even lyse the cells [144]. However, the extent of the ATP release, and how long the extracellular ATP molecules could exist outside the cells in the aquatic environment (with or without chlorine present), is unknown. Azam and Hodson [70] showed that ATP molecules are rather stable in abiotic environments, but the mechanism of its decomposition in natural waters is not obvious. These authors argued that decomposition by extracellular enzymes is unlikely, since the enzymes and the substrate are diluted too much in environmental samples [70]. Some studies showed uptake of extracellular ATP by bacteria [70, 73, 74], whereas Mempin and co-authors [75] argued that extracellular ATP molecules could not be taken up into the cell, but only be degraded on bacterial surfaces.

The understanding of ATP behavior in aquatic ecosystems, particularly during chlorination in engineered systems, is important for correct application of the ATP methodology and accuracy of cell viability determination with this approach. Moreover, it is important because extracellular ATP could contribute towards biological instability by supplying growth-supporting nutrients (carbon/phosphate) to bacteria. The main goals of the present study were: (i) to examine ATP release from bacterial cells during chlorination; (ii) to investigate the stability of extracellular ATP in the absence and presence of chlorine and a freshwater indigenous bacterial community; and (iii) to study extracellular ATP degradation/decrease in nutrient-limited environments.

4.2. Materials and Methods

4.2.1. ATP release during chlorination

Escherichia coli K-12 MG1655 strain was pre-incubated in 30 % Luria-Bertani (LB) medium at 30 °C for 24 h to reach stationary growth phase. Bacterial cells were separated from the medium by low-speed centrifugation (3000 rpm, 3 minutes) to avoid cell damage from high rotation rates. Centrifugation was repeated twice, with subsequent dilution in filtered (0.1 µm; Millex®-GP, Millipore) bottled mineral water. After the last step, the cell suspension was diluted in filtered (0.1 µm, Millex®-GP, Millipore) river water (Glatt river, Dübendorf, Switzerland) to a final concentration of approximately 3 x 10^6 cells mL^{-1}. The river water had a DOC content of 4 mg L^{-1} and a pH of 8.5. The suspension (25 mL) was thereafter subdivided into sterile Greiner tubes and treated with different chlorine concentrations. A sodium hypochlorite solution (6 – 14 %; Sigma) was diluted in nanopure water (deionized, 0.1 µm filtered) to make stock solutions, which were used to obtain final free chlorine concentrations from 0.04 to 22.4 mg L^{-1} (equivalent to 0.01 to 2.8 g-Cl_2 g-DOC^{-1}) in the experimental samples. All the samples were incubated for 5 minutes with chlorine, which was neutralized afterwards by adding 10 mM $Na_2S_2O_3$ with 1 % (v/v) ratio. Flow cytometric (FCM) intact cell counts (ICC), as well as total and extracellular ATP, were measured in untreated water and directly after chlorine quenching. All experiments were done in triplicate. The exact same experiment was also done with the indigenous microbial community in unfiltered river water (Glatt river, Dübendorf, Switzerland) without *E. coli* addition.

Additionally, heat-shock experiment was done in order detect whether the release of ATP, as seen with high chlorine concentrations, is similar when bacteria are rapidly and severely damaged with another aggressive disinfection method. For that, a sterile 15 mL Greiner tube with 10 mL of the filtered river water was heated in a water bath until boiling (100 °C). Then, 100 µL of an *E. coli* stock solution was added directly into the boiling water. The samples for total and extracellular ATP measurements were taken directly (< 10 seconds) after inoculation, then after 1, 2 and 5 minutes and put on ice prior analyses.

4.2.2. Stability of extracellular ATP in abiotic environments

Sterilized (121 °C) nanopure water (200 mL) was supplemented with a commercially available ATP standard (rATP 10 mM, Promega) to a final concentration of 0.3 nM. In a second experiment, 200 mL river water that is naturally rich in extracellular ATP (Chriesbach river, Dübendorf, Switzerland), was filtered (0.1 µm, Millex®-GP, Millipore) in order to obtain an ATP-containing bacteria-free sample of natural freshwater. In both cases, the water samples were incubated at 5 °C and 30 °C without any additional treatments, and the total and extracellular ATP were measured at the beginning of the experiment (0 h) and then after 4, 8 and 20 h. In order to study the stability of pure ATP in the presence of chlorine, the same free chlorine concentrations as above (section 4.2.1) were applied to a sterile nanopure water sample with 0.5 nM ATP standard with a total exposure time of 5 minutes. In addition, FCM total cell counts (TCC) and intracellular ATP measurements were made for the river water samples to assess possible microbial contamination through 0.1 µm pore size filters (Millex®-GP, Millipore) and subsequent regrowth during incubation. All measurements were done in triplicate.

4.2.3. Extracellular ATP and bacterial growth

A bacterial community adapted to batch growth was prepared by inoculating 400 µL of river water (Chriesbach river, Dübendorf, Switzerland) in sterile glass vial containing 40 mL filtered (0.2 µm, Millex®-GP, Millipore) river water and incubating it for 20 h (30 °C, 120 rpm).

This inoculum was subsequently added to filtered river water (1 L) and the sample was placed into a water bath (30 °C) equipped with a magnetic stirrer. ICC, extracellular ATP and total ATP were analyzed every 30 minutes for 12 h on manually collected samples (see the methods below).

4.2.4. Evaluation of extracellular ATP as a nutrient source

A series of batch growth experiments was performed to assess the possible relevance of extracellular ATP during bacterial growth. Assimilable organic carbon (AOC) free glassware was prepared as described previously [31]. Bottled mineral water (Evian, France) was used as the water matrix. This water simultaneously provided an indigenous microbial community for the growth assays (TCC = 9.83 ± 0.12×10^4 cells mL^{-1}), and was considered to be biologically stable (TOC = 82 µg L^{-1}; AOC = 2 µg L^{-1}). The bottled water was supplemented in 9 different combinations with minimal medium (MM; phosphate and nitrogen source), sodium acetate ($C_2H_3NaO_2$; carbon source), and ATP (potential carbon and phosphate source), to create different nutrient limitation/excess conditions for bacterial growth (Table 4.1). The MM was prepared as 999 mL of phosphate-nitrogen buffer solution (1.28 g L^{-1} Na_2HPO_4 * $2H_2O$, 0.3 g L^{-1} KH_2PO_4, 1.77 g L^{-1} $(NH_4)_2SO_4$), 1 mL of an acidified trace element solution (8 g L^{-1} $CaCO_3$, 1.15 g L^{-1} $MnCl_2$ * $4H_2O$, 0.146 g L^{-1} $CuSO_4$ * $5H_2O$, 0.13 g L^{-1} $CoCl_2$ * $6H_2O$, 0.4 g L^{-1} ZnO, 0.124 g L^{-1} H_3BO_3, 13.42 g L^{-1} $MgCl_2$ * $6H_2O$, 1.04 g L^{-1} Na_2MoO_4 * $2H_2O$ with 0.64 % HCl) and 3 mg L^{-1} Na_2EDTA. Samples with MM addition contained 3.4 mL of MM and 50 µL of $FeCl_3$ (10 mM) in 100 mL of the sample, thus providing all inorganic nutrients in excess. All samples were prepared in 150 mL AOC-free glass beakers filled with 100 mL of bottled water and then divided into 3 AOC-free glass vials with 20 mL of sample in each. The experimental combinations were as following (Table 4.1): (i) Untreated carbon-limited bottled water was used as negative control; (ii) Bottled water with only MM addition simulated a carbon-limited but phosphorus-rich sample; (iii) Bottled water was supplemented with 10 µM ATP (0.93 mg-P L^{-1}) final concentration to test whether ATP could be used as both carbon and phosphorus source; (iv) Bottled water with both MM and ATP (10 µM; as the sole carbon source) was tested if ATP could be utilized as carbon source; (v) Bottled water with acetate (0.93 mg-C L^{-1}) and MM was used as a positive control with no nutrient limitation; (vi) Finally, the bottled water sample with only acetate (0.93 mg-C L^{-1}) was investigated to show bacterial growth with phosphorus limitation. In addition, three further samples (vii-ix) were prepared to simulate a situation where ATP hypothetically was supposed to serve as a phosphorus source with C:P ratios of 1:1, 10:1, 100:1 with a fixed acetate concentration (0.93 mg-C L^{-1}). Samples were incubated at 30 °C with shaking (120 rpm) for 72 h. FCM-TCC and TOC measurements were performed before and after incubation.

Table 4.1.
Growth potential experimental overview. Compositions of the tested solutions (i-ix) with final concentrations of carbon (C) and phosphorus (P) of different origin (the source is shown in brackets) added to bottled mineral water. Concentrations of biological available carbon and phosphorus in the bottled water are unknown but considered to be growth limiting, thus C and P values are not shown in the table (-) [141]

	I	II	III	IV	V	VI	VII	VIII	IX
$P_{(MM)}$ (mg L^{-1})	–	10	–	10	10	–	–	–	–
$P_{(ATP)}$ (mg L^{-1})	–	–	0.93	0.93	–	–	0.93	0.093	0.0093
$C_{(acetate)}$ (mg L^{-1})	–	–	–	–	0.93	0.93	0.93	0.93	0.93
$C_{(ATP)}$ (mg L^{-1})	–	–	1.2	1.2	–	–	1.2	0.12	0.012

4.2.5. Fluorescent staining and FCM of water samples

Conventional FCM analysis was based on the methods described previously [76, 101, 111]. In short, for TCC staining, a working solution of 100x diluted SYBR® Green I (Invitrogen) was used. For ICC staining, propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution (SGPI) to a final PI concentration of 0.6 mM. Depending on the analysis, 1 mL of the sample was stained with SGPI or only SG at 10 µL mL^{-1}. Before analysis, samples were incubated 10 minutes at 37 °C (SG) or 15 minutes at 35 °C (SGPI). Prior to FCM analysis, the water samples were diluted (10 % v/v) with 0.22 µm filtered commercially available bottled water (Evian, France). FCM was performed using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. Fluorescence intensity was collected at Fl1 = 533 ± 30 nm, Fl3 > 670 nm, while sideward (SSC) and forward (FSC) scattered light intensities were collected as well. Measurements were performed at pre-set flow rate of 66 mL min^{-1}. All data were processed with the BD Accuri CFlow® software as described previously [111].

4.2.6. Adenosine triphosphate (ATP) analysis

Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA, USA) with minor adaptions from the method described elsewhere [67]. In short: a water sample (750 µL) and the ATP reagent (50 µL) mixed with 8 µL of 1 M $MgCl_2$ prior analyses were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were then combined and then the luminescence was measured after 20 s reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (rATP, 10 mM, Promega Corporation). For extracellular ATP analysis each sample was filtered through a 0.1 µm sterile syringe filter (Millex®-GP, Millipore), followed by analysis as described above. The intracellular ATP was calculated by subtracting the extracellular ATP from the total ATP for each individual sample. ATP was measured in triplicate for all samples.

4.2.7. Chlorine concentration determination

Free chlorine was measured using commercially available chlorine test kits (LCK 310; Hach-Lange) and a photometer (CADAS 30, Hach-Lange), which was developed according to ISO 7393-1-2-1985 and DIN 38408 G4-2 standards. Analysis was done as described in the user's manual.

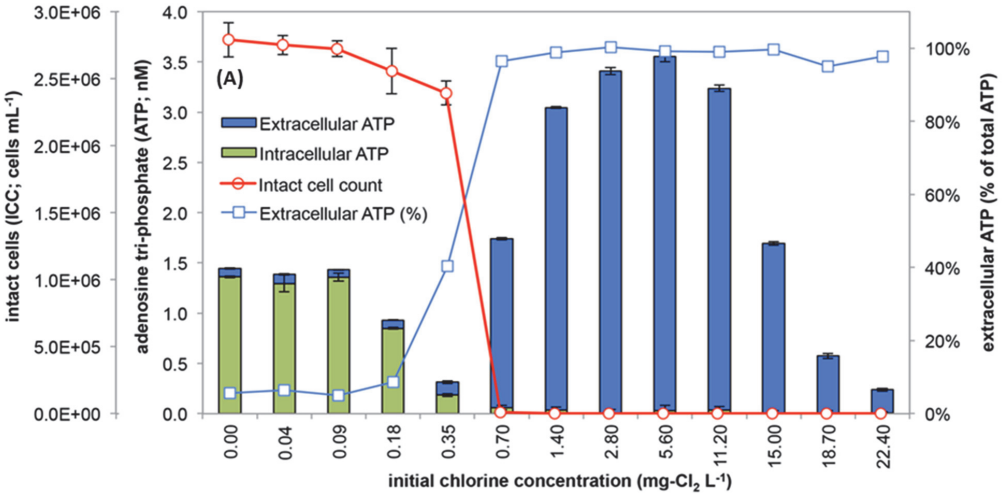
4.2.8. Total organic carbon (TOC) determination

TOC was measured as non-purgeable organic carbon with a Total Carbon Analyser TOC-5000(A) (Shimadzu, Reinach, Switzerland) equipped with a high-sensitive catalyst (High sense TC catalyst; Shimadzu). 20 mL of the sample was transferred into a C-free TOC vial and acidified with 200 µL HCl (2 M). After stripping CO_2 with CO_2-free air, three times 2 mL were injected into the TOC analyzer for measurement. The TOC concentration of the sample was then the average of the three measurements. To prepare a calibration curve a stock solution of potassium phthalate (TOC standard solution according to EN 1484-H3, 1997; from Merck, Darmstadt) was diluted with nanopure water to obtain solutions with carbon concentrations between 0 and 2 mg-C L^{-1}. The detection limit of the method was about 0.1 mg-C L^{-1}.

4.3. Results and Discussion

4.3.1. ATP release from bacteria during chlorination

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 (Fig. 4.1). Two different samples were exposed to chlorine (5 min). The starting *E. coli* suspension without chlorine addition had an intact cell concentration (ICC) of 2.8 ± 0.13×10^6 cell mL^{-1}, with 94 % intracellular ATP (1.36 ± 0.009 nM) and 6 % extracellular ATP (0.08 ± 0.005 nM) (Fig. 4.1A). Starting values for the measured parameters in river: ICC = 1.9×10^6 cells/mL, intracellular ATP = 0.169 nM (52.3 %), extracellular ATP = 0.154 nM (= 47.7 %). Exposure for five minutes to low initial free chlorine concentrations (0.04 – 0.09 mg-Cl_2 L^{-1}) did not significantly affect either the ATP concentrations or membrane integrity (ICC) of the bacterial cells. Progressively higher concentrations of chlorine (0.18 and 0.35 mg-Cl_2 L^{-1}) caused a clear decrease in both total and intracellular ATP with a minor impact on membrane integrity. At a dose of 0.18 mg-Cl_2 L^{-1}, the intracellular ATP of *E.coli* sample decreased to 0.85 ± 0.011 nM, while extracellular ATP remained unchanged, and ICC decreased slightly to 2.6 ± 0.17×10^6 cell mL^{-1}. At a dose of 0.35 mg-Cl_2 L^{-1}, the intracellular ATP decreased considerably (0.19 ± 0.014 nM), while the extracellular ATP showed a significant increase, comprising 40 % of the total ATP (0.13 ± 0.013 nM) (Fig. 4.1A). Simultaneously, the ICC showed a small but significant decrease down to 2.4 ± 0.09×10^6 cells mL^{-1}. According to the ICC values, the majority of bacteria remained intact at low chlorine concentrations during five minutes of exposure, similar to previous observations [91]. However, from the observed loss of intracellular ATP we suggest that the low chlorine concentrations clearly caused subcellular damage affecting membrane-bound functions. The simultaneous decrease of intracellular ATP and total ATP, while extracellular ATP was not affected, indicates that ATP was intracellularly hydrolyzed, and the ATP hydrolysis rate was higher than ATP synthesis.



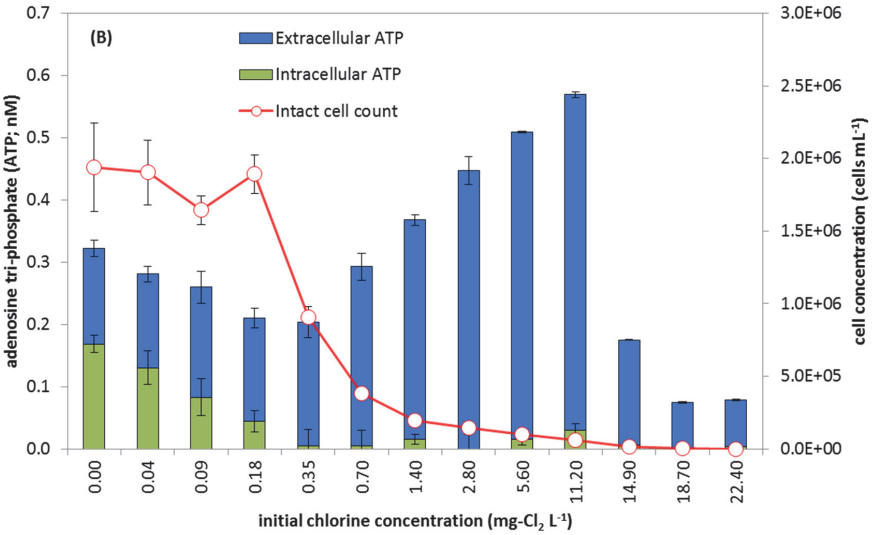


Fig. 4.1. Release of ATP during chlorination

(A) Chlorine disinfection (5 min, room temperature) of an *E. coli* pure culture suspended in river water with different hypochlorite doses. (B) Chlorine disinfection (5 min, room temperature) of river water (Glatt river, Dübendorf) samples with different hypochlorite doses. Stacked columns show the total ATP as the sum of intracellular ATP (green) and extracellular ATP (blue). The percentage of extracellular ATP at every chlorine dose is indicated (squares). Intact cell counts (ICC; circles) were measured with flow cytometry following SGPI staining. Error bars indicate standard deviation from triplicate experiments.

Initial chlorine concentrations of 0.7 mg-Cl_2 L^{-1} and higher resulted in clearly different behavior of ATP. Whereas intracellular ATP continued to decrease to insignificant levels (e.g., 0.06 ± 0.021 nM at 0.7 mg-Cl_2 L^{-1}), extracellular ATP (and therefore also total ATP) increased dramatically to 1.68 ± 0.01 nM, treated with 0.7 mg-Cl_2 L^{-1} and 3.52 ± 0.053 nM at 5.6 mg-Cl_2 L^{-1} (Fig. 4.1A). 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 increase of extracellular ATP is unlikely to be an artifact, since leakage of other cell constituents after chlorination was detected in previous studies as well [30, 133, 144], while Hammes and colleagues [76] observed similar ATP and ICC results during drinking water ozonation. Although the same experiment with indigenous river water community was not described in details, it shows similar tendency (Fig. 4.1B), suggesting that this behavior is universal for bacteria exposed to oxidative disinfection. From this data it is abundantly clear that extracellular ATP should be considered carefully when studying bacterial viability during chlorination disinfection, supporting previous observations in this regard [67, 77].

While the results show a clear link between the chlorine dose, ATP release and membrane damage, one aspect is still not completely clear. The experiment with the *E. coli* culture showed more than 2-fold higher total ATP (3.55 ± 0.003 nM) in the sample containing 5.6 mg-Cl_2 L^{-1} in comparison to the initial total ATP concentration of the untreated sample (1.44 ± 0.007 nM). This suggests that additional ATP was either synthesized or that there was an improved detection of the ATP following aggressive disinfection. A similar observation was shown in a previous study [76], where the total ATP after ozonation in the drinking water treatment plant

was also considerably higher than it was initially. In that study it was argued to be a methodological artifact, namely increased/improved ATP release as a result of ozonation process compared to the extraction capabilities of the ATP assay reagent [76]. While plausible, additional explanations should be considered. For example, chlorination can result in the inhibition of ATP-associated enzymes [145]. Thus in highly chlorinated samples, ATP hydrolysis is inhibited instantly, and this could lead to the detection of all ATP, part of which gets rapidly converted to energy for bacterial metabolism under normal and/or less stressful conditions. We examined this further with heat-shock experiments (Fig. 4.2), which showed that ATP measured after severe heat shock was also substantially higher than values measured in untreated control samples and similar to the total ATP values obtained with chlorination with 5.6 mg-Cl_2 L^{-1}. This suggests that the observed increased total and extracellular ATP concentrations are not specific only for oxidative stress, but are strongly linked to severe bacterial membrane damage. It is in our opinion highly unlikely that chlorination could promote functions of ATP synthases (enzymes concentrated in bacterial membrane responsible for ATP formation). Membrane damage caused by chlorine would rather result in dissipation of the proton motive force, which is essential for an ATP synthesis [146]. Additionally, it was also demonstrated in one of the previous studies that 0.4 mg-Cl_2 L^{-1} is able to stop phosphorus uptake completely [144].

As a final observation, the amount of extracellular (and therefore total) ATP decreased considerably at extremely high free chlorine concentrations (> 11.2 mg-Cl_2 L^{-1}) and reached 0.23 ± 0.013 nM (16 % of initial total ATP) at 22.4 mg-Cl_2 L^{-1}. This decrease in ATP in highly chlorinated samples is attributed to the oxidation of ATP molecules by chlorine (discussed in detail below).

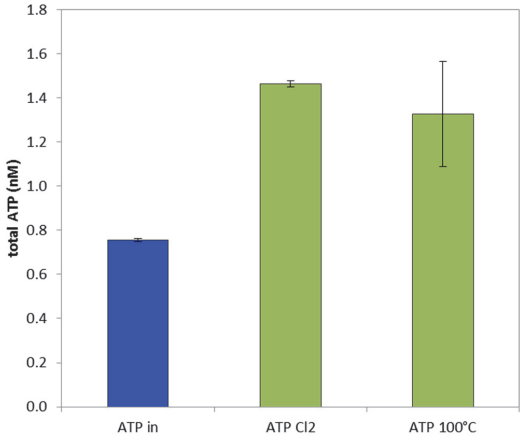


Fig. 4.2. ATP release from bacterial cells following high temperature (100 °C) heat shock compared to high concentration chlorination (5.6 mg-Cl_2 L^{-1})

ATP released after the heat shock was compared with the control samples: ATP in = the ATP concentration in untreated control samples; ATP Cl2 = similarly diluted samples chlorinated with 5.6 mg-Cl_2 L^{-1} for 5 minutes. The experiment was repeated thrice, the error bars correspond to standard deviation on triplicate samples.

4.3.2. Stability of extracellular ATP in cell-free environments

Supplemented extracellular ATP remained stable in sterile nanopure (filtered, deionized) water for at least 20 h at both 5 °C and 30 °C (Fig. 4.3A). In this experiment the initial concentration of extracellular ATP was 0.3 ± 0.003 nM, and after 20 h it was 0.35 ± 0.023 nM at 5 °C and 0.32 ± 0.005 nM at 30 °C, respectively. In contrast, a clear temporal decrease in naturally-occurring extracellular ATP was observed in 0.1 µm filtered river water (Fig. 4.3B), from 0.44 ± 0.004 nM to 0.16 ± 0.001 nM at 5 °C and from 0.31 ± 0.004 nM to 0.02 ± 0.002 nM at 30 °C. ATP decrease rates differed according to incubation temperature: $k = 0.051\ h^{-1}$ ($R^2 = 0.996$) at 5 °C and $k = 0.145\ h^{-1}$ ($R^2 = 0.999$) at 30 °C. This result differs from previous studies, where extracellular ATP decrease was not observed in filtered seawater and freshwater [70, 73]. However, we argue that the result could be explained by ATP decomposition due to extracellular enzymes naturally present in the river water. The presence and activity of extracellular enzymes (including phosphatases) in aquatic environments have been demonstrated previously [147–149], although Azam and Hodson [70] suggested that the high dilution of enzymes renders enzymatic ATP degradation improbable. Although 0.1 µm filtration was used to remove bacteria and test enzymatic degradation of extracellular ATP, it is noted that filtered natural water cannot be absolutely bacteria-free [150]. A small fraction of events (< 1 % of original water sample) was detected by FCM in the tested samples (Fig. 4.4), thus it should not be excluded that a part of extracellular ATP could be degraded by bacteria as well. Importantly, this is unlikely since bacterial growth was not observed during 20 h in this experiment (discussed further in Section 4.3.3 below).

To test the stability of extracellular ATP in the presence of chlorine, nanopure water that was supplemented with ATP and subsequently chlorinated, showed that extracellular ATP concentrations remained stable until an extremely high amount of chlorine (> 11.2 mg-Cl_2 L^{-1}) was added (Fig. 4.3C). The mean extracellular ATP in the chlorine dosage range of 0 – 11.2 mg-Cl_2 L^{-1} was 0.48 ± 0.012 nM (n = 10), which decreased until 0.05 ± 0.002 nM at a concentration of 22.4 mg-Cl_2 L^{-1}. This is further indication that extracellular ATP remains stable for at least five minutes at typical drinking water concentrations of chlorine, but that high concentrations of chlorine can oxidize ATP molecules, confirming the observations made above (Section 4.3.1).

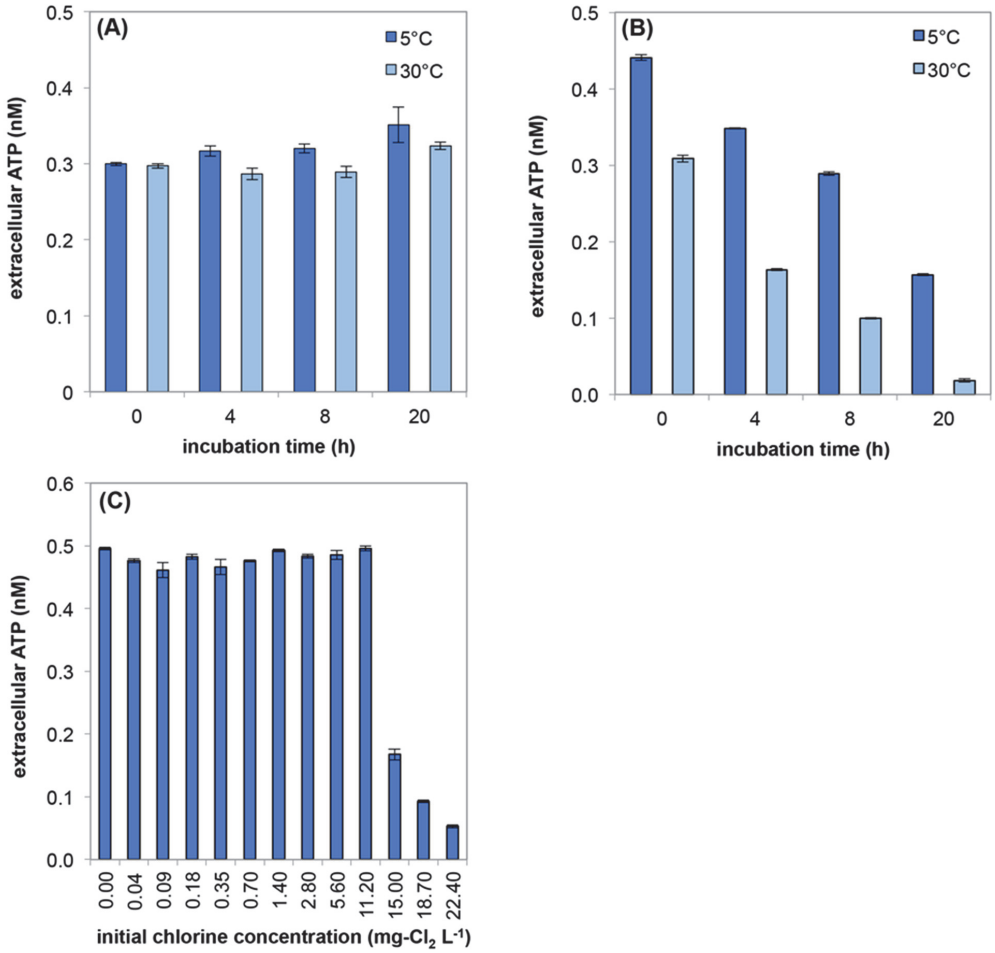


Fig. 4.3. Changes in extracellular ATP concentrations as a function of time (0 – 20 h), temperature (5 °C and 30 °C) and chlorine concentration in different aquatic environments

(A) sterile nanopure (deionized, filtered) water with added pure ATP; (B) filtered (0.1 µm) river water with natural-occurring extracellular ATP. (C) Changes in extracellular ATP concentrations, added to sterile nanopure water and exposed to different chlorine doses (5 minutes, room temperature). Total and extracellular ATP were measured for each sample to exclude any intracellular ATP influence on the results; total cell counts were measured in (B) to control if the decrease of ATP is related to bacterial growth (Fig. 4.4). Error bars indicate standard deviation from triplicate experiments.

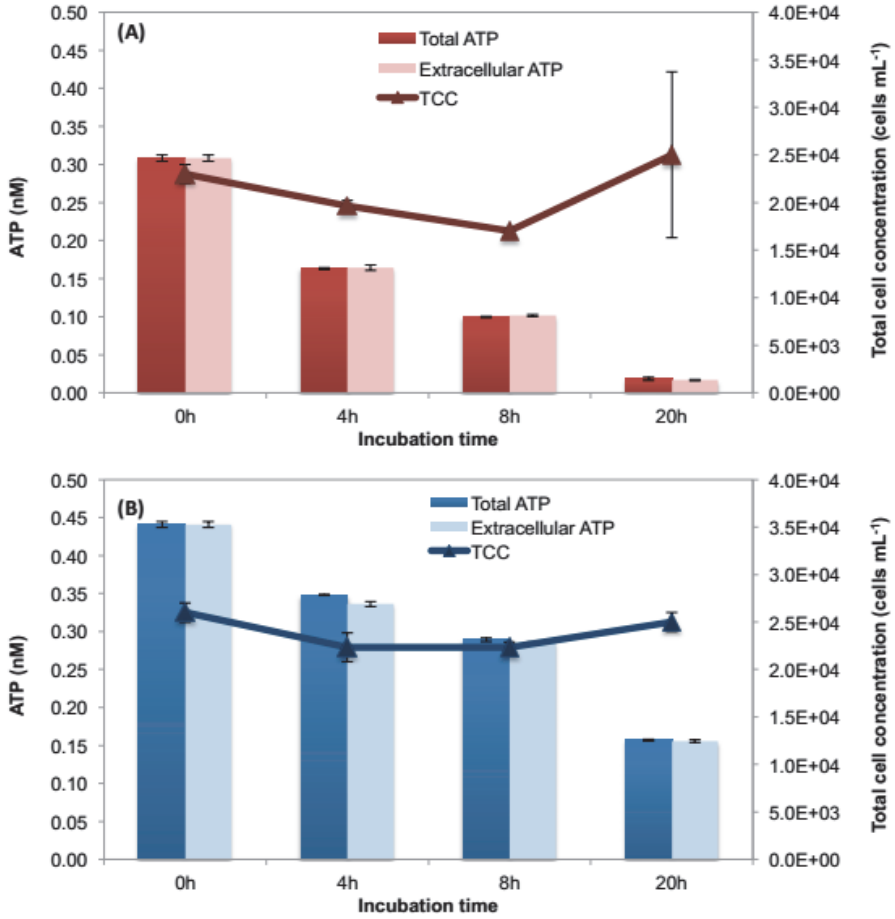


Fig. 4.4. Extracellular ATP and FCM total cell counts were measured to evaluate the possibility of ATP consumption by growing bacteria. Filtered (0.1 µm) river water incubated at 5 °C (A) and 30 °C (B). The data demonstrates that no bacterial growth occurred, whereas total and extracellular ATP were decreasing. Error bars indicate standard deviation on triplicate samples.

The speciation and activity of chlorine depend on the pH, which in turn could affect extracellular ATP degradation kinetics. Although pH was not specifically controlled during the experiments above, control experiments with the same river water showed that pH increases linearly but only slightly with higher sodium hypochlorite doses, e.g. from 8.54 without chlorine to 8.72 after 5 minutes exposure to 24 mg-Cl_2 L^{-1} of free chlorine (Fig. 4.5). Even the highest chlorine concentrations (8.5 – 24 mg L^{-1}) resulted in a minor pH increase (0.2 – 0.25 units), which would result in an increased ClO^- ratio in this pH range, and subsequently lower reactivity. It is therefore concluded that pH changes did not alter the chlorine speciation in a manner that would affect the experimental outcome. Since the most active chlorine species prevail at pH below 6.5, it is unlikely that the pH change contributed to faster ATP decrease in our experiments.

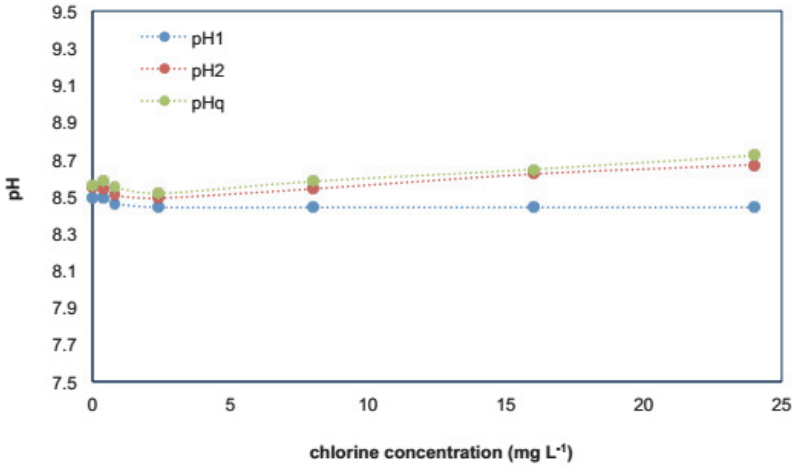


Figure 4.5. pH changes in river water during chlorination

Water samples (50 mL) were distributed into separate vials, and pH was measured before addition of hypochlorite (pH1), directly after addition and mixing with hypochlorite (pH2) and after 5 minutes of reaction and quenching (pHq).

As an additional control, we assessed the impact of the pH change on the ATP assay (Fig. 4.6). The data shows a small decrease (6 %) in measured extracellular ATP values as a result of a pH increase from 8.5 to 8.7 (see Fig 4.5), suggesting that this pH change had a minor impact on the present experiment. It is therefore concluded that the dramatic ATP decreases observed in Fig. 4.1 at high chlorine concentrations cannot be attributed to the minor pH changes at these concentrations (see Fig. 4.5). However, that also stressing the need for careful pH control in experiments where considerably pH changes are expected, and it is also evident that care should be taken with the interpretation of experimental set-ups where the pH can increase dramatically.

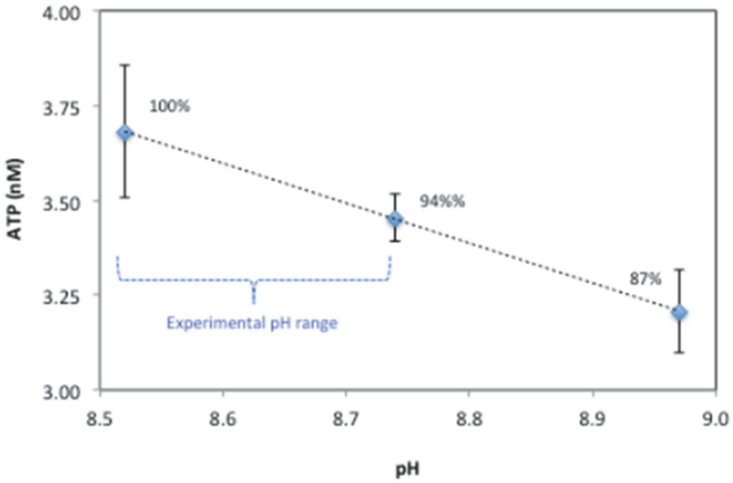


Fig. 4.6. The effect of increased pH on ATP measurements

The pH of three Tris buffer samples (10 mM; 100 mL) was adapted to respectively 8.52, 8.74 and 8.97. The samples were then aliquoted into triplicate Eppendorf tubes and the same amount of pure ATP was added to each (c.a. 3.75 nM). ATP was subsequently measured according to the protocol described in the Materials and Methods section. Error bars indicate standard deviation on triplicate samples.

4.3.3. Extracellular ATP stability in samples with bacterial growth

Rapid decrease of extracellular ATP occurred when incubated together with a growing bacterial community (Fig. 4.7). Initial ATP values at the beginning of the experiment were 0.185 ± 0.002 nM of extracellular ATP and 0.005 ± 0.005 nM of intracellular ATP (= 2.4 % of total), while the ICC was 2.5×10^4 cells mL^{-1}. A slight decrease in extracellular ATP was observed already after 2.5 - 3 h incubation, which coincided with a simultaneous intracellular ATP increase. After 8 h of incubation only about 8 % of the total ATP was extracellular (0.022 ± 0.007 nM). At this stage intracellular ATP reached almost a maximum (0.27 nM ± 0.007 nM) and remained stable for another 4 h until the end of the experiment. The combined increase in intracellular ATP and FCM ICC provide indisputable evidence that bacterial growth occurred (Fig. 4.7 & 4.9). The decrease of extracellular ATP was considerably faster ($k = 0.368\ h^{-1}$; $R^2 = 0.96$; Fig. 4.7, period = 2 – 8 h) in the presence of bacteria than without ($k = 0.145\ h^{-1}$; above). We therefor conclude that a considerable fraction of the extracellular ATP was degraded and/or taken up by growing bacteria. Extracellular ATP decomposition and/or uptake by bacteria was reported also in earlier studies [70, 71, 73–75]. The experiments above were all done with river water to illustrate the concept at high concentrations of naturally occurring extracellular ATP. To further assess the broader applicability of this data to drinking water, both bottled mineral water and non-chlorinated tap water were spiked with a high concentration of extracellular ATP and incubated overnight at 5 °C and 30 °C (Fig. 4.8). While the cold incubation (5 °C) showed no significant difference to the control sample, the 30 °C incubated samples showed clear ATP decreases for both bottled water (40 %) and tap water (15 %) (Fig. 4.8), albeit considerably smaller decreases in comparison with the river water experiment (above). Nevertheless, the results still suggest that similar biological processes are likely to occur in tap water.

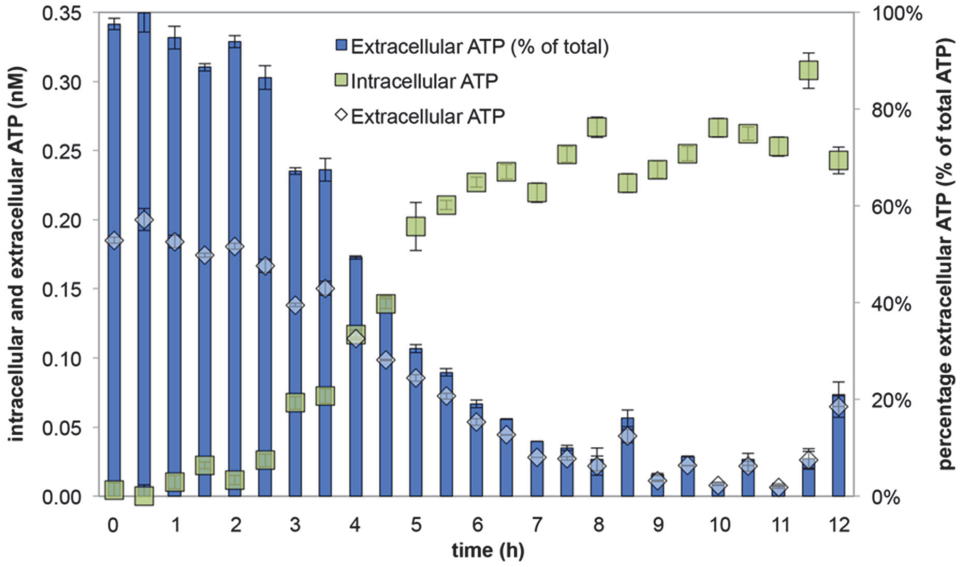


Fig. 4.7. Changes in intracellular and extracellular ATP during batch growth (12 h, 30 °C) of an indigenous river water bacterial community in filtered river water with natural-occurring extracellular ATP

Extracellular ATP (diamonds) decreases concurrent with increases in intracellular ATP (squares), thus completely reversing the ratio of extracellular to intracellular ATP (bars) over time.

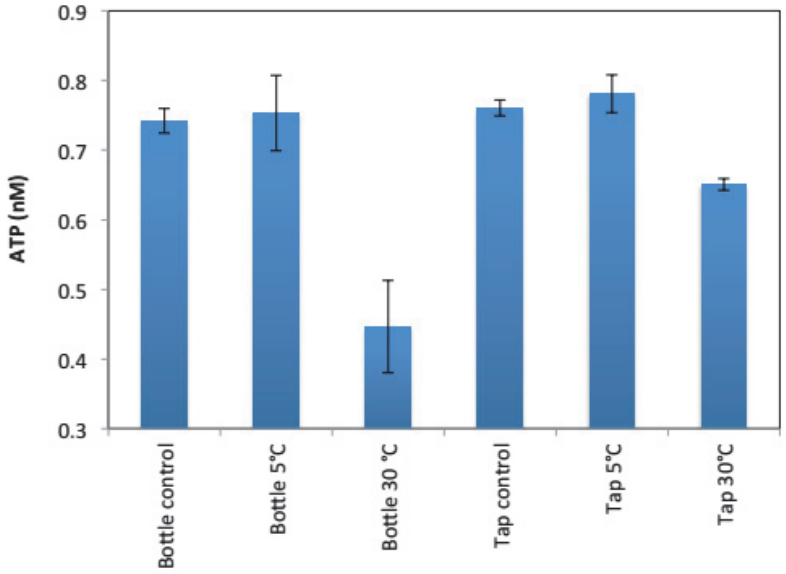


Fig. 4.8. Extracellular ATP decrease during 24 hours in bottled mineral water and non-chlorinated tap water

Pure ATP (c.a. 0.75 nM) was added to 100 mL water samples (in triplicate), measured prior to incubation (Bottle control & Tap control) and then incubated at either 5 °C or 30 °C for 24 hours, and measured again. Error bars indicate standard deviation on triplicate samples

4.3.4. Correlation between intracellular ATP and bacterial size

An interesting aspect of the growth study was that the increase in intracellular ATP was faster than the increase of intact cell counts (Fig. 4.7 & 4.9). The average rate constant of intracellular ATP increase during the first 8 h was $\mu = 0.51\ h^{-1}$, and the average growth rate constant for intact cell counts was $\mu = 0.32\ h^{-1}$ (Fig. 4.7). This discrepancy is partially explained by the change of the cell size/biovolume during bacterial growth. The relative cell size data (obtained from FCM forward scatter (FSC) signals) showed that ICC increased considerably in size during the first hours of incubation, reaching their maximum after 4 h. A decrease in the cell size was notable already at 5 h, while after 10 h of incubation the average cell size decreased to values almost equal to the start of the experiment. This corresponds with the previous studies that showed an increase in bacterial size during the first hours of bacterial growth and subsequent decrease [151, 152]. The study of Eydal and Pedersen [151] concluded that the cell volume influences ATP-per-cell values, and these are usually higher during exponential growth phase. The exact same observation is evident in Fig. 4.9, with the highest ATP-per-cell values of 1.51×10^{-9} nmole $cell^{-1}$ observed in early exponential phase with the highest growth rates of $0.48\ h^{-1}$, together with the largest relative cell size. This largely explains a rather weak correlation between intracellular ATP and ICC ($R^2 = 0.68$, $n = 25$) (Fig. 4.10). In contrast, ATP–per-cell values correlated strongly ($R^2 = 0.87$, $n = 25$) with relative cell size (Fig. 4.11) and intracellular ATP therefore correlated well with relative biovolume ($R^2 = 0.93$, $n = 25$; Fig. 4.12) showing a clear link between intracellular ATP, intact cells and cell size. Hammes and co-authors [67] previously showed a good correlation ($R^2 = 0.88$; $n = 102$) between intracellular ATP and cell biovolume for a variety of samples. This data shows the impact of cell size, in this case related to the physiological state of the cells (growth), on the interpretation of ATP data as well as the advantage of using FCM and ATP in concert.

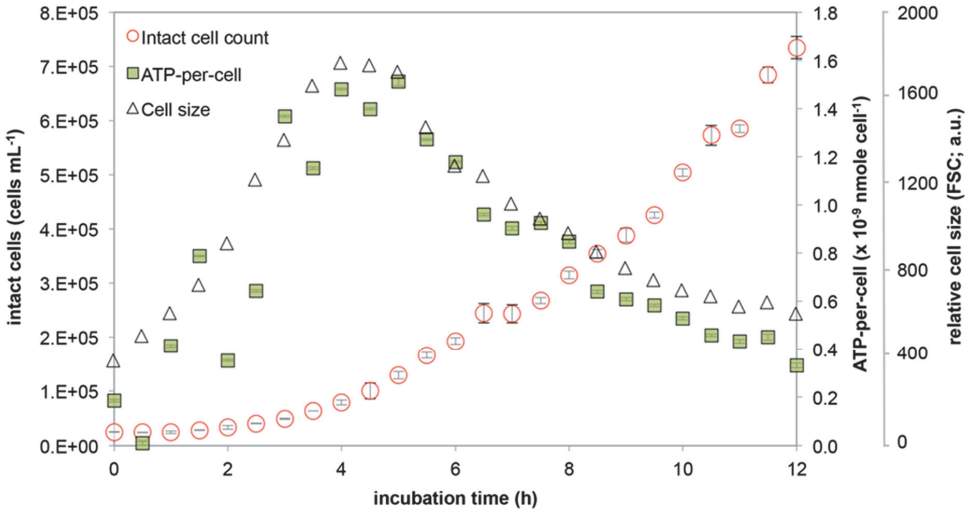


Fig. 4.9. Changes in bacterial variables during batch growth (12 h, 30 °C) of an indigenous river water bacterial community in filtered river water with natural occurring extracellular ATP

The intact cell concentration (circles) increases slower than the intracellular ATP (see Fig. 4.7). The relative cell size (triangles; measured as forward scattered (FSC) signals with FCM) peaks in the early exponential growth phase, at the same time as the ATP-per-cell (calculated as the intracellular ATP per intact cells).

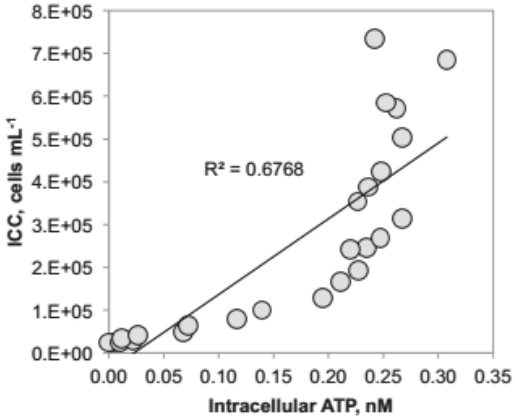


Fig. 4.10. A relatively weak correlation ($R^2 = 0.68$) between ICC and intracellular ATP during bacterial batch growth in natural river water sample (n = 25)

The samples were taken every 30 minutes during 12 h.

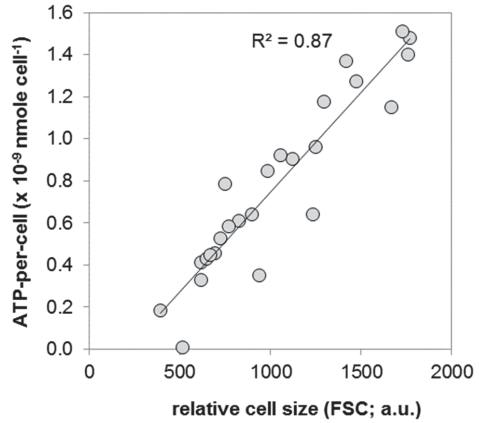


Fig. 4.11. A strong linear correlation (R^2 = 0.87, n = 25) between ATP-per-cell and relative cell size observed during batch growth (12 h, 30 °C) of an indigenous river water bacterial community in filtered river water

ATP-per-cell values were calculated as the intracellular ATP per intact cells. Relative cell size values are the forward-scattered (FSC) signals measured with FCM.

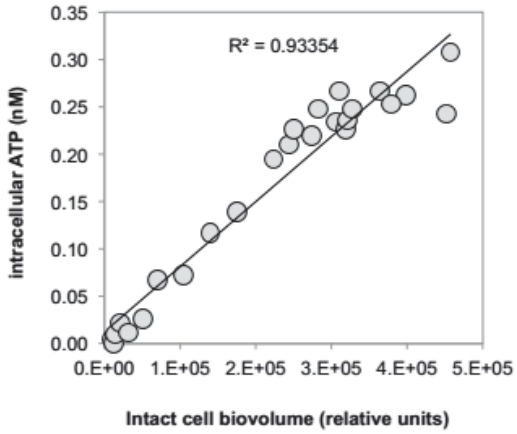


Fig. 4.12. A strong correlation (R^2 = 0.93) between intracellular ATP and intact cell biovolume during bacterial batch growth in natural river water sample (n = 25)

The samples were taken every 30 minutes during 12 h. The relative intact cell biovolume was calculated as a multiplication of relative cell size (from FCM forward scatter (FSC) data) and the FCM intact cell counts, assuming a linear correlation between FSC changes and cell size changes.

4.3.5. Role of the extracellular ATP in bacterial growth

To further investigate extracellular ATP degradation in the presence of bacteria, and particularly to understand which fraction of the ATP molecule is utilized by bacteria, a series of batch growth potential tests was performed. Carbon limited bottled mineral water was used as the water matrix, and different combinations of extracellular ATP, minimal media (MM) (as inorganic substrates) and sodium acetate (as AOC) were added to the water (Fig. 4.13; Table 4.1). It should be noted that these experiments were done at nutrient levels well in excess of

typical drinking water (ca. 1 mg-C L^{-1} acetate carbon) to allow clear observation of growth and to avoid miss-interpretation due to potential background contamination. Bacterial growth was insignificant in the samples without additional acetate (Fig. 4.13), which is considered as a typical AOC substrate [39]. The net growth was $7.8 \pm 1.8 \times 10^4$ cells mL^{-1} with the addition of ATP alone, $1.9 \pm 0.15 \times 10^4$ cells mL^{-1} with MM and $1.9 \pm 0.66 \times 10^4$ cells mL^{-1} when complemented with both ATP and MM, whereas negative control resulted in $2.1 \pm 0.73 \times 10^3$ cells mL^{-1}. Bacterial growth with the addition of ATP and MM alone or combined were statistically different from the negative control ($P < 0.05$), but the values were low in comparison to a net growth in the positive control sample (less than 3 %), meaning neither of these additions was a source of AOC. The positive control with 1 mg L^{-1} of acetate-C and excess P grew to a final concentration of $5.8 \pm 0.29 \times 10^6$ cells mL^{-1}, while without P-containing minimal media the growth was only $3.2 \pm 1.8 \times 10^6$ cells mL^{-1}, showing the cell concentration where P-limitation becomes relevant. Interesting results were obtained with samples where extracellular ATP was added together with acetate but without P-containing MM. The carbon-to-phosphorus ratios were chosen according to a rough estimation that the C:N:P ratio has to be approximately 100:10:1 for bacterial growth [32]. Bacterial growth in the samples with acetate-carbon and ATP-phosphorus in 1:1 and 10:1 ratios were not statistically different from a positive control (acetate-carbon + MM) ($P > 0.05$). Net growth was $6 \pm 0.45 \times 10^6$ cells mL^{-1} and $6 \pm 0.6 \times 10^6$ cells mL^{-1} respectively. This observation indicates that carbon is a limiting nutrient in these scenarios. This corresponds with a general C:N:P balance, by which phosphorus was in excess in these samples. In turn, a 100:1 C:P ratio resulted in less growth ($4.3 \pm 1.2 \times 10^6$ cells mL^{-1}). Since we showed that ATP-derived carbon did not support bacterial growth (see "MM, ATP" sample example), we conclude that bacterial growth was limited by phosphorus. Extracellular ATP was the only external source of phosphorus in the mentioned samples, thus the data clearly shows that bacteria utilized extracellular ATP as a phosphorus source in these experiments.

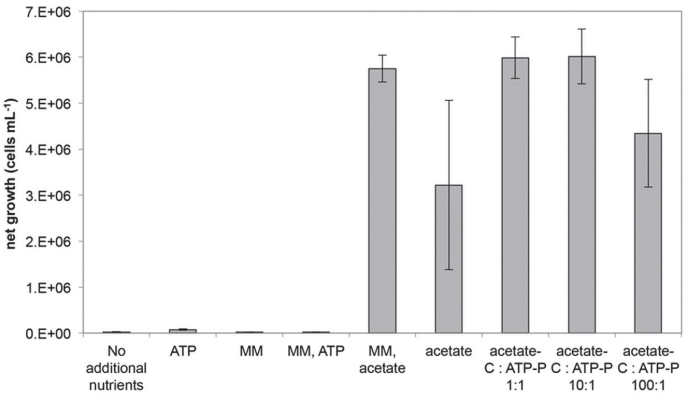


Fig. 4.13. The influence of extracellular ATP on bacterial growth in bottled mineral water, limited in carbon and/or phosphorous

The addition of neither ATP nor minimal media (MM), not a combination of both resulted in significant growth, while the addition of extracellular ATP together with acetate ($C_2H_3NaO_2$) overcame phosphorous limitation observed when acetate was added alone. Growth was measured with FCM after 3 days incubation. Error bars indicate standard deviation of triplicate experiments.

The practical implication of this should be considered. Fig. 4.1A showed that chlorination of 2.8×10^6 cells mL^{-1} of *E. coli* resulted in a maximum release of 3.6 nM extracellular ATP, comprising 0.328 µg-P L^{-1}, while a corresponding experiment with a river water bacterial community (1.9×10^6 cells mL^{-1}) released 0.54 nM extracellular ATP (= 0.049 µg-P L^{-1})

(Fig. 4.1B). In addition, previous studies in real drinking water treatment systems showed that ozonation of lake water (ca. 1×10^6 cells mL^{-1}) resulted in the release of 0.4 nM or 0.037 µg-P L^{-1} as ATP [64], while chlorination of GAC effluent (ca. 5×10^5 cells mL^{-1}) in the full-scale treatment plant of Riga (Latvia) released around 0.02 nM (= 0.002 µg-P L^{-1}) ATP [77]. These amounts of ATP-phosphorus could theoretically support bacterial growth on AOC in the range of 0.2 – 32.8 µg-C L^{-1}, estimated by an approximate C:P ratio of 100:1. According to the biological stability concept, the threshold AOC concentration in non-chlorinated and respectively chlorinated water is 10 µg L^{-1} and 100 µg L^{-1} [27, 28, 32, 39]. Hence, it is evident that ATP released from bacteria during chlorination 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. In our growth experiments the bacterial community was apparently not utilizing ATP as a source of AOC (Fig. 4.13), which is supported by TOC data before and after incubation (Fig. 4.14). This differs from previous studies, which suggested ATP-carbon uptake [70, 74], and could be related to the specific microbial community (i.e. composition, enzymatic activity, physiology) used in the study, or differences between short-term planktonic growth (i.e., growth potential assays in the present study) and long term growth in the presence of biofilms where more extracellular enzymes may be concentrated.

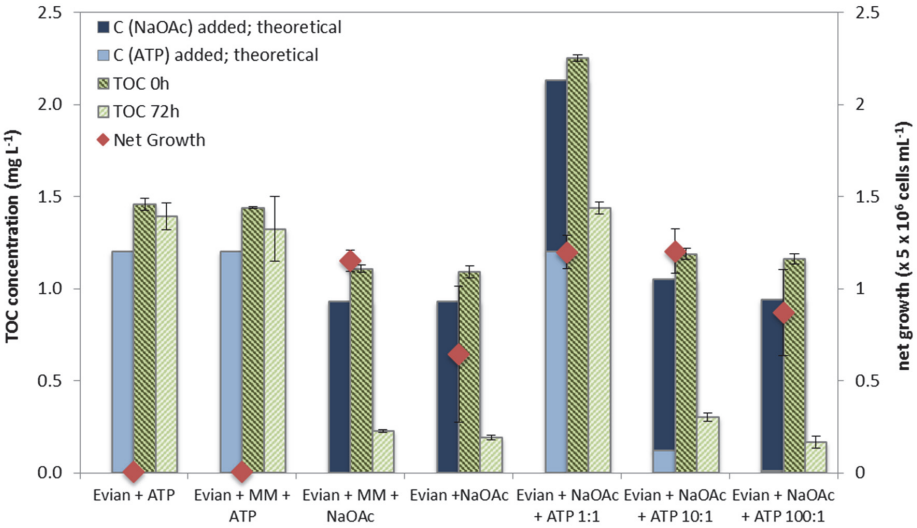


Fig. 4.14. Changes in carbon concentration as a result of bacterial growth

Blue columns show initial carbon concentration calculated as carbon added in form of ATP (light blue), or as sodium acetate carbon (NaOAc) (dark blue). Dark green columns represent actual (measured) initial total organic carbon (TOC), light green – TOC after 72h incubation. Evian was used a water matrix; MM = minimal media with excess nitrogen and phosphate. Cell growth was determined as an increase in flow cytometric TCC values after 72 h. Bacterial growth (red diamonds) and decrease of TOC were observed only in the samples with sodium acetate, thus indicating that ATP could not be consumed as a carbon source.

4.4. Conclusions of Chapter IV

- 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 release of ATP.
- 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.
- Extracellular ATP decline was considerably faster ($k = 0.368\ h^{-1}$; $R^2 = 0.96$) during growth of an indigenous bacterial community.
- 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.
- The overall results underline the necessity to specifically determine intracellular ATP during analyses of the chlorinated water samples.

5. CHAPTER V: IDENTIFYING THE UNDERLYING CAUSES OF BIOLOGICAL INSTABILITY IN A FULL-SCALE DRINKING WATER SUPPLY SYSTEM

5.1. Introduction

A strict definition of biologically stable water implies no changes in bacterial concentration and composition during drinking water distribution [1, 2], while a less strict definition would imply no adverse change in microbial water quality, such as pathogen proliferation. Although fluctuations in bacterial concentrations do not always indicate pathogen growth, unpredictable/unknown changes in biological water quality are undesirable. High bacterial concentrations might increase health risk, and cause problems with drinking water discoloration and corrosion [153]. Biological water quality can deteriorate as the result of external contamination and microbial growth due to poor initial water quality or insufficient disinfectant residuals, changes in water supply operation [154], seasonal fluctuations [18, 20], changes in water usage [77], influence of mixing zones and different water sources [17]. Moreover, periodic fluctuations in drinking water microbiology such as daily and seasonal changes complicate water monitoring and data interpretation [26, 60, 77, 155]. Long-term changes in bacterial concentrations are mostly attributed to seasonal fluctuations, which are at least partially caused by varying temperatures and resultant changes in bacterial abundance and activity [18, 20]. In contrast, short-term instability is normally caused by hydraulic disturbances due to changes in water consumption, which varies depending on time of the day, day of the week, and the purpose of the building [11, 19, 60, 77]. Therefore, biological stability should be considered on various time scales and specific investigations are needed to understand the root causes of instability.

Many previous studies investigated biological stability and the factors influencing bacterial growth in the distribution network [2, 18, 153, 156, 157]. Organic carbon, specifically biodegradable dissolved organic carbon (BDOC) and assimilable organic carbon (AOC), is considered to be the primary factor that promotes or limits growth of heterotrophic bacteria [23, 32, 41]. However, studies often do not consider inorganic nutrient limitation, or the potential for multiple limitations in the same sample. For example, phosphorus and other inorganic nutrient limitation were shown to be essential for biological stability in regions with high concentrations of organic carbon [21, 47, 49].

A number of exciting new methods to quantify and characterize bacterial biomass allowed to obtain more accurate and detailed information about biological instability *in situ*, such as biofilm formation rate (BFR) [18], adenosine triphosphate (ATP) measurements [18, 158], 16S rRNA gene pyrosequencing [16, 86], flow cytometric total cell concentration (TCC) [31, 111] and intact cell concentration (ICC) measurements [1, 77]. Nevertheless, available data on temporal bacterial fluctuations and especially long-term monitoring, which enable understanding of the influence of seasonal fluctuations and different water sources on temporal biological stability in a distribution network, is limited and contradictive. While large data sets obtained with cultivation-based technics showed clear seasonal/temperature dependence on bacterial growth [26, 90], only two from the six water treatment plants showed seasonal fluctuations of ATP [18]. Moreover, a recent two-year study of Prest and colleagues demonstrated only minor bacterial growth in the DN despite the significant seasonal fluctuations of temperature and bacterial counts in finished water [20]. Seasonal changes were investigated specifically for different water sources. However, van der Wielen and van der Kooij (2010) showed that anoxic groundwater was the less biologically stable and was influenced by seasons, but another study demonstrated that bacterial growth was the highest, when distributed water was originated from chlorinated surface water or mixed and water temperature was above 15 °C [17]. Spatial and short-term temporal (24 h) biological instability of the particular chlorinated drinking water distribution network was shown, but the reasons

were not identified [77]. Particularly some evidence was found of instability in mixing zones where treated surface water and treated groundwater meet. The time period of that study was limited to two weeks (in summer) [77], which does not allow the observation of seasonal fluctuations.

In this study we assessed microbial fluctuations in a complex full-scale distribution system supplied from three different water sources during 12 months at a weekly frequency using FCM TCC and ICC. Moreover, we used a modified approach to measure and evaluate biological stability in the treated and distributed water, based on the assessment of a range of growth-limiting/promoting compounds. The specific goals were to (1) investigate temporal biological (in)stability on a seasonal (long-term) scale, and (2) identify the growth limiting/promoting factors in the specific system.

5.2. Materials and Methods

5.2.1. Study site

Sampling was performed in the full-scale distribution network of Riga (Latvia) with a total length of about 1400 km. The network mainly consists of cast iron (80 %) and unlined iron (15 %) pipes. The city is supplied with drinking water from six water treatment plants (WTP) produced from both surface and groundwater (150 000 m^3 d^{-1}). Only the three major WTPs, which are continuously operated, were included in the sampling campaign. These WTPs include full-scale treatment of surface water and chlorination of artificially recharged and natural groundwater (Fig. 5.1), referred as WTP 1, WTP 2 and WTP 3 further in the text, accordingly. Sampling was done at all three WTPs before and after the final chlorination step (see residual chlorine concentrations in Table 5.1 and Fig. 5.2B). Additionally, raw water and samples after ozonation were taken at the surface water WTP. Network samples were taken at the locations linked to the individual WTPs (surface and groundwater WTPs) and potentially "mixed" zones. Natural and artificially recharged groundwater were defined as "groundwater" and were not specified as separate water sources for network samples due to close proximity of the WTP 2 and WTP 3, and inability to determine the exact source during distribution.

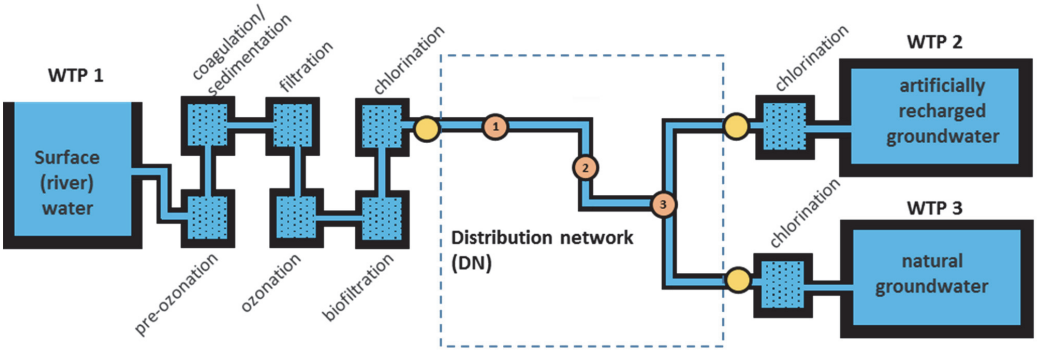


Fig. 5.1. A principal scheme of the investigated drinking water supply system

A city was supplied from three main water treatment plants (WTP), which involve full-scale treatment of surface water, and natural and artificially recharged groundwater. Samples were taken after chlorination (yellow circles) to investigate growth-promoting nutrients at WTPs. DN samples could be defined as surface water DN sample (1), mixed DN sample (2) and groundwater DN sample (3) according to supplying water sources. Samples 1 and 2 were analyzed weekly during one year, while samples 1 and 3 were measured to assess limiting nutrients in the DN. Additionally several samples were taken at WTPs (raw water, water after biofilters).

5.2.2. Long-term monitoring in drinking water distribution network

Long-term monitoring was performed at two locations in a distribution network (DN) with different water ages and different source water origins (Fig. 5.1). Source water origin and drinking water age were computed according to Riga DN hydraulic model on the Bentley WaterGEMS® platform and actual measurement data of the closest locations, provided by "Rīgas Ūdens" utility. The first location was a household tap in the area, where water was originated from treated surface water (80 – 100 % of surface water by modelling data). Drinking water at the second location, which is a tap in a public building on the university campus, was defined as mixed water: modelling data suggests 62 – 97 % groundwater during sampling time (data not shown). However, actual physically-chemical measurements < 2 km away from this location, provided by the water utility, indicate on surface water. Moreover, hydraulic data shows that this location could be supplied by water from a reservoir where groundwater and surface water could be literally mixed.

Sampling was performed every week during one year (April 2015 – April 2016). The taps were flushed (10 min with average flow rate 0.21 m^3 h^{-1}) before sampling to ensure that stagnation did not affect the measurements. Each sample was measured for ICC and TCC in triplicate. Additionally, the water temperature was measured in the tap on campus. Temperature and total chlorine data from WTPs were provided by SIA "Rīgas Ūdens" water supply provider (Fig. 5.2).

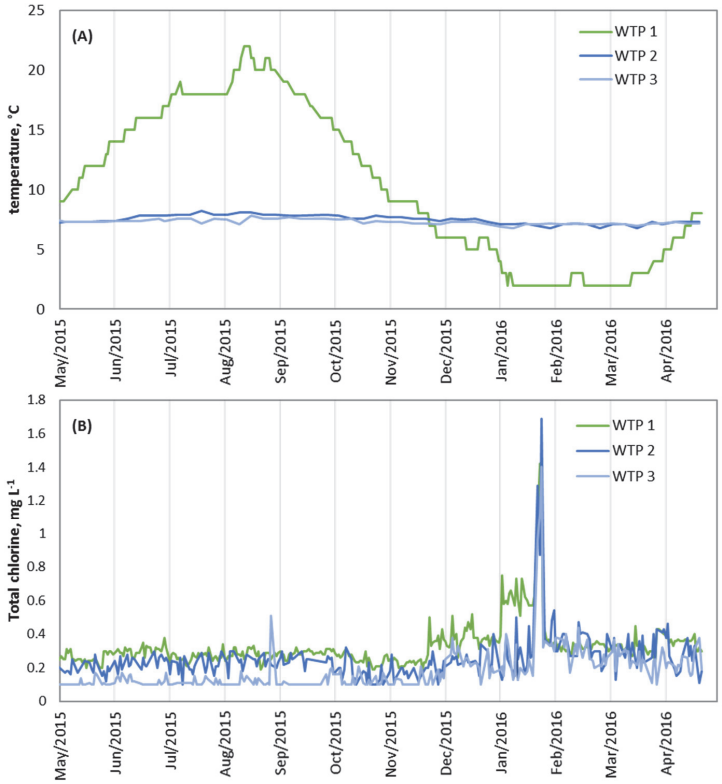


Fig. 5.2. Water temperature and total chlorine at three main drinking water treatment plants (WTP), provided by SIA "Rīgas Ūdens"

(A) Water temperature at WTP 1 (n = 325), WTP 2 (n = 52), WTP 3 – chlorinated natural groundwater (n = 53); Total residual chlorine at WTP 1 effluent (n = 372), WTP 2 (n = 248), WTP (n = 254).

5.2.3. Characterization of biological stability and identifying growth limitation compounds in drinking water

Samples were taken from various points in three WTPs as described before (Section 5.2.1, Fig. 5.1), and from two household taps, attributed to different water sources and treatments (treated surface water and chlorinated groundwater). Sampling was performed twice: in August 2015 (further in the text as "summer"), and in January 2016 ("winter"). The winter sampling campaign was conducted during a period of increased chlorination, which was a short-term (3-5 days) regular procedure aimed to disinfect water distribution pipes (Fig. 5.2B). Temperature, free and total chlorine measurements were conducted on site at the time of sampling (Table 5.1), while samples for FCM and growth potential were collected in AOC-free glass bottles and were analyzed within 8 h. A modified growth potential method (Section 5.2.4) was applied to the samples from the summer sampling campaign to identify growth-limiting compounds.

Table 5.1.

Free and total chlorine data (mg L^{-1}), and temperature measurements at three WTPs effluents, measured in summer (normal operation), and winter (increased chlorination) during our sampling campaign (1-year chlorine data demonstrated on Fig. 5.2B).

	August			January (increased chlorination)		
	Cl_2 Free	Cl_2 Total	t, °C	Cl_2 Free	Cl_2 Total	t, °C
WTP 1	0.03	0.21	20.4	0.73	1.05	1.3
WTP 2	0.06	0.36	8.9	0.93	1.31	8.1
WTP 3	0.11	0.22	9.1	0.92	1.17	8.1

5.2.4. Modified growth potential method

The method was modified from the method described in the recent study [22]. The following stock solutions were prepared and used in the experiment: 10 mM NaS_2O_3 as chlorine quenching solution (1 % v/v final concentration); 1 g-C L^{-1} sodium acetate $C_2H_3NaO_2$ as source of carbon (1 mg-C L^{-1} final concentration); 1.28 g L^{-1} $Na_2HPO_4 * 2H_2O$, 0.3 g L^{-1} KH_2PO_4 as phosphate source (0.1 % v/v final concentration); 1.77 g L^{-1} $(NH_4)_2SO_4$ as nitrogen source (0.1 % v/v); acidified trace element solution (8 g L^{-1} $CaCO_3$, 1.15 g L^{-1} $MnCl_2 * 4H_2O$, 0.146 g L^{-1} $CuSO_4 * 5H_2O$, 0.13 g L^{-1} $CoCl_2 * 6H_2O$, 0.4 g L^{-1} ZnO, 0.124 g L^{-1} H_3BO_3, 13.42 g L^{-1} $MgCl_2 * 6H_2O$, 1.04 g L^{-1} $Na_2MoO_4 * 2H_2O$ with 0.64 % HCl) (2 µL 100 mL^{-1} final concentration); 10 mM $FeCl_3$ (10 µL 100 mL^{-1} final concentration) and bottled mineral water (Evian, France) was used as natural bacterial inoculum (10^3 cells mL^{-1} (1 % v/v) final concentration). All samples were prepared in AOC-free glass bottles filled with 100 mL of bottled water and then divided into 3 AOC-free glass vials with 20 mL of sample in each. Direct growth potential was determined as sample incubation without any additives. General nutrient limitation was evaluated by addition of NaS_2O_3 (quenching of disinfectant) and a bacterial inoculum (bottled mineral water). Maximal growth potential was tested by addition of quenching solution, bacterial inoculum, and nutrients in excess relatively to 1 mg L^{-1} $C_2H_3NaO_2$. Finally, growth limitation by different nutrients was tested similarly to maximum growth without addition of the target nutrient source (Table 5.2). Samples were incubated at 30 °C with shaking (150 rpm) for 72 h. FCM-TCC measurements were performed before and after incubation.

Table 5.2.

Combinations of additives, used for modified growth potential method

	NaS_2O_3	inoculum	acetate	phosphates	nitrogen	iron
Direct growth	-	-	-	-	-	-
Quenching	+	+	-	-	-	-
C-limitation	+	+	-	+	+	+
P-limitation	+	+	+	-	+	+
N-limitation	+	+	+	+	-	+
Fe-limitation	+	+	+	+	+	-
Max growth	+	+	+	+	+	+

5.2.5. Fluorescent staining and FCM of water samples

FCM analysis was based on the methods described previously [76, 101, 111]. For TCC staining, a working solution of 100× diluted SYBR® Green I (SG) was used. 1 mL of the sample was stained with SG working solution at 10 µL mL^{-1} and incubated 10 minutes at 35 °C before analysis. For ICC staining, propidium iodide (PI; 30 mM) was mixed with the SYBR® Green I working solution (SGPI) to a final PI concentration of 0.6 mM. 1 mL of the sample was stained with SGPI at 10 µL mL^{-1} and incubated 15 minutes at 35 °C before analysis. Prior to FCM analysis, the water samples were diluted (10 % v/v) with 0.22 µm filtered commercially available bottled water (Evian, France). FCM measurements were performed on the CyFlow® SL, equipped with a blue 25 mW solid state laser emitting light at a fixed wavelength of 488 nm. Green fluorescence was collected at 520 ± 10 nm, red fluorescence above 630 nm, and high angle sideward scatter (SSC) at 488 nm. The trigger/threshold was set on the green fluorescence channel and data were acquired on two-parameter density plots, while no compensation was used. All data were processed with the FCM propriety software, and electronic gating was used to separate positive signals from instrument and water sample background [111].

5.2.6. Chlorine concentration determination

Free and total chlorine was measured using commercially available DPD reagent powder pillows (Hach®) and a portable photometer (DR 890, Hach®). Analysis was done as described in the user's manual.

5.3. Results and discussion

5.3.1. Spatial biological stability of groundwater and surface water during different seasons

Drinking water that originated from surface water was not biologically stable. An increase in cell concentrations was observed in the distribution network (DN) samples, when compared to surface water WTP (WTP 1) effluent, obtained on the same date (Fig. 5.3A, 5.3B): ICC increased from $6.27 \pm 0.48 \times 10^4$ cells mL^{-1} after chlorination to $2.92 \pm 0.05 \times 10^5$ cells mL^{-1} in DN in summer and from $1.11 \pm 0.17 \times 10^3$ to $9.95 \pm 0.99 \times 10^3$ cells mL^{-1} in winter; TCC changed from $4.51 \pm 0.06 \times 10^5$ cells mL^{-1} at the effluent to $5.12 \pm 0.12 \times 10^5$ cells mL^{-1} in DN in summer and from $7.64 \pm 0.15 \times 10^3$ to $6.69 \pm 0.12 \times 10^4$ cells mL^{-1} in winter. The lower absolute increase of ICC in winter could be explained by the low initial ICC, less bacterial growth due to the low temperature in general [26] and higher chlorine residuals during the winter sampling period. Intensified chlorination was applied during winter, which resulted in 0.73 ± 0.06 mg L^{-1} of free chlorine (1.05 mg L^{-1} as total chlorine) at the finished water and 0.23 ± 0.09 mg L^{-1} of free chlorine in the network. In comparison, free chlorine was 0.03 ± 0.006 mg L^{-1} (0.21 mg L^{-1} as total chlorine) after chlorination in summer, which is a normal chlorination practice in the

surface water WTP. According to a recent study and World Health Organization guidelines, 0.5 mg L^{-1} of free chlorine is a threshold for effective disinfection, and 0.2 mg L^{-1} at point of the delivery in low-risk systems [35, 36], thus bacterial growth in summer was not completely unexpected.

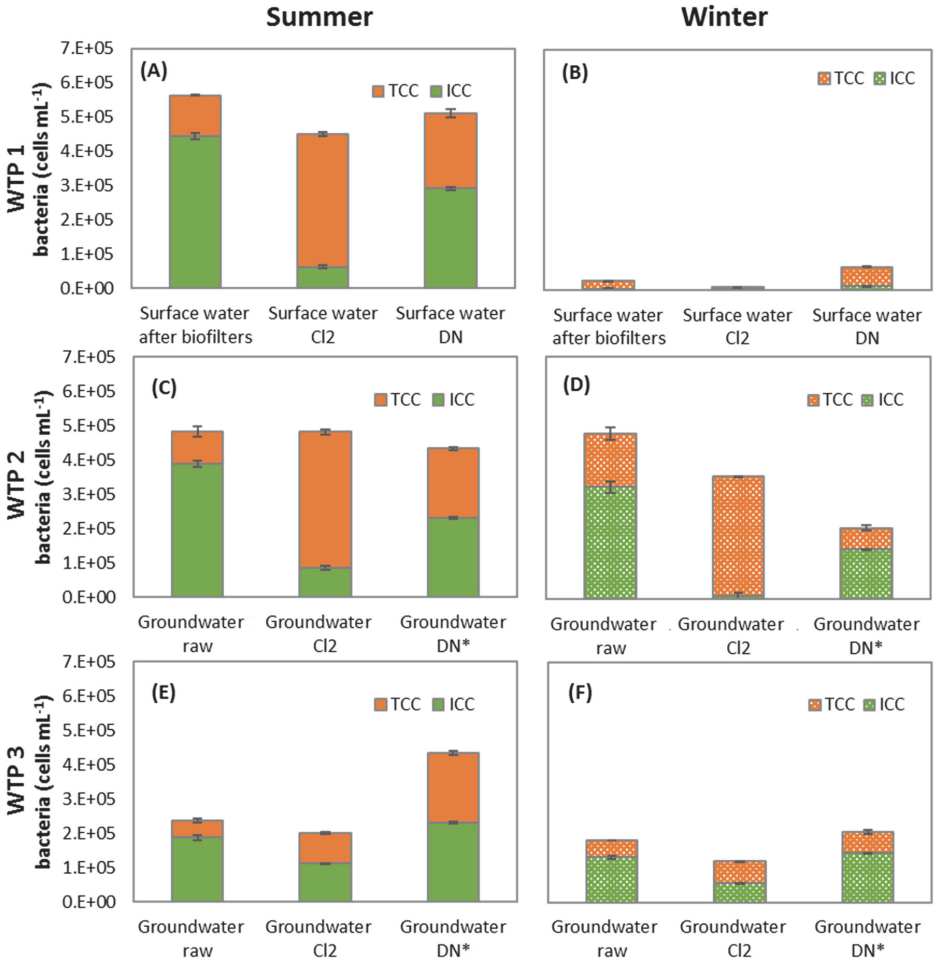


Fig. 5.3. ICC and TCC (the sum of ICC and damaged cell count), measured with FCM before ("after biofilters"; "raw") and after chlorination (Cl2) at the three different WTPs and network samples (DN)

The samples were measured in summer (A, C, E) and winter (B, D, F). Additionally, increased chlorine dose was applied during the winter sampling campaign. *groundwater DN sample is the same for both groundwater WTPs due to close location of both WTPs to each other, and thus inability determine the exact origin of the water.

Seasonal differences in cell concentrations were substantial in the treated surface water. Both TCC and ICC were at least 1 log lower in winter than in summer (Fig. 5.3A, 5.3B): TCC in the sample after biofiltration (before chlorination) was $5.63 \pm 0.02 \times 10^5$ in summer, whereas it was $2.63 \pm 0.02 \times 10^4$ cells mL^{-1} in winter (5 % of TCC in summer). ICC before chlorination was $4.44 \pm 0.1 \times 10^5$ cells mL^{-1} in summer, and $3.03 \pm 0.93 \times 10^3$ cells mL^{-1} in winter, which was 1 % of what was measured in summer. TCC and ICC after chlorination in summer were 4.51 ±

0.06×10^5 and $6.27 \pm 0.48\times10^4$ cells mL^{-1} respectively, and $7.64 \pm 0.15\times10^3$ and $1.11 \pm 0.17\times10^3$ cells mL^{-1} in winter (2 % of "summer" TCC and ICC) (Fig. 5.3). Seasonal differences were significant for raw surface water quality too. TCC and ICC of raw surface water in summer were $3.78 \pm 0.12\times10^6$ and $2.82 \pm 0.1\times10^6$ cells mL^{-1} respectively, and $1.28 \pm 0.01\times10^6$ and $1.08 \pm 0.03\times10^6$ cells mL^{-1} in winter, which was 34 % of TCC and 38 % of ICC in raw water samples in summer (Fig. 5.4). However, it is not likely that changes in raw water bacterial concentrations could influence biological quality of WTP 1 effluent. The biofiltration step in the WTP 1 is preceded by ozonation, which is known to inactivate/destroy the majority of bacteria [91, 158, 159]. Hence, bacteria detected after biofiltration and prior to chlorination most likely originated from the biofilters [158, 160]. Low bacterial numbers in the WTP 1 effluent during winter suggest that the low temperatures inhibited bacterial growth during biofiltration at the surface water WTP (Fig. 5.3, 5.4). These observations are similar to those reported recently by Prest and colleagues [20]. However, there is no clear consensus about influence of water temperature on amount of biomass in the biofilters. While some studies showed decreased amount of biomass on the filter media at low temperatures [161, 162], others did not observe differences in biomass levels at different temperatures [163, 164]. Hypothetically, the availability of nutrients could also explain changes in bacterial concentrations within different seasons. However, this is not likely to be a primary reason, since growth-promoting nutrients were detected after biofilters in winter (see section 5.3.5), which corresponds with some studies that showed decreased organic carbon and inorganic compounds removal in biofilters at colder temperatures [162–165]. Moreover, preceded ozone treatment is known to generate AOC and ATP, which could serve as a phosphorus source for bacteria [30, 141, 158]. Thus we argue that the seasonal effect on bacterial growth during biofiltration is mostly related to water temperature fluctuations.

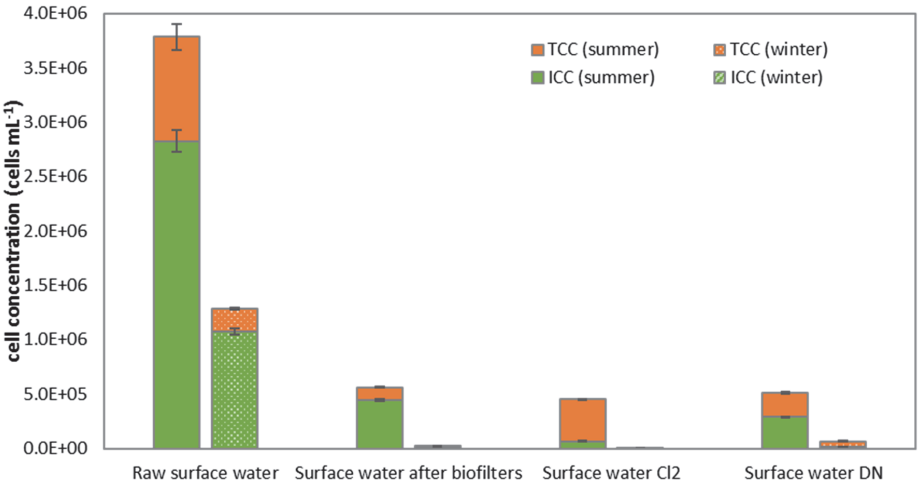


Fig. 5.4. ICC and TCC (the sum of ICC and damaged cells) of the samples from the WTP 1
Raw surface water, last treatment steps at WTP and DN sample were measured in August 2015 and January 2016. The figure represents actual data from Fig. 5.3A and 5.3B, and raw surface water values.

The biological quality of drinking water originating from chlorinated groundwater changed during distribution. The groundwater DN samples could have originated from both/either groundwater WTPs due to proximity of the latter, thus DN values cannot be compared to the individual raw water samples directly (Fig. 5.3C-5.3F). However, ICC values were higher in the network in comparison to both groundwater WTPs effluent values (Fig. 5.3). The difference between summer and winter was smaller in groundwater samples than in surface water. TCC in raw artificially recharged groundwater was not significantly different between summer and winter ($P > 0.05$), and was $4.83 \pm 0.15 \times 10^5$ cells mL^{-1} (99 % of TCC in summer). Seasonal difference was more significant in natural groundwater, where TCC in winter was 77 % of the summer TCC values ($2.36 \pm 0.06 \times 10^5$ cells mL^{-1}). ICC were 83 % of ICC in summer in artificially recharged groundwater and 70 % in natural groundwater ($3.89 \pm 0.01 \times 10^5$ and $1.87 \pm 0.08 \times 10^5$ cells mL^{-1} accordingly). These striking differences in seasonal fluctuations between groundwater and surface water bacterial counts are linked to changes of water temperature, which is one of the main factors influencing bacterial abundance and activity [18, 20, 26, 90]. Surface water was exposed to seasonal changes more than groundwater: at the WTP 1, water temperature during summer sampling was 20.4 – 21 °C and 0.8 – 1.3 °C at the time of winter sampling campaign. Treated surface water temperatures ranged between 2 – 22 °C during 12 months (n = 325), which corroborates significant seasonal temperature fluctuations (Fig. 5.2A). In contrast, groundwater temperature was more constant during the year, e.g. 8.7 – 10.6 °C at WTP 2 and WTP 3 in summer and 6.7 – 8.1 °C in winter during our sampling campaign, and 6.8 – 8.2 °C during 1-year weekly monitoring at both WTPs (n = 52 (WTP 2), n = 53 (WTP 3), Fig. 5.2A).

Our WTP data suggests that fluctuations of water temperature affect both initial bacterial concentrations and individual water treatment processes. Moreover, the results show that bacterial growth occurred in the DN and it is dependent on the season and the water source.

5.3.2. Temporal fluctuations of bacterial concentrations in the DN

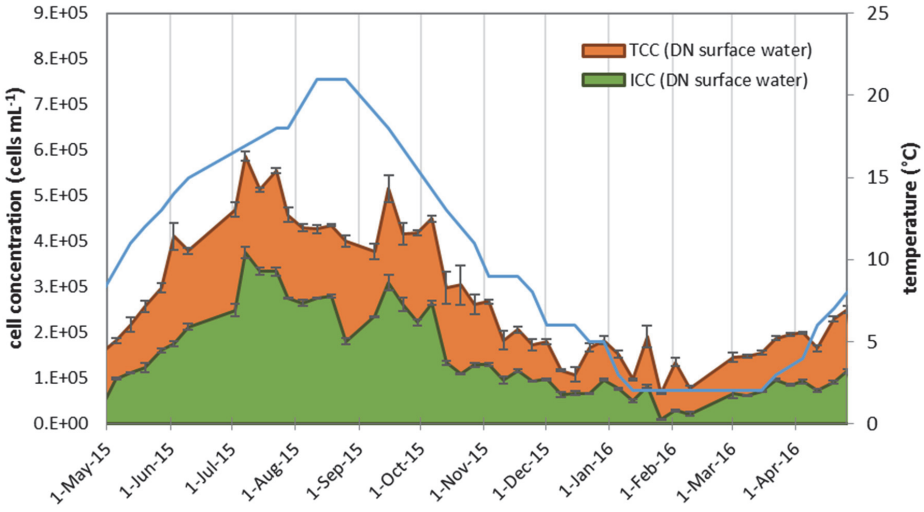


Fig. 5.5. Temporal bacterial fluctuations in the DN, which was supplied with treated surface water, and temperature of finished water at WTP 1 over long-term monitoring (n = 48)

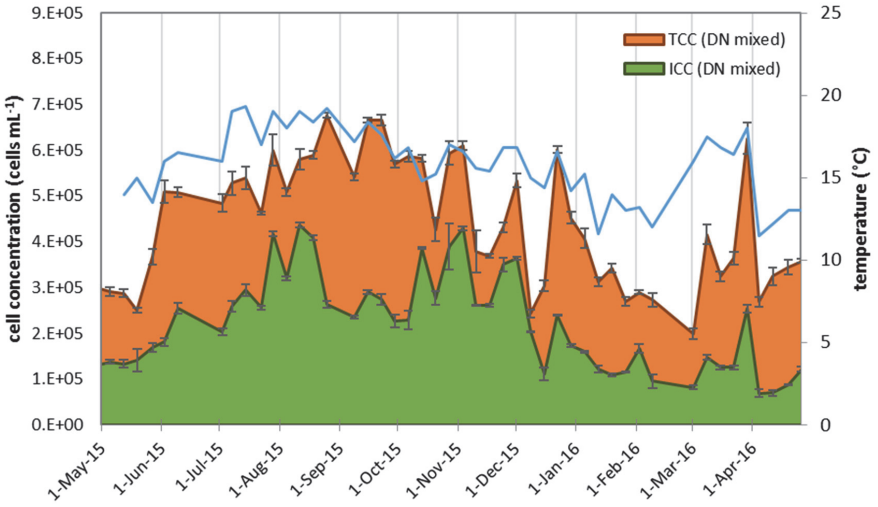


Fig. 5.6. Temporal bacterial cell fluctuations and tap water temperature over long-term monitoring of the drinking water network (DN) sample, which was originated from mixed water (n = 48)

Clear seasonal fluctuations in TCC and ICC were observed in the drinking water DN samples over 12 months (n = 48) (Fig. 5.5, 5.6). TCC values of the DN sample that originated from the WTP 1 varied from 0.67 to 5.87×10^5 cells mL^{-1}, with a mean value $2.75 \pm 1.41\times10^5$ cells mL^{-1} (n = 48) (Fig. 5.5), and ICC values varied from 0.1 – 3.75×10^5 cells mL^{-1}, with a mean value $1.46 \pm 0.95\times10^5$ cells mL^{-1} (n = 48). Generally higher bacterial concentrations were observed during summer: all ICC and TCC values that exceeded the average occurred between May to October, following a similar trend as a temperature at the WTP 1. A strong correlation between bacterial counts in the DN sample and water temperature at WTP 1 effluent was obtained for TCC ($R^2 = 0.82$; Fig. 5.7A) and ICC ($R^2 = 0.8$; Fig. 5.7B) (n = 37). The percentage ICC during the observation period was relatively stable: 50 ± 11 % (Fig. 5.8A) without sharp changes, except a drop due to intensified chlorination (end of January) (Fig. 5.2B).

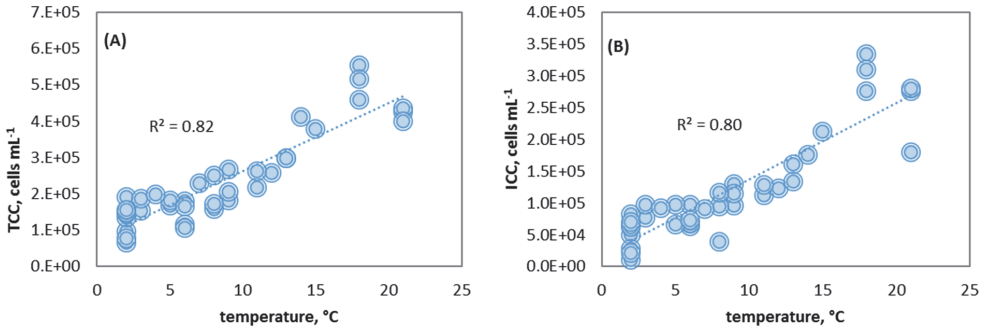


Fig. 5.7. Correlations between water temperature at surface water WTP 1 and total cell count (A) and intact cell count (B) in surface water DN sample from Fig. 5.5 (n = 37)

Our data corroborate previous studies, where generally higher bacterial concentrations were observed during summer or in warm water in comparison to cold water temperatures [18, 20,

26, 90, 166]. Particularly interesting example was demonstrated in the study of Prest and colleagues, where long-term monitoring was performed systematically in the DN with FCM, and a similar-appearing seasonal trend of bacterial concentrations was shown [20]. However, in contrast to the present study, these fluctuations were predominantly occurring at the WTP (during biofiltration), and only minor additional growth was observed in the network. In the current study, the data presented in Fig. 5.3 and Fig. 5.5 demonstrate that the dominant growth occurred in the distribution system and the extent of bacterial growth depended on the season.

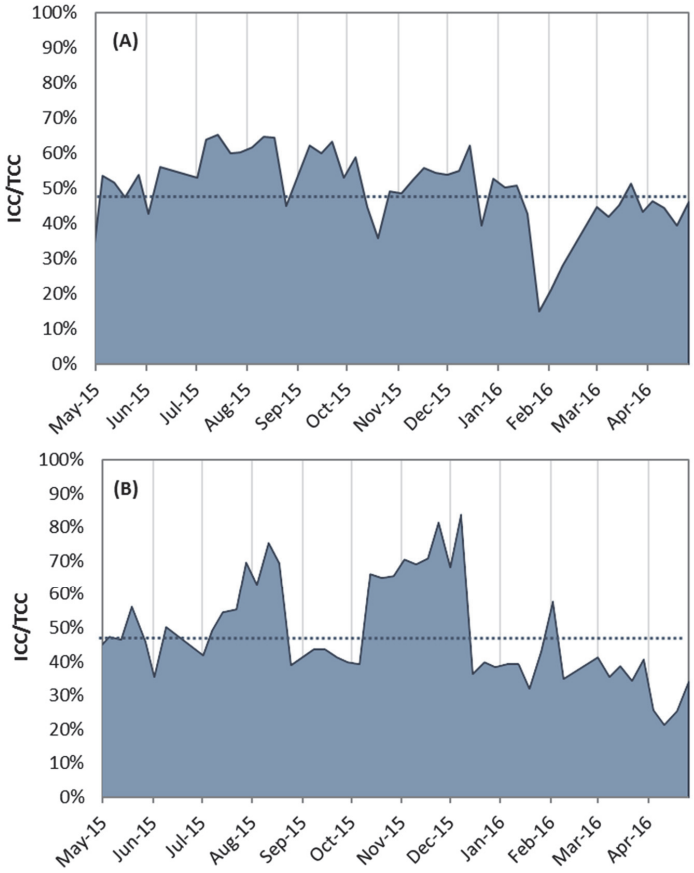


Fig. 5.8. Temporal variations of ICC/TCC during a long-term monitoring of the DN samples (n = 48), supplied by treated surface water (A) and mixed water (B)
Actual TCC and ICC are demonstrated on Fig. 5.5. and 5.6. A dotted line shows average ICC/TCC during observed period.

TCC and ICC values of the mixed DN sample were different from the surface water DN sample and showed less seasonal dependency. TCC ranged from 1.98 – 6.76×10^5 cells mL^{-1} (mean = $4.41 \pm 1.36\times10^5$ cells mL^{-1} (n = 48)) (Fig. 5.6), and ICC values varied from 0.44×10^5 – 4.37×10^5 cells mL^{-1} (mean = $2.20 \pm 1.05\times10^5$ cells mL^{-1}; n = 48), thus both TCC and ICC were significantly different from the surface water DN samples ($P < 0.05$). Differences between warm and cold seasons were not as clear as in the example of surface water sample. However, clearly lower TCC and ICC values were observed in a period from mid-January until April. Temperature in the tap ranged from 11.5 to 19.3 °C during the entire observation period, and

relatively weak correlations between tap water temperature and TCC (R^2 = 0.56) (Fig. 5.9A) and ICC (R^2 = 0.49) (Fig. 5.9B) were obtained. Similar correlations were obtained with water temperature at the WTPs: water temperature at WTP 1 (surface water) correlated with DN TCC (R^2 = 0.46; Fig. 5.9C), and ICC (R^2 = 0.42; Fig. 5.9D); while treated water at WTP 2 (groundwater) correlated with TCC and ICC with R^2 = 0.51 (Fig. 5.9E) and R^2 = 0.55 (Fig. 5.9F), respectively. Weaker correlation between bacterial counts and water temperature at WTP 1 in the mixed DN sample in comparison to the surface DN sample (Fig. 5.7) indicates that, in the mixed DN sample, changes in bacterial concentrations were less dependent on WTP 1 parameters. Therefore, biological water quality and bacterial growth were likely influenced from groundwater sources and/or were affected by longer water retention time (water age) than in the surface DN sample. For example, according to the modelling data, water age of surface water in the mixed DN sample site was 42 – 68 h during sampling time (groundwater water age was not available). In contrast, at the sampling time of surface water DN samples, theoretically calculated water age was ca. 32 – 50 h. Apparently, in the mixed DN sample, drinking water parameters should be more different from WTP 1 and higher bacterial growth should be expected than in surface DN sample.

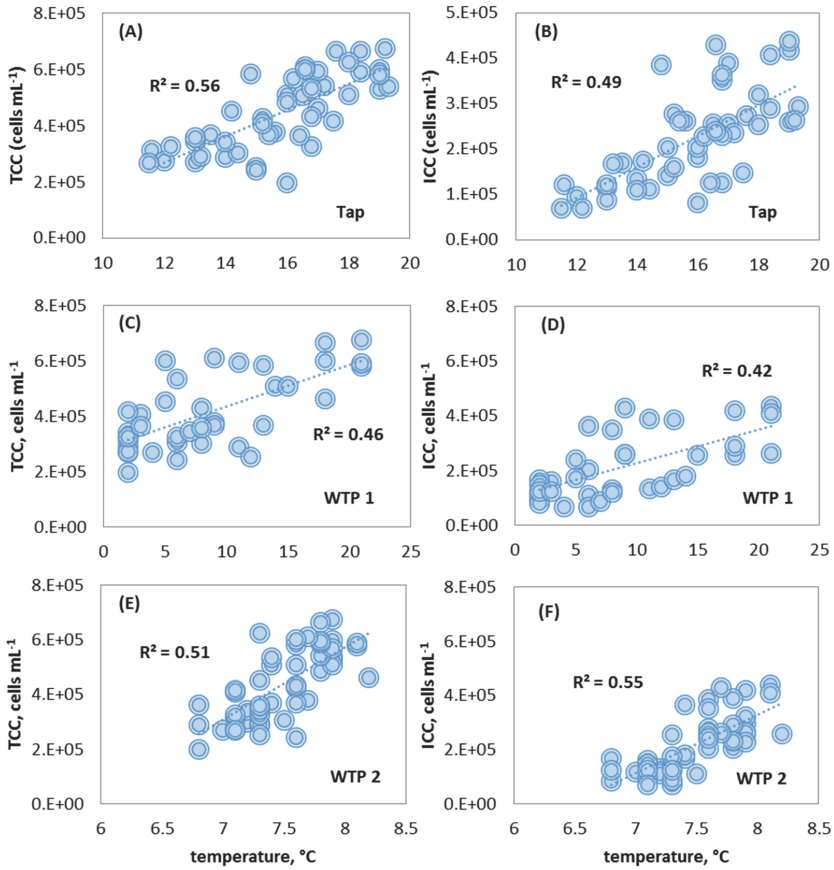


Fig. 5.9. Correlations between cell counts in mixed DN sample and water temperature
(A) correlation between tap water temperature and TCC (n = 46); (B) correlation between tap water temperature and ICC (n = 46); (C) correlations between TCC and WTP 1 (treated surface water) (n = 37); (D) ICC and WTP 1 (treated surface water) (n = 37); (E) TCC and WTP 2 (chlorinated groundwater) (n = 47); (F) ICC and WTP 2 (chlorinated groundwater) (n = 47). Bacterial concentrations correspond to the data from Fig. 5.6.

Moreover, in comparison to the surface water DN samples, the percentage ICC was less stable in the mixed DN sample with a mean value 49 ± 15 %, ranging from 21 – 84 % (Fig. 5.8B). Noticeable peaks in the percentage ICC were observed during mid-July to mid-August, and mid-October to mid-December. As a worst-case scenario, it could result from insufficient chlorination of groundwater: the % ICC of the peaks reached 84 %, which is similar to raw water parameters in groundwater (68 – 82 % ICC). However, it is not likely to be a cause, since bacterial concentrations in raw groundwater are generally stable, as was discussed before (Section 5.3.1). We believe that the increased ICC could be caused by bacterial growth in the sample. Increased bacterial growth could result from longer retention time in DN, including reservoirs, and/or water "mixing". For example, higher bacterial concentrations were observed in the "mixing" zones of the same DN before, as well as a significant increase of fixed biomass was observed in stagnated "mixed" water in the study of Brussels-Capital Region DN [17, 77]. As plausible, increase of ICC could be caused by changing hydraulic conditions. This could lead to bacteria detachment from biofilms due to increased shear stress, and afterward bacterial growth, which resulted from increased nutrient transport [95, 167, 168]. For example, an additional experiment showed that changing water consumption led to % ICC shift in the mixed DN sample during and after flushing the tap for 12 h (Fig. 5.10). % ICC in the tap water changed from 45 % (average before and during 12 h flushing) to 68 % (mean value of the measurements in the morning and afternoon the next 3 days) (Fig. 5.10A). Both TCC and ICC decreased during increased water usage (12 h flushing) (Fig. 5.10B), but TCC reached initial number by increased ICC when flushing was stopped, which indicates on bacterial growth in the network. Difference in water usage could be very likely the reason in our case as well, since there are very diverse types of the buildings in the neighborhood, namely other university buildings, supermarket, some residential buildings, a hotel and a sport center with a swimming pool, which have different daily and seasonal water consumption.

Although only limited data of the detailed long-term monitoring in distribution network is available, there is evidence that water biological parameters are affected by the season, and in certain cases these changes are inevitable [17, 20, 26, 90]. Thus seasonal fluctuations should be considered, when defining a baseline for bacterial parameters in water [60, 169], and operating water treatment plants (final disinfection).

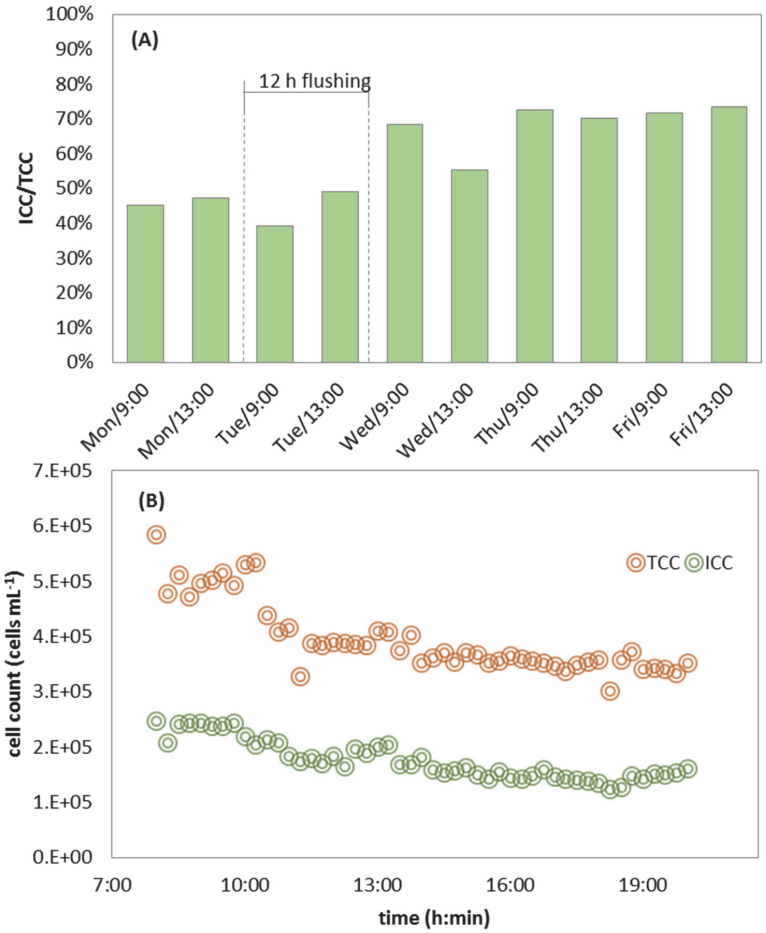


Fig. 5.10. Effect of water consumption on ICC, TCC and ICC/TCC in mixed DN water
(A) ICC/TCC ratio in morning (9:00) and early afternoon (13:00) hours, measured during a week; (B) TCC and ICC fluctuations during 12 h monitoring of continuously running water (corresponds to Tuesday from (A)).

5.3.3. Growth-limiting nutrients in the WTP samples from different water sources

Surface water and groundwater after treatment have different growth-promoting/growth-limiting properties. In this study we employed and expanded growth potential assay to assess for multiple limitations (Table 5.2, [22]). Bacterial growth was not detected in WTP 2 and WTP 3 after chlorination, when estimated as direct incubation growth, while growth in the WTP 1 effluent was $6.84 \pm 5.54 \times 10^4$ cells mL^{-1} (Fig. 5.11). If it is assumed that carbon is the limiting nutrient, this growth is equivalent to 6.8 µg AOC L^{-1} [170] in WTP 1 effluent. This value meet the requirements for biological stability of chlorinated drinking water (50 - 100 µg AOC L^{-1}), proposed by various authors [28, 32]. Higher bacterial growth in all samples was observed with addition of chlorine quenching solution ($Na_2S_2O_3$) and a bacterial inoculum ("Direct + Q + in" sample). While it was not significantly different from direct incubation growth in WTP 1 effluent, it resulted in $4.38 - 7.12 \times 10^5$ cells mL^{-1} increase in WTP 2 & 3. This indicates on growth limitation by residual chlorine in the initial sample, if compared to the direct incubation

results. However, absence of growth in WTP 1 effluent sample with neutralized chlorine indicates on nutrient limitation.

Phosphorus was the primary growth-limiting nutrient in the treated surface water sample (WTP 1). When all other nutrients were in excess, the available phosphorus in the water allowed $2.07 \pm 1.75 \times 10^5$ 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. 5.11A). Carbon was the second growth-limiting nutrient. However, the water contained sufficient organic carbon to promote 10-fold higher net growth than phosphorus ($2.06 \pm 0.22 \times 10^6$ cells mL^{-1}, equivalent to 206 µg AOC L^{-1}) when all other compounds were in excess. Phosphorus was considered as a main limiting nutrient in water in various countries, including Northern European countries and Japan, where high organic content is typical for natural water [47, 49].

In contrast, organic carbon was the primary growth-limiting nutrient in the treated groundwater samples. The amount of carbon in WTP 2 effluent could promote $9.42 \pm 0.47 \times 10^5$ cells mL^{-1} net growth (ca. 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 ($4.38 \pm 0.91 \times 10^5$ cells mL^{-1}, $P > 0.05$). Bacterial growth, obtained in conditions without additional phosphorus, was not statistically different from the samples with all nutrients added, which shows that phosphorus was not a growth-limiting element in either groundwater.

This data shows that water, distributed in the same network, has different and complementary growth-limiting nutrients, demonstrating the challenges faced by large water utilities, which use different water sources and treatment for drinking water production. This shows that it is critical to assess not only AOC [26, 28, 39], but rather carefully distinguish between different growth limiting compounds to understand biological instability [2, 22, 47].

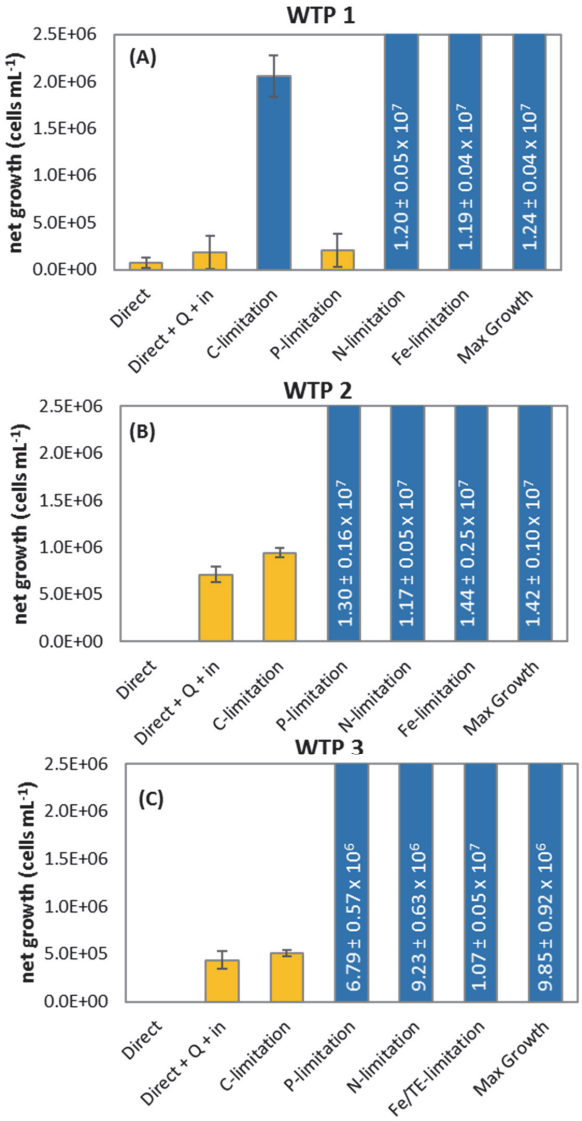


Fig. 5.11. Bacterial growth, represented as changes in total cell count (TCC) in the samples from WTP effluent after 72 h incubation

The growth potential approach was tested for effluent water after chlorination at three WTPs supplied with: (A) treated surface water; (B) artificially recharged groundwater and (C) natural 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.

5.3.4. Growth-limiting nutrients in the DN samples from different water sources

The biological stability of the drinking water changed during distribution. Water, which initially did not promote bacterial growth, was no longer biologically stable after distribution. Bacterial growth was $3.28 \pm 0.78 \times 10^5$ cells mL^{-1} in the surface water DN sample (Fig. 5.12A), and $2.48 \pm 0.79 \times 10^5$ cells mL^{-1} in groundwater DN sample, when estimated as direct incubation

growth (Fig. 5.12B). This was higher than the values obtained in the WTPs effluent, which could be partially explained by chlorine decay. However, particularly interesting is surface water DN sample, where growth was not limited only by residual chlorine at WTP 1. Bacterial growth in the DN sample indicates on availability of nutrients, which were absent in the WTP 1 effluent (Section 5.3.3). Thus transformation of nutrient composition and concentration should be considered as a plausible reason of growth potential changes.

Phosphorus became the primary growth-limiting nutrient in both surface water and groundwater DN samples. Phosphorus in the groundwater DN sample could promote only 1.19 ± 0.47×10^6 cells mL^{-1} growth (10-fold lower than in WTP sample). This indicates that phosphorus was either consumed by bacteria or transformed into other forms of phosphorus-containing compounds, which are not microbiologically available. Moreover, Rubulis and Juhna (2007) showed increased numbers of heterotrophic bacteria in a closed reactor biofilm a long time after phosphorus concentration decreased in the bulk water, and suggested that this could be explained by an ability of certain types of organisms to accumulate phosphorus, similarly to the mechanism used in biological phosphorus removal in wastewater [171, 172]. Although phosphorus remained the growth-limiting nutrient in surface water, it could promote 5-fold higher bacterial growth in water from the DN than directly after treatment: net growth without additional phosphorus in surface water DN sample was $9.76 \pm 3.29\times10^5$ cells mL^{-1}. There is no clear evidence of what could be the reason of increased biologically available phosphorus concentrations. However, several options should be considered. One of the most realistic explanations could be a direct mixture of groundwater and surface water in the reservoirs, which are used for compensation of water consumption fluctuations in the city. As was found out before, these water sources have different growth-promoting properties (Section 3.3). Additionally, phosphorus could be released from bacteria as a result of chlorination or other stress [141]. Other studies showed an increase of phosphorus in water as a result of cast iron pipes corrosion and its accumulation and release from biofilms [173, 174]. As plausible explanation of increased phosphorus concentrations could be phosphorus accumulation in the biofilms from initially phosphorus-unlimited groundwater, and its subsequent release.

Changes in the AOC concentration were observed in the groundwater sample, where 3-fold higher carbon was available after distribution than in WTP sample ($3.19 \pm 0.18\times10^6$ cells mL^{-1}). However, the AOC difference between the surface water samples before and after distribution was not significantly different ($P > 0.05$). Various hypotheses concerning increase of AOC concentrations could be considered. Several studies showed that AOC could be released as a result of chlorine oxidation of bacteria and complex organic matter [26, 28, 29]. Biofilms can play important role in providing nutrients, and particularly organic carbon, that are able to be consumed by bacteria. For example, a study on interactions between biofilms and humic substances showed that humics, which normally are not degradable by suspended bacteria, could serve as a sole carbon and energy source for bacteria in presence of biofilms [175]. Moreover, various extracellular enzymes potentially are able to degrade biofilm matrix during starvation [176].

Obviously, changes in growth-promoting parameters in the full-scale network cannot be explained by one single mechanism, it is rather a combination of various processes, occurring due to interaction between bulk water, biofilms, disinfection residuals and pipe materials. However, we also assume 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 risk of bacterial growth should be considered in such DN.

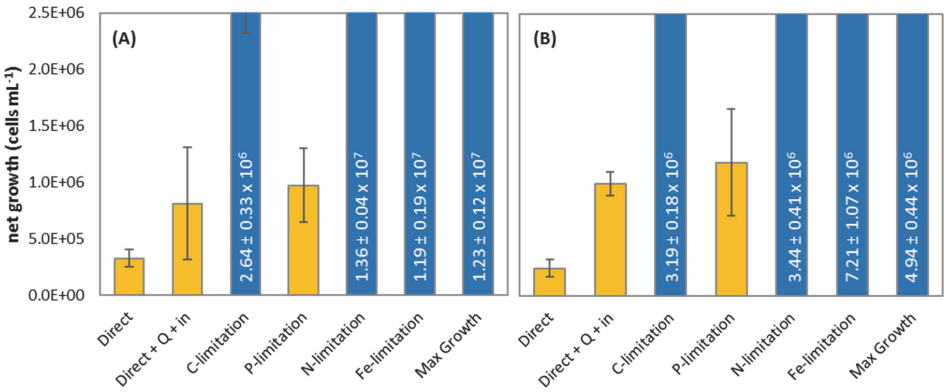


Fig. 5.12. Bacterial growth, represented as changes in total cell count (TCC) in the DN samples after 72 h incubation

The new growth potential approach was tested for drinking water DN samples, which are supplied principally from (A) treated surface water; (B) 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.

5.3.5. Effect of chlorination on nutrient release

Effect of chlorination on nutrient release differed depending on the water type. The growth potential measurements of the samples, taken before and after chlorination during summer sampling campaign, showed significant ($P < 0.05$) bacterial growth due to increased concentration of microbially available phosphorus at WTP 2, while increase of microbially available carbon was significant at WTP 3 (Fig. 5.13A). Changes of growth-promoting nutrients at WTP 1 due to chlorination were negative (concentration decreased) or not statistically significant ($P > 0.05$). During the winter sampling campaign, the only significant increase of growth-promoting nutrients was detected at WTP 2, where more phosphorus was produced after chlorination (Fig. 5.13B), while changes at WTP 1 and WTP 3 were negative of not statistically significant. Increased bacterial growth after chlorination in WTP 2 and WTP 3 samples during direct incubation with neutralized chlorine was observed (not shown), and corresponded with increase of carbon and phosphorus as discussed above (Fig. 5.13). Although increase of growth-promoting nutrients during chlorination was observed in other studies [92, 141], we do not have evidence or clear explanation of the differences in nutrient changes during chlorination between different samples. Hypothetically, water chemical (and biological) composition was different between surface and groundwater, thus chlorine oxidation of these compounds resulted in different products.

Interestingly, amount of microbially available carbon was generally higher in winter in all WTPs before and after chlorination. If converted to AOC, it was 603 µg AOC L^{-1}, 459 µg AOC L^{-1} and 835 µg AOC L^{-1} at WTP 1, WTP 2 and WTP 3 effluent, respectively. Similar trend was observed in the study of Prest and colleagues, where higher AOC was observed in winter, whereas bacterial growth was lower than in the summer [20].

Summarizing the observations from this section, influence of chlorination on availability of growth-promoting nutrients in the studied DN is following:

- WTP 1: no increased growth due to chlorination.
- WTP 2: no increased growth due to chlorination. Although phosphorus was produced during chlorination, water was carbon-limited.
- WTP 3: increased bacterial growth due to the carbon, produced during chlorination.

Hence, chlorination could lead to the increase of growth-promoting nutrients at certain conditions. However, overall results in this chapter indicate that nutrients, produced during chlorination, are not likely a major cause of biological instability in the studied DN. In terms of biological stability, chlorination posed a risk only at WTP 3, where increase of growth-limiting carbon was observed. 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. Moreover, water temperature plays a significant role on bacterial growth and biological stability in general. Our study showed that low water temperature resulted not only in low initial bacterial concentration, but it inhibited bacterial growth even with presence of growth-promoting nutrients.

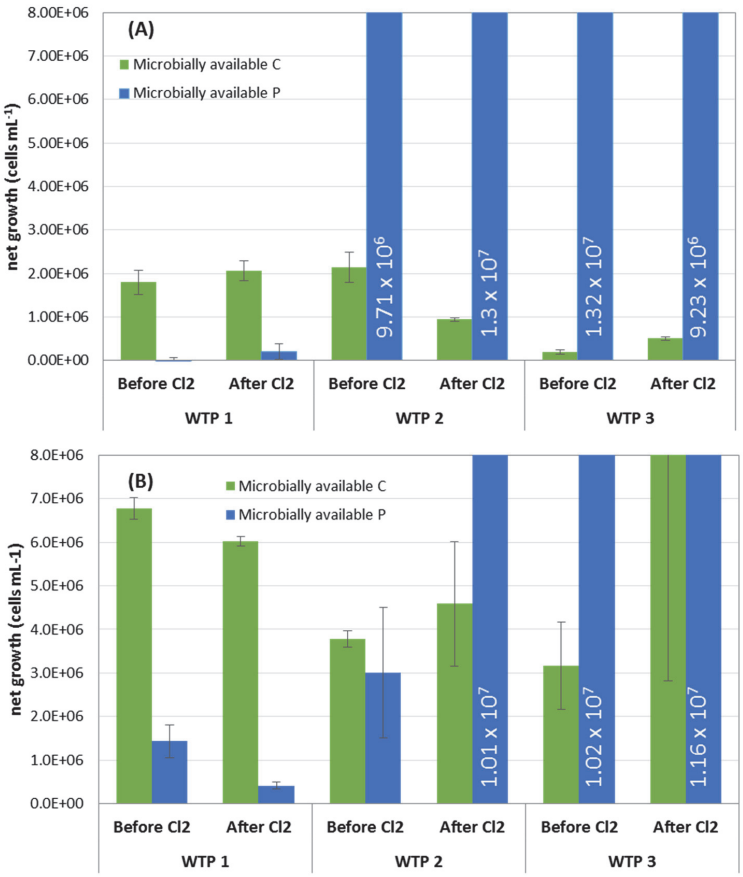


Fig. 5.13. Changes of microbially available carbon and phosphorus as a result of chlorination
The results are represented as changes in total cell count (TCC) in the samples from WTP effluent after 72 h incubation. (A) The samples were taken in summer during normal chlorination conditions; (B) the samples were taken in winter during intensified chlorination (Table 5.1).

5.4. Conclusions of Chapter V

- Seasonal instability was observed in drinking water samples, which was linked to bacterial growth in the network.
- Drinking water biological stability depended on the water source and its treatment.
- Phosphorus was the primary growth-limiting nutrient in the treated surface water sample.
- Carbon was the primary growth-limiting nutrient in the treated groundwater samples.
- Drinking water biological stability and amount of available nutrients changed during the distribution: treated surface water had up to five-fold more microbially available phosphorus during distribution, and treated groundwater had three-fold more assimilable organic carbon during distribution, but ten-fold less phosphorus.
- Increased risk of bacterial regrowth in the DN occurs if DN is supplied with different water sources with different growth-promoting parameters.

6. GENERAL DISCUSSION AND RECOMMENDATIONS

The purpose of this thesis was to investigate biological stability of drinking water in chlorinated distribution networks, with specific focus on the distribution system of Riga, Latvia. The DN of Riga is particularly interesting. Firstly, all water undergoes chlorination before entering the DN, which allows to study biological stability in a real full-scale chlorinated DN. Secondly, Riga DN consists of more than 1400 km of pipes. Large DN is advantageous for biological stability investigation, because water retention time is long enough to provide bacterial growth, if growth-promoting conditions are present in the network, which allows to evaluate spatial biological instability. Nevertheless, studying the large and complex DN is also challenging because of chemical, physical and biological processes interaction in DN during long water retention time. Such common processes as corrosion, increase of water temperature, water stagnation in dead-ends and/or reservoirs, detachment of biofilm bacteria and "mixing" of different water types because of uneven water consumption could affect bacterial growth, which should be considered during data interpretation. And finally, water of different origin and treatment is supplying the same DN. Different bacterial growth in different types of water and, accordingly, at different sites of the DN was expected, as far as differences in initial water quality and bacterial biomass after distribution regarding water origin and composition were shown before [17, 18, 47]. However, possible interaction between different water and its influence on bacterial growth within the same DN were not investigated. Therefore, more detailed investigation was necessary to understand the role of drinking water nutrient composition on biological stability in DN.

Biological stability has been a research topic for many years, but the emergence of new methods during the last years allowed more thorough and detailed investigations. Rapid bacteria enumeration methods gave a possibility to analyze larger amounts of samples, which gave a better understanding of the bacterial growth problem [1, 18, 177]. Moreover, novel molecular methods allowed to study not only changes of bacterial population in drinking water, but also to investigate interactions between bacterial communities [2, 178]. In terms of biological stability in chlorinated DN, appropriate bacteria viability methods are essential. FCM measurements of SGPI stained samples and ATP measurements were chosen as alternative to HPC – a conventional viable bacteria enumeration method. Although application of FCM-based viability methods and ATP measurements were applied for drinking water analyses before, discrepancies between the FCM protocols were found in literature, while ATP was never tested for chlorination specifically. Thus testing and optimization of the methods were needed in order to describe biological stability in chlorinated DN.

Finally, high quality drinking water has a societal value. Access to safe drinking water is a basic human right, which is "*essential to the realization of all human rights*"[179]. A task of water utilities includes clean drinking water production, its delivery with acceptable quality, regular and reliable water monitoring. Implementation of all these tasks will not only provide people with safe water, but could increase trust to the responsible institution and generally improve life quality. Distrust to drinking water quality in DN is a common problem, and water utilities should guarantee that water they provide is safe at every point of delivery to assure consumers of high drinking water quality. Although it is difficult to control drinking water quality in DN, it is essential to understand the processes, which could affect drinking water safety, in the particular DN to prevent water quality deterioration. Hereof, the reasons of biological water quality changes in the real full-scale chlorinated drinking water DN were investigated in this study, and novel bacterial viability methods were studied in details to substantiate selection of methods and approaches.

6.1. A need for new methods and importance of methods standardization

There is general consensus that conventional microbiological methods used in drinking water research are insufficient to assess biological stability. For example, "*assessment and monitoring of the level of microbial regrowth requires a method for the quantification of the biomass of all active bacteria*" [180]. Moreover, „*it is particularly useful to determine cultivation-independent bacterial viability/activity when disinfection is applied*" [2]. Considerable work has been done with cultivation-independent methods for drinking water bacteria viability analysis [18, 101, 110, 114, 177, 181]. Hence, biological stability of drinking water in distribution networks was evaluated with cultivation-independent bacteria determination methods – specifically FCM and ATP – and a modified growth potential approach during this PhD project. These methods were selected, because they could provide accurate information about total and viable bacterial biomass in water within a very short time. However, the majority of the recent studies with FCM and especially ATP has been done in non-chlorinated distribution systems [1, 18, 158, 166]. Therefore, special attention was paid to the optimization and testing of these viability methods for disinfection specifically, since viability is the main measure of chlorination efficacy.

The importance of using accurate and standardized protocols and proper data interpretation were discussed in chapters III and IV. A general pipeline for method optimization of SGPI fluorescent staining was developed during this PhD project. The pipeline included testing of various parameters, such as dye solvent, concentration of dye, staining time and temperature, which could severely affect staining outcome, if not used correctly. Often novel viability methods are sold as off-the-shelf kits with wide range of applications they could be used for, and the protocols not necessarily are optimized for drinking water specifically. Moreover, it was shown in this project that inactivation of bacteria, caused by several types of water treatment, cannot be detected with SGPI staining, and consequently with membrane integrity based methods in general. Therefore, our results highlight the significance and importance of understanding both the mechanism of the viability method as well as the mechanisms of cell death in order to avoid or minimize the potential method's pitfalls and artifacts. And in order to have reproducible data and compare data between different studies, it is imperative to standardize methods' protocols. Testing and optimizing methods for the target samples will provide deeper knowledge of the method and create confidence in the data.

Methods standardization is mandatory, if they are about to be implemented into drinking water monitoring process. Moreover, often this is a main obstacle, which prevents innovative methods from being used for routine analyses. However, it is only a matter of time, when cultivation-independent methods will be legally accepted for drinking water monitoring, because new methods and technologies are developing fast and the costs are decreasing, while limitations of cultivation based methods are significant. The fact that only very small percentage of viable drinking water bacteria could be cultivated, and that the results cannot be available before at least 24 h, impugns the value of the cultivation-based method, which aims to detect undesirable changes in drinking water quality and thus to prevent distribution of possible contamination. It is clear that more rapid and accurate methods should replace or at least supplement the existing cultivation-based methods, which are nowadays the only legally accepted methods for biological drinking water quality worldwide. SGPI staining coupled with flow cytometry is a promising tool for bacteria viability tests in drinking water, and in chlorinated water particularly. Large amount of chlorinated drinking water samples was analyzed with SGPI in the study of Gillespie and co-workers, and showed low % ICC, when free chlorine was > 0.5 mg L^{-1} (Fig. 6.1A) [36], which demonstrated considerable cell damage with these concentrations. Chlorine concentrations in Riga DN, obtained during the case study, presented in Chapter II of this thesis, were lower than in Scottish Water DN. Nevertheless, ICC and chlorine data support these results, showing similar trend for both free and total residual

chlorine (Fig. 6.1). The results indicate that chlorination causes membrane damage, which is detectable by SGPI staining mechanism.

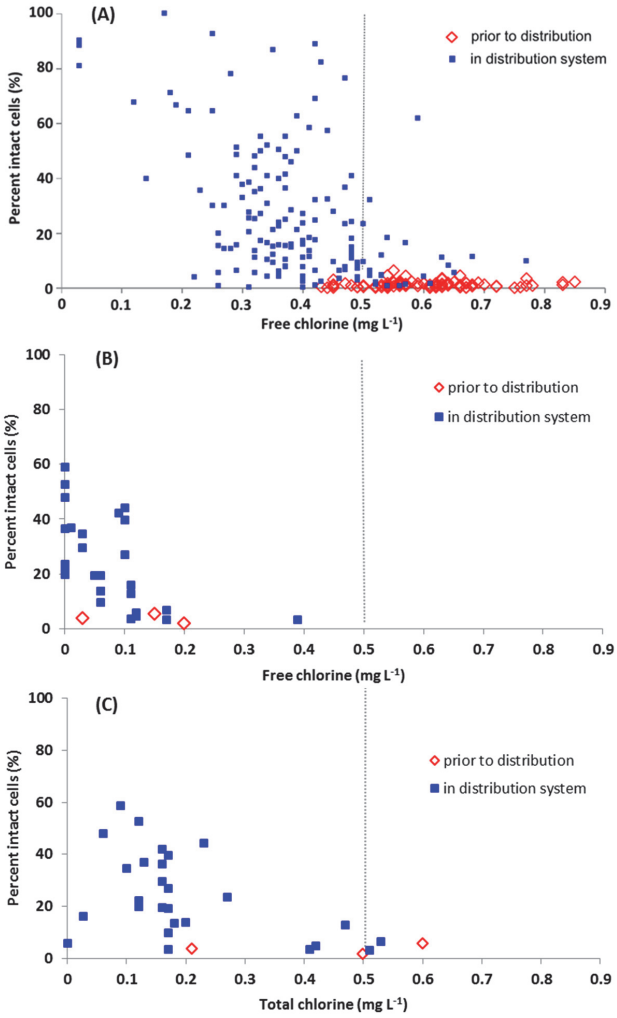


Fig. 6.1. Effect of chlorine concentrations on the % ICC in water DN

Effect of disinfectant concentrations on the relative proportions of intact cells in water distribution systems I (chlorinated), adapted from Fig.1 [36] (A); % ICC in Riga DN with different free chlorine residual (n = 30) (B) and total chlorine residual (n = 30) (C). A dotted line indicates a critical free chlorine concentration threshold, as was proposed in the study at Scottish Water DN [36].

Moreover, membrane damage is rather conservative parameter in terms of cellular death, or in other words, reliable indicator of cell death, which means that it is unlikely that PI-positive cells could remain viable. For example, membrane damage occurs with higher chlorine concentration in comparison with ATP decrease, as shown in Chapter IV (Fig. 4.1). This means that cell vital functions are disabled even before the membrane gets disrupted. This is important for control of chlorination efficacy before and during water distribution. Flow cytometry technique is advantageous not only because of its accurate measurements, but also from

practical prospective. The measurement itself takes less than 5 min, and the whole analysis does not exceed 20 min together with staining. A breakthrough example of FCM application was presented in studies of Besmer and colleagues, where staining and measurements were conducted fully automatically at the real drinking water treatment plant [19, 60]. Although fluorescent staining together with flow cytometry was used for drinking water microorganisms research just about one decade, it is clear that at the moment it is one of the most studied and developed cultivation-independent methods.

The importance of proper interpretation of the results is shown the best in Chapter IV, as the example of ATP measurements. Testing ATP method for water chlorination allowed better understanding of the mechanism of the cellular death and the mechanism of the method. The main conclusion of the chapter was that ATP was released from microorganisms as a result of chlorination. While at low chlorine concentration (< 0.35 mg-Cl_2 L^{-1}) intracellular ATP was decreasing, then at higher concentrations it was almost absent in intracellular form, but increased amount of ATP was detected in extracellular environment. The fact that this process coincided with membrane damage indicated on complete cell death at these chorine doses, where lower doses still allowed enzymatic activity. In terms of methodology, these observations emphasize that intracellular ATP should be distinguished from extracellular ATP to describe viable biomass and provides evidence that increased extracellular ATP, observed during the first case study (Chapter II) and the study of Hammes and colleagues [76, 77] due to oxidative stress, is not a method's artifact.

Another interesting conclusion was that extracellular ATP was used as a source of phosphorus for bacteria, suggesting a potential role of extracellular ATP in biological stability of drinking water. Although the ability of extracellular ATP to promote bacterial growth was demonstrated in the current project, it does not necessarily mean that chlorination will lead to biological instability. Since amount of released ATP is relatively low in terms of biological stability, a risk of bacterial regrowth due to ATP, released as a result of chlorination, is present only if water is phosphorus-limited. Moreover, low residual chlorine should be maintained in the network to ensure that bacteria growth is not inhibited by disinfectant. However, if chlorination is applied for drinking water supply systems, where above-mentioned statements are true, bacterial growth potential and chlorination necessity in general should be evaluated. 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, possible influence of ATP, released from biofilm bacteria, should be investigated.

Overall, ATP method proved to be appropriate for biomass characterization in chlorinated drinking water. The method is accurate and it takes about 5 min to analyze the sample. One can argue that it does not provide information on bacteria quantity, however, it could be used to monitor changes of biological water quality, and give additional data about chlorination process and biological stability.

6.2. Biological stability assessment in a full-scale drinking water supply system

Practical application of the methods, mentioned above, was demonstrated in a full-scale chlorinated drinking water distribution system in order to describe biological stability. ATP and/or FCM were measured at different sites in the DN, and randomly chosen samples were analyzed for short-term and long-term bacteria variations. The data identified both spatial and temporal biological instability in the network (Chapter II, V). Spatial instability was observed, when various samples from the DN were analyzed with ICC, and the lowest ICC were observed in the areas in close proximity to WTPs, while higher ICC were detected further in the network and in so-called mixed zones. Comparison of microbiological parameters of the water samples from DN and WTP indicated that these changes most probably were linked to bacterial

regrowth. However, microbiological quality of drinking water was not constant at particular location. Diurnal variations have been demonstrated on the example of DN sample, which was analyzed for 21 h. Bacterial concentration increased dramatically twice during the observation period, and both times numbers returned to low and more stable concentration. Interestingly, bacterial concentrations at the corresponding WTP were constant and significantly lower than at the DN sample. On the one hand, this was an additional evidence of bacterial regrowth in the DN. But on the other hand, it showed that fluctuations of bacterial counts at the single point is probably a combination of suspended bacteria and biofilm bacteria growth, where the latter could be detached in case of change of water consumption. This is the most probable reason of emerging peaks, however, lack of repetitions does not allow to understand whether these were regular or occasional changes. Moreover, explanation of these peaks was obstructed by the complexity of the DN, as was mentioned at the beginning of this chapter, which implies that increased numbers of bacteria could be caused by water "mixing" or potentially stagnated water from reservoirs. Although exact reasons of the diurnal bacterial fluctuations cannot be explained with available data, it is clear that microbiological parameters in DN are dynamic. This is corroborated by emerging studies on temporal bacterial dynamics, where short-term fluctuations in bacterial concentrations were detected [60, 111], indicating also that temporal dynamic is not typical just for the studied DN.

In turn, 1-year monitoring of two DN samples – one originating from surface water and the second representing "mixed" water sample – with FCM TCC and ICC demonstrated clear seasonal bacterial fluctuations, where bacterial numbers were mostly higher during warm time of the year than in winter. This could be explained by water temperature, which changed dramatically in surface water, thus affecting bacterial concentration and activity both in raw water and in biofilters. Data comparison between DN and WTPs showed that bacterial growth took place in the DN, and growth extent was higher during warm months (Fig. 6.2C, 6.2D). It is different from similar study of Prest and colleagues, where seasonal differences were related mostly to initial water quality, and growth in DN was minimal (Fig. 6.2A, 6.2B) [20]. Like the observations in short-term study, results from the long-term experiment showed that drinking water distribution system could not be considered as stable in terms of bacterial dynamics. Moreover, temporal fluctuations differ at different locations, and this should be taken into account, while evaluating monitoring data, to ensure good water quality.

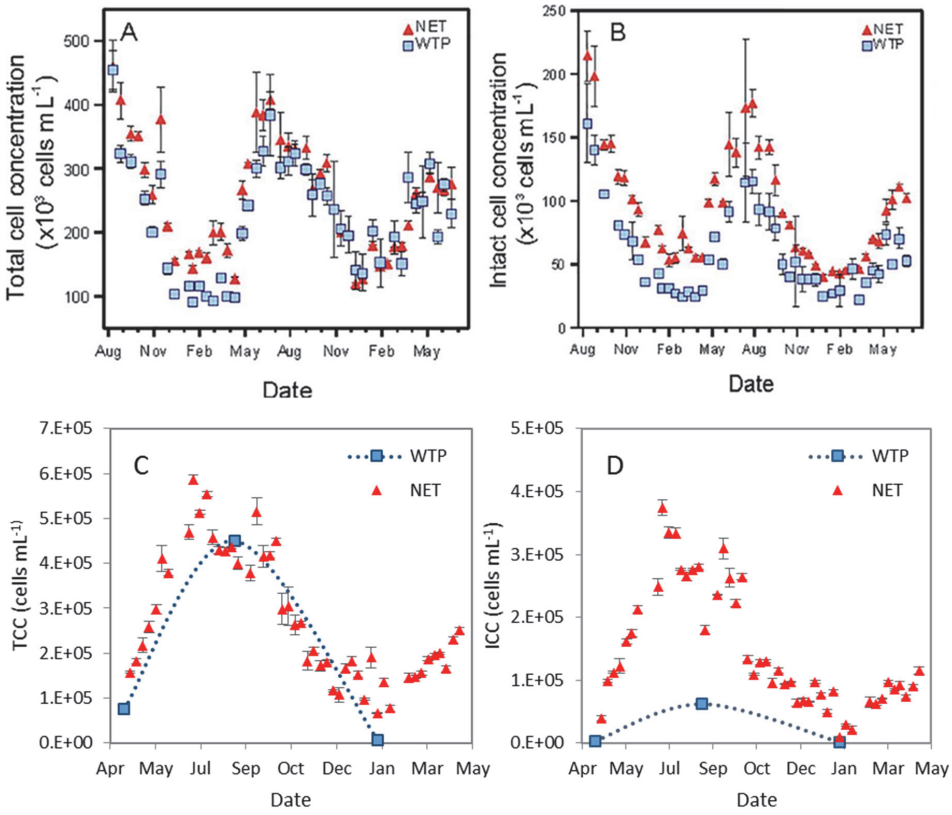


Fig. 6.2. Comparison of long-term TCC and ICC fluctuations between the study of Prest and colleagues (A, B) (adapted from [20]), and this project (C, D)

Only 3 WTP samples were analyzed within this project, so WTP curve at the figures C and D has very approximate pattern. Nevertheless, ICC differences between WTP and NET clearly demonstrated bacterial growth in the network.

A modified growth potential test was applied to determine growth-promoting nutrients in distributed drinking water. Bacterial growth prediction methods, used in previous studies, were based on determination of the single nutrient, usually organic carbon fractions (AOC, BDOC) or microbially available phosphorus, which assumed that other nutrients are not limited [21, 41, 92, 182]. A step forward in investigation of multiple nutrients within the same sample was presented in the new growth potential approach study of Prest and colleagues, where limitation by both organic and inorganic was investigated, however, specific inorganic compounds were not assessed [22]. Modification of the existing methods was needed for detection of various compounds by the same principle, thus providing direct data comparison between tested nutrients and ability to use the approach for compounds, others than were demonstrated in the project in general.

Interesting results were obtained with the new approach. It was found out that the studied DN was supplied with water with different growth-promoting properties: phosphorus was the primary growth-limiting nutrient in the treated surface water, while carbon was growth-limiting in the treated groundwater samples. Moreover, drinking water biological stability and amount of available nutrients changed during the distribution: treated surface water had more

microbially available phosphorus during distribution, and treated groundwater had more assimilable organic carbon, but less phosphorus, than it was before distribution. There is no clear answer why biological stability properties changed during distribution, because too many processes are influencing drinking water parameters in such a large DN with long water retention time. It could be likely related with the DN design and functioning, which could lead to nutrient transfer/exchange between different types of water. For example, groundwater and surface water could be pumped into the same reservoir, where direct water mixing could take place. Water from different sources could be also "mixed" in the pipes, when changes in water consumption could lead to changes of predominating water. Therefore, different types of water with different but complementing growth-promoting nutrients could increase a risk of drinking water instability in the DN.

This study demonstrated that chlorination is not a main reason of biological instability in Riga DN, but it creates favorable conditions for bacterial growth. Chlorination led to increase of phosphorus in WTP 2 and carbon in WTP 3, while no changes in bacterial growth was observed in WTP 1. Moreover, only carbon, produced at WTP 3, could influence biological stability, since produced phosphorus was not limiting at WTP 2. However, residual chlorine concentration during normal system operation was too low to prevent bacterial growth in the DN. Chlorine disinfection at WTPs led to 41 – 86 % ICC decrease during normal operation in summer and 56 – 97 % during enhanced chlorination in winter. Although bacterial composition was not studied during this project, it was shown in other studies that chlorine and chloramine disinfection alters bacterial composition [86, 181]. Thus removing large part of bacteria could influence natural bacterial competition in water, which could be favorable for growth of specific bacterial species.

The results from this project indicate that bacterial growth problem was caused by changes in nutrient composition in the DN. Interestingly, finished water was biologically stable regardless its origin, and growth was limited either by carbon or phosphorus. However, bacterial growth was no longer limited by nutrients in the DN, and growth potential was different at different network locations. Since this rather unique feature of Riga DN, i.e. supply by water with different growth-promoting nutrients, was discovered, transfer of nutrients could be the reason of these changes in the DN. Future research is needed to understand the path of nutrients. More locations should be included into the sampling campaign for growth potential tests, and hydraulic model should be considered to assess water retention time and type of water. Additionally, ability of biofilms to accumulate and then release nutrients could be tested.

6.3. Recommendations and implications for water utilities

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 to apply for routine drinking water monitoring analysis in addition or, ideally, instead of HPC, since in case of real biological contamination danger, these would provide much faster response and give a possibility to prevent distribution of contaminant. Ability of SGPI staining and ATP method to detect bacterial damage caused by chlorination is useful for characterization of disinfection process, whereas ATP could also give instant information on growth potential prediction. Moreover, the methods could generate a lot of data within short time, which would provide better knowledge about drinking water system performance. Nowadays a certain interest is present 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. Collection of large amount of data is essential for development of the 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 safety by individual water utilities. The reasons of 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 [51, 52]. Interestingly, despite the well-known limitations of the HPC method, Counsil 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*" [51], while suggesting HPC as bacteria determination method. Ideally, legislation of cultivation-independent methods should be evaluated. It should be noted that neither SGPI nor the ATP method could identify specific (pathogen) microorganisms. Nevertheless, pathogen and opportunistic bacteria should be distinguished, and importance of the latter should be understood. While pathogens in water are mostly associated with external contamination (usually fecal pollution) and cause infections, opportunistic bacteria is often a part of a natural water microbial composition, and in normal concentrations should not cause diseases in a healthy host. However, they could pose a certain risk for weakened immune system, when proliferate, thus bacterial growth is undesirable. Therefore, control of total and viable bacterial concentration and assessment of biological stability in drinking water DN is of great importance.

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 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 seasonal tendency, however, it also differs depending on the location and water source. Seasonal effect should be taken into consideration while monitoring DN samples: the same bacterial concentrations could be normal during warm season, but it would indicate on contamination, if measured in winter. Additionally, WTP performance could be optimized both from safety and economy prospect. For example, speaking about only biological quality of drinking water, chlorination could be minimized or cancelled for surface water treatment in winter, when bacterial concentrations and growth in biofilters are low. Similarly, ozonation intensity could be reduced, when less microorganisms and organics are present in raw water. In contrast, with presence of high amount of bacteria and during hot weather, chlorination could be intensified to prevent bacterial growth in DN.

An interesting finding about different growth limitations in different water sources supplying the same DN emphasize the need to test several nutrients, which could relate to biological stability concept. As was observed in the present study, different water can contribute to different growth limitations, which implies the potential regrowth 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 treatments at groundwater WTPs, for example, biofiltration. In 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 Riga DN, chlorination only removed large part of bacteria during disinfection, whereas residual chlorine was too low to prevent bacterial growth further in DN. Although amount of released nutrients during chlorine disinfection was insignificant in terms of growth potential, 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 [31, 158]. Before making decision, various actions should be undertaken. The main goal of disinfection is to kill pathogens, this is why absence of pathogens before distribution have to be checked and ensured. To be sure that stopping chlorination would not lead to pathogen and bacterial growth problems, cancellation of chlorination could be realized by gradual reducing chlorine dose, with enhanced monitoring of DN water samples. In contrast, amount of chlorine could be increased to solve a problem of bacterial growth in DN. However, it should be understood which type of bacteria proliferates in DN and whether it poses any risk.

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

Summarizing observations from our study, we generally conclude that chlorination does not ensure biological stability of drinking water in DN. Understanding of growth risks regarding water origin, 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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