BIOLOGICAL STABILITY
IN DRINKING WATER DISTRIBUTION SYSTEMS

A novel approach for systematic microbial water quality monitoring

Emmanuelle PREST

BIOLOGICAL STABILITY
IN DRINKING WATER DISTRIBUTION SYSTEMS

A novel approach for systematic microbial water quality monitoring

Proefschrift
ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof. Ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op
13 november 2015 om 12:30 uur

door

Emmanuelle Ingrid Elisabeth Denise PREST

Ingénieur chimiste
École Nationale Superieure de Chimie de Montpellier, Frankrijk.
geboren te Parijs, Frankrijk

Dit proefschrift is goedgekeurd door:

Promotor	Prof. dr. J.S. Vrouwenvelder
Promotor	Prof. dr. dr. h. c. ir. M.C.M. van Loosdrecht
Copromotor	Dr. F. Hammes

Samenstelling promotiecommissie:

Rector Magnificus	Voorzitter
Prof. dr. J.S. Vrouwenvelder	Technische Universiteit Delft, Promotor
Prof. dr. dr. h. c. ir. M.C.M. van Loosdrecht	Technische Universiteit Delft, Promotor
Dr. F. Hammes	Eawag, Copromotor

Onafhankelijke leden:

Prof. dr. J.C. Block	Université de Lorraine, Nancy, France
Prof. dr. J.P. van der Hoek	Technische Universiteit Delft
Prof. dr. G.J.W. Euverink	Rijksuniversiteit Groningen
Dr. A.J. Pinto	University of Glasgow, United Kingdom
Prof. dr. ir. L.C. Rietveld	Technische Universiteit Delft, reserve

The work presented in this thesis has been performed at the Delft University of Technology, in close collaboration with and financially supported by: Evides Waterbedrijf, Eawag (Swiss Federal Institute of Aquatic Science and Technology), and King Abdullah University of Science and Technology.

ISBN: 978-94-6259-812-6

Printed by Ipskamp Drukkers, Enschede

Contents

Summary

Challenges to achieve biological stability in drinking water distribution systems

Drinking water is distributed from the treatment facility to consumers through extended man-made piping systems. The World Health Organization drinking water guidelines (2006) stated that "Water entering the distribution system must be microbiologically safe and ideally should also be biologically stable". The biological stability criterion refers to maintaining the microbial drinking water quality in time and distance from the point of drinking water production up to the point of consumption. However, uncontrolled growth of indigenous bacteria during water transport can result in the deterioration of aesthetic aspects of water, such as taste, colour, and odour, in exceeding of guideline values, and/or in technical problems. Controlling bacterial growth in piping systems and premise plumbings is very challenging (Chapter 2), and changes in drinking water microbial characteristics are often measured in networks distributing water with or without residual disinfectant such as chlorine, monochloramine or chlorine dioxide. In the Netherlands, drinking water is distributed without detectable residual disinfectant. Quantitative and qualitative knowledge on the indigenous bacterial communities and microbiological processes taking place during drinking water distribution is limited and in-depth investigations are required.

New opportunities with novel analytical methods

One reason for the lack of knowledge on bacterial growth controlling factors in drinking water distribution systems is that methods for characterizing drinking water bacterial communities are still relying heavily on culture-based techniques such as plate counts, developed more than 130 years ago. The conventional cultivation-based methods have major limitations: only a minute fraction (<0.1 %) of drinking water bacteria is detected, which is not representative of the drinking water bacterial community, and results are obtained only after a minimum of two days. During the last decade, new cultivation-independent techniques have emerged for the characterization of water bacterial communities. Among them, flow cytometry (FCM) enables the rapid detection and counting of all bacterial cells in water (within 15 minutes), and provides information on bacterial cell properties such as viability. Besides, high-throughput sequencing methods (e.g. 454-pyrosequencing or Illumina) enable characterization of the total bacterial community composition and structure at various taxonomic levels. FCM and high-throughput sequencing methods offer new perspectives for better

and faster water microbiology monitoring and for increased understanding of the complex bacterial dynamics occurring during drinking water distribution up to the point of consumption (Chapter 2).

Method development

The primary goal of this study was to develop a methodological approach, based on advanced analytical methods, for the assessment of biological stability in drinking water distribution systems. A standardized, rapid and simple FCM method was shown to be highly reproducible and sensitive for total and intact bacterial cell enumeration. Changes in bacterial community characteristics could be detected based on bacterial cell concentrations and FCM fluorescence fingerprints, which are characteristic of each water sample (Chapter 3). Changes in fluorescence fingerprints were proven to be a rapid indication for changes in bacterial community composition, by comparing FCM and 16S rRNA gene pyrosequencing data obtained from the same drinking water samples. Combining the two methods enabled both quantitative and qualitative characterization of water bacterial communities (Chapter 4). An integrated approach was proposed for the assessment of bacterial growth-controlling factors in drinking water and for the evaluation of the impact of full-scale distribution conditions on bacterial growth extent. The approach combines (i) characterization of autochthonous bacterial communities in water samples collected at several locations in full-scale drinking water distribution systems, using FCM and high-throughput sequencing methods, (ii) comparison of changes in bacterial abundance recorded during water distribution and during controlled laboratory bacterial growth tests, and (iii) stepwise assessment of bacterial growth limitations in drinking water using straightforward bacterial growth potential tests (Chapter 5).

Application of developed methodological approach to a full-scale drinking water system

The developed methodological approach was applied to a Dutch full-scale drinking water treatment and distribution system operated without detectable disinfectant residual. Spatial and temporal variations were studied on short-term (hour, day, week) and long-term (seasonal) time-scales, and bacterial growth-limiting factors were investigated. Bacterial growth in the produced drinking water was limited both by organic carbon and inorganic nutrients (Chapter 5). Large seasonal variations in bacterial cell concentrations were recorded at the treatment effluent, which were congruent with water temperature fluctuations. Changes in bacterial community characteristics in the distribution system were minor compared to temporal variations in the treatment effluent (Chapter 6). However, all studies univocally showed that changes in bacterial community abundance, viability and/or community composition occurred during water distribution in the well-maintained network

(Chapters 4, 5, 6 and 7). Changes were not detected with conventional bacterial detection methods. In-depth analysis of bacterial community composition in water samples, using pyrosequencing, showed that the core bacterial community did not change during water distribution, whereas high dynamicity was found in rare taxa (Chapter 7). Different bacterial cell concentrations were measured in the full-scale system and after incubation of the same water under controlled conditions, highlighting the effect of distribution conditions (e.g. temperature, pipe material, residence time) on drinking water microbial quality (Chapter 5). The results suggest that the extent of bacterial growth at one specifically studied location in the distribution system was not determined by the concentration of assimilable organic carbon in the treatment effluent. Likely not only one single parameter can be considered as controlling factor of microbial growth in drinking water distribution systems (Chapter 6).

Recommendations

From these observations, it is recommended to study microbial dynamics in drinking water distribution systems using a combination of controlled laboratory growth potential tests and *in-situ* characterization of the drinking water bacterial communities in the distribution network, which includes both spatial and temporal investigations. Applying such an approach to individual systems would provide better understanding of microbial dynamics during drinking water production and distribution, enabling (i) rapid and sensitive drinking water monitoring, (ii) effective corrective and maintenance actions and (iii) funded decisions for the optimization of water treatment production and/or distribution conditions to control bacterial growth in drinking water distribution systems. In this regard, the recent emergence of on-line flow cytometers will promote flow cytometry as an ideal monitoring method, for the rapid detection of system failure and targeted maintenance management.

Samenvatting

Uitdagingen voor realiseren biologische stabiliteit in drinkwaterdistributienetwerken

Drinkwater wordt van de zuiveringsinstallatie naar consumenten gedistribueerd via een fijnmazig leidingnetwerk. De Wereldgezondheidsorganisatie richtlijnen voor drinkwater (2006) geven aan dat "water dat het drinkwaterdistributiesysteem in gaat microbiologisch veilig moet zijn en idealiter ook biologisch stabiel". Het biologische stabiliteit criterium houdt in dat de microbiologische drinkwaterkwaliteit gewaarborgd moet worden van productie tot consumptie. Echter, groei van de aanwezige bacteriën tijdens het watertransport kan resulteren in (i) verslechteren van esthetische aspecten van het water, zoals smaak, kleur en geur; (ii) overschrijden van richtwaarden; en in (iii) technische problemen. Bacteriegroei in het ondergrondse netwerk en in gebouwen is een uitdaging (Hoofdstuk 2), zowel met als zonder aanwezigheid van een desinfectiemiddel (chloor, mono-chloramine, chloordioxide) in het water worden veranderingen in de microbiële populatie in het drinkwater vastgesteld. In Nederland wordt drinkwater gedistribueerd zonder aanwezigheid van een desinfectiemiddel. De kwantitatieve en kwalitatieve kennis over de aanwezige bacteriepopulatie en de microbiële processen die plaats vinden tijdens drinkwaterdistributie is beperkt en vereist diepgaand onderzoek.

Potentie nieuwe analytische methoden

Een reden voor het gebrek aan kennis over de factoren die de groei van bacteriën in drinkwaternetwerken bepalen is dat de methodes om de bacteriepopulatie in drinkwater te karakteriseren nog steeds zijn gebaseerd op cultivatie-technieken zoals uitplaten, die meer dan 130 jaar geleden ontwikkeld werden. Deze conventionele cultivatie-gebaseerde methoden en richtlijnen hebben aanzienlijke nadelen: (i) slechts een minieme fractie (< 0.1%) van het aantal drinkwaterbacteriën worden gedetecteerd en de (ii) de resultaten zijn pas beschikbaar na minimaal twee dagen. De afgelopen tien jaar werden nieuwe cultivatie-onafhankelijke technieken ontwikkeld om bacteriepopulaties in water beter te karakteriseren. Eén van die technieken, *flowcytometrie* (FCM), laat toe om snel (in 15 minuten) alle bacteriën in water te detecteren en te tellen. Verder levert deze techniek informatie over bepaalde eigenschappen van de bacteriecellen, zoals levensvatbaarheid. Bovendien bieden *high-throughput sequencing* methoden (bv. 454-

pyrosequencing of Illumina) de mogelijkheid om de bacteriepopulatie te karakteriseren op verschillende taxonomische niveaus. FCM en high-throughput sequencing methoden bieden nieuwe perspectieven voor betere en snellere monitoring van de microbiologie in water en voor een beter begrip van de complexe bacteriële dynamiek tijdens de distributie drinkwater naar de consument (Hoofdstuk 2).

Ontwikkeling van de methoden

Het belangrijkste doel van dit werk was om een methodologische benadering te ontwikkelen, gebaseerd op geavanceerde analytische methoden, voor het bepalen van de biologische stabiliteit in drinkwaternetwerken. Een gestandaardiseerde, snelle en eenvoudige FCM methode voor het tellen van totale en intacte bacteriecellen bleek zeer reproduceerbaar en gevoelig. Veranderingen in de eigenschappen van de bacteriepopulatie konden vastgesteld worden op basis van de concentratie bacteriecellen en de FCM fluorescentie-fingerprint die karakteristiek is voor elk drinkwatermonster (Hoofdstuk 3). Door FCM en 16S rRNA gen pyrosequencing-gegevens van dezelfde watermonsters te vergelijken werd aangetoond dat veranderingen in de fluorescentie-fingerprint een snelle indicatie zijn van veranderingen in de samenstelling van de bacteriepopulatie. De combinatie van de twee methodes maakte het mogelijk om de bacteriepopulatie in water zowel kwantitatief als kwalitatief te karakteriseren (Hoofdstuk 4). Voor de beoordeling van de factoren die bacteriegroei in drinkwater bepalen en voor de evaluatie van de impact van condities in praktijk distributienetwerken op de mate van bacteriegroei werd een geïntegreerde aanpak voorgesteld. Deze aanpak combineert (i) karakterisatie van de autochtone bacteriële populatie in watermonsters verzameld op verschillende plaatsen in een praktijk drinkwaternetwerk, gebruikmakend van FCM en high-throughput sequencing methoden, (ii) vergelijking van veranderingen in de bacteriecel-concentratie vastgesteld tijdens waterdistributie en tijdens gecontroleerde bacteriële groeitesten in het laboratorium, en (iii) een stapsgewijze beoordeling van de bacteriële groeibeperkingen in drinkwater gebruikmakend van eenvoudige bacteriële groeipotentietesten (Hoofdstuk 5).

Toepassen van de ontwikkelde methodologische benadering op een praktijk drinkwaternetwerk

De ontwikkelde methodologische benadering werd toegepast op een Nederlandse praktijk drinkwaterzuivering en netwerk dat drinkwater distribueert zonder detecteerbaar residueel disinfectiemiddel. De spatiale en temporele variatie werd bestudeerd over korte termijn (uur, dag, week) en lange termijn (seizoenen), en de limiterende factoren voor bacteriegroei werden onderzocht. De bacteriegroei in het geproduceerde drinkwater werd door zowel organische koolstof

als door anorganische nutriënten gelimiteerd (Hoofdstuk 5). In het effluent van de drinkwaterzuiveringsinstallatie werden grote seizoenvariaties gemeten in de concentratie bacteriecellen. Deze variaties kwamen overeen met de watertemperatuurschommelingen. De veranderingen van de eigenschappen van de bacteriepopulatie in het distributienetwerk waren echter klein in vergelijking met de temporele variaties in het effluent van de zuivering (Hoofdstuk 6). Alle studies toonden eenduidig aan dat veranderingen in de bacteriecel-concentratie, levensvatbaarheid en/of populatiesamenstelling voorkomen in goed onderhouden drinkwaterdistributienetwerken (Hoofdstukken 4, 5, 6 en 7). Deze veranderingen werden niet gedetecteerd met conventionele bacteriedetectiemethoden. Een diepgaande analyse van de samenstelling van de bacteriepopulatie in watermonsters, gebruikmakend van pyrosequencing, toonde aan dat de kern van de bacteriepopulatie niet veranderde tijdens drinkwaterdistributie, maar dat diverse zijpopulaties echter een aanzienlijke dynamiek hadden (Hoofdstuk 7). Er was een verschil in, enerzijds, de concentratie bacteriecellen die werden gemeten in het prakrijknetwerk en, anderzijds, na incubatie van hetzelfde water onder gecontroleerde omstandigheden. Dit verschil werd verklaard door het effect van de condities in het netwerk (bv. temperatuur, leidingmateriaal, verblijftijd) op de microbiologische drinkwaterkwaliteit (Hoofdstuk 5). De resultaten suggereren dat de mate van bacteriegroei zoals bestudeerd op een specifieke plaats in het netwerk, niet bepaald wordt door de concentratie assimileerbare organische koolstof in het effluent van de zuivering. Waarschijnlijk is er niet één enkele parameter die beschouwd kan worden als de factor die bacteriegroei bepaald in drinkwaterdistributienetwerken.

Aanbevelingen

Op basis van bovenstaande resultaten wordt aanbevolen om de microbiële dynamiek in drinkwaternetwerken te bestuderen met een combinatie van groeipotentietesten in het laboratorium en *in-situ* karakterisering van de bacteriepopulatie in het netwerk, met aandacht voor zowel spatiale als temporele variaties. Toepassing van deze benadering op individuele netwerken zal een beter begrip opleveren van de microbiële dynamiek tijdens de productie en distributie van drinkwater. Dat zal leiden tot (i) een snelle en gevoelige monitoring van de drinkwaterkwaliteit, (ii) doeltreffende maatregelen bij verstoringen en onderhoud en (iii) gefundeerde beslissingen voor optimalisatie van drinkwaterproductie en/of omstandigheden in het netwerk om bacteriegroei in drinkwaternetwerken te beheersen. In deze context, de recente ontwikkeling van on-line flow cytometrie zal flow cytometrie promoten als ideale monitoring tool voor de snelle detectie van systeemverstoring en uitvoer van gericht onderhoud.

Chapter 1

Introduction and thesis outline

1.1 Providing direct access to safe and clean drinking water

1.1.1 Access to drinking water: situation and criteria

The United Nations general assembly has recognized in 2010 the human right to water and sanitation, thus the right to access safe, clean, sufficient, and affordable water for personal and domestic use (United Nations general assembly, July 2010). In 2010, about 50% of the world population was provided water directly from transport pipes into premises (Figure 1.1; Unicef and World Health Organization, 2012). Though direct access to safe and clean drinking water from household taps is often taken for granted in developed nations, continuous supply of good drinking water quality is the result of extensive water treatment and well-protected water distribution conditions. These are intended to produce and provide to consumers water which is free from micro-organisms and chemical substances representing a threat to human health, and which has an acceptable taste, color and odor for consumption.

1.1.2 Bacteria in drinking water

Bacteria are ubiquitous in aquatic environments, and are naturally present in drinking water. These indigenous organisms are closely related to both safety and quality of drinking water.

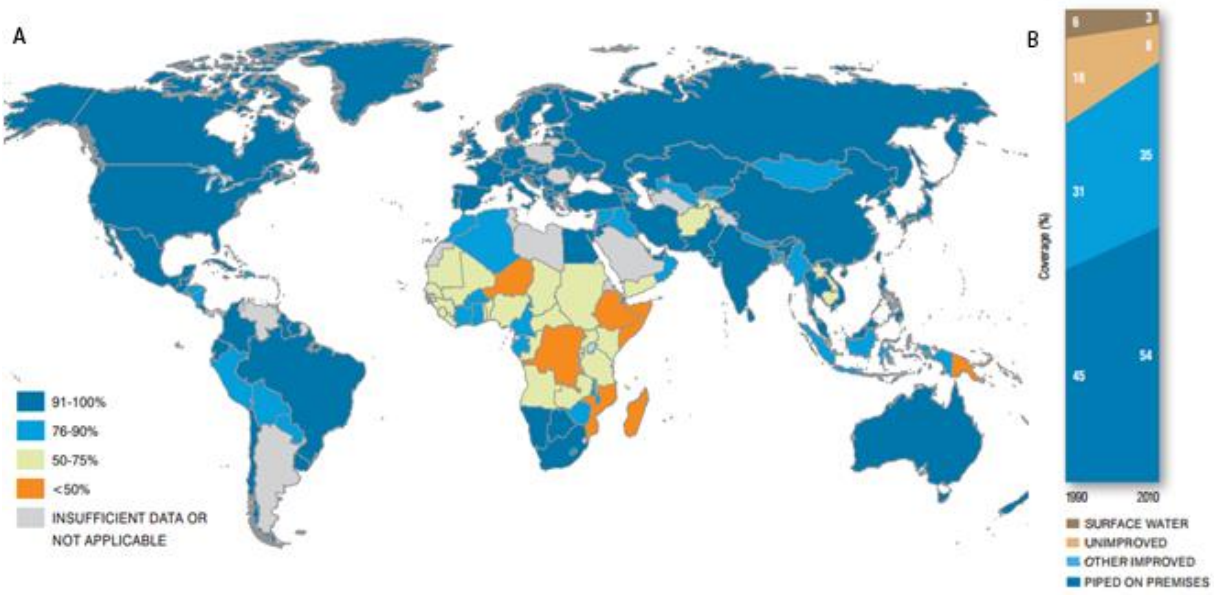


Figure 1.1. (A) World map of the proportion of the world population using improved drinking water sources in 2010. (B) Trend in the proportion of the global population using piped drinking water on premises, other improved drinking water sources, unimproved sources, and surface water, between 1990 and 2010 (Unicef and World Health Organization, 2012)**.**

A number of bacterial species present in aquatic environments can cause human diseases. A typical example is *Vibrio cholera*, the causative agent for the cholera disease. In 1854, John Snow identified drinking water as a transmission agent responsible for an epidemic of cholera in London. Ever since this discovery, sanitation measures have permitted to significantly reduce the occurrence of water-borne diseases in developed countries. As an example, the number of deaths caused by cholera in the Netherlands dropped from 21000 in 1866 to 0 at present time (Buitenswerf-van der Molen, 2007). Presently, water treatments target to produce drinking water causing less than 1 infection per 10,000 people per year (Waterleidingbesluit, 2001). However, there is growing concern about the emergence of environmental pathogens able to grow or re-contaminate drinking water after treatment. A typical example is the pathogen *Legionella pneumophila*, which can cause Legionnaire's disease when inhaled from water droplets from showers, bubble baths or cooling systems. These pathogens are of particular concern for human populations with weak immune systems, such as infants, elderly people or people with compromised immune systems.

The large majority of drinking water bacteria are however not harmful for human health. It is known that drinking water contains a large number of indigenous bacterial cells, in the range of 10^3 to 10^6 cells/mL (e.g. Hammes et al., 2010a). Notably, aquatic bacteria have been recognized to be useful for the production of drinking water at the treatment plant. As an example, bacteria in biological filters used for water treatment enable the removal of unwanted compounds from water, particularly organic compounds responsible for taste, colour and odour of the water (Rittman and Huck, 1989; Servais et al., 1994). Nevertheless, when present in excessive amounts, these organisms can be responsible for unpleasant taste, odour and turbidity of drinking water (van Lieverloo et al., 2002a; Vreeburg et al., 2004).

1.1.3 Drinking water production

Water treatment processes aim at producing safe and clean drinking water. Very various types of water sources are available throughout the world, including deep ground waters, infiltrated ground waters, spring waters, surface waters (rivers, lakes) and seawater. Water sources can differ greatly in terms of chemical and microbial properties. Besides, depending on the location, the raw waters are potentially contaminated with toxic compounds and/or faecal organisms. Water treatment strategies are therefore adapted to each water source, and also differ between countries (Rittmann and Huck, 1989). The treatment strategies range from direct distribution of the source water with or without a chemical disinfection step, for example in the case of clean spring waters from mountainous regions in Switzerland, to extensive combinations of treatment steps, for example in the case of surface water in the Netherlands.

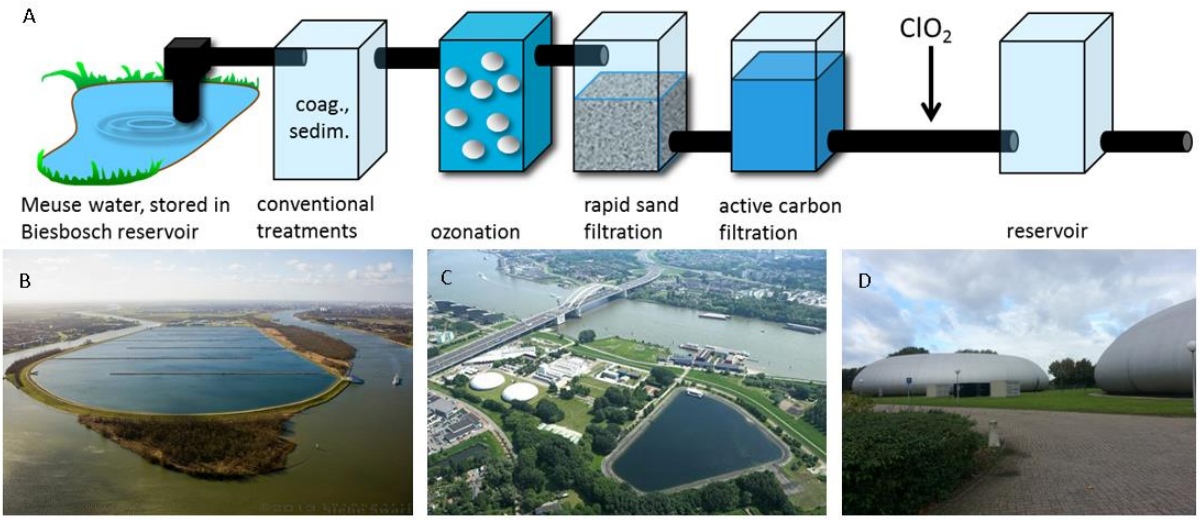


Figure 1.2. Example of a combination of treatment steps applied to produce drinking water using the river Meuse as water source (Kralingen, Rotterdam area, The Netherlands). (A) Overview of water treatment train, (B) aerial view of raw water reservoir (Biesbosch, The Netherlands), (C) aerial view of water treatment plant, (D) drinking water reservoirs.

The diverse treatments usually aim at (i) inactivating pathogenic organisms, usually using chlorine dosage, ozonation or UV irradiation, (ii) removing chemical toxic compounds, usually by active carbon filtrations, (iii) improving the turbidity, taste, odour and colour of the water, usually using conventional coagulation, flocculation and sedimentation steps, and/or rapid or slow sand filtration. A typical example of an extensive treatment train is given in Figure 1.2, as applied in the Netherlands for the production of drinking water using the river Meuse as water source. Each of the treatment steps can considerably affect the bacterial community initially present in the raw water, and influence the characteristics of the bacteria present in the produced drinking water.

1.1.4 Drinking water distribution

Once safe and clean drinking water is produced, water must be transported to consumers via underground piping systems. In most places, the water distribution systems cover large distances to provide water to far-off places in extended cities and/or to different small towns and remote villages. In the Netherlands, the total length of water distribution pipes is exceeding 110,000 km. As an example, the Dutch water treatment plant described in the previous section provides water to 530,000 inhabitants, with a total annual water production of 33 million m^3, distributed over an area of 120 km^2 through 1450 km pipe length. The distribution systems are typically composed of complex interconnections of pipes of different diameters, ranging from very large transmission mains (400-1600 mm diameter), to distribution mains (30-400 mm) and service pipes (20-25 mm), as represented in Figure 1.3A. Because of the system's complex structures, the water trajectory is far

from being linear. Furthermore, drinking waters produced at different treatment plants, and with different chemical and microbial characteristics, are often mixed in parts of the distribution systems. The water flow through the water distribution system is also fluctuating with time due to e.g. changing water consumptions over daily and monthly time periods. Finally, very diverse pipe materials are in use in drinking water distribution systems, and typically include cement, cast iron and/or plastic materials (Figure 1.3B).

The presence of bacteria in drinking water networks has been recorded in numerous systems throughout the world, whether residual disinfectants are applied or not. Bacteria are present in the bulk water, but also within sediments and in the form of biofilms attached to the inside of distribution pipe walls (e.g. Liu et al., 2014). Bacteria in the drinking water enter the distribution system, and can be significantly affected by the complex hydraulic conditions and the diverse pipe materials in the distribution piping networks. Because of the complexity of distribution systems, it is very challenging to control the behavior of bacteria in drinking water during its distribution, and thus to maintain the same microbial water quality from the treatment plant outlet to the consumer.

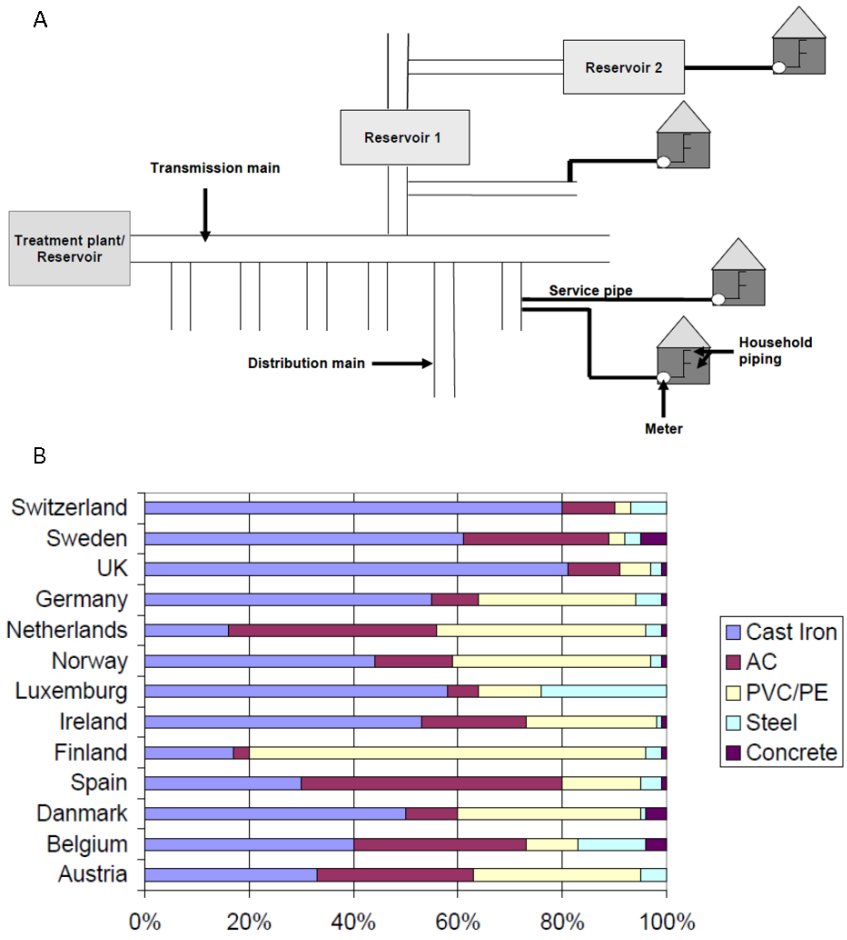


Figure 1.3. (A) Schematic layout of a drinking water distribution systems (Lautenschlager, 2011). **(B) Overview of pipe materials in use in European countries for drinking water distribution** (Vreeburg, 2007a)**.**

1.2 Biological stability of drinking water

1.2.1 The concept of biological stability

In 2006, the World Health Organization stated that "water entering the distribution system must be microbiologically safe and ideally should also be biologically stable" (WHO, 2006). The concept of biological stability of drinking water refers to the objective to maintain the same water quality from the drinking water treatment plant outlet up to the consumer's tap, from a microbial point of view. Simply viewed, this would mean that any of microbial-related parameter of water should not change during drinking water distribution (Figure 1.4).

1.2.2 Problems associated with biological unstable water

In 1984, Rittman and Snoeyink stated that: "A biological stable water does not support the growth of microorganisms to a significant extent, whereas an unstable water supports high numbers of microbes in distribution systems if sufficient disinfectant is not used". Effectively, uncontrolled growth of indigenous bacteria in drinking water has been associated with aesthetic and operational-related problems (Figure 1.5). Modification of aesthetic aspects of drinking water during its distribution are the object of 40% to 80% numerous customer complaints to drinking water providers, and include altered taste and colour, increased turbidity, and/or presence of invertebrates in water (Polychronopolous et al., 2003; Vreeburg and Boxall, 2007b). Besides, microbially-induced operational problems such as corrosion or fouling of distribution pipes represent major investment and maintenance costs for water utilities.

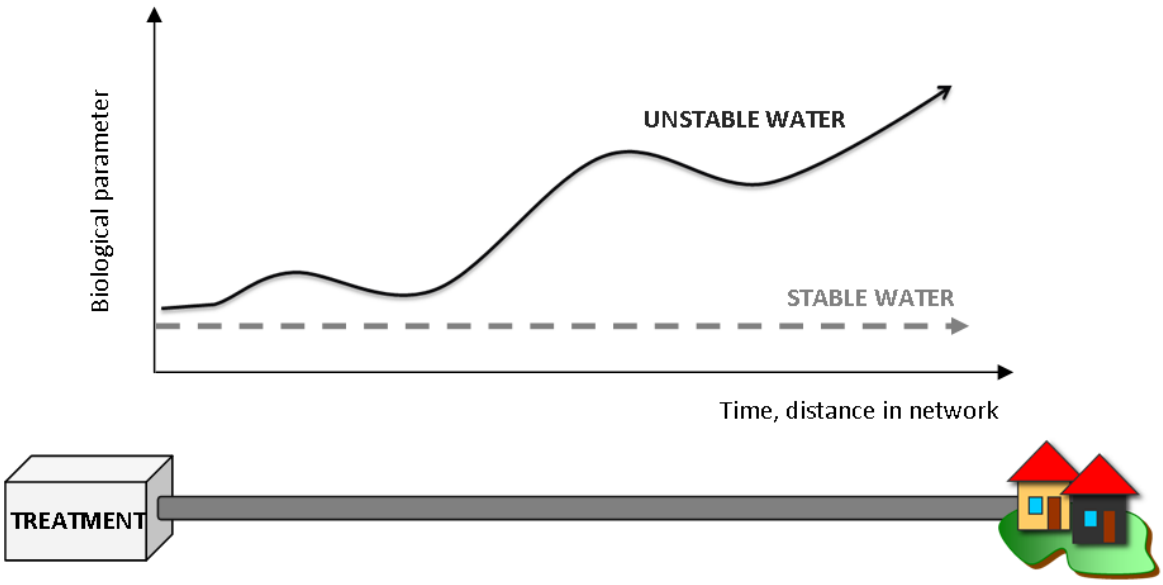


Figure 1.4. Schematic definition of biological stable and unstable drinking water.

Figure 1.5. Example of problems related to biological unstable water. (A) Brown water emerging from household tap (http://www.lu354.com/the-colors-of-tap-water/). **(B) *Assellus aquaticus*, an invertebrate organism commonly found in drinking water distribution systems** (https://www.kuleuven-kortrijk.be/kulakbiocampus/insecten-ongewervelden/index.htm). **(C) Biofilm in drinking water pipe** (Jost Wingender, http://www.anti-ocker.de/en/node/18).

In the Netherlands, re-investment costs for distribution systems alone are in the range of €20 billion for the entire country. Investment costs on distribution pipelines represented approximatively 50% of water utilities investments (de Moel et al., 2006). Finally, legal guidelines for microbial parameters in use for the control of microbial water quality might be exceeded (Anonymous, 1998; Sartory et al., 2004; Uhl and Schaule, 2004).

1.2.3 Strategies to limit bacterial growth in drinking water distribution systems

To avoid microbial-related aesthetic and operational problems, water utilities aim to limit bacterial growth in distribution systems, by applying one of the two main strategies. In many countries, including the USA, Canada, Australia, and European countries, a secondary disinfection step is applied in water leaving the treatment plant, to prevent bacterial growth during water distribution. Addition of disinfectants in drinking water has however raised health concerns, as these have been shown to react with organic compounds in water, resulting in the formation of carcinogenic by-products (US national cancer institute, 1976; Hogan et al., 1979). Concentrations of the dosed disinfectants are therefore kept the lowest possible. In such conditions, disinfectant residuals are often depleted in part of the distribution system, thereafter leaving the possibility for organisms to grow in the system (LeChevallier et al., 1996; Niquette et al., 2001). Alternatively, few European countries, including the Netherlands, Switzerland, Austria and Germany use extensive treatment strategies to limit nutrients in water that can serve as food source for bacteria to grow. In addition, water is distributed in well-maintained distribution systems using as much as possible materials that do not promote growth of bacteria during water distribution (van der Kooij, 2000; 2003). Both approaches have been shown to be effective to limit bacterial growth to a certain extent, but microbial changes in water microbial quality have been recorded in distribution systems of several

countries applying one or the other approach. Typically, uncontrolled bacterial growth occurs when residual disinfectants are depleted, at remote locations and/or during warm periods (LeChevallier et al., 1996; Uhl and Schaule, 2004; van der Wielen and van der Kooij, 2010; Nescerecka et al., 2014). There is therefore a need for in-depth understanding of microbial dynamics in drinking water distribution systems, for a better control of microbial water quality from treatment to tap in future.

1.3 Monitoring of biological stability of drinking water

1.3.1 Heterotrophic plate counts, the traditional method for bacterial enumeration

The lack of knowledge on the microbial dynamics in drinking water distribution systems is partially due to the monitoring methods traditionally applied for water analysis. Drinking water microbiology has remained an extremely conservative field, and most water utilities still employ worldwide both cultivation-based methods and guidelines proposed by Robert Koch 120 years ago. The method was initially developed for counting microorganisms from air, soil and water, by cultivation on nutrient media solidified with gelatine. It is known nowadays as the heterotrophic plate count (HPC) method, and is used as a general indicator of microbiological water quality. Additionally, it has been adapted for the detection of indicator organisms for faecal contamination such as *E. coli* and *Enterococci*. The method is included worldwide in legislations for drinking water quality. Typically, it is advised to maintain the counts of HPC below 100 colony forming units (CFU) per millilitre of water, while no faecal indicator should be found in 100 to 250 mL of water (Anonymous, 1998). Furthermore, the HPC method is still employed in numerous research publications on microbiological drinking water quality.

Though the HPC method has been the object of numerous improvements in terms of growth medium and conditions, there is evidence that only a minute fraction of bacterial cells present in drinking water are able to grow on cultivation media (Figure 1.6; Hoefel et al., 2003; Hammes et al., 2008). The information provided by the HPC method is therefore not representative of the entire bacterial community in drinking water, and the relevance of the plate count method for the study of microbial dynamics in drinking water distribution systems can be questioned. Furthermore, the method is labour-intensive and requires 3 to 5 days before analytical results are available. Consequently, unexpected events such as uncontrolled bacterial growth or pipe breakage would only be detected after a few days. The HPC method is thus obviously not appropriate as a rapid analytical tool for water quality surveillance.

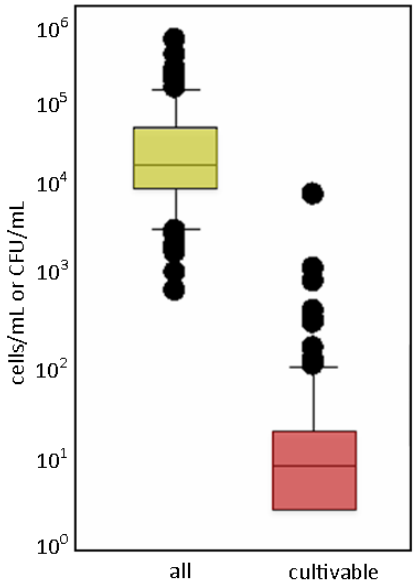


Figure 1.6. Comparison of total and cultivable bacterial cells in drinking water samples, as measured with flow cytometry and heterotrophic plate counts. The methods were applied to 90 drinking water samples collected in 2011 in Switzerland from drinking water systems distributing water treated from different raw water types and distributed in chlorinated and non-chlorinated networks (unpublished data).

1.3.2 Traditional indicators for biological stable water

Based on the statement of Rittman and Snoeyink (1984) that biological stable water does not support the growth of microorganisms, laboratory-scale bacterial growth tests under controlled conditions have been developed to evaluate the growth-promoting properties of water. These include the determination of the fraction of organic carbon that can be used by bacteria as food source. One method, often referred to as assimilable organic carbon (AOC) method (van der Kooij, 1982, 1985a) is based on the measurement of the growth of two pure bacterial strains in a pasteurized water sample. Alternatively, the biodegradable dissolved organic carbon (BDOC) method measures the consumption of dissolved organic carbon (DOC) by the autochthonous bacteria in a water sample (Servais et al., 1987, 1989). The ability of water to promote the growth of bacteria into a biofilm, has also been proposed via the biofilm formation rate (BFR) method (van der Kooij, 1995). The AOC, BDOC and BFR methods have been associated with guideline values that should not be exceeded for considering water as biologically stable, and are meant to provide support to water utilities to minimize bacterial growth during distribution. However, laboratory-scale bacterial growth-based methods are predictive parameters and do not provide information on what is effectively happening in the studied network.

1.3.3 New opportunities with emerging methods

Adequate analytical methods are required for representative characterization of bacterial communities in drinking water, both (i) for water utilities to monitor and rapidly detect changes in microbial water quality, and (ii) for research purposes to study microbial communities in drinking water distribution systems.

Rapid developments in microbial methodologies have taken place during the last decade. Among them, flow cytometry has been proposed as an ideal tool for drinking water monitoring (Hammes et al., 2008, 2010a). The method is rapid, easy and sensitive and enables the detection of all bacterial cells in a water sample. This cultivation-independent method was first introduced in the medical field but has been adapted for drinking water analysis during the past 10 years. Besides, high-throughput sequencing methods such as 16S rRNA gene 454-pyrosequencing enable in-depth description of microbial communities in drinking water. The latter provides information on the composition and structure of bacterial communities in water, as well as information on functionality of the present organisms (Liu et al., 2014; Pinto et al., 2014).

In 2011, at the start of the work presented in this thesis, the number of studies using flow cytometry and high-throughput sequencing methods for the study of microbial communities in drinking water was limited in literature. However, these have gained considerable attention, and the number of publications has increased significantly in the past 4 years (Table 1.1). The two methods have large potential to provide more insight in microbial dynamics in drinking water distribution systems and represent new opportunities for monitoring and research purposes.

Table 1.1. Comparison of the number of published studies before 2011 and before May 2015, using flow cytometry and high throughput 16S rRNA gene sequencing methods for the description and evaluation of microbial dynamics in full-scale drinking water treatment and distribution systems*.

Method applied	2011	2015
Flow cytometry	4	14
Cell counting	*6*	*13*
Fingerprints	*0*	*1*
High-throughput 16S rRNA gene sequencing methods	2	20

*The numbers were obtained using a literature search on *Scopus* using the following key words: (1) drinking water AND distribution systems AND Flow cytometry; (2) drinking water AND distribution systems AND 16S rRNA gene sequencing OR pyrosequencing. The publications reporting studies in pilot distribution systems and laboratory experiments have not been taken into account.

1.3.4 Short introduction to flow cytometry

1.3.4.1 Basic principle of flow cytometric measurements

Flow cytometry is a cultivation-independent method enabling the enumeration of particles in suspension in a liquid sample. The measurement is based on hydrodynamic focusing principle, which enables to suspend particles in a single line in a flow stream (Figure 1.7). Each particle in suspension passes one after the other through a laser beam, thus generating scattered light. In the case of fluorescently-labelled bacterial cells or auto-fluorescent algae, fluorescent light is generated as well. The fluorescent light is collected through filters that enable to select for wavelengths of interest for selective detection of bacteria. Detectors collect scattered and fluorescent light intensity for each single particle flowing through the flow cell for a determined volume of water sample. Flow cytometric measurements of a liquid sample provide therefore information on bacterial single-cell level, which in turn can be used for cell counting.

1.3.4.2 Detection and characterization of bacterial cells using flow cytometry

Enumeration of bacterial cells by flow cytometry requires labelling cells with fluorescent dyes prior to the measurement, to differentiate bacterial signal from abiotic particles and instrument background. A large number of fluorescent dyes are available, each of them targeting different features of bacterial cells and thus providing information on specific functions of the cells (Hammes et al., 2011). In this thesis, two fluorescent dyes have been used, namely SYBR Green I and Propidium iodide.

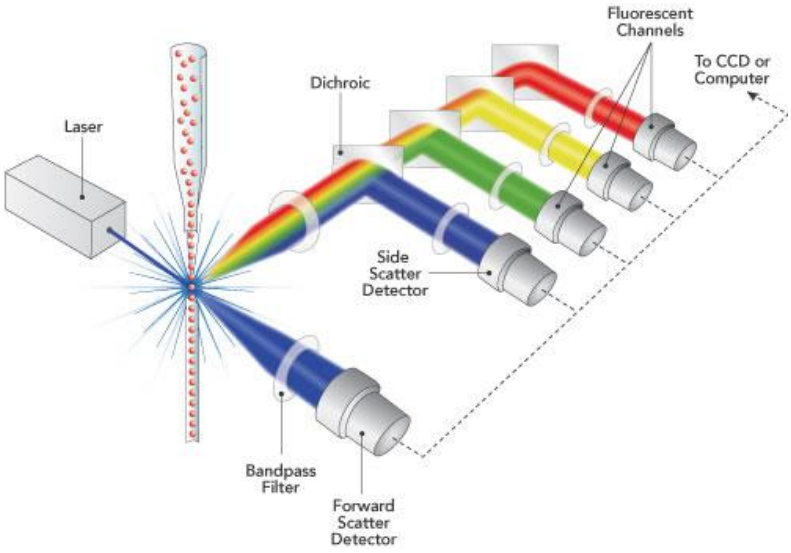


Figure 1.7. Schematic principle of flow cytometric measurements (http://www.semrock.com/flow-cytometry.aspx).

SYBR Green I has the property to penetrate all bacterial cells, independently from their activity or viability state, and to bind to the nucleic acids of the cells (Zipper et al., 2004). When excited, SYBR Green I emits green fluorescent light (520 nm). Flow cytometric measurements following bacterial staining using SYBR Green I therefore provides the total number of bacterial cells contained in a specific water volume. On the other hand, propidium iodide only penetrates bacterial cells with a damaged cell wall, due to the larger size of the molecule (Figure 1.8A). Propidium iodide emits red fluorescent light (617 nm). When the two dyes are added simultaneously to a water sample, both dyes are enabled to penetrate damaged bacterial cells. However, green fluorescence produced by the SYBR Green I stain is reduced due to the presence of the propidium iodide, and bacterial cells with damaged cell walls emit increased red fluorescence intensities compared to bacterial cells with intact membranes. Consequently, it is possible to classify bacterial cells based on their membrane integrity, using a two-dimensional dot plot to visualize the bacterial signals, and to count intact and damaged bacterial cells independently (Figure 1.8B). This combination of fluorescent dyes is often called “live/dead” staining (Boulos et al., 1999; Berney et al., 2007). However, this terminology is not precise enough, as intact cells are not necessarily alive, and cases of damaged cells that have been able to recover their cell wall have been recorded. Subsequently, the measurements will be referred to as total, intact and damaged cells in this thesis.

In addition to cell counting information, flow cytometric measurements following bacterial staining with SYBR Green I alone or combined with Propidium iodide provide so called “fingerprints”. The fingerprints correspond to the fluorescence intensity distribution recorded for a water sample (Figure 1.8C).

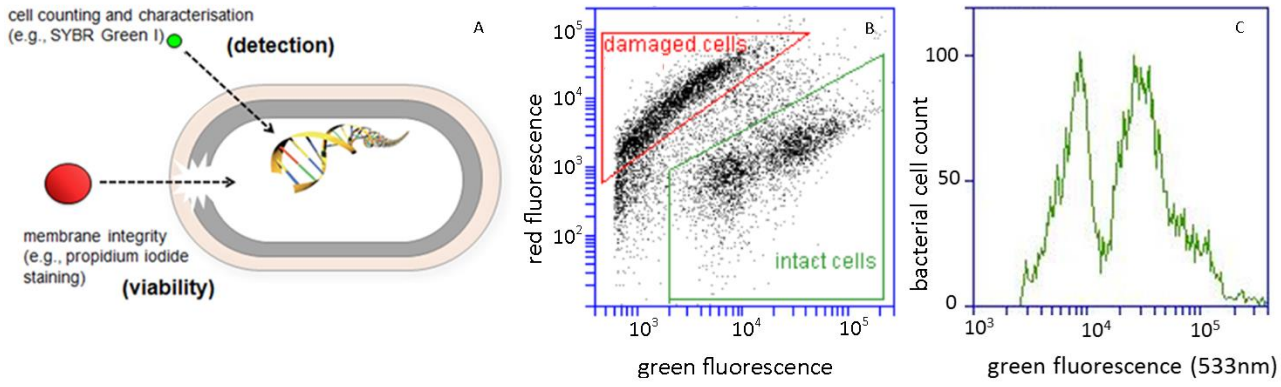


Figure 1.8. (A) Principle of dye penetration of SYBR Green I and Propidium iodide into a bacterial cell (adapted from Hammes et al., 2011)**. (B) Differentiation between intact and damaged bacterial cells on the flow cytometric dot plot provided by the flow cytometric software. Each dot represents one particle detected by the flow cytometer. The coloured boxes are used to distinguish bacterial signal from inorganic particles and instrument background. (C) Typical example of fluorescence fingerprint obtained after measurement of a water sample stained with SYBR Green I.**

1.3.4.3 State of the art of flow cytometry applied to drinking water at start of phD project

In 2011, at start of the work reported in this thesis, flow cytometry had mainly been applied to drinking water using the total and intact cell counting information, for evaluation of water treatment processes or changes in microbial water quality during distribution (Hoefel et al., 2005; Berney et al., 2008; Hammes et al., 2008, 2010a). The use of fluorescence fingerprints had already been suggested by other authors, for characterization of microbial communities in natural waters and/or engineered systems (Mueller et al., 2003; Bombach et al., 2010; Pomati et al., 2011), but had not been used for drinking water characterization purposes (Table 1.1).

1.4 Objectives

The overall goal of this thesis was to define and evaluate new methodological strategies to study biological stability of drinking water in distribution systems, based on the newly available analytical methods flow cytometry and high-throughput 16S rRNA gene based sequencing. In this thesis, main focus was on flow cytometry, but the combination of flow cytometry with high-throughput sequencing methods has been explored. The combination of the methods has been the object of collaborative work with Joline El-Chakhtoura (phD candidate at KAUST), which led to two joined publications included in this thesis.

Specific objectives were to:

(i) provide a detailed overview of factors regulating bacterial growth in drinking water, and influencing biological stability during water distribution;
(ii) propose adequate, targeted combinations of methods for microbial community characterization;
(iii) optimize the proposed methods, and specifically the flow cytometric method, for rapid and accurate detection of changes in microbial drinking water quality;
(iv) elaborate a comprehensive strategy to study biological stability in drinking water distribution systems;
(v) apply and evaluate the developed tools and strategy to a full-scale distribution system.

1.5 Thesis outline

Chapter 2 reviews current knowledge on biological stability of drinking water and on microbial dynamics in drinking water distribution systems. Factors affecting bacterial growth in drinking water are described in detail (e.g., organic and inorganic nutrients, bacterial competition processes, habitat, other organisms), as well as how these factors are affected by water treatment and distribution conditions. The traditionally applied and emerging methods for studying drinking water microbial communities and biological stability are described. Moreover, the current definitions and approaches to assess biological stability are discussed.

Chapter 3 describes how flow cytometry can be used for the sensitive detection of changes in microbial communities in drinking water systems. Specific attention is given to flow cytometric fingerprints.

Chapter 4 investigates how flow cytometry can be combined with 16S rRNA gene pyrosequencing, and highlights the information gained from the combined methods.

Chapter 5 explores a new approach for studying biological stability in drinking water distribution systems, combining on-site measurements (with methods described in chapters 3 and 4), and laboratory-scale investigations based on batch growth potential tests using flow cytometry. The new approach was tested on a full-scale drinking water distribution system.

Chapter 6 describes a long term monitoring study (2-years) on a full-scale drinking water distribution system, for which the developed tools were applied, and highlights approaches for the study of microbial dynamics in distribution systems.

Chapter 7 provides an example of data which can be obtained using pyrosequencing. The technique was applied for the study of short-term dynamics (hour, day week time-scales) in the same distribution system as investigated in chapter 6.

Chapter 8 summarizes the obtained results and the proposed integrated approach for the study of microbial dynamics during water treatment and distribution of drinking water. In addition, potential application fields are discussed, for drinking water monitoring and for future research opportunities.

The thesis outline is summarized in Figure 1.9. The thesis is structured as a paper dissertation and consists of a succession of scientific papers, except for the introduction and part of the outlook chapters. Repetitions between chapters are consequently unavoidable. Small adaptations have been made to improve the chapters.

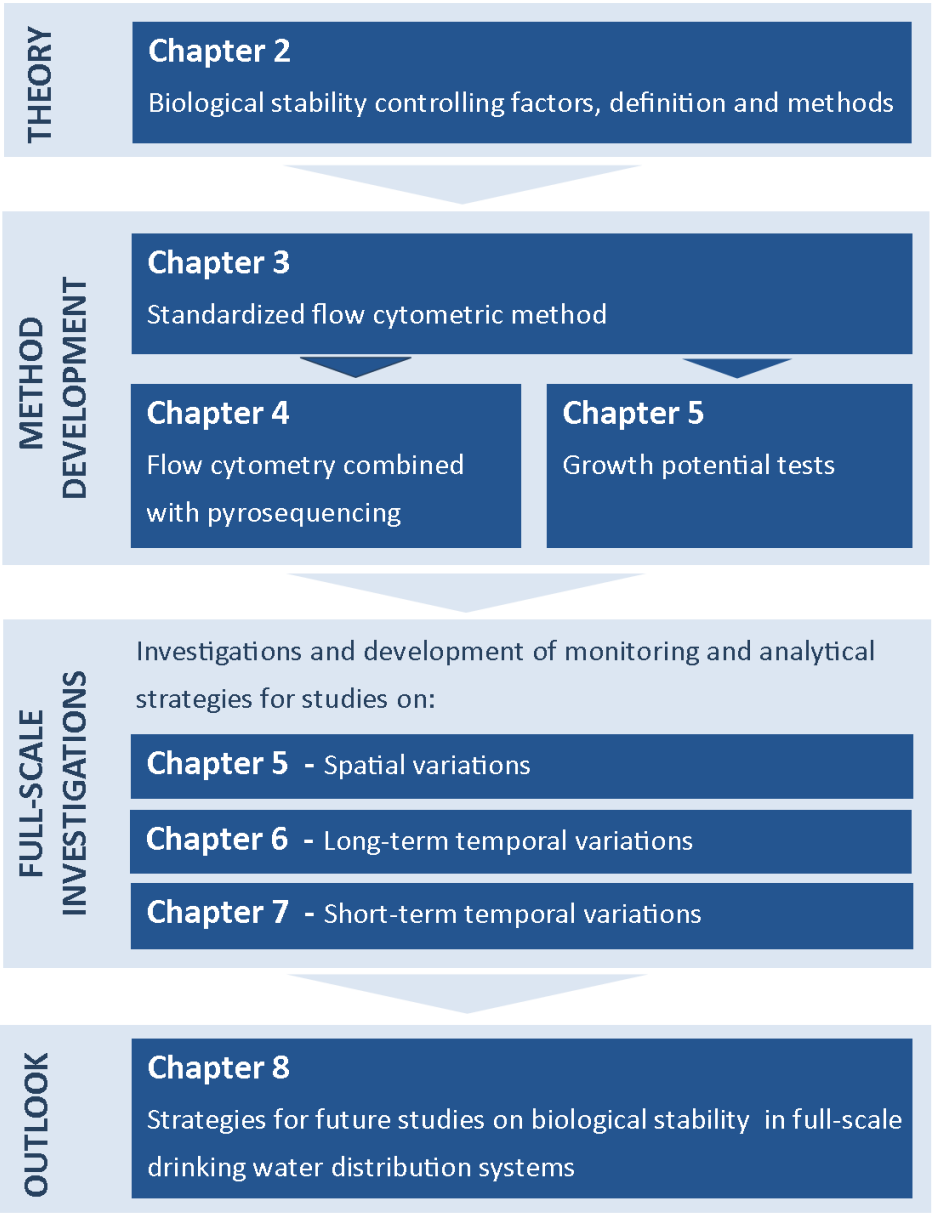
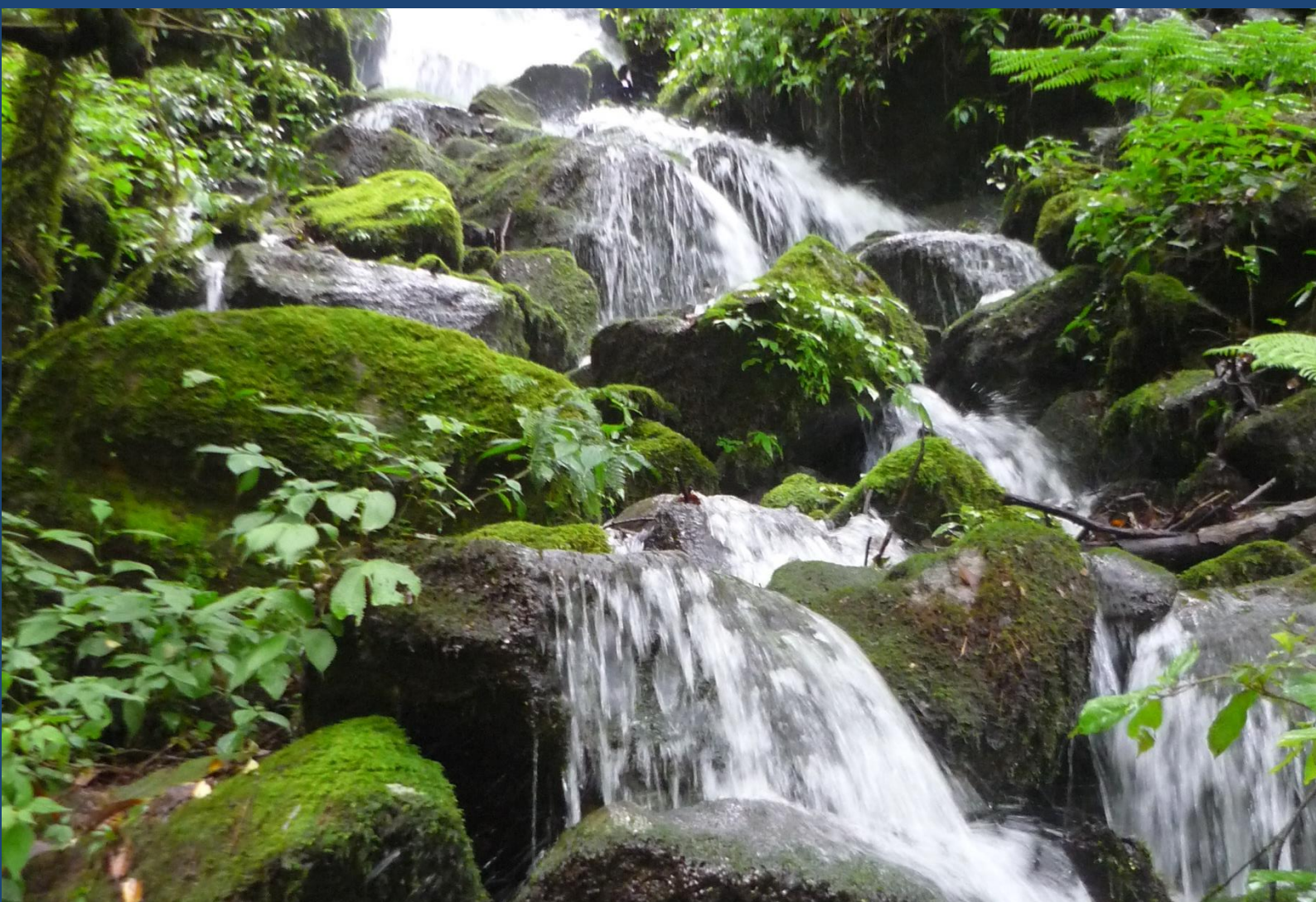


Figure 1.9. Summary of thesis structure.

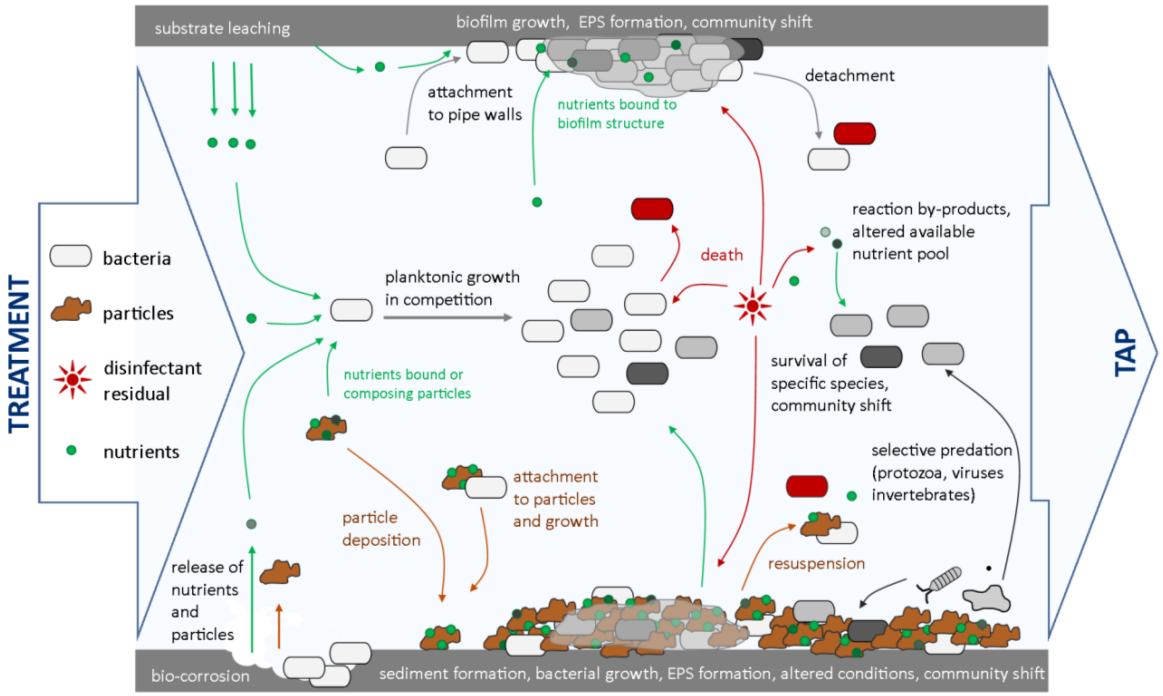
THEORETICAL BACKGROUND



Chapter 2

Biological stability of drinking water:

controlling factors, definitions and methods



This chapter has been submitted for publication as: E.I. Prest, F. Hammes, M.C.M van Loosdrecht, J.S. Vrouwenvelder. Biological stability of drinking water: controlling factors, definitions and methods.

Abstract

Biological stability of drinking water refers to the concept of providing consumers with drinking water of same microbial quality at the tap as produced at the water treatment facility. However, avoiding changes in microbial community characteristics (abundance, composition, viability) during distribution in water mains and premise plumbing is challenging, and uncontrolled growth of bacteria during water distribution can lead to hygienic, aesthetic and/or operational problems. Drinking water contains a diverse microbiome, with various microorganisms competing for limited available biodegradable nutrients. Bacterial growth and interactions are regulated by various factors, including (i) type and concentration of available organic and inorganic nutrients, (ii) type and concentration of residual disinfectant, (iii) presence of predators such as protozoa and invertebrates, (iv) prevailing environmental conditions, such as water temperature, and (v) spatial location of microorganisms, i.e. bulk water, sediment and/or biofilm. Water treatment and distribution conditions in water mains and premise plumbing affect each of these factors and thus can significantly affect bacterial community characteristics in distribution systems. Improved understanding of interactions between bacteria in distribution systems and influence of environmental conditions is needed for better control of drinking water microbial communities during drinking water production and distribution. This article provides a review of (a) existing knowledge on biological stability controlling factors, and (b) how controlling factors are affected by drinking water production and distribution conditions. In addition, (c) the concept of biological stability is discussed in light of experience gained from well-established and new analytical methods, enabling high throughput analysis and in-depth characterization of bacterial communities in drinking water. We discuss and propose how knowledge gained from novel techniques will improve design of water treatment and distribution systems in order to maintain good microbial quality of drinking water up to consumer's tap. A new definition and methodological approach for biological stability is proposed.

2.1 Introduction

The World Health Organization stated in 2006 that "Water entering the distribution system must be microbiologically safe and ideally should also be biologically stable" (WHO, 2006). There is general consensus that the term 'biological stability' in this context refers to the concept of maintaining the microbial water quality from the point of drinking water production up to the point of consumption (Rittman and Snoeyink, 1984; van der Kooij, 2000). Unwanted changes in the microbial quality of drinking water can have adverse effects on the distribution system and the consumers. For example, during distribution, excessive growth of bacteria can lead to the deterioration of drinking water quality in terms of safety (e.g., pathogens), consumer's perception (e.g., discolouration) and operational aspects (e.g., biocorrosion) (Szewzyk et al., 2000; Vreeburg et al., 2004; Sun et al., 2014). Changes in microbial water quality are a result of complex interactions between various organisms (bacteria, but also viruses, protozoa, higher organisms), regulated by access to the available growth-limiting nutrients, response to environmental conditions such as water temperature, presence of potential residual disinfectants and other inhibitory substances, attachment of bacteria to pipe walls, particle deposition, sediment re-suspension and biofilm formation. The aim behind the concept of biological stability is that minimum change in the water quality is occurring during drinking water distribution. To achieve this and limit bacterial growth during transport, drinking water is distributed in numerous countries with disinfectant residuals, using different substances (e.g. free chlorine, chlorine dioxide, monochloramine) at varying concentrations (Servais et al., 1995; LeChevallier et al., 1996; Gillepsie et al., 2014). Adverse health effects of disinfection by-products and altered water taste have however led several countries to opt for water distribution without the addition of disinfectant to the produced drinking water (Vital et al., 2012a; Lautenschlager et al., 2013; Chapter 4). In the latter case, minimum change in the water quality is achieved in the first place by controlling the water quality with the best water treatment strategy, and secondly by well-controlled distribution conditions (van der Kooij, 2003).

A number of methods to assess the bacterial growth-supporting properties of water have been developed during the last three decades to provide support to water utilities for the improvement of water treatment and distribution conditions in the context of biological stability (van der Kooij et al., 1982; Servais et al., 1989). In addition, several studies have addressed the effect of individual distribution-related factors on changes in drinking water quality (Table S2.1 in supplementary information). Concomitantly with methodological and experimental advances in this field, the definitions of biological stability, as well as methods and approaches to address the concept have evolved (Rittman and Snoeyink, 1984; Sibille, 1998a; van der Kooij, 2000, 2003; Lautenschlager et al.,

2013). In the recent years, high-throughput analytical and molecular methods have emerged, enabling detailed characterization of bacterial communities in water (for review, see Douterelo et al., 2014), and distribution networks have been examined with an increasingly ecology-oriented approach, in which interactions between organisms are investigated (Berry et al., 2006; Proctor and Hammes, 2015).

The objective of the present chapter is to review the existing knowledge, future challenges and emerging ideas that aim to achieve and monitor biological stability of drinking water in full-scale distribution systems. We examine the existing definitions and approaches to address biological stability, and highlight the information gaps.

2.2 Problems associated with bacterial growth in drinking water distribution systems

The presence of bacteria in drinking water *per se* is not an issue, as long as no pathogenic organisms are present: there are bacteria in drinking, even in relatively high numbers (10^3 to 10^6 cells/mL), without consequences on human health (Hoefel et al., 2005; Hammes et al., 2008; Vital et al., 2012a). However, unwanted and/or excessive bacterial growth in drinking water distribution systems can cause deterioration of microbial water quality during storage and transport. Firstly, a number of hygienically relevant opportunistic pathogens such as *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Mycobacteria*, *Aeromonas hydrophila, Klebsiella pneumoniae* and *Campylobacter* have the capacity to grow at low nutrient concentrations in drinking water distribution systems and/or in households (Szewzyk et al., 2000; Flemming et al., 2002a; Vital et al., 2008, 2012b; Wang et al., 2013a). In addition to bacterial species, certain protozoa have pathogenic properties (e.g. *Acanthamoeba, Cryptosporidium, Giarda lamblia*), or act as hosts for pathogenic bacteria such as *Legionella pneumophila* (Bichai et al., 2008; Thomas and Ashbolt, 2011; Wang et al., 2013a), while enteric viruses were recognized to cause water-born gastrointestinal or other viral illness (e.g., noroviruses, Hepatitis A virus) (Wingender and Flemming, 2011). Secondly, aesthetic deterioration of drinking water represents up to 80% of consumer complaints to water utilities (Polychronopolous et al., 2003; Vreeburg and Boxall, 2007b). Turbid or discoloured water is the result of particles in suspension (Vreeburg et al., 2004), which can originate from excessive growth of non-pathogenic bacteria within drinking water distribution systems, attached to particles, sediments or biofilms. These can be re-suspended in the water and cause yellowish coloured water (Gauthier et al., 1999; Vreeburg and Boxall, 2007b). Red or black coloured water can be the consequence of iron particles and manganese precipitates (Sly et al., 1990; Seth et al., 2004), which can be partially produced by

bio-corrosion of iron pipes (Sun et al., 2014) or manganese oxidizing or reducing organisms (Cerrato et al., 2010). Moreover, specific bacteria produce molecules affecting taste and odour of water. Typical examples are actinomycetes, which produce geosmin, responsible for an earthy-muddy water taste (Srinivasan and Sorial, 2011), and bacteria involved in the sulphur cycle (e.g. sulphate reducing or oxidizing bacteria) that can promote a sulphur-based odour (Scott and Pepper, 2010). Besides, yeast, fungi and algae have also been recorded in drinking water and some of these organisms have been associated with taste and odour complaints (Block et al., 1993; Sibille et al., 1998a; van der Wielen and van der Kooij, 2013). In addition, bacteria represent the start of a trophic chain, and high bacterial numbers would result in the occurrence of protozoa and of invertebrates such as crustaceans (e.g. *Asellidae*), worms (e.g., annelida) or snails (e.g, mollusca) in distribution systems (van Lieverloo et al., 2002a; Christensen et al., 2011). The presence of invertebrates and particularly of the large *Asellus aquaticus* (2 to 10 mm long; Christensen et al., 2011) in household taps is negatively perceived by consumers (van Lieverloo et al., 2002a). Thirdly, operational problems were related with bacterial activity, such as fouling of concrete pipes due to growth of bacteria to high numbers in the form of a biofilm (Flemming, 2002b; Allion et al., 2011), or biocorrosion of cast-iron pipes promoted by e.g. sulfate-reducers and iron-oxidizers (Lee et al., 1980; Emde et al., 1992; Sun et al., 2014). The replacement of damaged distribution pipes related to microbial processes represents one major financial investment for water utilities. Finally, growth of cultivable heterotrophic bacteria can result in non-compliance with regulatory guidelines on e.g., heterotrophic plate counts (HPC) or *Aeromonas* counts (Anonymous, 1998; Sartory et al., 2004; Waterleidingbesluit, 2001). For example, HPC measured in drinking water sampled at long residence times in a distribution system in Germany during a warm summer (water temperatures above 20°C) were excessively high, sometimes exceeding the German guideline value of 100 CFU/mL, while HPC values in the treatment effluent were below 5 CFU/mL (Uhl and Schaule, 2004). Similarly, Lautenschalger et al. (2010) showed that HPC in water stagnated in premise plumbing of 6 out of 10 studied houses were higher than the recommended HPC value in Switzerland (300 CFU/mL), which was the result of increased HPC numbers during stagnation (up to 580 fold higher than in flushed tap water). Achieving biological stability and providing good drinking quality water to consumers require therefore not only to produce clean and safe water, but also to limit changes in the bacterial community during drinking water distribution that would lead to uncontrolled growth up to high bacterial cell numbers and to the occurrence of unwanted microorganisms.

2.3 Deeper look into microbial dynamics in drinking water

In this section, factors affecting bacterial growth in drinking water are reviewed. Each factor is examined in view of its relevance for achieving biological stability, i.e. of its influence on shaping and/or modifying the bacterial community characteristics (bacterial abundance, viability and community composition). An overview of primary conditions for bacterial growth and influencing factors on bacterial competition processes is given in Figure 2.1.

2.3.1 Effect of nutrient concentration and composition

The composition and concentrations of individual substrates in drinking water are inherently related to biological stability, by limiting or promoting bacterial growth in water. In the first place, concentrations of available organic and inorganic nutrients govern the extent of bacterial growth (Figure 2.2). Heterotrophic organisms constitute the majority of bacteria in drinking water, and draw their energy from degradation of organic carbon compounds. Due to bacterial elemental composition (ratio C:N:P), organic carbon is most often the growth limiting compound and thus is particularly important for biological stability.

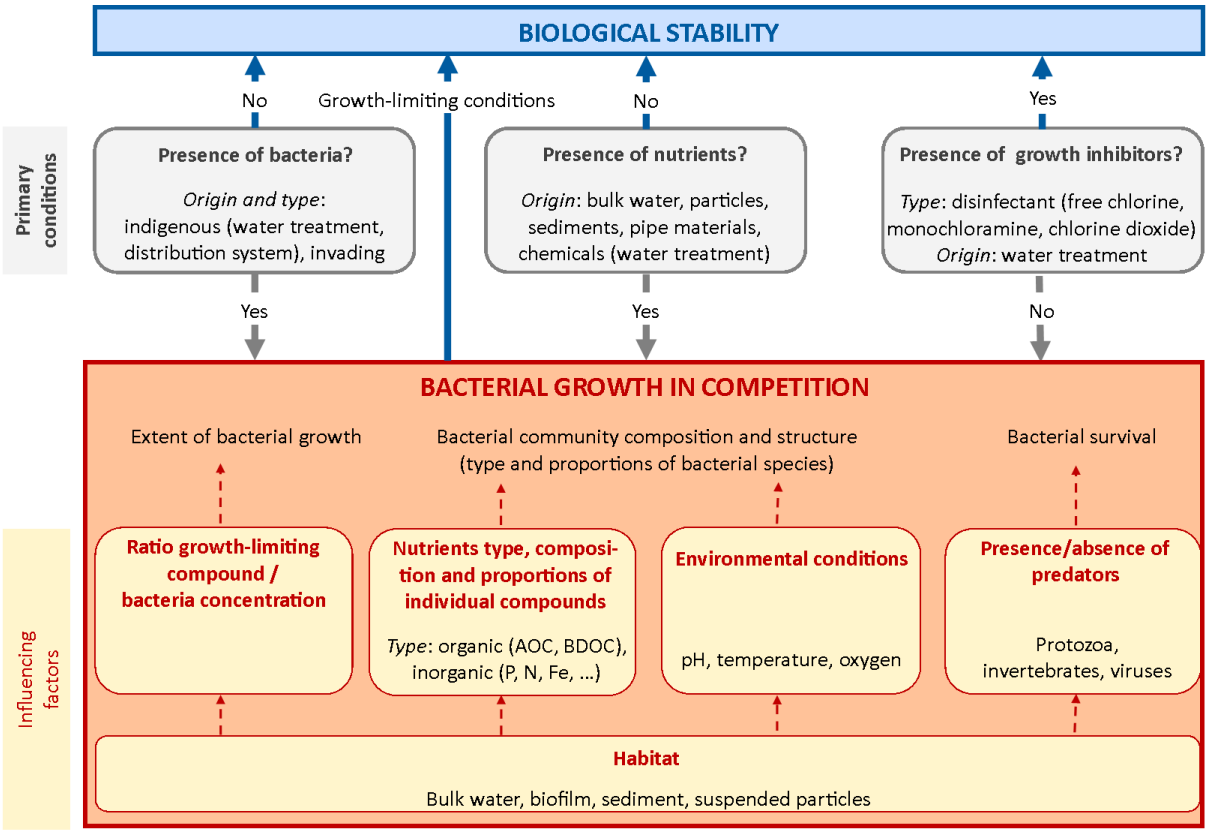


Figure 2.1. Overview of primary conditions for bacterial growth and influencing factors of bacterial competition processes.

Biodegradable organic matter (BOM) comprises a broad spectrum of different organic carbon compounds ranging from simple organic acids and sugars to complex polymeric substances such as humic compounds (Münster, 1993; Schmidt et al., 1998). Only a fraction of the dissolved organic carbon (DOC) can be utilized by bacteria as energy source for growth. Concentrations of available organic substrate typically range between 1 and 300 µg C/L when estimated by assimilable organic carbon (AOC) methods (typically 0.1 to 10% of DOC) or range between 40 and 800 µg C/L when estimated by biodegradable dissolved organic carbon (BDOC) methods (1 to 30% of DOC) (data compiled from references listed in Table S2.1 in supplementary information). Typical yield values for heterotrophic bacteria are between 4.6 x 10^6 – 20 x 10^6 cells/µg C (van der Kooij and Hijnen, 1985b; Hammes and Egli, 2005), which implies that an organic carbon concentration as low as 1 µg/L is sufficient to promote the growth of 10^3-10^4 cells/mL (van der Kooij et al., 1980, 1982, 1985a; Vital et al., 2012a). In the context of regulatory guidelines for HPC, typically in the range of 10^2-10^3 cells/mL, producing stable water is therefore challenging. Inorganic nutrients such as phosphorus, nitrogen or other trace elements (iron, magnesium, copper, potassium…), are also required for heterotrophic growth, though in considerable smaller amounts than organic carbon (Ihssen and Egli, 2004). Very low concentrations in any essential inorganic compounds will result in heterotrophic bacterial growth limitation, as observed in waters with highly elevated organic carbon concentrations (Miettinen et al., 1997). Studies have however essentially focussed on organic carbon limitations so far and it is still unclear whether bacterial growth limitations in inorganic elements, including phosphate limitations, but also other elements, are frequent in drinking water systems.

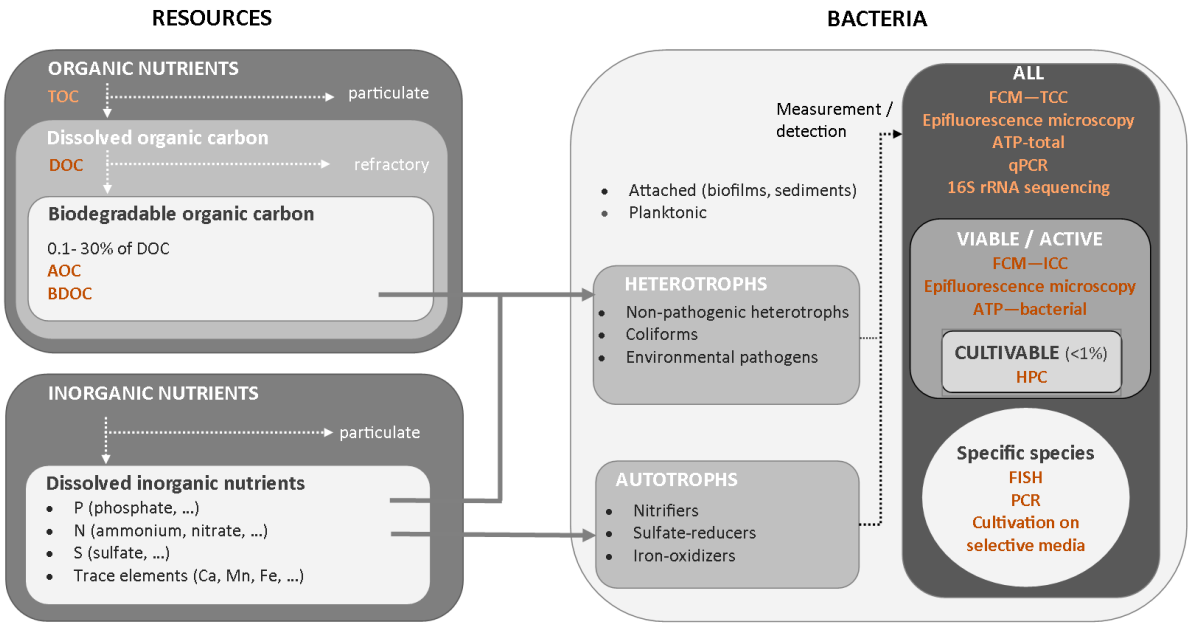


Figure 2.2. Overview of resources available for different types of bacteria and of characterization methods of organic nutrients and bacterial communities in water.

While concentrations of individual substrates present in water define the growth-limiting substrate and control the extent of bacterial growth, the type of individual organic and inorganic substrates determines the type of organisms present in water. A typical example is the presence of methane-oxidizing bacteria in deep ground waters containing high concentrations of methane (de Vet et al., 2009; Lin et al., 2012). Though it is generally accepted that heterotrophic bacteria constitute the large majority of bacteria found in drinking water, presence of autotrophic organisms such as nitrifying, sulfate-reducing or iron-oxidizing bacteria has also been recorded in different drinking water systems (Rittman and Snoeyink, 1984; Pepper et al, 2004). For example, ammonium oxidizing bacteria such as *Nitrosomonas* and *Nitrospira* are found in treated deep-ground waters rich in ammonium (de Vet et al., 2009), while sulfate-reducers (e.g., *Desulfovibrio* and *Desulfotomaculum*) and iron-oxidizers (e.g., *Gallionella*, *Leptothrix*, and *Sphaerotilus*) were associated with microbially induced corrosion processes (Emde et al., 1992; Sun et al., 2014). Dosage of monochloramine as residual disinfectant during drinking water transport was also shown to cause growth of ammonium oxidizing (e.g. from genus *Nitrosomonas*) or nitrite oxidizing bacteria (Wolfe et al., 1990; Lipponen et al., 2002). Clear data are lacking on the contribution of autotrophic growth in the total bacterial production and in the occurrence of aesthetic or operational related problems. Insights in functions of specific bacterial species in the water eco-system, and compounds and conditions required for their development would be a major step forward in the understanding of controlling factors of drinking water biological stability.

Besides type and concentrations of available substrates, composition and proportions of individual organic and inorganic compounds are essential parameters in the competition processes regulating bacterial growth, and are therefore essential to the concept of biological stability. Competition is a complex interplay between bacterial species, controlled by nutrient composition and proportion in water, physico-chemical parameters such as water temperature or pH, and specific kinetic capabilities of individual species (Figure 2.1). As discussed above, drinking water contains numerous different nutrients at very low concentrations of individual compounds (Schmidt et al., 1998; Sibille, 1998a; Wong et al., 2002). In such environment, bacteria are able to use simultaneously several nutrients for growth (Ihssen and Egli, 2004; Egli, 2010). The composition and concentration of nutrients defines an ecological niche, in which bacteria that have an overlap in substrate utilization spectrum will compete for available substrate (Hansen and Hubel, 1980; Fredrickson and Stephanopoulos, 1981; Vital et al., 2012b). Therefore, composition and proportions of individual organic and inorganic compounds shape the bacterial community composition and structure, which would be affected by any disturbance in the nutrient pool (Gottschal et al., 1979). The complex nutrient composition in drinking water typically results in the presence of a large diversity in

autochthonous bacterial species (Pinto et al., 2012; Yin et al., 2013; Liu et al., 2014), well adapted to survival and proliferation in oligotrophic environments. Bacterial communities with high richness and evenness have been shown to be potentially more resistant against biological invasion and environmental stress (Wittebole et al., 2009; De Roy et al., 2013; Van Nevel et al., 2013a). One explanation may be the broad substrate utilization spectrum and the large range of functionality and metabolisms covered by bacteria. Based on these observations, one could argue that a drinking water containing a highly diverse bacterial community with high evenness would have a higher chance to remain stable during water distribution where conditions are changing (cf. details in section 2.4). The role of complex bacterial competition processes for nutrients in drinking water and of bacterial diversity, richness and evenness for biological stability requires further research.

2.3.2 Effect of growth-inhibiting substances

The question of applying a disinfectant residual in water is central in the context of biological stability. Increased bacterial abundance in water has been observed when a residual disinfectant is partially or fully depleted in drinking water distribution systems (Servais et al., 1995; Nescerecka et al., 2014), due to reaction with bacterial cells, natural organic matter (NOM), particles, sediments and biofilms (Rossman et al., 1994; Gauthier et al., 1999; Campos and Harmant, 2002). Disinfectant threshold concentrations for bulk bacterial growth to occur are dependent on water quality and type of disinfectant applied. For example, LeChevallier et al. (1996) reported the occurrence of high numbers of bacteria of the coliform group in systems maintaining free chlorine concentrations below 0.2 mg/L and monochloramine concentrations below 0.5 mg/L, when AOC concentrations were above 100 µg/L. More recently, Gillepsie et al. (2014) showed that drinking water distribution areas with free-chlorine concentrations below 0.5 mg/L were related to higher intact cell concentrations in bulk water than for areas with higher disinfectant concentrations. Moreover, biofilm development cannot be avoided at disinfectant concentrations used in drinking water systems (LeChevallier et al., 1987; Revetta et al., 2013; Wang et al., 2014).

Addition and depletion of disinfectants in water has been shown to influence bacterial community composition and structure. Shifts in bacterial community and lower bacterial diversity were found in various systems after chlorination (Norton and LeChevallier, 2000; Roeder et al., 2010). Such shifts can be due to different resistance to chlorine of different bacterial species (Knochel, 1991; Abu-Shkara et al., 1998; Chiao et al., 2014), resulting in partial disappearance of the bacterial community after chlorine addition (Figure 2.3).

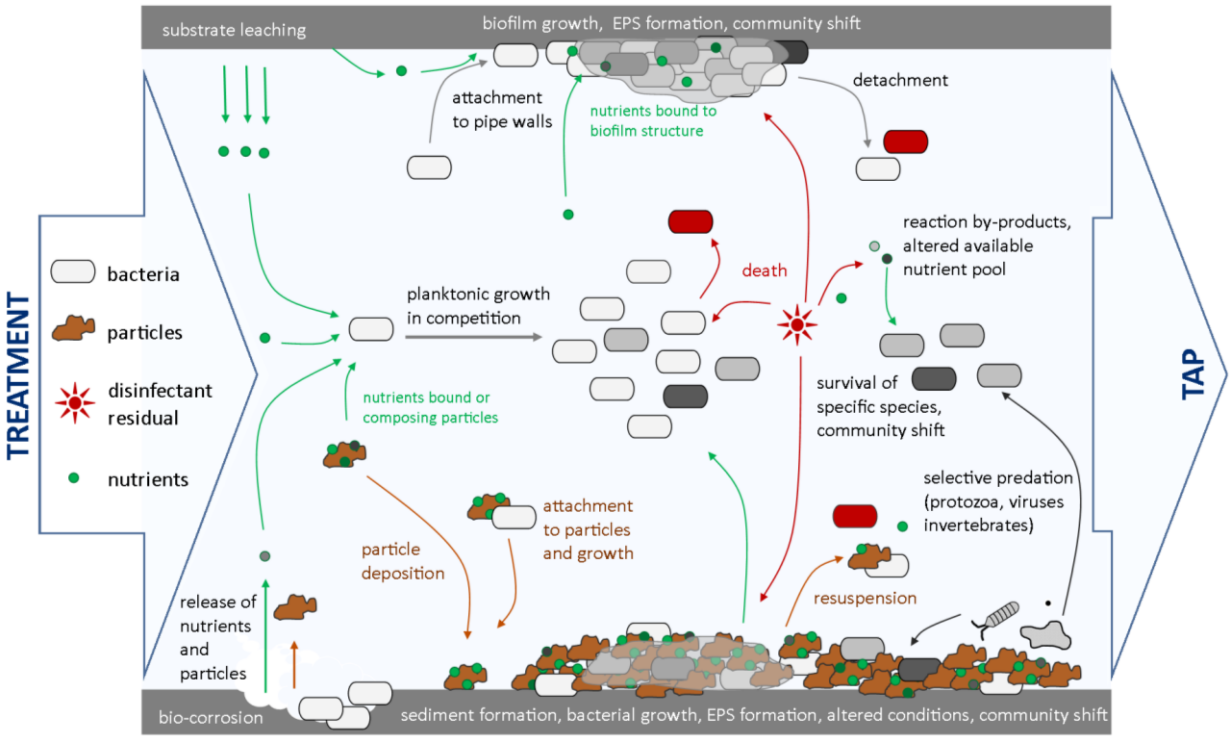


Figure 2.3. Overview of microbial dynamics in a distribution pipe section. Influences of pipe material, hydraulics, residual disinfectant, and bacterial predators on bacterial growth and community shifts are highlighted.

Chiao et al. (2014) have shown with laboratory experiments that genera such as *Dechloromonas* and *Acidovorax* were most sensitive to monochloramine compared to highly resistant genera such as *Geobacter* or *Legionella*. One possible consequence is that a lower substrate range is covered by the remaining bacterial community, meaning that more niches are available for bacterial growth once disinfectant is depleted. The available substrate pool can also be modified by reaction of residual disinfectants with NOM, resulting in formation of low molecular weight assimilable organic carbon compounds (Reckhow, 1990; Fass et al., 2003), which may subsequently cause a shift in bacterial community composition. Disinfectants such as monochloramine have also been shown to support the growth of specific bacteria, in this case nitrifying bacteria in distribution systems (Lipponen et al., 2002).

2.3.3 Effect of other organisms in drinking water distribution systems

The importance of bacterial growth control by other organisms than bacteria (e.g. protozoa, invertebrates, viruses) present in drinking water distribution systems is still unclear. Bacteria represent the start of a trophic chain in drinking water, and are subject to predation by organisms such as protozoa, which in turn are targets for invertebrates (Figure 2.3) (Sibille, 1998b; van Lieverloo et al., 2002a). Selective grazing by protozoa is likely to affect bacterial abundance and community

composition (Wang et al., 2013a). The presence of protozoa in drinking water systems has been reported in concentrations ranging from 5×10^4 to 7×10^5 protozoa/L (Sibille, 1998a,b), and has been linked to the presence of bacteria (Servais et al., 1995). Moreover, both protozoa and invertebrates excrete inorganic and organic nutrients, that are utilizable by bacteria and therefore modify the pool of available nutrients for bacterial growth (Sherr and Sherr, 2002; Wang et al., 2013a).

2.3.4 Location of bacterial cells: in water, sediment and biofilm

Bacteria attached to surfaces, such as pipe surfaces, deposited particles/sediments, and suspended particles, grow in a significantly different environment in comparison with conditions in bulk water (Figure 2.3), which has considerable influence on abundance, growth rates and composition of the bacterial community (Boe-Hansen et al., 2002a; Liu et al., 2014).

2.3.4.1 Characteristics of drinking water biofilms

Biofilms are initiated by adsorption of bacterial cells to a surface, followed by production of extracellular polymeric substances (EPS) by attached cells, and bacterial proliferation within the formed biofilm (Figure 2.3). Biofilm cell density in drinking water distribution systems can vary significantly with cell numbers in the range of 10^4 to 10^8 cells/cm^2, and adenosine tri-phosphate (ATP) concentrations in the range of 10^2 to 10^4 pg ATP/cm^2, (Boe-Hansen et al., 2002b; Wingender and Flemming, 2004; Långmark et al., 2005; Liu et al., 2014). The EPS structure offers a protective environment against disinfectant residuals and against grazing organisms, and binds organic and inorganic compounds (LeChevallier et al., 1988; Flemming and Wingender, 2010). Due to extracellular enzymes in the EPS, biofilm bacteria can utilize complex organic substrates, such as humic acids that are not easily biodegradable and usually not used by bulk water bacteria (Camper, 2004; Flemming and Wingender, 2010). Availability of additional nutrients creates new ecological niches and thus enables growth of different microorganisms than present in bulk water. Liu et al. (2014) reported that about 12% of the total bacteria (operational taxonomic units; OTUs) found in biofilms were not shared with the bulk water. Shift in bacterial community between suspended and attached phases are furthermore influenced by specific pipe materials applied in drinking water distribution networks (see details in section 2.4). Studies have shown that young biofilms display similar characteristics to the bulk water bacterial communities, while mature biofilms displayed lower growth rates and lower community richness, indicating a different bacterial community (Boe-Hansen et al., 2002b; Martiny et al., 2003).

2.3.4.2 Characteristics of drinking water sediments

Sediment formation is the result of particle deposition under favourable hydraulic conditions (Vreeburg et al., 2008) (Figure 2.3), and distribution networks can contains as much as 3000 mg/m loose deposits (Barbeau et al., 2005; Vreeburg et al., 2008). Deposited particles offer a favourable environment for bacterial growth, as (1) they provide a large surface area, (2) are usually composed of organic compounds and also (3) contain inorganic substrates (e.g. Ca, Fe, Mn) (Gauthier et al., 1999; Zacheus et al., 2001). Liu et al. (2014) found that sediments may favour the growth of specific bacterial species, for example bacteria involved in iron and arsenic cycling (e.g. *Rhodoferax* spp. and *Geobacter* spp.). Particle sedimentation combined with biofilm formation and EPS production consolidates the sediment structure, which expands as long as sediments are not re-suspended during high hydraulic peaks (cf. details in section 2.4). During sediment expansion, anoxic or anaerobic conditions are likely to be created, providing a selective environment for the growth of bacteria not found in the bulk water phase (e.g. *Rhodoferax* spp. and *Geobacter* spp., or bacteria from actinomycetes group) (Zacheus et al., 2001; Liu et al., 2014). Sediments were shown to contain large amounts of biomass, in the range of 700 to 4000 ng ATP/g loose deposit (Liu et al., 2014) and up to 10^{11} cells/g (Barbeau et al., 2005) and to harbour the largest bacterial diversity, compared to bulk water and biofilm phases, with 29% of the total bacteria which were not shared with the bulk water (Liu et al., 2014). Sediments can be the source of hygienic, aesthetic and operational problems, as they offer a protective environment for bacteria to grow, particularly for undesirable organisms (Gauthier et al., 1999), and iron-oxidizing bacteria shown to increase corrosion processes (Sun et al., 2014). Furthermore, sediments can be the source of coloured water when re-suspended into the bulk water (Vreeburg and Boxall, 2007b) and host invertebrates (Christensen et al., 2011).

2.3.4.3 Interactions between biofilm, sediment and bulk water phases

Mechanisms of interactions between biofilm, sediment, and bulk water bacteria have been investigated to estimate to which extent biofilms and sediments affect the bacterial community in bulk water in terms of abundance and community composition and structure, thus how these interactions might affect biological stability. Biofilms have long been considered as containing the largest fraction (up to 95%) of bacterial cells in distribution systems (Flemming et al., 2002a,b). However, the sediment phase has been largely overlooked due to sampling difficulties (Liu et al., 2013a). A recent study has shown that 98% of bacterial cells were situated in both biofilms and sediments, of which 60 to 90% were actually situated in the sediment phase (Liu et al., 2014). It was first assumed that the majority of bulk water bacteria originate from biofilm detachment

(LeChevallier et al., 1987; van der Wende et al., 1989), rather than bacterial growth in the bulk water phase. However, this hypothesis was challenged by a study by Boe-Hansen et al., (2002a), which demonstrated higher bacterial activity and growth rates in bulk water than in biofilm (0.30 day^{-1} in bulk water compared to 0.048 day^{-1} in biofilm). The study showed that bacterial production in the bulk water constituted 37% of the total bacterial production in a model drinking water system. Recently, Liu et al. (2014) detected different bacterial community compositions in bulk water and biofilm, and Henne et al. (2012) found significantly different core communities in both water and biofilm sampled from full-scale and long used distribution systems. From these observations, it was suggested that bulk water bacteria function as a seed bank for biofilms and sediments (Henne et al., 2012; Liu et al., 2014), each phase thereafter developing its own bacterial community, with different predominant species depending on specific environmental conditions. However, interactions between bacteria in water, sediment and biofilm phases are still unclear. Detachment of biofilms and re-suspension of sediments would arguably contribute to bacterial cell concentrations and community composition in the bulk water (cf. details on hydraulic conditions in section 2.4.2). Moreover, bacterial cells in biofilm and sediments would compete with bulk water bacteria for available nutrients. As the largest proportion of bacteria is present in the two phases, substantially fewer nutrients would remain available for bulk water bacteria. Consequently, the role and interactions between bulk water, biofilms and sediments should be taken into account when considering biological stability in drinking water distribution systems.

2.4 Biological stability in drinking water: implications for treatment and distribution

Both water treatment and distribution conditions can significantly affect biological stability in drinking water distribution systems, by shaping bacterial community characteristics and/or modifying bacterial growth environment. Achieving biological stability therefore requires to (1) produce biological stable water, i.e. a water that does not support bacterial growth, considering the composition of its nutrients and its bacterial community, and (2) distribute water in conditions that do not promote changes in the microbial community, in full-scale systems as well as in households (Figure 2.4).

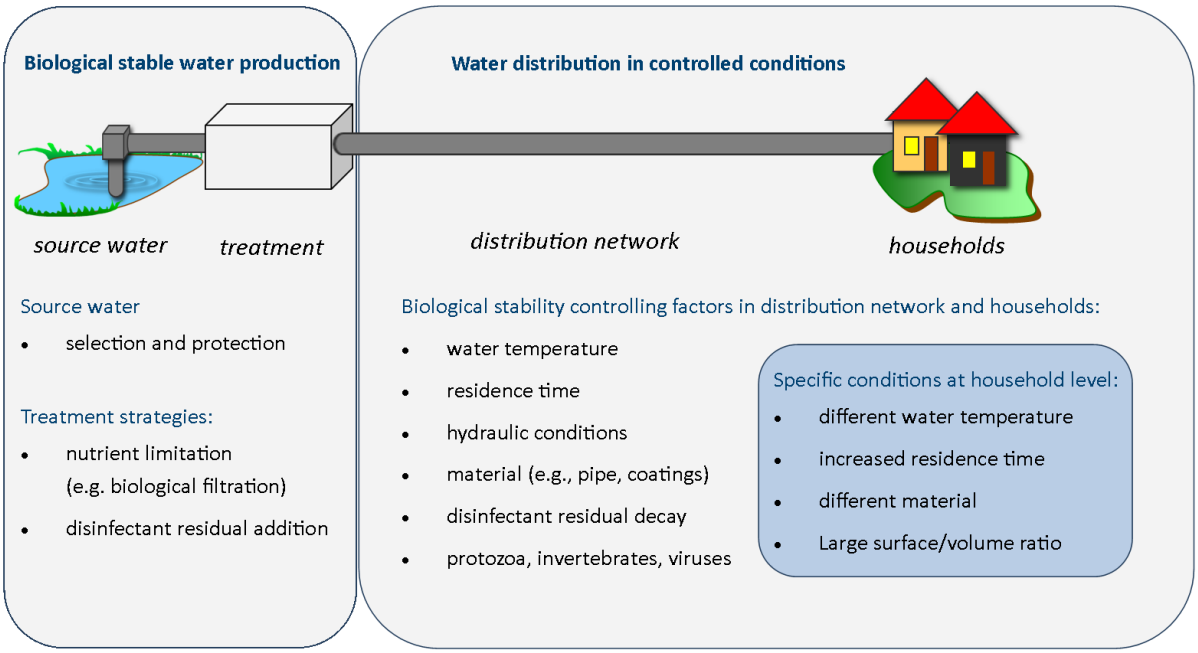


Figure 2.4. Biological stability components: source to tap overview of critical parameters controlling biological stability in drinking water systems.

2.4.1 Treatment strategies for the production of biological stable water

2.4.1.1 Water sources and treatment strategies

Water treatment strategies are adapted to the characteristics of raw water, which can be very diverse. Deep ground water typically contains very low bacterial cell concentrations (10^3-10^4 cells/mL), are often anaerobic and contain low organic nutrients (e.g., AOC below 10 µg ac-C/L) but potentially high methane (e.g, 0.01 to 9 mg/L) and ammonium concentrations (e.g, 0.2 to 5 mg/L) (van der Kooij et al., 1982; de Vet et al., 2009; Hedegaard and Albrechtsen, 2014). Surface waters, on the other hand, typically contain high bacterial cell numbers (10^5-10^6 cells/mL) and relatively high concentrations of organics (e.g., AOC in the range of 5 to 150 µg Ac-C/L) (van der Kooij, 1990; Hammes et al., 2010a; van der Wielen and van der Kooij, 2010), while numerous in-between situations can be found, with e.g. infiltrated water or phreatic aerobic ground water (van der Kooij et al., 1982, 1985a). Treatment strategies typically aim to inactivate hygienically relevant organisms, remove micro-pollutants, improve aesthetic aspects (turbidity, taste and odour), and prevent bacterial growth during water distribution. Therefore, combinations of different treatment types are applied. In several European countries, stable water is produced from surface water using extensive multistep treatments, without the use of residual disinfectant. As an example, treatment trains applied in Zurich and Amsterdam include one or several disinfection steps (e.g., ozonation) and a combination of biological filtration processes (e.g., rapid sand filter, slow sand filter, activated carbon

filter and/or dune infiltration) (Hammes et al., 2010a; Vital et al., 2012a). The choice of treatment strategies and combinations are crucial in the production of biological stable water, as it determines (i) composition and concentration of individual organic and inorganic nutrients, and (ii) bacterial community characteristics (abundance, activity, community composition).

2.4.1.2 Effect of oxidation processes

Oxidation processes, such as chlorination and ozonation or UV/H_2O_2 advanced oxidation, are commonly applied as primary disinfection strategies for bacterial inactivation (Bernarde et al., 1967; Hoefel et al., 2005; Ramseier et al., 2011a). Oxidative treatments often result in modification of the substrate composition (van der Kooij et al., 1989; Schmidt et al., 1998; Okuda et al., 2009; Ramseier et al., 2011b; Sarathy and Mohseni, 2009). As an example, Vital et al. (2012a) reported an increase from 20 to 120 µg AOC/L after ozonation, while DOC concentration did not change, indicating a clear change in the composition of organic compounds in water. In this regard, Hammes et al. (2006) showed that 60 to 90% of the AOC formed after ozonation of Lake water was composed of organic acids. Primary disinfection processes with high ozone or free chlorine dosage also typically result in inactivation of the entire bacterial community (Hammes et al., 2008, 2010a), thus leaving room for new microorganisms to colonize the treated water and consume the altered nutrient pool. Overall, oxidative processes create highly unstable water due to combined effects of (i) increased nutrients availability and (ii) absence and/or inactivation of bacterial cells.

2.4.1.3 Effect of biological filtration processes

Biological filtration processes are applied worldwide and are usually implemented after primary disinfection processes (van der Kooij, 1992; Prévost et al., 1998; Gauthier et al., 1999; Hammes et al., 2010a; Pinto et al., 2012). Biological filtration processes are believed to be an essential step for production of biological stable water (Rittman and Snoeyink, 1984; van der Kooij, 1990; Smeets et al., 2009). Different types of filtration technologies, applied for diverse purposes, can act as biological filtration. These include active carbon, rapid or slow sand filtrations, or soil infiltration. When water flows through biological filters, bacterial cells attach to filter particles (Servais et al., 1994; Velten et al., 2007) and consume substrates provided by the water, resulting in formation of a diverse bacterial community. Pinto et al. (2012) recorded the presence of 14 different phyla in the effluent of a rapid dual media filter. Bacterial community abundance, activity and composition in filters depends on nutrient composition of water flowing through the filter, resulting in the presence of different organisms being able to metabolize different types of substrate, and on environmental parameters

such as temperature (cf. section 2.3; Fonseca et al., 2001). Bacterial cells regularly detach from the filters, and bacteria are found in water after biological filtration typically in bacterial cell concentrations ranging between 10^4 and 10^5 cells/mL (Servais et al., 1994; Hammes et al., 2010a; Lautenschlager et al., 2014). Studies have shown that biological filtration processes shape the bacterial community composition in treated water (Pinto et al., 2012; Lin et al., 2014), which is only slightly modified during water distribution (Henne et al., 2012). Successive biological filtration steps of different types are often applied in order to consume fast degradable and more complex organic compounds. As an example, Lautenschlager et al. (2014) showed in a multi-step treatment plant in Zürich that low molecular weight humic substances were mainly removed during rapid sand filtration, while polysaccharides were degraded in subsequent slow sand filter. This can be attributed to residence time of water in the slow sand filter, which can be up to 50 times longer than in rapid sand filter (Huisman and Wood, 1974). Application of successive biological filtration steps thus gradually shapes both the nutrient pool and bacterial community characteristics in treated water (Lautenschlager et al., 2014). Changing raw water quality or temperature variations potentially affect bacterial community characteristics in treated water in time, including bacterial community composition (Pinto et al., 2014) and cell concentrations (Chapter 6). Application of biological filtration processes has two main advantages: (i) growth-supporting organic and inorganic nutrients in treated water are considerably reduced (up to 80 to 90% AOC removal) (van der Kooij, 1987; Hijnen and van der Kooij, 1993; Servais et al., 1994) and (ii) a very diverse autochthonous bacterial community is released in treated water, therefore covering a large substrate utilization spectrum and contributing to biological stability of water (cf. section 2.3).

2.4.1.4 Membrane filtration

In recent years, application of membrane filtration methods rather than chemical additions to water, have been proposed as an alternative primary or secondary disinfection approach (Sibille, 1997, 1998a; Vrouwenvelder et al., 2004). Several membrane types can be distinguished based on their removal capacity, and include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmonsis (RO) (Mallevialle et al., 1996). The advantage of this approach is that it lowers significantly bacterial cell concentration in treated water, by removing over 99.5% of bacterial cells (Liikanen et al., 2003), without producing any by-products that have potential adverse health effects, or contribute to available nutrients for bacterial growth. Moreover, membrane filtration considerably reduces the amount of suspended particles entering the distribution system, thus lowering the potential for sediment deposition (Vreeburg et al., 2008) and associated problems (cf. sections 2.3.4 and 2.4.2.3).

The use of filtration methods, however, potentially leads to production of unstable water (Okabe et al., 2002). While the majority of bacterial cells are removed, a significant fraction of nutrients remain in water (Liu et al., 2013b). In a controlled laboratory study, Meylan et al. (2007) showed that nanofiltration retained 97% to 99% of polysaccharides, humic substances, and low molecular weight organic compounds, but only 88% to 94% of low molecular weight neutrals and hydrophobic organic compounds. Consequently over 90% of BDOC is retained (Escobar and Randall, 2001a; Vrouwenvelder et al., 2004), while a large proportion of AOC passes through NF membranes, ranging between 5% and 90%, depending on the cut-off value and the influent water quality (Liikanen et al., 2003; Meylan et al., 2007). As no system remains sterile (Liu et al., 2013b, Liikanen et al., 2003), these nutrients are fully available for new bacterial growth in the distribution system. In such case, water retains a certain level of growth potential, as highlighted by Liikanen et al. (2003) who observed an HPC increase of over a log unit during incubation of the permeate of nanofilters for 20 days. One option to reduce significantly the growth potential is to use reverse osmosis (RO). RO is used for production of high quality drinking water, not only for seawater desalination but also within large scale treatment utilities e.g. in the Netherlands (Kruithof et al., 2001). However, despite an efficient bacterial cell and organic compound removal (over 99.5% rejection of bacterial cells, and over 90% of total organic carbon (TOC) rejection), Park and Hu (2010) showed that not all AOC is retained by RO membranes, with about 80% AOC rejection efficiency. As a consequence, significant regrowth occurred, from 50 bacterial cells/mL in the RO permeate up to about 10^3 cells/mL in the bulk water after 20 days residence time in a model distribution system fed with RO permeate. Additionally, a biofilm containing 8×10^3 cells/cm^2 was formed after 20 days. Bacterial cell numbers remained however extremely low in comparison with a similar system fed the same tap water without prior RO treatment (2×10^5 cells/mL in bulk water, and 7×10^5 cells/cm^2 in biofilm). In summary, membrane filtered water remains unstable due to the permeability of certain compounds through the membrane, the lowest growth potential being obtained by membranes retaining the highest proportion of nutrients (e.g., RO).

2.4.1.5 Ion exchange

Ion exchange (IEX) has also been proposed as an additional step in full-scale conventional drinking water distribution system to improve colour of final water and biological stability (e.g., Heijman et al., 1999). Implementation of IEX as pre-treatment in a pilot drinking water treatment plant has been shown to significantly reduce organic content of final water, reducing DOC of final water by 50% and AOC by 60%, compared to a control treatment lane without IEX (Grefte et al., 2011). Biofilm formation rate of produced water was subsequently reduced by 70%. This can be explained by the

fact that IEX is efficient in removing low molecular weight organics (Bolto et al., 2002), that are composing the AOC fraction. However, water treated by IEX retains a certain degree of growth potential: in another study, IEX enabled to reduce DOC by 67% and total cell concentration by about 50% (Liu et al., 2013b), but bacterial growth was detected in the treated water incubated for 24h, with an increase in ATP of 23% and in total cell concentration of 41%.

2.4.1.6 Secondary disinfection

In many cases, a secondary disinfection is applied to the produced drinking water before distribution, typically with free chlorine, chlorine dioxide or monochloramine (van der Kooij, 1992; LeChevallier et al., 1996; Prévost et al., 1998; Batté et al., 2006; Pinto et al., 2012). The aim of secondary disinfection is either to prevent bacterial growth in drinking water distribution systems, or to reduce HPC values below the guideline value. In both cases, application of a disinfectant alters significantly the bacterial community, lowering the diversity and richness, which has consequences on the growth potential of the water once disinfectant residual is depleted (cf. section 2.3).

2.4.2 Factors influencing biological stability in distribution networks

Water distribution conditions can have a considerable impact on biological stability (Figure 2.4). Various factors influence microbial processes described in section 2.3, including water temperature, residence time, hydraulic conditions, pipe material, and/or disinfectant residual decay. An overview of microbial dynamics in a drinking water pipeline section is provided in Figure 2.3.

2.4.2.1 Pipe materials

Pipe material composition influences biofilm development on pipe surfaces (Figure 2.3). A single distribution system typically includes diverse materials such as metal pipes (e.g. cast iron, stainless steel), cement, and/or synthetic polymers (e.g., PVC), on which widely different growth rates, bacterial densities and community compositions were measured (Niquette et al., 2000; Yu et al., 2010; Wang et al., 2014). Several studies have demonstrated that iron pipes allow the highest bacterial densities, with up to 45 times higher bacterial biomass fixed on iron coupons than on plastic materials (Norton and LeChevallier, 2000; Niquette et al., 2000). Corrosion of iron pipes leads to the release of particles and deposit formation (Figure 2.3), on which organic and inorganic compounds adsorb, and which act as attachment sites where bacteria are protected from disinfectant residuals (Camper, 2004; Morton et al., 2005). Synthetic polymeric pipe materials such as cross-linked poly-ethylene (PEX), polybutylene (PB), or polyvinyl-chloride (PVC), were shown to release biodegradable

organic substances (Figure 2.3), modifying the available nutrient source for bacteria to grow (Skjevrak et al., 2003; Bucheli-Witschel et al., 2012). Even if stable water is produced at the treatment plant, release of additional nutrients into water can promote biological instability. As an example, van der Kooij and Veenendaal (2001) reported an increase by up to 200% in ATP concentration after incubation of water with plastic materials (e.g., PVCp) compared to the incubation of the same water alone. Pipe materials influence the bacterial community predominantly during its first stage of development (Martiny et al., 2003; Henne et al., 2012). Consequently, construction of new distribution systems or replacement of pipe segments in old distribution systems profoundly affects the biological stability of drinking water for a period of time that can reach up to several years before a stable system is reached again (Martiny et al., 2003).

2.4.2.2 Hydraulic conditions

Hydraulic changes in distribution systems are frequent and play a major role in interactions between bulk water, sediment and biofilm phases (Figure 2.3). Low water consumption periods result in low flow velocities or even water stagnation in reservoirs and parts of distribution systems, enabling particle deposition, increased residence time, and offering favourable conditions for bacterial growth to occur (Gauthier et al., 1999; Zacheus et al., 2001; Liu et al., 2013a,b). On the other hand, hydraulic peaks caused by high consumption periods, fire-fighting actions, pipe flushing or system malfunctioning such as pipe breaks, unavoidably result in increased biofilm detachment and possible sediment re-suspension (Lehtola et al., 2006; Vreebrug and Boxall, 2007). This increases bacterial dispersal in the network water and will modify bacterial abundance and community composition in the bulk water, thus affecting biological stability (Figure 2.3). Hydraulic peaks can cause release of hygienically relevant organisms (Torvinen et al., 2004; Wingender and Flemming, 2011), and serious aesthetic issues such as presence of invertebrates in the bulk water (van Lieverloo et al., 2002a), or colour deterioration of water at the point of consumption (Vreeburg et al., 2004). To avoid such problems, Vreeburg et al. (2009) have proposed to adapt distribution system design, to control hydrodynamic conditions in distribution systems and limit particle deposition, sediment formation and avoid dramatic hydraulic peaks.

2.4.2.3 Water temperature

Water temperature is an essential factor influencing bacterial growth kinetics and competition processes. Drinking water temperatures typically range between 3 and 25 °C in European countries, (Kerneis et al., 1995; Niquette et al., 2001; Uhl and Schaule, 2004), and fluctuate seasonally within a

single drinking water distribution system (Chapter 6). As an example, Kerneis et al. (1995) recorded water temperatures of 8 °C in January and 22 °C in July of the same year at the effluent of a treatment facility in France. Elevated water temperatures have often been associated with increased bacterial abundance in drinking water distribution systems (Servais et al., 1992; Kerneis et al., 1995; Francisque et al., 2009; Liu et al., 2013a), and with higher numbers in indicator organisms such as coliforms or *Aeromonas* (Burke et al., 1984; Volk and Joret, 1994; LeChevallier et al., 1996). Francisque et al. (2009) recorded 5 times more occurrences of HPC concentrations above 100 CFU/mL at temperatures above 18 °C than at lower temperatures. In addition, water temperature can also affect bacterial community composition, by providing competitive advantages to specific bacterial species in defined temperature ranges, including pathogenic species (Vital et al., 2007, 2012b). For example, Vital et al. (2012b) showed that the maximum growth rate and competitive fitness of *E. coli* grown with an indigenous drinking water community increased with temperature in the range of 12 to 30 °C. There is therefore increased chance for problems associated with bacterial growth in summer periods, such as hygienic risks, deterioration of aesthetic aspects of water, malfunctioning of water installations, exceeding of legal guidelines for e.g. heterotrophic plate counts (cf. section 2.2). In this regard, specific attention is given to the influence of global warming on future drinking water quality, as average water temperatures are expected to increase, concomitantly with longer periods at higher temperatures (Levin et al., 2002). Moreover, significant seasonal shifts in bacterial community composition (Pinto et al., 2014) and abundance (Chapter 6) have been reported in effluents of treatment utilities. Though the cause of such variations is not clear at this stage, seasonal variations in water temperature could well be involved in bacterial community characteristics of water entering the distribution system. Further research would be needed to determine to which extent these changes affect bacterial competition processes within the drinking water distribution system.

2.4.2.4 Residence time

Residence times can reach up to a few days within a distribution system (Kerneis et al., 1995), leaving time for bacterial growth to occur. Residence times depend on (i) distance from treatment plant, up to 100 km or more in the case of extended cities or remote villages (Cook et al., 2014), (ii) pipe diameters, varying from a few meters in water mains down to a few millimetres in service pipes, and (iii) water flow velocity caused by water consumption, that vary daily and over one week. The latter also influences additional residence time of water within reservoirs at the treatment outlet and/or within distribution systems. Residence time in a given system can therefore vary significantly and the challenge is to maintain biological stability independently of time. In general, higher bacterial

abundances were observed at higher residence times (Maul et al., 1985a; Kerneis et al., 1995; Uhl and Schaule, 2004; Nescerecka et al., 2014). In the case of chlorinated water, increased bacterial abundance at long residence time is often congruent with decay of disinfectant residual (Servais et al., 1995). However, a decrease in bacterial abundances and/or activity at long residence times has also been reported occasionally (van der Wielen and van der Kooij, 2010), possibly be due to substrate limitations. A few recent studies have shown that the core bacterial community composition was not affected by residence time (Hwang et al., 2012; Pinto et al., 2014).

2.4.2.5 Residual disinfectant decay

While biological stability could theoretically be achieved by maintenance of sufficient disinfectant residuals at all points of a distribution system, this is challenging to achieve. Many of the factors mentioned in this section contribute to disinfectant decay within distribution systems. For example, dissolved nutrients in bulk water, as well as EPS and organic and inorganic nutrients adsorbed on biofilms and sediments can react with the disinfectant (Campos and Harmant, 2002; Gauthier et al., 1999), resulting in lowered concentrations or even absence of residual disinfectant at long residence times (Maul et al., 1985a; Kerneis et al., 1995). This phenomenon is increased in pipe sections with small diameter, in which the surface to volume ratio is higher, thus increasing contact of water with pipe materials and/or biofilms and sediments. Prévost et al. (1998) have observed a faster decay in 150 mm pipes than in water mains. Chemical reactions are further affected by water temperature, also modifying the disinfection capacity in time (Bernarde et al., 1967; Urano et al., 1983). Loss of disinfection residuals undoubtedly results in biological instability and bacterial regrowth (Servais et al., 1992; LeChevallier et al., 1996; Nescerecka et al., 2014).

2.4.2.6 Construction, operation and maintenance practices

Good practices for construction, operation and maintenance of water distribution systems are also essential to maintain biological stability in distribution systems (Smeets et al., 2009). A code for hygiene during pipe installation was developed by water utilities in the Netherlands (van Lieverloo et al., 2002b). Besides, maintenance of sufficient pressure in the system for protection against intrusion of external water, and good maintenance of pipelines to maintain physical integrity and limit leakages, are essential to prevent external contamination. In this way, intrusion of both external organisms and organic and inorganic nutrients is avoided, that would profoundly affect the nutrient pool and bacterial community composition in drinking water.

2.4.3 Is biological stability compromised at household level?

Premise plumbing conditions can cause biological instability and compromise water quality in the last meters prior to consumption. Water stagnation in household pipes has been shown to result in significantly increased bacterial abundance in the water (up to 3 fold increase) and in a shift in bacterial community composition (20 to 100% turnover) (Pepper et al., 2004; Lautenschlager et al., 2010; Lipphaus et al., 2014). Water quality within households is influenced by the same parameters as in the distribution system, i.e. water temperature, residence time, pipe material, hydraulics, disinfectant decay, and interactions between bulk water, sediments and biofilms. However, conditions within households are more extreme (Figure 2.4). Average water temperatures are generally higher in households than in the distribution system, sometimes reaching 20 °C or more, due to pipes installed through heated rooms or nearby heat sources (Lautenschlager et al., 2010; Lipphaus et al., 2014). Bacterial growth is supported in biofilms and bulk water due to both warmer water temperatures and long residence times in household pipes. On average, water is stagnant in pipes for 23 hours per day (Proctor and Hammes, 2015), and in practice this varies from a few hours up to some weeks in cases such as holiday houses or seasonal hotels. Measured changes in microbial community abundance and composition were dependent on stagnation time (Manuel et al., 2010; Lautenschlager et al., 2010). One of the challenges in maintaining good water quality up to the consumer's tap is that pipe materials choice and replacement are left to the house owner responsibility, and are often not well controlled. A large variety of pipe materials are often found in households and differ from the ones found in distribution mains. These include copper, plastic and elastomeric materials, which are sometimes not in accordance with regulations for use in drinking water (Regulation EC No 1935/2004, 2004; Regulation EU No 10/2011, 2011). Typical examples are flexible plastic materials such as shower tubes or small rubber fittings, including ethylene-propylene-diene-monomer (EPDM), which have considerable bacterial growth promotion potential (Bucheli-Witschel et al., 2012). The effect of pipe material on bacterial growth is further increased by significantly smaller pipe diameters in households than in distribution networks, resulting in increased contact between bulk water bacteria and biofilm and/or pipe material, and in faster disinfectant decay (Servais et al., 1992; Rossman et al. 1994; Prévost et al., 1998). Finally, consumer's taps can be the source of water back-contamination by organic and inorganic nutrients and/or bacteria. Better regulations of pipe materials in use in premise plumbing would help a better control of microbial processes in the last meters before the tap (Flemming et al., 2014; Proctor and Hammes, 2015). However, it is still unclear if household conditions could promote uncontrolled bacterial growth and significant biological instability following a biological stable drinking water distribution system.

2.5 How is biological stability assessed?

There are three types of indicators for biological instability of a drinking water system. Firstly, indirect signs of instability for water utilities are customer complaints about taste, colour, turbidity and/or odour, and the deterioration or malfunctioning of water installations, due to fouling or (bio-) corrosion. Secondly, biological stability/instability is traditionally predicted based on growth-promoting properties of treated water and/or materials in contact with water, and associated with guideline values (Table S2.1 in supplementary information). Thirdly, direct detection of changes in microbial community characteristics within distribution systems is indicative for instability. This section reviews existing and emerging methods for predicting and monitoring biological stability in drinking water distribution systems (Figure 2.2).

2.5.1 Predictive methods

2.5.1.1 Evaluation of growth promoting properties of drinking water and associated guidelines

A range of predictive methods have been developed to assess growth promoting properties (growth potential) of drinking water and are traditional indicators for biological stability (van der Kooij et al., 1982; Servais et al., 1989; Hu et al., 1999; Ross et al., 2013). Initial focus of these methods was on biodegradable organic carbon and included the assessment of assimilable organic carbon (AOC). The method was first developed by van der Kooij and co-workers (1982, 1985a), who proposed to follow the growth of two bacterial strains (*Pseudomonas fluoresc*ens strain P17 and *Sprillum* strain NOX), specifically selected for their versatility in organic compound utilization, thereafter complimented with an additional bacterial strain *Flavobacterium johnsoniae* strain A3 for utilization of more complex organic carbon compounds (Sack et al., 2009). The AOC method has been the subject of multiple subsequent variations (Werner, 1984; Hambsch et al., 1992; LeChevallier et al., 1993; Haddix et al., 2004; Hammes and Egli, 2005; Ross et al., 2013). However, AOC assays by definition focus on easily available substrates, and it is likely that refractory biodegradable compounds are not measured. The refractory fraction of biodegradable organic carbon is a potentially relevant parameter related to growth potential of drinking water, as it can be used by biofilm-bacteria in distribution systems (Camper, 2004; Flemming and Wingender, 2010). In this regard, the biodegradable dissolved organic carbon (BDOC) method, initially proposed by Servais and co-workers (1987, 1989), uses the indigenous bacterial community from the water sample to consume the available organic carbon during a long period of time (4 weeks in the initial method), and organic carbon consumption is followed by DOC measurements. This method was improved, with the use of

an indigenous bacterial community attached to sand obtained from a drinking water treatment plant (Joret et al., 1986, 1988), and with the use of continuously flowing systems instead of batch tests (Lucena et al., 1990; Ribas et al., 1991). The test time was reduced in this way from 4 weeks to 5 days (Joret et al., 1986, 1988) and to a few hours only (Lucena et al., 1990; Ribas et al., 1991). In general, higher BDOC values than AOC values are measured in drinking water. For example, Volk and LeChevallier (1999) reported BDOC values in the range of 0.15 to 0.75 mg/L and AOC values in the range of 0.10 to 0.33 mg/L in treated surface waters. Systematic application of the methods for full-scale studies showed that little AOC uptake occurred and HPC values remained below guideline values (100 cfu/mL) during distribution of non-chlorinated waters with an AOC level below 10 µg Ac-C/L (van der Kooij et al., 1992). In chlorinated water, statistically fewer occurrences of coliforms were observed at AOC concentrations below 100 µg Ac-C/L than in waters with higher AOC concentrations (LeChevallier et al., 1996). No decrease in BDOC was observed during distribution of waters with a BDOC levels below 150 µg C/L in chlorinated distribution systems (Servais et al., 1992; Volk and Joret, 1994). These different AOC and BDOC values have been associated with no/limited bacterial growth and have been subsequently used as guidelines for biological stable water (Van der Kooij, 1987; LeChevallier et al., 1992).

Both AOC and BDOC methods focus primarily on organic carbon as the only growth-limiting substrate in drinking water. However, other compounds were identified as possible microbial growth controlling substances (organic carbon, ammonium, manganese, iron) (Rittman and Snoeyink, 1984; van der Kooij et al., 1985a; States et al., 1985; Miettienen et al., 1997). Mittienen et al. (1997) have demonstrated that drinking waters can also be limited in other nutrients (e.g. phosphate) and have adapted the AOC method by adding phosphate into the water sample before the growth test. The method, however, still focuses on AOC determination, though other elements might be more critical to describe and understand microbial dynamics in full-scale drinking water systems. In a recent study of our group (Chapter 5), we have proposed to evaluate the growth potential of a water sample by considering (1) no pre-treatments (growth that water can support as such) and (2) by systematic step-wise addition of single substrates or combinations of substrates, for identification of growth-limiting substances. For more hygienic related questions, a pathogen growth potential (PGP) was also recently developed in which selected pathogenic bacterial strains are inoculated into a pasteurized and filtered water sample, and bacterial growth is followed by flow cytometric measurements (Vital et al., 2010).

AOC assays assess planktonic growth, while BDOC assay focus on organic carbon removal. However, water properties can also support growth of bacteria attached to surfaces, and a large number of

other devices have been used to study the development of biofilms in drinking water distribution systems. These include annular reactors (e.g., Volk and LeChevallier, 1999), Propella® reactors (e.g., Lehtola et al., 2006), flow cell systems (e.g., Manuel et al., 2010), and Robbins devices (e.g., Kalmbach et al., 1997). An extensive overview of available systems has been provided by Gomes and co-workers (2014). Many of these devices comprise coupons of defined materials such as copper, PVC or cement. Materials can have an influence on growth of biofilm, which can be studied by defined growth tests, as detailed below. Alternatively, van der Kooij and co-workers (1995) proposed to quantify the potential of water to form biofilms on inert materials. Subsequently, additional guideline values for biological stable water have been introduced, such as biofilm formation rates (BFR) which should be kept smaller than 10 pg ATP/cm^2.d in produced drinking water (van der Kooij et al., 1995).

2.5.1.2 Growth promoting properties of materials in contact with water

Materials in contact with drinking water can profoundly influence growth potential. A number of assays have been developed to assess this effect, and the approach varies from country to country. For example, a standardised method in the United Kingdom measures consumed dissolved oxygen as an indicator for bacterial growth on 150 cm^2 material during 7 weeks (Colbourne and Brown, 1979; British Standards 6920, 2000). In Germany, formation of biomass on 800 cm^2 material surface is measured as the volume of slime produced during 12 weeks (ÖNorm B 5018-1,2, 2002) and in the Netherlands, biofilm formation potential (BFP) is measured on 50 cm^2 material (van der Kooij and Veenendaal, 1994) with ATP analysis. The latter test was further adapted to estimate the potential of tested material to promote bacterial growth both on its surface (biofilm) and in the bulk water in contact with it (biomass production potential; BPP) (van der Kooij and Veenendaal, 2001). More recently, Bucheli-Witschel and co-workers (2012) have developed a new test package which requires 14 days and that measures the BPP (quantified with FCM) as well as the AOC fraction in water after a series of high-temperature standardised migration assays. Though assessment of growth-promoting properties of materials in contact with water is a useful decision-making tool for selection of appropriate materials, it is challenging to assess growth-promoting properties of materials already installed in networks. The latter can be largely affected by long-term aging in specific conditions in distribution systems, including continuous flow and presence of biofilms or specific degrading organisms.

2.5.2 Direct indicators of change

Distribution conditions can significantly affect bacterial growth in distribution systems (cf. section 2.4). To evaluate the distribution effect, the best approach is to directly characterize bacterial communities in the system, by assessing bacterial abundance, viability and community composition.

2.5.2.1 Bacterial abundance

An increase in bacterial abundance is a clear sign of biological instability, and can be measured as a change in specific bacteria, bacterial groups or in the total bacterial community (Servais et al., 1992; LeChevallier et al., 1996; van der Wielen and van der Kooij, 2010; Nescerecka et al., 2014). Specific detection usually focuses on hygienically relevant organisms such as *Legionella*, *Mycobacterium*, *Pseudomonas aeruginosa*, total coliforms, *Enterococcus* and *E. coli* (Tallon et al., 2005). In the Netherlands, the presence of *Aeromonas* is also included in legislation and often tested as an indicator for biological stability (Waterleidingbesluit, 2001). Specific detection is traditionally performed by cultivation on selective media, and more recently with molecular-based techniques such as quantitative polymerase chain reaction (qPCR) or with the use of specific antibodies (Schets et al., 2002; Rompré et al., 2002; Tallon et al., 2005; Hügler et al., 2011; Douterelo et al., 2014). Besides specific detection, cultivation is also used worldwide in drinking water monitoring for heterotrophic plate counting (HPC) methods (Pepper et al., 2004; Uhl and Schaule, 2004; Allen, 2004; Batté et al., 2006). There is more or less universal agreement that the fraction of bacterial cells detected by HPC methods is less than 1 % of the total bacterial concentration in drinking water (Staley and Konopka, 1985; Hoefel et al., 2003; Hammes et al., 2010a; Epstein et al., 2013). Quantification of all bacterial cells in a water sample is achieved by cell labelling with fluorescent dyes (e.g., SYBR Green I or DAPI) and subsequent detection by either epifluorescence microscopy (Servais et al., 1992; Niquette et al., 2001) or flow cytometry (FCM) (Hammes et al., 2008; Hoefel et al., 2003). FCM enables extremely fast water analysis with limited sample handling (Hammes et al., 2008; Chapter 3), as well as possibility for automation (Hammes et al., 2012; Besmer et al., 2014). The use of FCM has revealed an increase in total cell concentrations in the range of 11% to 23% in drinking water distributed without disinfectant residual (Hammes et al., 2010a; Chapter 4) and up to 300% in household taps after stagnation (Lautenschlager et al., 2010). While all methods above are well adapted for enumeration of suspended bacterial cells, they cannot be applied directly for enumeration of bacteria attached to particles, sediments, or biofilms. Pre-treatments such as ultrasonication or physical removal (e.g. swabbing or scratching the surface) are required to detach and suspend bacteria (Boe-Hansen et al., 2002b; Magic-Knevez and van der Kooij, 2004).

2.5.2.2 Bacterial viability and activity

A large number of viable drinking water bacteria are not detected with cultivation methods as a result of being either so-called uncultivable bacteria (Kaeberlein et al., 2002; D'Onofrio et al., 2010; Epstein, 2013) or in the so-called "viable but non cultivable" (VBNC) state (Oliver, 2005; Hammes et al., 2011). It is particularly useful to determine cultivation-independent bacterial viability/activity when disinfection is applied. Viability of all bacterial cells can be assessed by cell labelling with fluorescent dyes targeting specific features of bacterial cells related to bacterial viability such as cell membrane integrity (e.g., propidium iodide), membrane potential (e.g., $DiBAC_4(3)$), respiratory activity (e.g., CTC), and subsequent detection with epifluorescence microscopy or flow cytometry (Prévost et al., 1998; Hoefel et al., 2005; Berney et al., 2008; Ramseier et al., 2011a; Hammes et al., 2011). As an example, Berney et al. (2008) have shown that water sampled at a household tap after non-chlorinated water distribution contained 1.7×10^5 cells/mL, of which about 70% had intact, polarized membranes, but only 20% displayed esterase activity. Noticeable biological instability has been detected in a chlorinated drinking water distribution system, in which the total bacterial cell concentration increased from 1.62×10^5 to 1.07×10^6 cells/mL and the percentage of cells with intact membranes increased from 3 to 59 % (Nescerecka et al., 2014). Besides the above single-cell methods, adenosine-tri-phosphate (ATP) is a useful bulk water measurement of biological activity, as ATP is present only in living cells (Karl et al., 1980). Typically, total ATP concentrations in the range of 0.8 to 12 ng ATP/L are found in drinking water (Lautenschlager et al., 2010; Van der Wielen and van der Kooij, 2010). For increased specificity in the method, it is possible to distinguish between intracellular and extracellular ATP and to estimate the average amount of bacterial ATP per cell when combined with FCM quantification (Hammes et al., 2010b; Vital et al., 2012a). ATP measurements have successively been applied in several case studies to assess biological stability (Van der Wielen and van der Kooij, 2010; Vital et al., 2012a; Nescerecka et al., 2014; Liu et al., 2014). A few alternatives for bacterial activity measurements in drinking water have been proposed (Staley and Konopka, 1985), including the measurement of H^3-Leucine or H^3-thymidine incorporation (Servais et al., 1992; Boe-Hansen et al., 2002a).

2.5.2.3 Bacterial community composition

A change in microbial community composition is indicative of instability (Lautenschlager et al., 2013; Pinto et al., 2014; Chapter 7). Molecular methods for this purpose are predominantly based on DNA extraction and PCR amplification (e.g., of 16S rRNA gene) and are roughly classified into either fingerprinting methods or high-throughput sequencing methods (Read et al., 2011; Douterelo et al.,

2014). Fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) provide insight in the bacterial community composition, without identification of specific bacterial groups or species. Fingerprinting methods are useful for a quick comparison between bacterial community in different water samples, and have been applied to study e.g., the effect on bacterial communities of different pipe materials (Yu et al., 2010; Roeder et al., 2010) or of water stagnation and flushing of household taps (Manuel et al., 2010; Lautenschlager et al., 2010). Recently, non-molecular techniques such as flow cytometric fingerprints have been shown to be indicative of the bacterial community composition, and have been useful for detection of community shifts after changing environmental conditions or after water distribution (De Roy et al., 2012; Koch et al., 2013a; Chapter 4).

High-throughput 16S rRNA gene based sequencing methods, including 454-pyrosequencing, Illumina, or Ion-torrent, enable taxonomic and/or functional classification of organisms present in drinking water at various phylogenetic levels. These techniques evolved rapidly in the past decade and are consequently used in increasing numbers of studies on drinking water (e.g., Hwang et al., 2012; Wang et al., 2013b, 2014; Pinto et al., 2014; Lin et al., 2014; Liu et al., 2014; Roeselers et al., 2015). High-throughput sequencing techniques also enable the study of specific bacterial groups such as ammonia-oxidizing, iron-oxidizing or sulfate-reducing bacteria (Gomez-Alvarez et al., 2013; Sun et al., 2014).

2.6 Evaluation of current definitions and approaches for biological stability assessment

2.6.1 Existing definitions of biological stability

The first definition of biological stability was formulated by Rittman and Snoeyink in 1984: "*A biological stable water does not support the growth of microorganisms to a significant extent, whereas an unstable water supports high numbers of microbes in distribution systems if sufficient disinfectant is not used*". This definition essentially focused on the properties of water leaving treatment facilities. However, concomitantly with research developments on microbial dynamics in drinking water treatment and distribution systems (Table S2.1 in supplementary information), it became clear that not only the properties of water, but also distribution conditions could significantly affect bacterial growth in drinking water distribution systems (cf. section 2.4).

Table 2.1. Summary of advantages (+) and drawbacks (-) of current approaches for assessment of biological stability of drinking water.

	Predictive approach *AOC, BDOC, BFR, BPP guidelines values*		**Direct assessment approach** *No change in microbiological-related parameters*
+	Useful decision-making tools	-	Lack for guideline values and specified methods
-	Guidelines sometimes too strong	-	No clear value for what is an acceptable change
-	Distribution system complexity not considered	+	Both water and system stability are considered
-	No evaluation of what really happens	+	Direct evaluation of what really happens
-	Guideline values dependent on application or not of disinfectant residuals	+	Applicable to any system (w/o disinfectant residuals)
-	Focus on heterotrophic growth	+	All bacterial types considered
-	Focus on bacterial abundance	+	All characteristics of bacterial community considered (abundance, viability, composition)

New definitions were proposed taking into account combinations of parameters: Sibille (1998a) pointed out the importance of organic matter and of predation by protozoa (Table S2.1 in supplementary information, definition Nr. 2), while van der Kooij (2000) considered both the properties of treated water and of piping material as critical points: *"Biostability is defined as the inability of water or a material in contact with water to support microbial growth in the absence of a disinfectant".* With the emergence of high-throughput methods such as flow cytometry or 16S rRNA gene based sequencing methods, sensitive detection of changes in bacterial community characteristics led to a broader definition of biological stability (Lautenschlager et al., 2013): "*Biological stability would imply no changes occurring in the concentrations and composition of the microbial community in the water during distribution*".

The last two definitions strongly rely on available methods, and shaped the two main current strategies for assessment of biological stability, namely (i) prediction and (ii) direct assessment of changes in bacterial community characteristics during water distribution. Both approaches have major advantages and drawbacks, as detailed in this section and summarized in Table 2.1.

2.6.2 Evaluation of predictive approaches

One usual approach to evaluate biological stability is the prediction of changes that could potentially occur during water distribution, based on controlled laboratory-scale methods (e.g. AOC, BDOC, BFR, BPP tests; van der Kooij et al., 1982; LeChevallier et al., 1993; van der Kooij and Veenendaal, 2001; Bucheli-Witschel et al., 2012) and/or modelling of microbial dynamics during water distribution (Servais et al., 1992, 1995; Dukan et al., 1996; Srinivasan and Harrington, 2007). The approach is based on the definition of biological stability provided by van der Kooij (2003), who subsequently

proposed to use combinations of tools for (i) assessment of biological stability of water by evaluating treated water growth-promoting properties based on both organic carbon content (AOC method) and biofilm promoting properties (BFR method), (ii) assessment of growth-promoting properties of materials in contact with water (e.g. biomass production potential (BPP) test) and (iii) modelling effects of water quality parameters and distribution systems conditions on microbial activity. In this way, both effects of water growth potential and distribution conditions are evaluated, and the effect of each individual parameter, such as water temperature, residence time, residual disinfectant decay, or hydraulic conditions can be modelled or tested individually (Dukan et al., 1996; van der Kooij, 2003). Thorough application of predictive methods and statistical evaluation of large datasets from distribution systems (van der Kooij et al., 1992; Servais et al., 1995; van der Kooij et al., 1995; LeChevallier et al., 1996) have provided guideline values (e.g. for AOC, BDOC or BFR or BPP parameters) that are helpful decision-making tools for water utilities for optimization of water treatment trains for production of biological stable water and of distribution (cf. Table S2.1 in supplementary information and section 2.5).

However, predictive approaches do not cover all aspects of bacterial growth-controlling factors in drinking water distribution systems. As an example, AOC tests by definition do not evaluate autotrophic growth or limitations in any other nutrient that organic carbon, unless the method is adapted and specifically targets inorganic nutrient limitation. Besides, conditions in full-scale distribution systems are complex, with the conjunction of numerous factors that are specific to each and every distribution system: structure and length of distribution systems, water consumption and temperature profiles, combination of pipe materials, history of water treatment implementations, pipe replacement, and/or maintenance actions such as pipe flushing. These factors would influence the bacterial community that colonize drinking water distribution systems (cf. details in sections 2.3 and 4). As a result, prediction does not necessarily reflect what actually occurs during water distribution system, as many factors are likely to affect bacterial dynamics.

2.6.3 Evaluation of direct assessment approaches

Another approach to assess biological stability relies on measurement of changes in bacterial community characteristics directly in distribution systems (Hammes et al., 2010a; Lautenschlager et al., 2013), and is based on the definition of biological stability provided by Lautenschlager et al. (2013). This approach has the major advantage to directly evaluate what occurs in drinking water distribution systems and/or at household levels, and to take into account both effects of treated water and of distribution conditions. Besides, the approach is applicable to any drinking water

distribution system, whether disinfectant residuals are present or not, and all bacterial types are considered, including heterotrophic and autotrophic bacteria. Finally, absence of change in bacterial community characteristics refers to any parameter related to microorganisms in water, i.e. not only abundance, but also viability, activity and composition and structure of the microbial community.

However, the absence of a defined toolbox and clear guideline values represents a major difficulty for water utilities to implement the direct approach. There is currently no specification of which parameters should be measured, as there is no unified method to describe and quantify microorganisms and particularly bacterial communities in water. Most likely different conclusions could be obtained from different methods, as they target part or all the bacterial community, and/or specific features related to bacterial activity or viability (cf. details in section 2.5), and direct comparison of results from different studies is difficult. More importantly, there is a major knowledge gap, related to the "degree of acceptable change" or the degree of instability that would cause problems such as deterioration of water aesthetic aspects or of installations. During studies performed with flow cytometry, small changes in bacterial cell abundance have been detected in drinking water distribution systems (from $9.5 \pm 0.6 \times 10^4$ to $1.3 \pm 0.1 \times 10^5$ cells/mL; Lautenschlager et al., 2013; from $3.5 \pm 0.2 \times 10^5$ to $4.3 \pm 0.4 \times 10^5$ cells/mL; Chapter 4). While Hwang et al. (2012) and Pinto et al. (2014) have shown that the core microbial community does not change with distance in the water distribution system, an extreme dynamicity in rare taxa was highlighted in a study from our group (3 to 4 % of the total community; Chapter 7). These relatively small variations in bacterial community characteristics were not related to any loss in water quality, and it is unclear from these studies if such a change in bacterial abundance and/or community composition should be considered as a problem, or more broadly as biological instability. One essential question is whether universal guideline values can be proposed for e.g. bacterial cell concentrations and/or extent of acceptable change, or whether the degree of acceptable change is specific to each and every drinking water distribution system.

2.6.4 New definition of biological stability

In essence, a biological stable drinking water is a water where the microbial community does not change during distribution, or at least not to a degree that affects negatively consumer's safety and aesthetic perception or cause technical system failure, neither on spatial nor on temporal scales, at any drinking water distribution location including the point of use and consumption. From a pure scientific perspective, biological stability would imply that no change in the microbial community characteristics (abundance, viability, community composition) is detected in time or distance in the

distribution system. Practically, current knowledge still does not allow for stating specific guideline values of acceptable change in the water quality.

2.7 Conclusion

The production and distribution of biological stable water should be a non-negotiable goal for water utilities with the perspective of providing the same water quality to the consumer than what is produced at the treatment facility. This can only be achieved by a full control of microbial processes during water treatment and distribution. Research in the past 30 years has significantly increased the knowledge on factors driving changes in the microbial water quality during drinking water distribution. These findings have led to the implementation and improvement of new water treatment strategies, distribution system designs, and good operation and maintenance practices. However, there are still large knowledge gaps, and the change in microbial water quality is not systematically avoided. Emerging analytical and molecular methods, such as flow cytometry and high-throughput sequencing methods, open new ways for increased understanding of drinking water distribution pipeline ecology and for drinking water monitoring. The concept of biological stability is evolving concomitantly with methodological and research developments. Sensitive detection of change in microbial community characteristics is enabled by the novel flow cytometric and high-throughput sequencing methods, but the question of acceptable degree of change in the bacterial community in drinking water still needs to be unravelled in the context of biological stability.

SUPPLEMENTARY INFORMATION – Chapter 2

Table S2.1 - Legends and definitions

* indicates the tools used as predictive methods.

Abbreviations:
AOC: assimilable organic carbon; ATP: adenosine-tri-phosphate; BDOC: biodegradable dissolved organic carbon; BFP: biofilm formation potential; BFR: biofilm formation rate; BPP: Biomass production potential; DGGE: Denaturing gradient gel electrophoresis; DOC: dissolved organic carbon; DVC: direct viable count (epifluorescence microscopy, staining with CTC); HPC: heterotrophic plate count (the medium is indicated between brackets); FISH: fluorescence in situ hybridization; ICC: intact cell concentration (Flow cytometry); MAP: microbially available phosphate; PCR: polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; TCC: total cell concentration (Flow cytometry); TDC: total direct counts (epifluorescence microscopy, staining with acridine orange or DAPI); TOC: total organic carbon; T-RFLP: Terminal restriction fragment length polymorphism

AOC methods
1. Van der Kooij et al., 1982, 1985a: Sample autoclaving, inoculation with pure strains P17 and NOX, bacterial growth assessment by plate counting.
2. LeChevallier et al., 1993: Sample autoclaving, inoculation with pure strains P17 and NOX, bacterial growth assessment by ATP measurement.
3. Miettinen et al., 1999 : Sample autoclaving, inoculation with pure strains P17 and NOX, nutrients addition (mixture of several elements needed for bacterial growth), bacterial growth assessment by plate counting.
4. Hammes and Egli, 2005 : sample filtration, inoculation with natural bacterial community, bacterial growth assessment by flow cytometry.

Definitions:
1. “A biological stable water does not support the growth of microorganisms to a significant extent, whereas an unstable water supports high numbers of microbes in distribution systems if sufficient disinfectant is not used”.
2. “A biological stable water is a water for which bacterial growth, organic matter consumption and predation are not significant”.
3. “Biostability is defined as the inability of water or a material in contact with water to support microbial growth in the absence of a disinfectant”.
4. “Biological stability would imply no changes occurring in the concentrations and composition of the microbial community in the water during distribution”.

Table S2.1. Selection of relevant publications on biological stability of drinking water and/or on microbial growth in distribution systems, with an overview of study focus, considered microbial growth controlling factors, study approach and methods, and if applicable, proposed guideline values and definition for biological stability (BS).

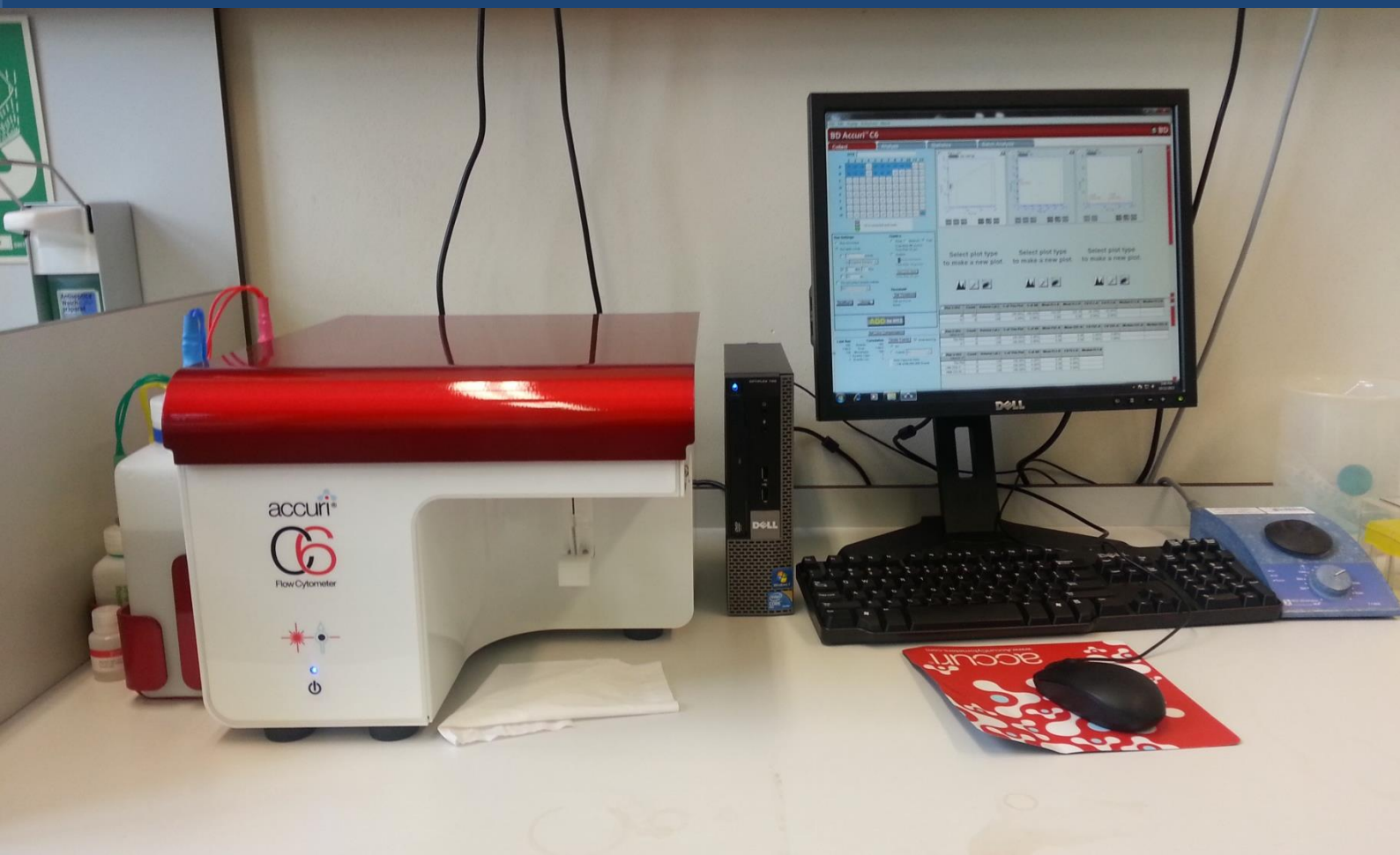
Authors	**Focus**					**Considered microbial growth controlling factors**		**Approach and methods**			**Guideline value? Definition BS?**
	Study focus	Temporal/ spatial variations?	Residual disinfectant ?	Type of organisms	Organisms location	nutrients	Other factors	Approach microbial study	Microbial analysis	Other methods	
Rittmann, Snoeyink, 1984	Treated water	-	General	All organisms, focus on heterotrophic + autotrophic bacteria	Suspended	All, focus on NH^{4+}, BOM, Mn	-	-	-	-	No guideline value Definition Nr. 1
Maul et al., 1985a / Kerneis et al., 1995	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	Heterotrophic bacteria	Suspended	-/ BOM	Disinfectant concentration, residence time, temperature	Assessment	HPC (R2A)	-/BDOC	No guideline value No definition
Van der Kooij et al., 1992	Treated water + distribution effect	Spatial + temporal (seasonal)	No	Heterotrophic bacteria	Suspended	BOM	Residence time, distance	Assessment	HPC(PCA) total coliforms total ATP	AOC[1]	AOC < 10 µg Ac-C eq/L No definition
Servais et al., 1992, 1995	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	All bacteria, focus on heterotrophic bacteria	Suspended + fixed (biofilms)	BOM	Disinfectant concentration, temperature, pipe diameter	Assessment, prediction*	TDC ^{3}H thymidine incorporation	BDOC Models*	BDOC < 150 µg/l No definition
LeChevallier 1992, 1996	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	Heterotrophic bacteria, focus on coliforms	Suspended	BOM	Disinfectant concentration / type, rainfall temperature	Assessment	HPC (R2A) total coliforms	AOC[1,2]	AOC < 50 µg Ac-C eq/L, Temp <15°C , AOC < 100 µg Ac-C eq/L No definition
Volk, Joret, 1994	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	All bacteria, focus on coliforms	Suspended	BOM	Disinfectant concentration, temperature	Assessment, prediction*	Total coliforms TDC	BDOC, Models*	No definition Temp < 15°C BDOC < 0.15 mg/L Free Chlorine > 0.10 mg/L

Authors	Focus					Considered microbial growth controlling factors		Approach and methods			Guideline value? Definition BS?
	Study focus	Temporal/ spatial variations?	Residual disinfectant ?	Type of organisms	Organisms location	nutrients	Other factors	Approach microbial study	Microbial analysis	Other methods	
Miettinen et al., 1997	Treated water	Spatial	General	Heterotrophic bacteria	Suspended	All, focus on MAP	-	Prediction	HPC (R2A)	AOC[3] (nutrient addition)	No guideline value No definition
Prévost et al., 1998	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	All bacteria, focus on heterotrophic bacteria	Suspended	BOM	Disinfectant concentration, residence time, pipe surface/ volume ratio, temperature	Assessment	HPC TDC DVC ^{3}H thymidine incorporation	BDOC	No guideline value No definition
Sibile, 1998a	Treated water + distribution effect	Spatial	General	Heterotrophic bacteria, protozoa	Suspended + fixed (biofilms)	BOM	Protozoa, sediments, disinfectant concentration, residence time, temperature	-	-	-	No guideline value Definition Nr. 2
Van der Kooij, 2000, 2003	Treated water + distribution effect	Spatial	General	Heterotrophic bacteria, incl. undesirable bacteria	Suspended + fixed (biofilms, sediments)	BOM	Pipe material temperature, residence time, flow velocity	Assesment, prediction*	HPC (R2A) total ATP PCR FISH	AOC[1]* BFR* BFP* BPP* Models *	AOC < 10 µg Ac-C eq/L BFR < 10 pg ATP/cm^{2}.d Definition Nr. 3
Niquette et al., 2001	Treated water + distribution effect	Spatial	Yes	Heterotrophic bacteria	Suspended + fixed (biofilms)	BOM	Disinfectant concentration, residence time, temperature	Assessment	TDC 3H thymidine incorporation	BDOC	BDOC < 0.25 mg C/L No definition

Authors	**Focus**					**Considered microbial growth controlling factors**		**Approach and methods**			**Guideline value? Definition BS?**
	Study focus	Temporal/ spatial variations?	Residual disinfectant ?	Type of organisms	Organisms location	nutrients	Other factors	Approach microbial study	Microbial analysis	Other methods	
Lehtola et al., 2006	Treated water + distribution effect	-	Yes	All bacteria, focus on heterotrophic bacteria	Suspended + fixed (biofilms, sediments)	BOM, MAP, Iron, copper	Sediments, pipe material, flow velocity, temperature	Assessment	TDC HPC (R2A) total coliforms RT-PCR (viral analysis)	TOC, AOC[3], MAP	No guideline value No definition
Pepper et al., 2004	Treated water + distribution effect + household effect	Spatial	Yes	Heterotrophic + autotrophic bacteria	Suspended	-	-	Assessment	HPC (R2A) identification of specific bacterial groups	-	No guideline value No definition
Batte et al., 2006,	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	Heterotrophic + autotrophic bacteria, bacterial indicators	Suspended + fixed (biofilms)	BOM,	Disinfectant concentration, residence time, temperature	Assessment	HPC (R2A) total coliforms enterococci	BDOC	No guideline value No definition
Srinivasan, Harrington, 2007	Treated water	Spatial	Yes	Heterotrophic + autotrophic bacteria	Suspended	AOC, NH^{4+}	Disinfectant concentration and type	Prediction*	HPC	Models*	No guideline value No definition
Manuel et al., 2010	Treated water + distribution effect + household effect	Temporal	Yes	All bacteria, focus on heterotrophic bacteria	Suspended + fixed (biofilms)	BOM	Stagnation, unsteady flow rates	Assessment	TDC HPC (R2A) FISH	TOC	No guideline value No definition
Lautenschlager et al., 2010	Household effect	Temporal	No	All bacteria	Suspended	-	Stagnation: residence time, temperature,	Assessment	TCC and ICC total/free ATP HPC DGGE	AOC[4]	No guideline value No definition

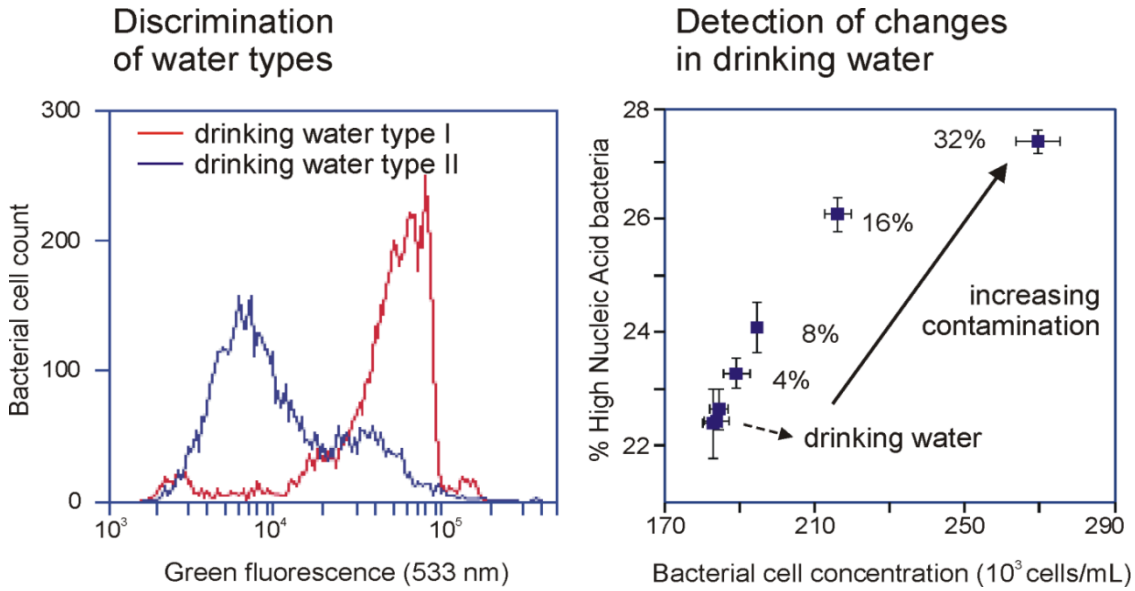
Authors	**Focus**					**Considered microbial growth controlling factors**		**Approach and methods**			**Guideline value? Definition BS?**
	Study focus	Temporal/ spatial variations?	Residual disinfectant ?	Type of organisms	Organisms location	nutrients	Other factors	Approach microbial study	Microbial analysis	Other methods	
Lautenschlager al., 2013	Treated water + distribution effect	Spatial + temporal	No	All bacteria	Suspended	BOM	Residence time, temperature	Assessment	TCC and ICC HPC total/free ATP DGGE Pyrosequencing	AOC^{4}	No guideline value Definition Nr. 4
Nescerecka 2014	Treated water + distribution effect	Spatial + temporal (short-term)	Yes	All bacteria	Suspended	BOM	Disinfectant concentration, residence time	Assessment	TCC and ICC total/free ATP HPC (PCA)	DOC, AOC^{1}	No guideline value No definition
Pinto et al., 2012, 2014	Treated water + distribution effect	Spatial + temporal (seasonal)	Yes	All bacteria	Suspended	BOM, NH^{4+}, PO_4^{3-}, SO_4^{2-}	Distance, temperature, pH	Assessment	Pyrosequencing	-	No guideline value No definition
Liu et al., 2013a, b, 2014	Treated water + distribution effect	Spatial + temporal (seasonal)	No	All bacteria	Suspended + fixed (biofilms, sediments, suspended solids)	BOM	Pipe material, disinfectant concentration and type, distance, temperature	Assessment	HPC (R2A) TCC total/free ATP *Aeromonas* Pyrosequencing	-	No guideline value No definition
Wang et al., 2014	Treated water + distribution effect	Spatial	Yes	All bacteria, + eukaryotes, focus on opportunistic pathogens	Fixed (biofilms)	-	Pipe material disinfection concentration and type, residence time	Assessment	Pyrosequencing T-RFLP	-	No guideline value No definition

METHOD DEVELOPMENT



Chapter 3

Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method



This chapter has been published as: E.I. Prest, F. Hammes, S. Kötzsch, M.C.M van Loosdrecht, J.S. Vrouwenvelder (2013). Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. *Water Research* 47 (19), 7131-7142.

Abstract

Flow cytometry (FCM) is a rapid, cultivation-independent tool to assess and evaluate bacteriological quality and biological stability of water. Here we demonstrate that a stringent, reproducible staining protocol combined with fixed FCM operational and gating settings is essential for reliable quantification of bacteria and detection of changes in aquatic bacterial communities. Triplicate measurements of diverse water samples with this protocol typically showed relative standard deviation values and 95% confidence interval values below 2.5% on all the main FCM parameters. We propose a straightforward and instrument-independent method for the characterization of water samples based on the combination of bacterial cell concentration and fluorescence distribution. Analysis of the fluorescence distribution (or so-called fluorescence fingerprint) was accomplished firstly through a direct comparison of the raw FCM data and subsequently simplified by quantifying the percentage of large and brightly fluorescent high nucleic acid (HNA) content bacteria in each sample. Our approach enables fast differentiation of dissimilar bacterial communities (less than 15 minutes from sampling to final result), and allows accurate detection of even small changes in aquatic environments (detection above 3% change). Demonstrative studies on (a) indigenous bacterial growth in water, (b) contamination of drinking water with wastewater, (c) household drinking water stagnation and (d) mixing of two drinking water types, univocally showed that this FCM approach enables detection and quantification of relevant bacterial water quality changes with high sensitivity. This approach has the potential to be used as a new tool for application in the drinking water field, e.g. for rapid screening of the microbial water quality and stability during water treatment and distribution in networks and premise plumbing.

3.1 Introduction

Flow cytometry (FCM) was introduced in the field of aquatic microbiology as a cultivation-independent tool to enumerate and assess suspended bacterial communities (Robertson and Button, 1989; Vives-Rego et al., 2000; Gasol and Del Giorgio, 2000). FCM used in combination with fluorescent stains reveals important information such as total cell concentration, bacterial viability, bacterial characteristics or bacterial identity in water samples (Veal et al., 2000; Wang et al., 2010; Hammes et al., 2011). A wide range of fluorescent stains and fluorescently labeled compounds is available, targeting a specific characteristic or component of the cell, such as membranes, nucleic acids or proteins (Vives-Rego et al., 2000; Gasol and Del Giorgio, 2000).

During the past decade, FCM has emerged as a tool for rapid assessment of drinking water quality (Hoefel et al., 2003, 2005; Phe et al., 2005; Berney et al., 2008). One of the most straightforward applications, namely the determination of total cell counts (TCC) using FCM in combination with nucleic acid targeting stains, was shown repeatedly to be useful for monitoring of treatment processes or detection of changes in drinking water quality (Hammes et al., 2008; Lautenschlager et al., 2010; Vital et al., 2012; Ho et al., 2012; Kötzsch and Egli, 2013). The TCC method was recently standardized via an inter-laboratory ring trial and officially accepted as a guideline method for drinking water analysis in Switzerland (SLMB, 2012). However, the capability of the TCC method extends beyond cell counting. Each measurement provides multi-variable information at single cell level by recording the fluorescence intensity and light scatter signals, thereby creating a so-called “fingerprint” of the bacterial community that can be analyzed in different ways (Hammes et al., 2012; De Roy et al., 2012). The fingerprint information can be valuable in practice for detection of changes that are not reflected in the cell concentration.

Some fluorescent stains such as DAPI or SYBR® Green I bind preferentially to nucleic acids (Robertson et al., 1989; Zipper et al., 2004). The fluorescence intensity of such stains is directly related to the amount of nucleic acids present in the treated sample (Zipper et al., 2004) when the nucleic acids are easily accessible by the stain (e.g. free DNA solutions). In theory, the fluorescence intensity recorded for one labeled bacterial cell should be directly related to its nucleic acid content, which is dependent on both the type of bacteria as well as its physiological state (Müller et al., 2000; Günther et al., 2008). As an example, increased fluorescence intensity was recorded during regrowth of pure lab-cultured bacterial communities due to duplication of DNA (Müller et al., 2000). When considering natural samples, indigenous bacterial cells have widely different amounts of nucleic acids (different genome sizes) and can differ in their physiological states, which will have an impact on the

fluorescence intensities when bacteria are stained. Because of this heterogeneity, analysis of indigenous communities generates a unique fluorescence distribution or fluorescence fingerprint, made of multiple clusters of bacteria, distinguished from each other by differences in fluorescence intensity (Wang et al., 2008; Günther et al., 2008).

Here we argue that the quantitative use of both cell concentration data and fluorescence distribution information is only meaningful if a highly reproducible labeling of the cells is achieved during staining. While this notion seems self-evident, an overview of current literature suggests that staining procedures are not necessarily applied (or at least described) in a reproducible manner in practice. Multiple studies do not take in account critical aspects of staining such as temperature, incubation time and FCM settings, and particularly the concept of a stringent reproduction of staining and measurement protocols for allowing correct comparison of samples (e.g. Gasol et al., 1999; Lebaron et al., 2001; Hoefel et al., 2005; De Roy et al., 2012) (discussed in detail below).

The aim of the present study was to determine (i) whether flow cytometric methodology can be improved by using a reproducible staining procedure and (ii) whether a simple data collection and analysis strategy, using the combination of cell concentrations and fluorescence fingerprints, is suitable for sensitive detection of changes in the microbial communities of natural aquatic environments in general, and specifically for drinking water (distribution networks). Studies were done to assess whether flow cytometry can sensitively detect and quantify (a) regrowth, (b) contamination of drinking water with wastewater, (c) household stagnation and (d) mixing of two drinking water types. Potential practical applications of flow cytometry for the water field are discussed.

3.2 Materials and methods

3.2.1 Staining protocol

Water samples were stained according to the standardized protocol proposed in the Swiss guideline for drinking water analysis (SLMB, 2012). In short, samples (500 µL) were pre-heated to 35 °C (5 minutes) and then stained with 10 µL mL^{-1} SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), and incubated in the dark for 10 minutes at 35 °C before measurement. Where necessary, samples were diluted just before measurement in filtered (0.22 µm; Millex-GP, Millipore) bottled mineral water (EVIAN, France), so that the bacterial concentration measured with the flow cytometer (FCM) was always less than $2x10^5$ cells mL^{-1}. Unless stated otherwise, this staining protocol was strictly applied in order to achieve comparable data.

3.2.2 Flow cytometric measurements

Measurements were 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 and forward scattered light intensities were collected as well. The FCM is equipped with volumetric counting hardware, calibrated to measure the number of particles in 50 µL of a 500 µL sample. Measurements were performed at pre-set flow rate of 35 µL min^{-1}. All data were processed with the BD Accuri CFlow® software, and electronic gating was used to separate positive signals from instrument and water sample background. A threshold value of 500 was applied on the green fluorescence channel (FL1). Unless stated otherwise, the instrument settings and electronic gates were kept the same for all samples in order to achieve comparable data.

3.2.3 Gating strategy

Density plots of green fluorescence (FL1; 533 nm), and red fluorescence (FL3; >670 nm) allowed for optimal distinction between the stained microbial cells and instrument noise or sample background (Figure 3.1A, Hammes and Egli, 2005, 2010c). An electronic gate on the green/red fluorescence density plot was used to select the signals of bacteria (Figure 3.1A) and the selected data from the bacterial gate were subsequently visualized on a green fluorescence histogram for further analysis (Figure 3.1B). The green fluorescence channel was chosen, since it collects fluorescence at 533 nm, which is closest to the emission maximum of SYBR® Green I (520 nm). The typical two-cluster pattern of low (LNA) and high (HNA) nucleic acid content bacteria, usually observed in natural samples (Lebaron, 2001; Wang et al., 2009; Vila-Costa et al., 2012), was used in the gating strategy by applying additional gates on the green fluorescence histogram to distinguish LNA and HNA communities (Figure 3.1B). The exact same gates and gating strategy was applied for all samples in the form of a fixed template to allow direct comparison between measured samples (SLMB, 2012).

3.2.4 Comparison and quantification of fluorescence distribution

The green fluorescence intensity histogram data, generated as a result of SYBR Green I staining of the bacterial cells (Figure 3.1B), was used as the main FCM “fingerprint” for the bacterial community. For a first comparison, the available FCM software (BD Accuri CFlow®) was used to directly overlay raw data from different samples. Secondly, gates applied on the green fluorescence histogram (Figure 3.1B) were used to calculate the number of HNA cells and express it as percentage of the total cell number (LNA and HNA cells).

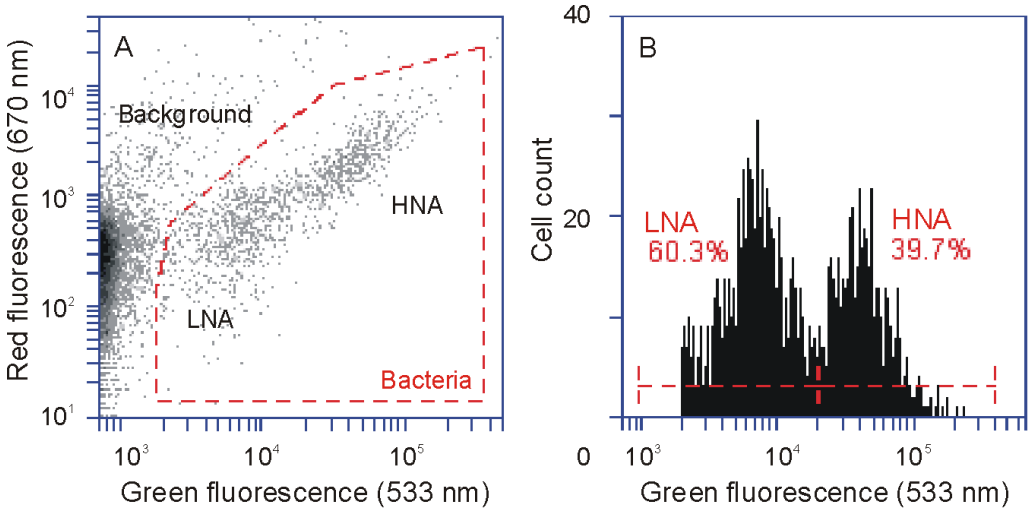


Figure 3.1. Data collection and gating strategy. (A) Selection of bacteria in a typical drinking water sample using a fixed gate (red dotted line) on the green/red fluorescence density plot (for exclusion of background). (B) Selection of low and high nucleic acid (LNA and HNA) content bacterial clusters on the resulting green fluorescence histogram of selected data (from "Bacteria" gate in Figure 3.1A). The LNA and HNA percentages indicate the distribution of the overall bacterial community into these two clusters. Exactly the same gates were used for all measurements.

The gates for LNA and HNA content cells were set based on experimental data from the present study (discussed below) and previous experience (Wang et al., 2009). The distribution between LNA and HNA, expressed as the percentage of HNA was chosen as a simplified, single value to describe the fluorescence fingerprint of the community. Straightforward comparison of data from different water samples was subsequently made based on the measured total cell concentration and the percentage of HNA cells.

3.2.5 Repeatable staining and analysis of aquatic bacteria

The repeatability of the FCM method was assessed by separately staining and measuring 10 discrete sub-samples of the same natural drinking water community (non-chlorinated tap water, Dübendorf, CH). The staining protocol, FCM settings and gating strategy described in section 3.2.1 to 3.2.3 were strictly applied for all sub-samples. Subsequently, the influence of two critical staining parameters, namely sample staining temperature and staining time, was studied by deliberate variations from the staining protocol. Aliquots of a same tap water sample (non-chlorinated tap water, Dübendorf, CH) were stained with 10 µL mL^{-1} SYBR® Green I at respectively 4, 22 and 35 °C. Each sub-sample was pre-heated or pre-cooled to the experimental temperature before addition of the stain. For each temperature, the staining of triplicate aliquots was tested after 5, 10 and 15 minutes of staining time respectively.

3.2.6 Discrimination of waters of different origins

Water samples were collected from seven separate drinking water distribution networks located in the Canton of Zürich (CH), three different commercial brands of bottled mineral water (Evian, Vittel, OK), three separate river/creek samples (Chriesbach river and Glatt river, Dübendorf, CH, Schachenbach, Bonstetten, CH), three separated rainwater collection/treatment ponds (Dübendorf, CH) and one wastewater treatment plant effluent (Dübendorf, CH). The staining protocol, FCM settings and gating strategy described in sections 3.2.1 to 3.2.4 were strictly applied for all sub-samples, with the exception that for a direct comparison of the fluorescence fingerprints, the measurement was stopped when 6000 cells were counted within the defined bacterial gate (Figure 3.1A).

3.2.7 Detection of changes in aquatic bacterial community

3.2.7.1 Contamination of drinking water by wastewater

Wastewater treatment effluent was used to simulate a contamination event in drinking water. Wastewater effluent was collected from a treatment plant in Horstermeer (NL), while drinking water (without residual disinfectant) was collected from a household tap in Haarlem (NL). Both the wastewater and tap water samples were first analyzed to determine their total cell concentration and fluorescence fingerprints (as described in sections 3.2.1 to 3.2.4). The wastewater sample was then spiked into sub-samples of the tap water in such a way that respectively 1, 2, 4, 8, 16 and 32% of the total cell concentration of the spiked sample were originating from the wastewater sample. Triplicate aliquots were then stained and analyzed as described in sections 3.2.1 to 3.2.4.

3.2.7.2 Mixing of two drinking water types

Two different drinking water samples were used to simulate changes in a mixing zone in a drinking water distribution network. Both drinking waters were prepared from surface water at two different locations (Leiduin and Haarlem, NL) and contained no residual disinfectant. Both samples were first analyzed to determine their total cell concentration and fluorescence fingerprint (as described above). The samples were then mixed with each other into 6 sub-samples in such a way that respectively 0, 20, 40, 60, 80, and 100% of the total cell concentration of the mixed sample were originating from one of the original samples. Triplicate samples were then stained and analyzed as described in sections 3.2.1 to 3.2.4.

3.2.7.3 Overnight stagnation of drinking water in premise plumbing

A household tap (Dübendorf, CH), receiving non-chlorinated drinking water, was opened after overnight stagnation (14 hours) and the flowing water was sampled immediately after opening the tap, after 30 s, 1 min, and at regular intervals during 6 hours. The flow rate of the constantly running tap was about 180 L h^{-1}. Each sample was stained and analyzed as described in sections 3.2.1 to 3.2.4.

3.2.7.4 (Re)growth of indigenous bacterial communities

River water (Chriesbach, Dübendorf, CH) was diluted 10-fold in filtered (0.22 µm) river water (same source) to a final cell concentration of $3x10^5$ cells mL^{-1}. Approximately 0.5 mg L^{-1} assimilable organic carbon (AOC) was added as nutrient (Luria-Bertani (LB) medium; 10000x final dilution) (Hammes et al., 2010d; Vital et al., 2010). A 250 mL aliquot of this sample was incubated at 30 °C for 8 hours with continuous stirring. Samples (3 x 500 µL) were collected at 1 h intervals and then stained and analyzed as described in sections 3.2.1 to 3.2.4.

An overview of the experimental conditions is shown in Table 3.1.

Table 3.1. Experimental conditions of the flow cytometry studies on change detection and characterization of bacterial communities in water.

Study	Staining time (min)	Staining temperature (°C)	Water types studied	Figure	Results section
Repeatable staining and analysis of aquatic bacteria					
- Effect standardized staining protocol	10	35	Drinking water	3.2	3.3.1.1
- Effect staining protocol variation	5, 10, 15	4, 22, 35	Drinking water	3.3	3.3.1.2
Discrimination waters from different origin	10	35	Drinking water, bottled water, river water, pond water, wastewater	3.4	3.3.2
Detection of changes in aquatic bacterial communities					
- Contamination of drinking water by wastewater	10	35	Drinking water, wastewater treatment effluent	3.5	3.3.3.1
- Mixing of two drinking water types	10	35	Drinking water from two different sources	3.6	3.3.3.2
- Overnight stagnation of drinking water in premise plumbing (household)	10	35	Drinking water sampled from tap in time	3.7	3.3.3.3
- Regrowth of indigenous bacterial community	10	35	River water with dosed substrate	3.8	3.3.3.4

3.3 Results

3.3.1 Repeatable staining and analysis of aquatic bacteria

3.3.1.1 Repeatable results with a strict staining protocol

Staining and analyzing indigenous bacterial communities according to a strict protocol provides highly repeatable data on cell concentrations and on fluorescence distribution (Figure 3.2). The fluorescence distribution histograms (533 nm) obtained after analysis of ten sub-samples from the same water were visually identical (Figure 3.2A). Moreover, quantitative analysis of the raw data resulted in relative standard deviation (RSD) values of respectively 1.15% (total cell counts) and 1.53% (percentage of HNA cells; %HNA), and in 95% confidence intervals of 0.71% (total cell counts) and 0.95% (%HNA) (n = 10) (Figure 3.2B). In fact, for all measurements described in the present study (50 samples), the RSD on triplicate samples was on average below 2.5% in cell counts and %HNA cells, and the 95% confidence interval values were 2.5% on average for both parameters. Figure 3.2C demonstrates how identical samples would cluster together on a combined plot of cell concentration and fluorescence distribution, which can subsequently be used for comparing different water samples.

3.3.1.2 Adverse results from staining protocol variation

Deviation from a fixed staining protocol negatively impacts the accuracy of the obtained data (Figure 3.3). Visual comparison of the fluorescence distribution histograms obtained after measurement of the same sample stained for 10 minutes at 4, 22 and 35 °C (Figure 3.3A) and quantification of the data (Figure 3.3B and 3.3C) show that the fluorescence distribution and cell concentrations are strongly affected by variations in both staining temperature and staining time. Significantly lower cell concentrations were measured when staining was performed at a low temperature (4 °C), opposed to high temperature (35 °C). For each temperature, increasing cell concentrations were found with increasing staining time, although the impact of staining time was less pronounced than that of staining temperature (Figure 3.3B and 3.3C). The variations in staining protocols clearly show that reliable and comparative results can only be obtained when using an accurate and reproducible staining protocol as shown in Figure 3.2. Therefore, a fixed staining protocol (section 3.2.1) and fixed analysis and gating settings (sections 3.2.2 and 3.2.3) were applied for all samples to maintain consistency in the analysis and to allow direct comparison between different samples.

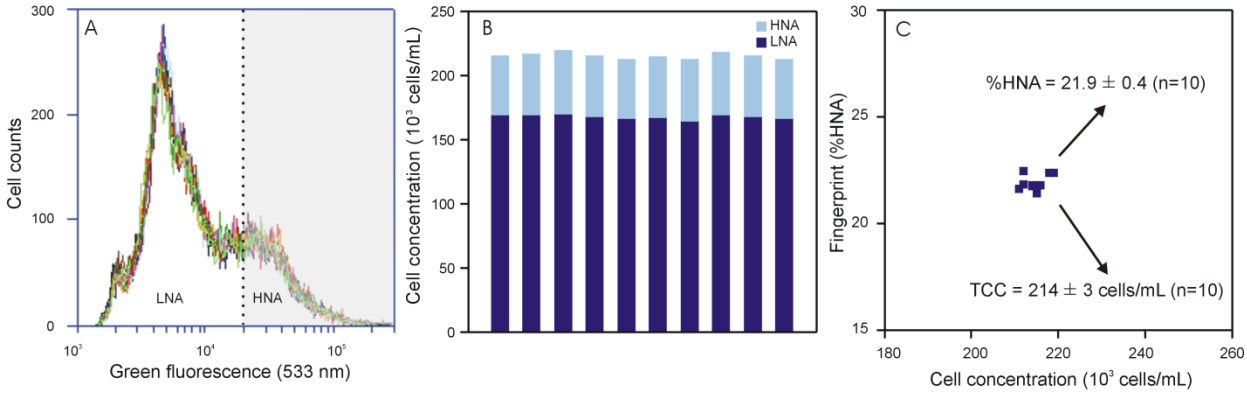


Figure 3.2. High repeatability in (A) fluorescence distribution and (B) cell concentrations resulting from the flow cytometric measurement of 10 sub-samples of a same drinking water using a fixed staining and analysis protocol. The fluorescence distribution and cell concentration information can be combined (C) for direct comparison of different water samples. Different colored lines in Figure A represent raw data from individual measurements and the vertical dotted line indicate the fluorescence intensity used for separation of LNA and HNA cells. HNA = high nucleic acid content bacteria; LNA = low nucleic acid content bacteria; TCC = total cell concentration.

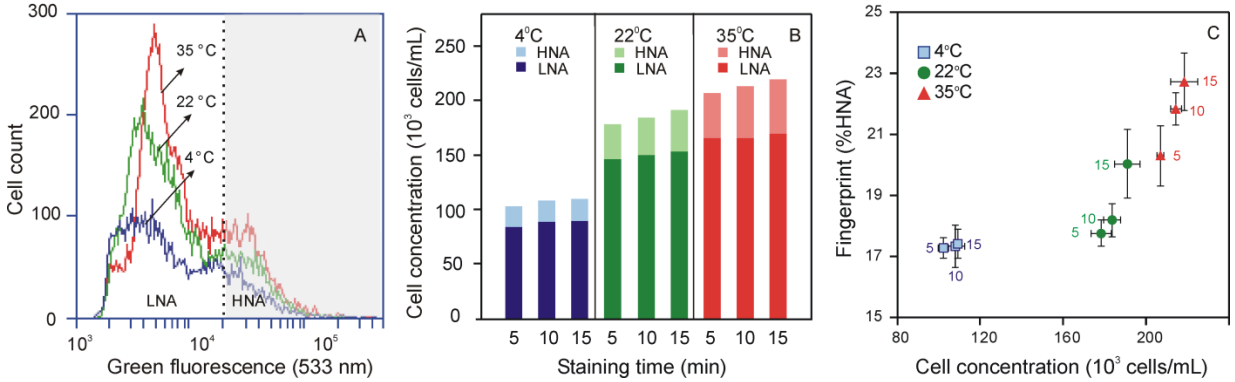


Figure 3.3. Adverse effects of non-optimal staining procedures on the repeatability of FCM measurements, demonstrated with sub-samples of the same water sample. (A) Direct comparison of green fluorescence histograms obtained after staining the subsamples at different temperatures (4, 22, 35 °C) and at a fixed staining time (10 min). (B) Combined effects of staining temperature and contact time on cell concentrations of total, LNA and HNA cells. (C) Combined results of total cell concentration and %HNA, where numbers next to the markers indicate the incubation time (min) of each sample, and error bars indicate the error on triplicate samples (n = 3).

3.3.2 Discrimination of waters from different origin

When using a reproducible staining protocol, water samples from different origins (tap, bottled, river, pond and waste- waters) differed considerably in terms of fluorescence distribution and cell concentrations (Figure 3.4). For example, bottled water had a higher percentage of HNA cells compared to tap water, while drinking water samples taken from different distribution networks had clear differences in both cell concentrations (ranging from $1x10^3$ to $4x10^5$ cells mL^{-1}) and fluorescence fingerprints in most cases (Figures 3.4 and S3.1 in supplementary information).

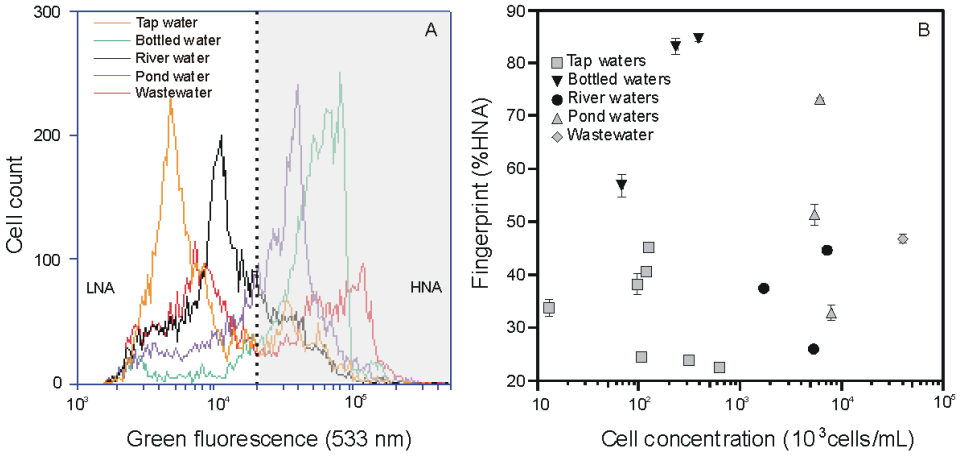


Figure 3.4. Comparison of different water samples based on fluorescence distribution and total cell concentration. (A) Overlay of fluorescence distribution of one example of each water type, where the number of measured bacterial cells were kept constant to allow direct comparison of the flow cytometric fingerprints. (B) Combined results of cell concentration and %HNA. Error bars indicate the error on triplicate samples (n = 3).

However, in some cases samples had very similar cell concentrations and fluorescence fingerprints, and were therefore difficult to distinguish from one another (Figure S3.1 in supplementary information). Cell concentrations of drinking waters (both tap and bottled waters) were in the range of 10^3 to 10^5 cells mL^{-1}, while higher cell concentrations were recorded in river, pond and waste-waters (one to two log unit higher values). The fluorescence fingerprints of representative examples of each water type, and the results of additional drinking water samples are given in Figures S3.1 and S3.2 in supplementary information. Most water samples displayed the typical LNA and HNA bacterial clusters commonly observed with FCM in aquatic samples, although a small percentage of samples (e.g. Figure S3.2E in supplementary information) showed no such discrimination, and some samples were dominated by one of the clusters (e.g. Figure S3.2B and S3.2C). The separation between the LNA and HNA clusters was approximately at the same green fluorescence intensity (for our flow cytometer around 2 x 10^4 a.u.) for all samples, which was the experimental basis for selecting the LNA and HNA gating positions (section 3.2.3). While the differentiation of samples based on only cell concentrations and the %HNA is a rather simplistic approach, it should be noted that all 17 water samples shown in Figure 3.4 were clearly and significantly distinguishable from one-another.

3.3.3 Detection of changes in aquatic bacterial communities

Laboratory scale studies have been performed to demonstrate the effect of (a) contamination of drinking water by wastewater, (b) mixing of two drinking water types, (c) overnight stagnation of drinking water in premise plumbing (household), and (d) (re)growth of indigenous bacterial communities in drinking water.

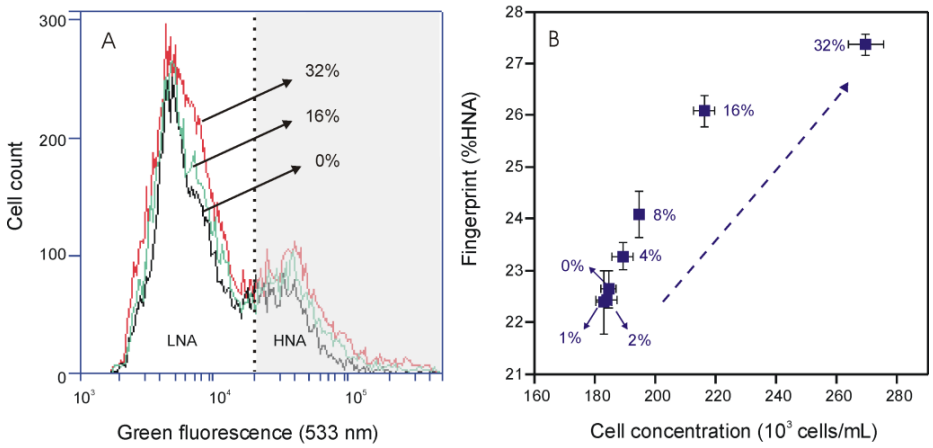


Figure 3.5. Detection of tap water contamination with wastewater by fluorescence distribution and total bacterial cell concentration. (A) Direct comparison of the green fluorescence histograms of the tap water and the tap water contaminated with 16% and 32% wastewater bacteria. (B) Combined results of cell concentration and %HNA, where the numbers next to the markers indicate the percentage of total cells originating from the contaminating wastewater, and error bars indicate the error on triplicate samples (n = 3). Arrow on figure B indicates increasing contamination.

3.3.3.1 Contamination of drinking water by wastewater

Contamination of drinking water by wastewater was detected by changes in both the total cell concentration and the percentage of HNA cells (%HNA) when more than 2% of the total cells in the mixed sample originated from the wastewater (Figure 3.5). A gradual increase in cell concentration was observed in the drinking water with increasing contamination level (increased percentage of wastewater). This increase in cell concentration was linked to a gradual change in the fluorescence fingerprint with an increasing percentage of the HNA cells. The overlay of the green fluorescence histograms of samples with different levels of contamination (Figure 3.5A) suggests that visual observation of the fingerprints does not allow an easy detection of change when the level of contamination is below 16%. Therefore, the quantification of these fingerprints (in our study expressed as %HNA cells) is essential for sensitive detection of change (Figure 3.5B).

3.3.3.2 Mixing of two drinking water types

A particularly valuable application of the standardized flow cytometric method could be the detection of mixing zones in water distribution networks. Mixing of two drinking waters from different locations (referred to as water type 1 and type 2) was tested in different proportions and could be detected in all the mixed samples (Figure 3.6). The two drinking waters were both prepared from surface water and had similar characteristics. However, water type 1 had a slightly lower cell concentration ($1.5x10^5$ cells mL^{-1}) than water type 2 ($1.8x10^5$ cells ml^{-1}) and had a lower percentage of HNA cells (14%, compared to 24% in water type 2).

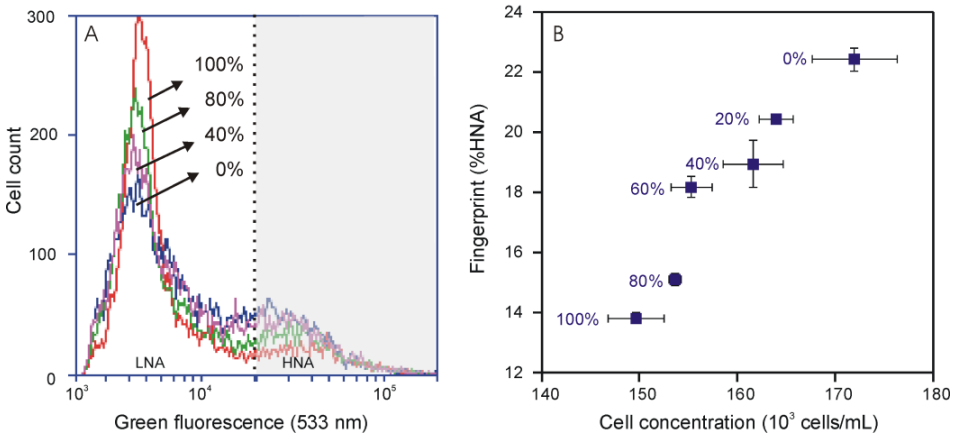


Figure 3.6. Detection of the mixing of two different drinking water samples by fluorescence distribution and total cell concentration. (A) Direct comparison of the green fluorescence histograms of the mixed water samples in different proportions (0, 40, 80 and 100% of drinking water type 1) show that the number of LNA cells increases when the proportion of water type 1 increases. (B) Combined results of cell concentration and %HNA. Numbers next to the markers indicate the percentage of water type 1. Error bars indicate errors on triplicate samples (n = 3).

Increased proportions of the water type 2 in water type 1 therefore resulted in a gradual change in fluorescence distribution visually observed (Figure 3.6A) through a clear increase in cell count in the LNA fraction and quantitatively translated as a decrease in the percentage of HNA cells. A gradual decrease in cell concentration was simultaneously observed (Figure 3.6B).

3.3.3.3 Overnight stagnation of drinking water in premise plumbing

Changes in fluorescence distribution and cell concentration were clearly observed in drinking water that stagnated overnight in household pipes. Water was sampled after overnight stagnation from the same household tap, immediately after opening the tap, after 30 s, 1 min, and at regular intervals during 6 hours. The first water sample displayed a significantly higher cell concentration than all following samples (Figure 3.7A and 3.7C). The cell concentration dropped dramatically from $5x10^5$ to $1x10^5$ cells mL^{-1} within the first 30 seconds of flushing (i.e. the first 1.5 L) and decreased gradually over one hour (200 L) until it reached about $8x10^4$ cells mL^{-1}. This cell concentration was maintained over the next 5 hours (about 1000 L) with less than 2% variation. The large difference in cell concentration between the first sample after tap opening and all other samples was clearly related to a different fingerprint, with a significantly higher HNA cell percentage (80% instead of 34 to 39% in all other samples; Figure 3.7B and 3.7C). When the cell concentration stabilized, some variation was still observed in %HNA (4% variation, Figure 3.7B and 3.7D), showing the additional value of using the combination of the two parameters.

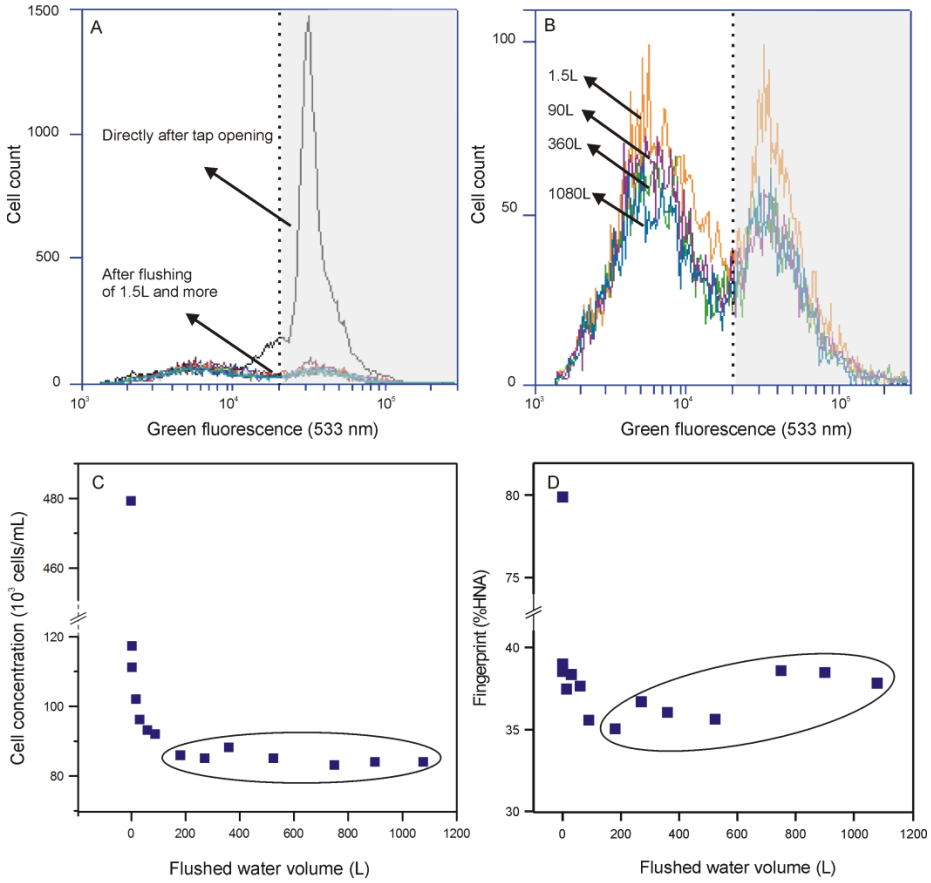


Figure 3.7. Drinking water tap flushing after overnight stagnation. Comparison of green fluorescence histograms of samples taken at different time intervals: (A) directly after tap opening and after flushing of at least 1.5 L; (B) after flushing of 1.5, 90, 360 and 1080 L. Development with flushed water volume of (C) total cell concentration and (D) %HNA cells. Both cell concentrations and fingerprints showed a distinct change in microbial water quality in the first 1.5 liter after tap opening. Cell concentration stabilization in time is shown in an oval in figure C. The corresponding points are indicated in an oval in figure D.

Both cell concentrations and fingerprints indicate that water stagnation in the household induced changes in drinking water microbial characteristics. The strong increase in cell concentration during overnight stagnation suggests that the change is linked to either bacterial regrowth or detachment of the biofilm attached to the pipe walls at household level or both regrowth and detachment.

3.3.4 (Re)growth of indigenous bacterial community

A (re)growth test was performed with river water to determine the effect of (re)growth on the FCM fingerprints. Figure 3.8B shows that the increase in total cell concentration over time correlated with an increase in the HNA cell concentration. Comparison of the green fluorescence histograms obtained in time (Figure 3.8A) show a clear change in the HNA cell fraction through the course of the experiment (Figure 3.8B), while the LNA fraction remained fairly stable.

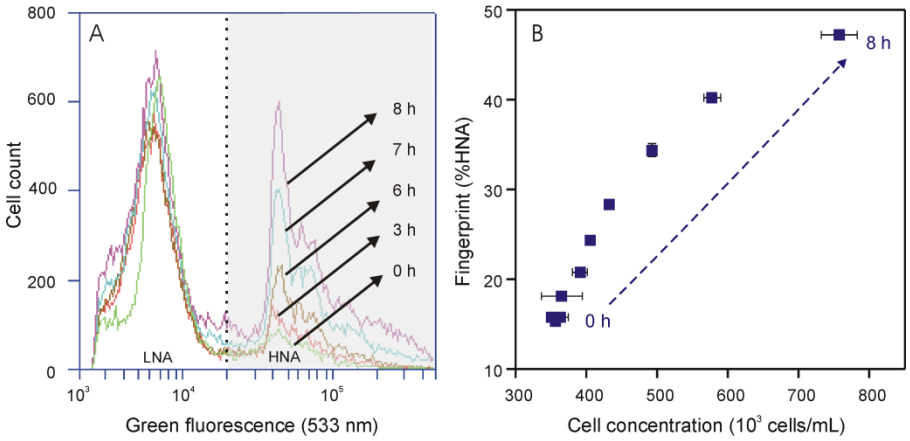


Figure 3.8. (Re)growth detection of indigenous bacteria in river water by fluorescence distribution and total cell concentration. (A) Direct comparison of the green fluorescence histograms of samples taken at 0, 3, 6, 7 and 8 hours from start of the experiment showing an increase in HNA cells. (B) Combined results of cell concentration and %HNA. The dashed arrow direction indicates increasing incubation time. Error bars indicate errors on triplicate samples.

3.4 Discussion

Flow cytometry (FCM) was proposed previously as an alternative tool to cultivation-dependent heterotrophic plate counts (HPC) for monitoring of microbial drinking water quality (Hoefel et al., 2003; Hammes et al., 2008; Vital et al., 2012). In the present study, a FCM approach was developed and evaluated with respect to the characterization of indigenous aquatic bacterial communities in real water samples and the ability to detect changes in such communities that are relevant for drinking water monitoring. The method, partially based on the recently standardized Swiss guideline method (SLMB, 2012), uses reproducible staining and analysis of bacteria with a common nucleic acid stain to obtain (i) the total cell concentration and (ii) fluorescence intensity distribution (or fluorescence fingerprint) of the water sample. A fast and instrument-independent approach was proposed for straightforward and quantitative comparison of the fluorescence fingerprints from different samples, based on the differentiation between the low- and high nucleic acid (LNA and HNA) content bacterial clusters (Figure 3.1B; Figure 3.4 and S3.2 in supplementary information). We demonstrated that when a reproducible staining and analysis procedure is strictly followed for all samples, the FCM method was (i) highly repeatable (Figure 3.2), (ii) allowed discrimination between water samples from different origins (Figures 3.4, S3.1 and S3.2 in supplementary information) and (iii) enabled detection of small variations in bacterial communities due to e.g. contamination (Figure 3.5), mixing of two drinking water types (Figure 3.6) or (re)growth (Figures 3.7 and 3.8).

3.4.1 Need for a reproducible staining and analysis protocol

FCM total cell count (TCC) analysis is a relatively novel method, used for diverse applications including analysis of seawater (Gasol and Del Giorgio, 2000), wastewater (Günther et al., 2008; Muela et al., 2011) and drinking water (Hammes et al., 2008). As a consequence, a range of different staining protocols can be found in literature, using different stains, stain concentrations, incubation times and incubation temperatures (Gasol et al., 1999; Lebaron et al., 2001; Berney et al., 2008; Hammes et al., 2012). Additionally, nearly no standardized or controlled approach is used with respect to instrument settings and data analysis (i.e. FCM gating strategies) (Hammes and Egli, 2010c). Nonetheless, it is logic that correct comparison of samples requires reproducible methodology. In this regard, an inter-laboratory ring trial showed that different instrument brands and users deliver comparable results when the same staining and measurement procedures were strictly applied (SLMB, 2012). However, the importance of the reproducibility of the staining protocol on a same instrument had not been studied or demonstrated in detail until now. Our study clearly showed that the staining procedure (staining incubation time and temperature) is critical for both total cell concentration measurements and fluorescence fingerprints and that a clearly defined protocol, followed precisely, is needed for a meaningful comparison of different water samples and for sensitive detection of changes in bacterial communities (Figures 3.2 and 3.3). A striking example is the incubation temperature. We showed that particularly colder staining temperatures adversely affect the staining reaction (Figure 3.3), supporting previous data in this regard (Hammes et al., 2012). While fixing the staining temperature may appear extremely logic, nearly all previously published studies described staining at either unstated temperatures (e.g. Gasol et al., 1999) or at “room temperature” (e.g. Lebaron et al., 2001), which can be a source of considerable variations between different seasons, locations or times of the day. This effect is aggravated by the fact that water samples are typically transported and stored at < 5 °C before measurement (Lebaron et al., 1998; Hoefel et al., 2005; Berney et al., 2008). Staining the sample at room temperature (c.a. 22 °C) directly following cold storage and without pre-heating would result in a considerably colder, and thus inaccurate, staining process (Figure 3.3). The final staining protocol in the present study comprises 5 min pre-incubation (35 °C), followed by staining and a further 10 min incubation (35 °C). The rationale behind these conditions was as follow: The pre-incubation step warms the sample to the selected staining temperature before the stain is added to ensure a homogenous staining reaction. The pre-incubation time (5 min) was selected based on the time it takes for 500 µL of cold samples (5 °C) to reach 35 °C (data not shown), and should be tested and adapted for larger sample volumes. The incubation temperature was selected from specific experiments (e.g. Figure 3.3, present manuscript), previous data (Hammes et al., 2012) and the Swiss guideline method (SLMB,

2012). The rationale is that higher temperatures lead to improved staining, but too high temperatures should be avoided due to potential cell damage. The stain incubation time (10 min) was selected based on own experiments (e.g. Figure 3.3) and previous studies (SLMB, 2012; Hammes et al., 2012), to ensure a stable fluorescence pattern. However, it is important to note that the purpose of the present study was not to optimize the TCC staining protocol further with respect to incubation time and temperature, as this was already done in a number of studies and "optimal" staining conditions may arguably differ for different microbial communities. Here we simply demonstrate the critical importance of using the exact same staining and analysis protocol for all samples in order to obtain a sensible and correct comparison. This does not only affect TCC quantification; a sensitive FCM fingerprinting approach can only be reliable when it is coupled to a precise and reproducible staining protocol.

3.4.2 The added value of fluorescence fingerprints

Bacterial cell concentration measurements with FCM were previously shown to be sensitive, with a quantification limit as low as 100 – 1000 cells mL^{-1} (Hammes et al., 2008; Hammes et al., 2012). However, the sensitivity of FCM to detect changes in cell concentrations is not based on absolute values, but rather relative to the initial cell concentration of the analyzed water. The present study showed that a change in cell concentration larger than 3%, thus slightly above than the average 95% confidence interval, could be detected (Figures 3.2 and 3.5). However, in field situations, changes in the microbial community might occur without necessarily being reflected in the cell concentration (e.g. community turnover resulting from continuous attachment and detachment of cells from the pipe biofilm during distribution of drinking water). It has been suggested previously that such changes can be detected in the FCM patterns (De Roy et al., 2012). Therefore, we introduced a fingerprinting approach in order to gain complementary information to the cell concentration data. For the fingerprinting approach, fluorescence distribution was preferred to scattered light parameters, based on a better differentiation between signal and background in the case of small water bacteria (Hammes and Egli, 2010c; De Roy et al., 2012; Figure S3.3 in supplementary information), and green fluorescence was selected specifically, since it is detected closest to the SYBR Green I emission maximum. The obtained fluorescence raw data (e.g. Figure 3.4A) show that even if different water samples are normalized with respect to the cell concentrations, they often demonstrate different fluorescence distributions, allowing characterization and differentiation of samples using these fingerprints. Figures 3.5 to 3.8 demonstrate clearly that changes in samples are also clearly reflected in the FCM fingerprints. It is logic that the FCM fingerprints reflect microbial changes in water samples: in the present study, the fingerprints were based on the fluorescence

intensity of individual cells, which in turn relates to the concentration of SYBR Green I molecules bound to nucleic acids in each cell. Hence, in case the staining protocol allowed complete dye penetration into cells, the fluorescence intensity should relate to the concentration of DNA and RNA in cells. The latter is dependent on both the type of bacteria as well as the physiological state of cells (Müller et al., 2000; Günther et al., 2008; Vila-Costa et al., 2012), and the principle has been used in FCM analysis previously to distinguish between cells with high and low nucleic acid content (Lebaron et al., 1998; Wang et al., 2009; Vila-Costa et al., 2012). Moreover, it has been shown that changes in flow cytometric data often correspond to changes in the microbial community composition assessed with molecular fingerprinting tools (De Roy et al., 2012; Lautenschlager et al., 2013; Koch et al., 2013b).

3.4.3 A straightforward approach to quantify FCM fingerprints

As seen from Figure 3.4, the fluorescence fingerprints of samples contain information that is not necessarily easy to describe or quantify, particularly when changes in samples are small (Figure 3.5 and 3.6). Previous approaches have used principle component analysis or other statistical means to assess FCM patterns (Pomati et al., 2011; Boddy et al., 2001; De Roy et al., 2012), and recently Koch and co-workers (2013a) described an advanced fingerprinting method for indigenous microbial communities based on the combination of scatter and fluorescence signals. While this type of approach can indeed provide detailed information, it often requires complex additional data analysis steps that render the fingerprint information difficult to use for practical applications (e.g. rapid drinking water monitoring), or incompatible for comparison when different instruments were used for data collection. We attempted to quantify (but also simplify) the information from the fluorescence distribution plots for easy, instrument-independent comparisons of the data. For the latter we used the differentiation between LNA and HNA bacterial communities in FCM plots, which was shown elsewhere, to render reproducible data when different instruments and different users were processing the same samples (SLMB, 2012). Differentiation between LNA and HNA bacteria can also provide additional information on samples. The almost systematic presence of distinct LNA and HNA clusters was previously observed during flow cytometric measurements in a wide range of aquatic environments (Robertson and Button, 1989; Li et al., 1995; Gasol et al., 1999; Lebaron et al., 2001; Wang et al., 2009). LNA bacteria were first considered as being not active or even dead (Lebaron et al., 2001, 2002; Phe et al., 2005), but were shown thereafter to be viable and active in low nutrient environment (Servais et al., 2003; Longnecker et al., 2005; Bouvier et al., 2007; Wang et al., 2009). Several studies showed that the HNA cluster is more dynamic and sensitive to changes than the LNA cluster. As an example, indigenous HNA bacteria were damaged faster by chlorination

(Ramseier et al., 2011a). It was also previously suggested that growth (particularly in stagnating drinking water) might occur preferentially in the HNA-fraction of the microbial community, leading to changes in the LNA:HNA ratios (Lebaron, 2001; Gasol et al., 1999; Lautenschlager et al., 2010; Vila-Costa et al., 2012; Kötzsch and Egli., 2013). A comparison of several water types showed that the position and the distribution of cells in the LNA and HNA groups vary from sample to sample (Figure S3.2 in supplementary information), but the separation of the two clusters was always at a stable fluorescence intensity (c.a. $2x10^4$ a.u. for our flow cytometer, Figure 3.2). Based on these observations, we chose the percentage of HNA cells as a straightforward quantification of the FCM fingerprints, and combined it with the total cell concentration, for rapid characterization of water samples (e.g. Figure 3.4B). Figures 3.5 to 3.8 show that the description of the fingerprint information with only the percentage of HNA cells enabled quantification of changes in the fingerprints during batch growth of aquatic indigenous communities and in stagnating drinking water, and related to a change in community composition, due to either contamination or mixing of two samples. The important aspect of the fingerprinting strategy described herein is the use of reproducible sample processing coupled with straightforward data analysis with fixed gating positions, enabling fast and easy data handling for in-practice applications such as drinking water monitoring. The method presented herein should be seen as a rapid screening tool for a fast identification of changes in samples, and which can then be complimented by advanced flow cytometric fingerprinting methods (Koch et al., 2013a,b), or by more time consuming methods such as molecular community analysis (Pinto et al., 2012) or specific pathogen/indicator screening.

3.4.4 Application value for drinking water utilities and laboratories

The developed method is sensitive, rapid and easy and therefore has potential to be used as a routine tool for monitoring the drinking water quality at the treatment outlet and during distribution. The implementation of a flow cytometric based total cell count (TCC) method in the Swiss guidelines (SLMB, 2012) reflects the interest of the drinking water utilities and quality controlling laboratories for accurate and reproducible alternative monitoring tools to conventional cultivation-based methods. The combination of the cell concentration and the FCM fingerprint information would allow a further step in the sensitive detection of changes, for example during water distribution. This approach has the potential to be used as indicator for biological stability/activity in networks (Vital et al., 2012a). Moreover, the fast detection of change using flow cytometry would enable a quick and targeted reaction in case of dramatic events, e.g. contamination. A completely automatized and standardized procedure is under development by combining the staining and processing method with on-line flow cytometers (Hammes et al., 2012). From a research perspective, the method can be

used for understanding biological processes in natural environment, e.g. understanding the role that the two LNA and HNA communities play in natural environments. Based on fast detection of change using flow cytometry, more elaborate and time consuming methods (e.g. molecular techniques like 454-pyrosequencing) could be used to further evaluate the changes in bacterial populations.

3.5 Conclusions

- A reproducible bacterial staining method is required for obtaining reliable and accurate flow cytometric results with respect to total cell counts and fluorescence fingerprints.
- Such an approach allows sensitive and accurate (i) detection of changes and (ii) characterization of bacterial communities in water systems.
- The proposed data handling strategy (quantification of fluorescence fingerprints) is fast, simple and easy to standardize using fixed gating positions for low (LNA) and high (HNA) nucleic acid containing bacterial communities and the use of one parameter only (%HNA bacteria).
- Small changes in aquatic environments such as (re)growth, contamination, stagnation and mixing of water types can be detected with both cell concentrations and flow cytometric fingerprints.
- Evidently, the flow cytometric method has considerable application potential for bacterial water quality monitoring and control and for early and on-line detection of changes during e.g. water treatment and in drinking water distribution networks.

SUPPLEMENTARY INFORMATION – Chapter 3

S3.1 Discrimination of different water types

a. Comparison of fingerprints and cell concentrations of various drinking waters

A total of 90 drinking water samples were collected from 11 drinking water utilities situated in various regions of Switzerland (in a total of 18 distribution networks). The samples were representative of a wide range of water types and treatments. The samples were transported to the laboratory under cold storage and analyzed on the day of sampling. Each sample was stained using a precise replication of the protocol described in section 3.2.1.

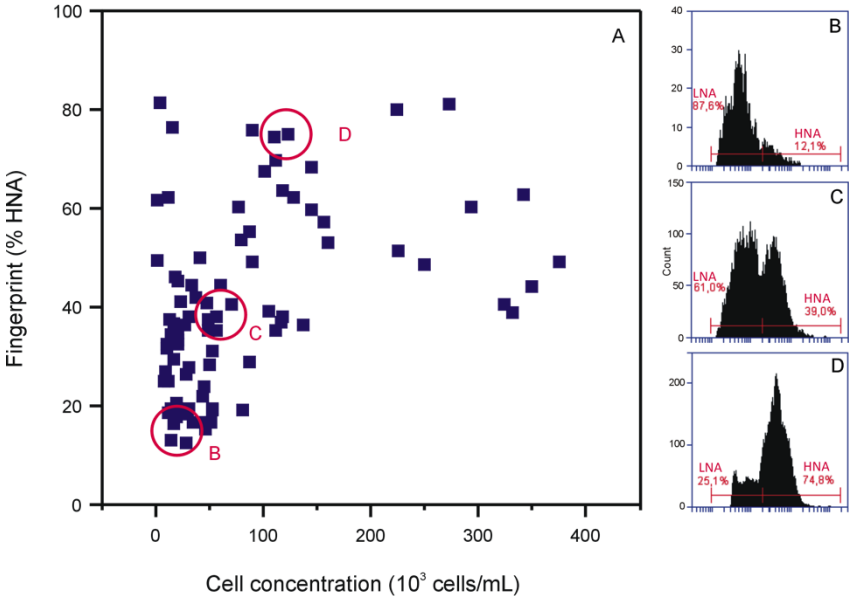


Figure S3.1. Comparison of 90 drinking water samples from various Swiss distribution networks sampled in the period September-October 2011. Each sample can be discriminated from the others based on bacterial cell concentration and percentage HNA cells (A). Histograms (B, C and D) illustrate the clear differences in fingerprints found for the samples indicated in (A) with circles labeled B, C, and D.

b. Comparison of fingerprints of different water types

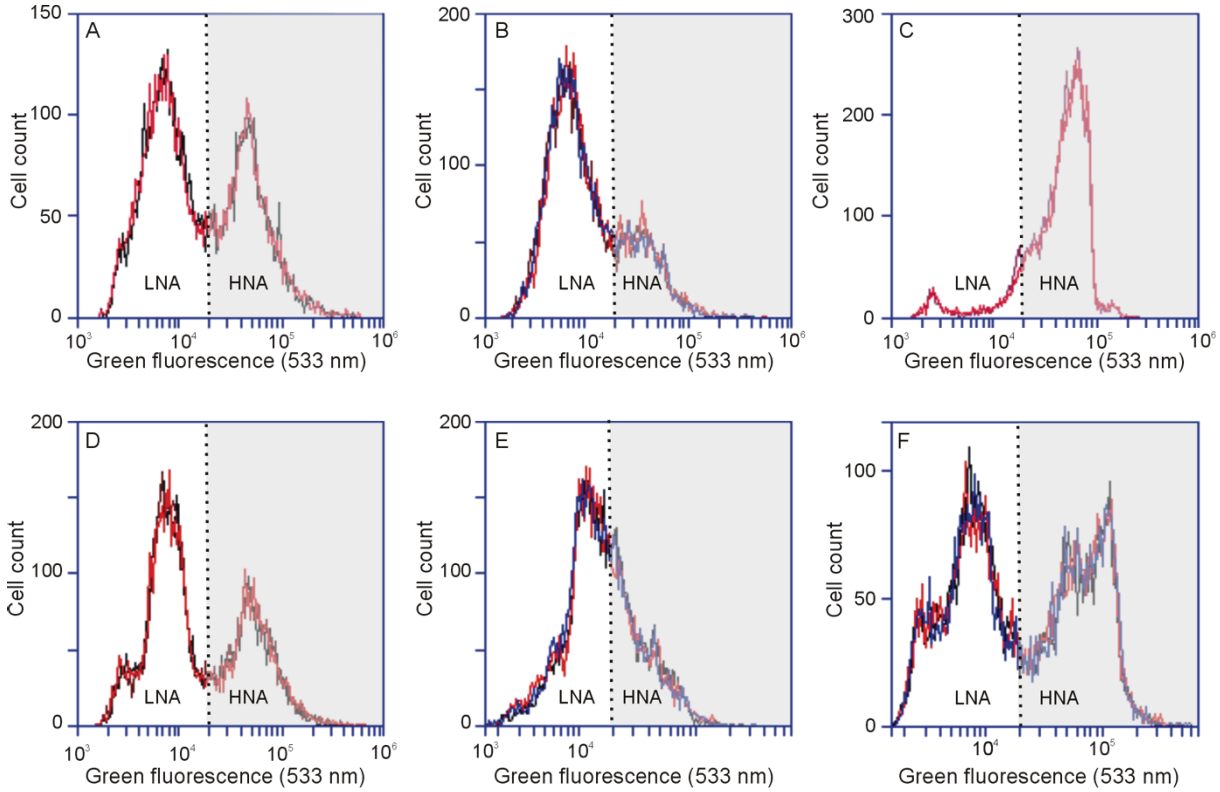


Figure S3.2. Green fluorescence histograms of various water samples measured in triplicate: (A), (B) drinking water from two different locations, (C) bottled water, (D) river water, (E) pond water and (F) wastewater. Each colored line corresponds to one of the triplicate measurements. LNA and HNA regions were separated at a fluorescence intensity of 2.10^4 arbitrary units (a.u.) for all samples.

S3.2 Selection of fluorescence vs. scatter parameters

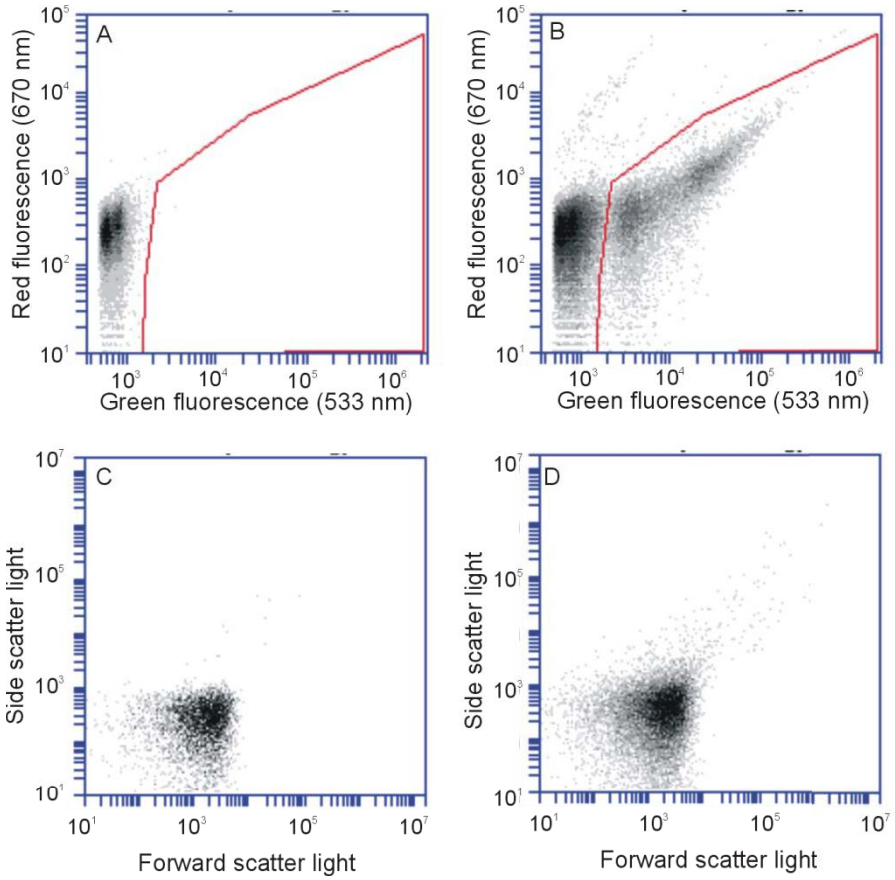
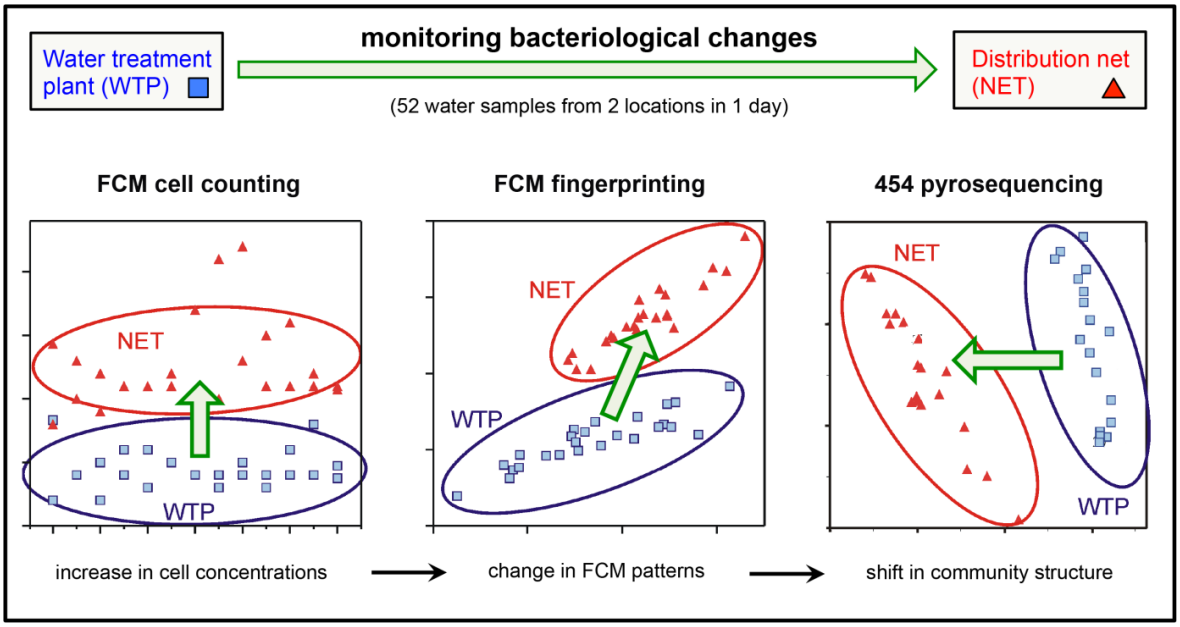


Figure S3.3. Comparison between fluorescence and scatter parameters for distinction between background and signal. Green and red fluorescence intensity of (A) non-stained tap water filtered through a 0.22 µm pore size filter and of (B) stained tap water. The red gate is used to distinguish between signal and background. Forward and side scattered light intensity of (C) non-stained tap water filtered through a 0.22 µm pore size filter and of (D) stained tap water.

Figure S3.3 shows that a good differentiation is obtained with fluorescence parameters between background and signal, while these overlap significantly when considering only scatter parameters.

Chapter 4

Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization



This chapter has been published as: E.I. Prest, J. El-Chakhtoura, F. Hammes, P.E. Saikaly, M.C.M. van Loosdrecht, J.S. Vrouwenvelder (2014). Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization. *Water Research* 63, 179-189.

Emmanuelle Prest has contributed to the study design and organization, water sampling, flow cytometric analysis and data interpretation. Joline El-Chakthouta (KAUST) has contributed to sequencing sample preparation, and sequencing data analysis and interpretation. The manuscript has been written by Emmanuelle Prest.

Abstract

The combination of flow cytometry (FCM) and 16S rRNA gene pyrosequencing data was investigated for the purpose of monitoring and characterizing microbial changes in drinking water distribution systems. High frequency sampling (5 min intervals for 1 h) was performed at the outlet of a treatment plant and at one location in the full-scale distribution network. In total, 52 bulk water samples were analysed with FCM, pyrosequencing and conventional methods (adenosine-triphosphate, ATP; heterotrophic plate count, HPC). FCM and pyrosequencing results individually showed that changes in the microbial community occurred in the water distribution system, which was not detected with conventional monitoring. FCM data showed an increase in the total bacterial cell concentrations (from 345 ± 15 × 10^3 to 425 ± 35 × 10^3 cells mL^{-1}) and in the percentage of intact bacterial cells (from 39 ± 3.5 % to 53 ± 4.4 %) during water distribution. This shift was also observed in the FCM fluorescence fingerprints, which are characteristic of each water sample. A similar shift was detected in the microbial community composition as characterized with pyrosequencing, showing that FCM and genetic fingerprints are congruent. FCM and pyrosequencing data were subsequently combined for the calculation of cell concentration changes for each bacterial phylum. The results revealed an increase in cell concentrations of specific bacterial phyla (e.g., *Proteobacteria*), along with a decrease in other phyla (e.g., *Actinobacteria*), which could not be concluded from the two methods individually. The combination of FCM and pyrosequencing methods is a promising approach for future drinking water quality monitoring and for advanced studies on drinking water distribution pipeline ecology.

4.1 Introduction

Drinking water should be biologically stable in order to limit unwanted bacterial growth within distribution systems. Bacterial growth can cause operational problems such as pipeline bio-corrosion or fouling, resulting in maintenance issues and customer complaints, and in the worst-case hygienically-related problems. It is therefore important to rapidly identify distribution systems areas with bacterial growth issues in order to undertake early maintenance actions. However, the occurrence of such situations may also require long-term improvement of the distribution conditions and maintenance strategies, which can only be achieved with an in-depth understanding of microbial dynamics in distribution pipelines. There is therefore a need for rapid, sensitive and accurate tools for microbial monitoring but also a need for quantitative and qualitative tools for detailed characterization of microbial communities in water samples.

The value of *flow cytometry (FCM)* for assessment and monitoring of total and intact bacterial cell concentrations during drinking water treatment and distribution has been highlighted before (Hoefel et al., 2003; Hammes et al., 2008, 2010a; Ho et al., 2012; Lautenschlager et al., 2013; Liu et al., 2013c). The method is easy and rapid, with results obtained in 15 min from sampling. Moreover, FCM is quantitative, highly reproducible (less than 5% error) and sensitive (detection of change down to 3% from initial value) (Chapter 3). In addition, correctly performed FCM measurements also generate so-called fluorescence fingerprints (De Roy et al., 2012; Koch et al., 2013c), which are unique to each sample and apparently dependent on the bacterial community composition and DNA content (De Roy et al., 2012; Vila-Costa et al., 2012; Koch et al., 2013a, 2013c; Müller, 2010). FCM fingerprints thus provide information on the bacterial community characteristics that is not obtained with FCM cell counting alone. The combination of FCM cell counting and fluorescence fingerprinting can have value for both monitoring purposes and for advanced studies in distribution pipelines, by providing rapid and quantitative information on the bacterial community characteristics, also revealing changes that are not reflected in the total cell concentration (e.g. a bacterial community turnover due to continuous attachment and detachment from pipe wall biofilms; Liu et al., 2013a). A recent laboratory-scale study has shown that the fingerprints can be quantified and used in combination with the total cell concentration for accurate detection of events affecting the bacterial community in water (Chapter 3). However this approach has not yet been tested on real, full-scale drinking water distribution systems, where changes may well be less pronounced than those created under controlled laboratory conditions. S*equencing methods* have also gained considerable interest for microbial community characterization during drinking water treatment and distribution (Henne et al., 2008; Pinto et al., 2012; Liu et al., 2013d). Pyrosequencing is a high-throughput sequencing

technology that provides insight on the microbial community composition (identity) and structure (proportion). It does not require labelled primers/nucleotides or gel electrophoresis and allows a large number of samples to be pooled (Ronaghi, 2001; Fakruddin and Chowdhury, 2012). This technique has recently been applied for the identification of species present in water during treatment (Wakelin et al., 2011; Pinto et al., 2012) and distribution (Henne et al., 2008; Hong et al., 2010; Hwang et al., 2012; Lin et al., 2013; Liu et al., 2013c; Lautenschlager et al., 2013). The studies using pyrosequencing have proved the value of identifying bacterial groups, for the evaluation of e.g. disinfection (Hwang et al., 2012) or residence time (Lautenschlager et al., 2013) effects on bacterial community composition. Pyrosequencing can therefore provide meaningful qualitative information on drinking water distribution pipeline ecology.

Combining highly quantitative FCM data with detailed qualitative pyrosequencing data could provide adequate tools for both monitoring and detailed investigations of full-scale drinking water treatment and distribution systems. To date, only few recent studies have applied both FCM and pyrosequencing. The studies were either applied to different fields than drinking water (e.g. seawater bacterial community identification, Vila-Costa et al., 2012) or were lab-scale batch experiments under controlled conditions (Bombach et al., 2010). Two recent studies applied both methods on full-scale drinking water systems, one focusing on the characterization of particle associated bacteria (Liu et al., 2013d), the other exploring the variations in bacterial community characteristics in a distribution network (Lautenschlager et al., 2013). The latter study showed that relatively small changes in bacterial cell concentration and community composition can occur during water distribution and can be detected using FCM and pyrosequencing individually.

The objective of this study was to evaluate the combination of FCM bacterial cell counting, newly-developed FCM fingerprinting and 454-pyrosequencing data for the detection and characterization of microbial changes occurring in full-scale drinking water distribution systems. For this purpose, we moved a step forward from previous studies by (i) evaluating the complementary nature of data derived from these methods, particularly comparing FCM fingerprints with pyrosequencing-derived genetic fingerprints and (ii) combining data sets obtained independently by the two methods, for the generation of new quantitative information on the bacterial community composition. To provide statistical credibility to the approach and solid comparison of the methods, a large amount of samples were taken at high frequency (52 samples on the same day) from only two locations in a full-scale distribution system.

4.2 Materials and methods

4.2.1 Sampling

The study was performed on the large-scale drinking water treatment plant of Kralingen (Rotterdam area, The Netherlands) and its corresponding distribution network. The annual drinking water production at Kralingen is 40 x 10^6 m^3/year. Surface water is treated at Kralingen by coagulation, flocculation and sedimentation followed by ozonation, dual medium filtration, and granular active carbon filtration. Chlorine dioxide (0.1 mg L^{-1}) is added at the end of the treatment and the water is collected in a reservoir before distribution. The chlorine dioxide concentration in the reservoir effluent water is below detection limit. The water is thereafter distributed in a well-maintained drinking water distribution network operating without residual disinfectant, which can be considered as representative for other locations in many industrialised countries. Samples were taken at the treatment outlet of the drinking water treatment plant and at one location in the network. Sampling was specifically limited to only two locations to enable the collection of a large amount of similar samples at high frequency, thus providing a solid basis for comparison between the datasets. The network location was selected based on preliminary studies indicating that microbial characteristics of the water differed from the treatment outlet water, thus allowing an evaluation of the capacity of the analytical methods to detect events affecting the microbial community in drinking water. Samples were collected from the two locations every 5 min during 1 h. This procedure was performed in the morning from 08:00 to 09:00 and repeated in the afternoon of the same day from 13:00 to 14:00 (Figure 4.1), to evaluate variations in water quality on short time-scales (morning vs. afternoon). The residence time of the water in the system at the network location was approximately two days. However, the residence time was not taken in account in the sampling scheme, as the available tools to estimate residence time provide only rough estimations, and sampling of the “same water” at both locations could therefore not be accurately achieved. The sampling taps at both locations were opened at least 1 h before sampling and were running continuously during the entire sampling period. At each sampling time, water was collected in separate bottles for each parameter to be measured, i.e. adenosine tri-phosphate (ATP), heterotrophic plate count (HPC), total and intact bacterial cell concentrations and fluorescence fingerprints (flow cytometry), TOC, pH, conductivity, temperature and 454-pyrosequencing. Bottle materials and measurement procedures are described below for each parameter. Water temperature was 20.9 +/- 0.1°C at the treatment outlet and 22.7 +/- 0.1°C at the network location. The water samples were transported on ice, stored at 4°C until analysis and processed within 24h.

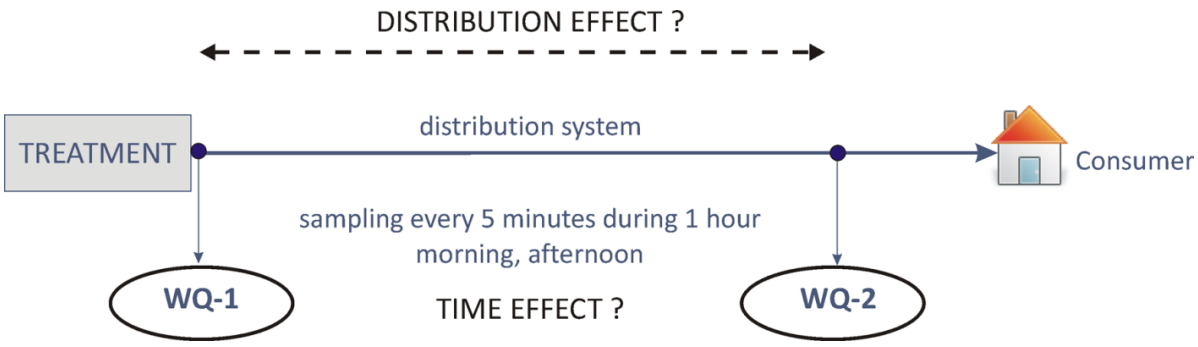


Figure 4.1. Experimental scheme for evaluation of flow cytometry and 454-pyrosequencing for the detection and characterization of microbial changes in full-scale drinking water distribution systems. Drinking water samples were taken at the treatment outlet and at one position in the distribution network, every 5 min during 1 h. The sampling was performed once in the morning and repeated once in the afternoon on the same day. Such sampling enables detection and characterization of (i) short-term temporal changes at each sampling location (time effect) and (ii) spatial changes by comparison of water samples taken at both locations (distribution effect). The study was performed in a full-scale well-maintained drinking water distribution network. WQ: water quality.

4.2.2 Conventional parameters

For HPC and ATP measurements, water was collected in HD-PE plastic bottles containing 2 mL L^{-1} of a mixed solution of sodium thiosulfate (20 g L^{-1}) and of nitrilotriacetic acid (25 g L^{-1}), as routinely applied by accredited laboratories for drinking water analysis in the Netherlands. HPC was measured by Aqualab Zuid (Werkendam, NL), according to the Dutch standard procedure (NEN-EN-ISO 6222, 1999). In short, 2 mL of the sample were transferred to a sterile Petri-dish and mixed with about 20 mL yeast extract agar. The agar was kept at 44°C before plating. The samples were incubated at 22°C for 3 days. ATP was measured by Het Waterlaboratorium (Haarlem, NL), as described previously by Magic-Knevez and van der Kooij (2004). The ATP measurement is based on the emission of light resulting from the reaction between the ATP molecule and a luciferin/luciferase reagent (LuminATE, Celsis). For total ATP determination, ATP was first released from suspended cells with nucleotide-releasing buffer (LuminEX, Celsis), while this step was not performed for assessment of free ATP. The intensity of the emitted light was measured using a luminometer (Celsis Advance^{TM}) that was calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure given by the manufacturer. The detection limit of the method was 1 ng ATP L^{-1}. Bacterial ATP concentrations were calculated by subtracting free ATP from total ATP concentrations.

Drinking water samples were collected in PET bottles without headspace for pH and conductivity analysis and in glass bottles containing sulphuric acid (8 mol L^{-1}, 0.2 mL in 100 mL bottle) for TOC analysis. The three parameters were measured by Aqualab Zuid (Werkendam, NL). The water temperature was measured directly on site.

4.2.3 Flow cytometry (FCM)

Drinking water was collected in HD-PE bottles containing 2 mL L^{-1} of a mixed solution of sodium thiosulfate solution (20 g L^{-1}) and of nitrilotriacetic acid (25 g L^{-1}) for FCM measurements. Water samples were stained according to the standardized protocol proposed in the Swiss guideline for drinking water analysis (SLMB, 2012) for the determination of total bacterial cell concentrations. In short, samples (500 µL) were pre-heated to 35 °C (5 minutes) and then stained with 10 µL mL^{-1} SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), and incubated in the dark for 10 minutes at 35 °C before measurement. For the assessment of intact bacterial cell concentrations, a working solution containing SYBR® Green I (1:100 dilution in DMSO; Molecular Probes) and propidium iodide (0.3 mM) was prepared. The same protocol as described above was used for the staining. Flow cytometric measurements were performed, as described in Chapter 3, 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. The FCM is equipped with volumetric counting hardware, calibrated to measure the number of particles in 50 µL of a 500 µL sample. Measurements were performed at pre-set flow rate of 35 µL min^{-1}. A threshold value of 500 a.u. was applied on the green fluorescence channel (FL1).

Data analysis was performed using the BD Accuri CFlow® software, following the procedure described in Chapter 3. Briefly, bacterial signal was selected using electronic gating on density plots of green fluorescence (FL1; 533 nm), and red fluorescence (FL3; >670 nm). The selected data was subsequently visualized on a green fluorescence histogram, which was used as the main FCM "fingerprint" for the bacterial community. Analysis of the fingerprints was based on the separation, using fixed electronic gates, of the typical two-clusters formed by the low (LNA) and high (HNA) nucleic acid content bacteria, as described in Chapter 3. Quantification and straightforward comparison of fingerprints from different water samples was made using the percentage of HNA cells (compared to total cells) and the relative nucleic acid content (calculated from the green fluorescence distribution). The fingerprinting approach is discussed in supplementary information (calculation of the relative nucleic acid content and comparison with similar fingerprinting strategies, Figure S4.1.1).

4.2.4 Bacterial community analysis with 16S rRNA gene pyrosequencing

Water samples (2 L) were collected in HD-PE bottles containing 2 mL L^{-1} of a mixed solution of sodium thiosulfate solution (20 g L^{-1}) and nitrilotriacetic acid (25 g L^{-1}). Each sample was filtered through a 0.2 µm-pore-size Isopore membrane filter (Merck Millipore, Billerica, MA) within 5 hours of sampling. The filters were stored at -20 °C until processing. Genomic DNA was extracted from the collected biomass using the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. Bacterial 16S rRNA genes were amplified with the bacteria-specific forward primer 515F (5'-LinkerA-Barcode-GTGYCAGCMGCCGCGGTA-3') and reverse primer 909R (5'-LinkerB-CCCCGYCAATTCMTTTRAGT-3'). A single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (QIAGEN, Valencia, CA) was performed for each DNA sample (triplicate reactions). The PCR conditions are described in supplementary information. Pyrosequencing was carried out on the Roche 454 FLX Titanium genome sequencer (detailed processing procedure is described in supplementary information). Multidimensional scaling (MDS) was performed with the Bray-Curtis coefficient using the R statistical package to ordinate the pyrosequencing operational taxonomic unit (OTU) data. An analysis of similarity (ANOSIM; Ramette, 2007) tool was used to examine statistical differences between samples using the Bray-Curtis measure of similarity (vegan package within the R statistical software).

Table 4.1. Overview of study focus, methods and data analysis procedures applied for the detection and characterization of variations in bacterial community in time and during drinking water distribution in a full-scale distribution network.

Focus	Methods	Data analysis approach	Figure	Results section
Rapid detection of change with bacterial cell concentrations and conventional tools	FCM: total and intact cell concentrations	Direct cell concentration data	4.2	4.3.1
	ATP, HPC	Direct measurement	4.3	
Comparison of flow cytometric and genetic fingerprints	FCM: fingerprints	Processed FCM fingerprints	4.4A	4.3.2
		Unprocessed FCM fingerprints	4.4C	
	454 pyrosequencing	MDS graphs	4.4B	
		Phylum relative abundance	4.4D	
Combination of FCM and pyrosequencing data for quantitative characterization of change	FCM: TCC 454 pyrosequencing	Combined data of TCC and relative phyla abundance	4.5	4.3.3

FCM: flow cytometry; TCC: total cell concentration; ATP: Adenosine-triphosphate; HPC: heterotrophic plate counts; MDS: multidimensional scaling.

4.3 Results

High frequency sampling of drinking water was performed at the treatment plant outlet and at one location in the distribution network to provide strong statistical basis for (i) the evaluation of variations in bacterial cell concentration and community composition on short time-scales (morning vs. afternoon) at each location and (ii) the comparison of the microbial community characteristics (cell concentration, viability, community composition) at the two locations based on a large data set. Table 4.1 gives an overview of the methods and data processing approaches used for the analysis of the results presented in the paper. Results of each test can be found in the figures and sections noted.

4.3.1 Rapid detection of change in bacterial cell concentrations

FCM total bacterial cell concentrations and percentage of intact bacterial cells data revealed changes in the drinking water samples both in time (morning vs. afternoon) and between the two locations (Figure 4.2). These changes were not detected with conventional analysis (heterotrophic plate counting, HPC; and adenosine tri-phosphate, ATP; Figure 4.3, and Figures S4.3.1 and S4.3.2 in supplementary information). Short-term temporal changes in cell concentrations were limited in general. Reproducible total cell concentrations were measured over one hour at the treatment plant outlet during each of the two sampling periods, with less than 5 % variation, which is in the range of FCM measurement error (Chapter 3). However, a small but significant difference ($t=3.88$, $p=0.0007$, based on an independent-sample t-test) in total cell concentrations was measured between the treatment plant outlet water sampled in the morning ($355 \pm 17 \times 10^3$ cells mL^{-1}, n=13) and in the afternoon ($336 \pm 9 \times 10^3$ cells mL^{-1}, n=13) of the same day. At the network location, also less than 5% variation in total cell concentration was detected over one hour in the morning ($406 \pm 13 \times 10^3$ cells mL^{-1}, n=13), but a clear peak in total cell concentration was observed in the network in the afternoon (between 13:30 to 13:55, from 410×10^3 to 520×10^3 cells mL^{-1}). The peak might be due to increased water consumption during lunch-time in the building where the samples were taken, possibly resulting in bacterial cell re-suspension from e.g. sediments in the piping network into the bulk water (Tsai, 2005; Lehtola et al., 2006; Nescerescka et al., 2014). Reproducible percentages of intact cells were also measured during 1 h at both locations during the two sampling periods (less than 5 % variation), except for a few data points at the treatment plant outlet and in the network.

Differences in cell concentrations between the two locations were more pronounced than these temporal variations: total bacterial cell concentrations clearly increased between the treatment plant and the network location (n=26 on each location, increase from 345 ± 15 × 10^3 to 425 ± 35 × 10^3 cells mL^{-1}), as well as the percentage of intact bacterial cells (increase from 39 ± 3.5 % to 53 ± 4.4 %). The direct inference from this data is that significant microbial growth, or contamination from an external source, occurred during water distribution.

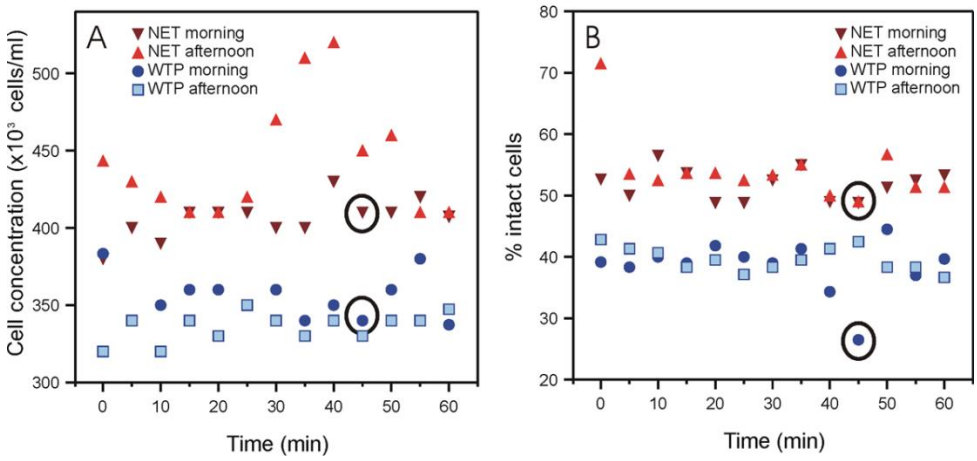


Figure 4.2. Variations over 1 h, in the morning (08:00-09:00) and in the afternoon (13:00-14:00), of drinking water (A) total bacterial cell concentration and (B) percentage of intact bacterial cells at the treatment outlet (WTP) and at the network location (NET). Drinking water samples were taken every 5 min during each sampling period. The circles indicate the two samples that were analysed in detail in figures 4.4C, 4.4D and 4.5. The standard error for the flow cytometric measurements is less than 5%.

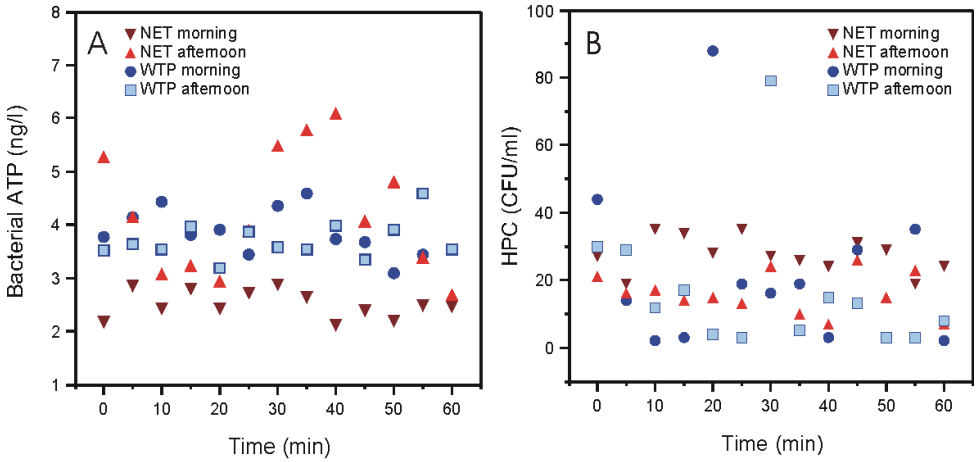


Figure 4.3. Variations over 1 h, in the morning (08:00-09:00) and in the afternoon (13:00-14:00), of (A) bacterial adenosine-tri-phosphate (ATP) and (B) heterotrophic plate count (HPC) of drinking water at the treatment outlet (WTP) and at the distribution network location (NET). Drinking water samples were taken every 5 min during each sampling period.

4.3.2 Comparison of flow cytometric and genetic fingerprints

The change in bacterial cell concentrations detected between the treatment outlet and the network location was clearly related to a change in FCM fingerprints and this was reflected in a change in bacterial community composition as well. All fingerprint data points were reproducible for each location, at each sampling period, with less than 5 % error on the percentage of HNA cells and the relative nucleic acid content, which is within the range of the flow cytometric measurement error (Figure 4.4A; time sequence is provided in Figure S4.4.1 in supplementary information). Figure 4.4A shows that samples from the two locations displayed distinct FCM fingerprints, as the treatment plant and network data clustered separately. The network samples (n=26) had higher high nucleic acid (HNA) values, with average %HNA values of 43.5 ± 2.1 % compared to 35.8 ± 1.7 % for the treatment plant (n=26). Differences between the morning and afternoon samples were reflected as well in the FCM fingerprints, although less pronounced than the changes between the two locations. The FCM fingerprints of the treatment outlet samples in the morning and afternoon (n=13 for each time period) were located in different areas of the graph, although slightly overlapping (34.6 ± 1.4 %HNA in the morning and 36.8 ± 1.3 %HNA in the afternoon). More overlap in FCM fingerprints between the morning and afternoon samples of the network was observed (42.5 ± 2.6 %HNA in the morning and 44.5 ± 5.2 %HNA in the afternoon). For both locations, the differences between morning and afternoon could be considered as significant based on independent-samples t-tests (t=-4.04, p=0.0005 for the treatment plant outlet and t=-2.90, p=0.008 for the network).

The FCM fingerprinting data were subsequently compared to the results of the 454 pyrosequencing (Figure 4.4B), and a similar trend was observed. The multidimensional scaling (MDS) graph indicates the degree of similarity in bacterial community composition between samples. A difference in community structure was clearly observed between the two water sampling locations, as well as between the morning and the afternoon treatment outlet samples, while the network samples displayed more overlap between morning and afternoon. The differences in bacterial community composition were found to be statistically significant in all cases (Table 4.2), based on analysis of similarity (ANOSIM).

To illustrate the relation between the FCM and the genetic fingerprints, a detailed comparison was made between two samples taken at the same time (8:45) at each location on the basis of unprocessed FCM fingerprints (Figure 4.4C) and phyla-level community analysis (Figure 4.4D). The samples had clearly different cell concentrations, FCM fingerprints and community compositions, as indicated in Figures 4.2, 4.4A and 4.4B. Comparison of the unprocessed fingerprints showed that the network samples contained more cells with high fluorescence intensity, i.e. high nucleic acid content

(HNA) cells, which was reflected by a higher %HNA value in Figure 4.4A. Such increase in HNA cells can be due to the growth of specific bacterial species (Müller et al., 2010; Vila-Costa et al., 2012). The relative abundance analysis of the pyrosequencing data allowed for characterizing the change between the two samples and revealed that the main bacterial phylum present in both water samples was *Proteobacteria*. The percentage of *Proteobacteria* increased during distribution from 47.7 to 57.4 % of the total bacterial population. Deeper analysis of this phylum at the class level showed that this increase was mainly due to an increase in the percentage of *Betaproteobacteria* (from 12.5 to 32.8 % of the total population, data not shown).

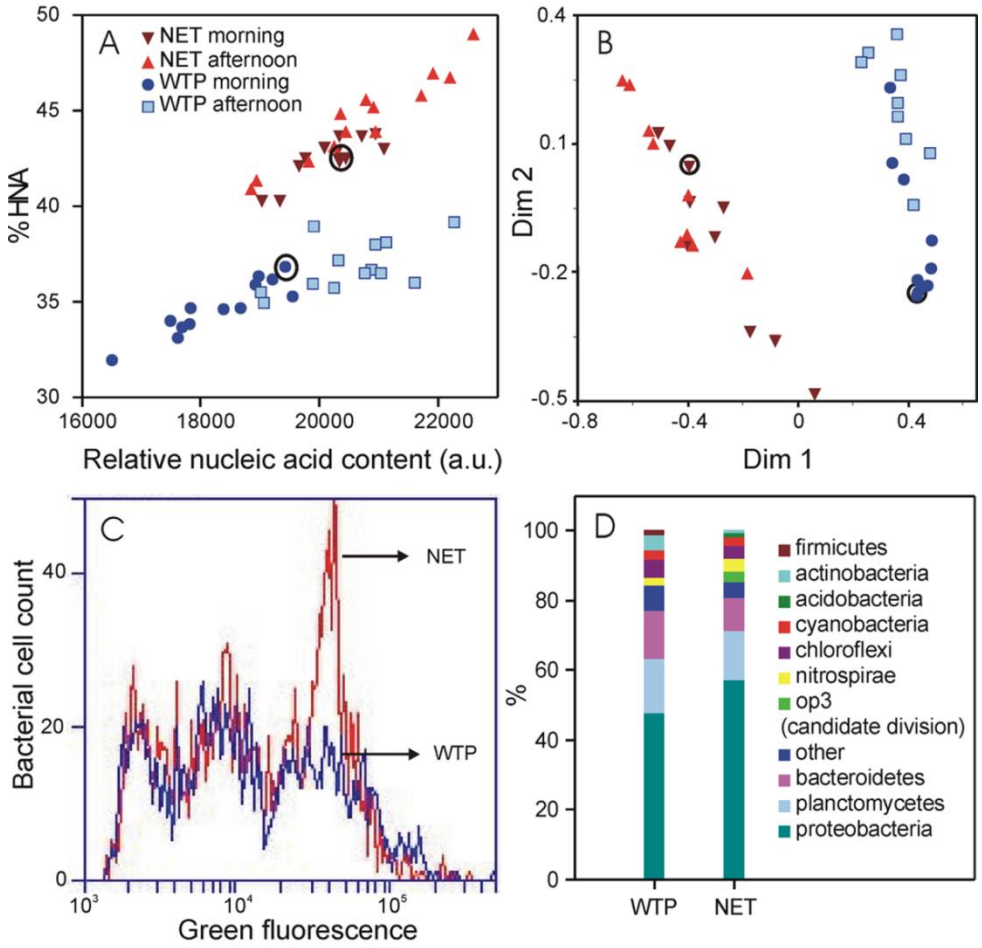


Figure 4.4. Comparison of processed (A) flow cytometric fingerprints and (B) genetic fingerprints (molecular data, multidimensional scaling graph) of bacteria in the drinking water samples taken at the treatment outlet (WTP) and at the distribution network location (NET) in the morning (08:00-09:00) and in the afternoon (13:00-14:00). Drinking water samples were taken every 5 min during each sampling period. The circles in Figures 4.4A and 4.4B indicate two samples, taken at 8:45 at both locations, that were studied in detail in Figures 4.4C, 4.4D and 4.5. (C) Overlay of the flow cytometric green fluorescence distribution histograms (unprocessed fingerprints) of the two samples. (D) Comparison of relative abundance of phyla present in the two samples. %HNA: percentage of high nucleic acid cells compared to the total population.

Table 4.2. Results of analysis of similarity (ANOSIM) for determination of statistical significance of differences between community composition of water samples, as analysed with pyrosequencing. Data sets were obtained from water samples taken at the treatment plant outlet (WTP) and in the distribution network (NET) at different times of the day (morning and afternoon). A p-value lower than 0.01 indicates a statistically significant difference. The R value indicates whether the separation between the two data sets is strong (R>0.75: well separated; R>0.5: separated but overlapping; R<0.25: barely separable).

Data set 1	Data set 2	P value	R value
WTP (all samples)	NET (all samples)	0.0001	0.9756
NET morning	NET afternoon	0.0008	0.3296
WTP morning	WTP afternoon	0.0091	0.3268

4.3.3 Combination of FCM and pyrosequencing data for quantitative characterization of change

The combination of FCM bacterial cell concentrations and relative abundance of bacterial phyla obtained with pyrosequencing enabled a quantitative evaluation of the contribution of each bacterial phylum to the observed change between the samples compared in Figures 4.4C and 4.4D. The obtained absolute cell concentration information is particularly useful since the total bacterial cell number was different in the two samples, thus making direct comparison of the samples difficult when only based on the relative abundance obtained from 454 pyrosequencing data. Figure 4.5 gives the absolute change in bacterial cell concentration of the different phyla detected, calculated from the total cell concentration of each sample as determined with FCM, and the relative abundance of different phyla obtained using 454 pyrosequencing.

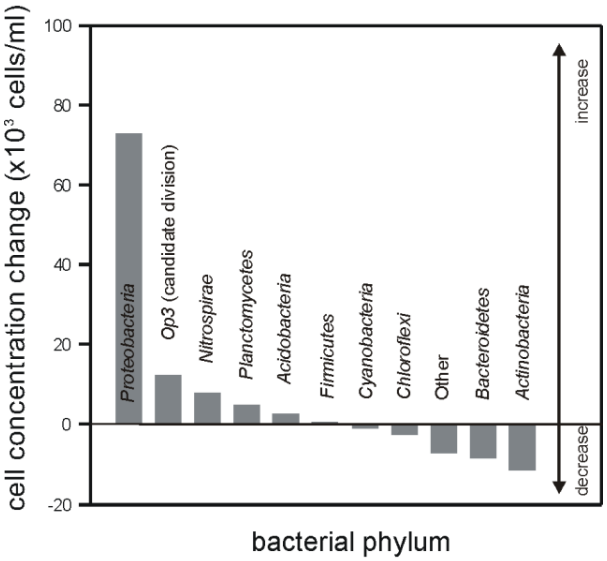


Figure 4.5. Absolute total bacterial cell concentration change per phylum between the treatment outlet and the distribution network location for the samples taken at 8:45. The total cell concentration increase between the two locations was 70 ×10^3 cells/ml.

The figure shows that *Proteobacteria* have increased in number during distribution, and that the change is significant compared to all other detected phyla (increase of 73×10^3 cells mL^{-1}, while the maximum change in other phyla was 12×10^3 cells mL^{-1}). Although most bacterial phyla have increased in cell concentration, other phyla, such as the *Actinobacteria* decreased in cell concentration (decrease of 11×10^3 cells mL^{-1}), which could not be observed based on the relative abundance data alone.

4.4 Discussion

High frequency sampling was performed in a full-scale drinking water distribution system in order to investigate variations in the microbial characteristics of the water on short time-scales (morning vs. afternoon of the same day) at two locations and compare them with each other. Flow cytometry (FCM) and 16S rRNA gene pyrosequencing independently showed the microbial concentration and community structure varied slightly in time on the same location and was clearly differing from one location to the other (Figures 4.2 and 4.4), while no detectable difference was observed with conventional methods (ATP, HPC; Figure 4.3, Figures S4.3.1 and S4.3.2 supplementary information). The large number of analysed samples (52) from a limited number of locations provided a strong basis to consider the changes as statistically significant. The detection with FCM was rapid and quantitative, based on bacterial cell concentration, percentage of intact bacterial cells and FCM fingerprints (Figures 4.2A, 4.2B and 4.4A). Analysis with pyrosequencing enabled extensive characterization of the detected change in bacterial community composition (Figure 4.4D). Importantly, comparison of the two methods showed that the bacterial community structure and FCM fingerprints are congruent (Figure 4.4A and 4.4B). The combination of the data from the two methods provided quantitative description of change in bacterial composition, which could not be obtained by either method separately (Figure 4.5).

4.4.1 Rapid and informative detection of microbial changes with flow cytometry

FCM is an appropriate method for rapid detection of changes in microbial community characteristics. Results can be obtained within 15 minutes from sampling and were shown to be reproducible (less than 5% error) and sensitive (detection of change in a sample down to 3% of the initial value) on both cell counting and fingerprints (Hammes et al., 2008; Chapter 3). Moreover, FCM measurements using only two fluorescent dyes (SYBR® Green I and propidium iodide) provide a detailed set of information on the detected change: an increase or a decrease in total bacterial cell concentration is a straightforward indication that events have occurred that affect the microbial community (which can

have multiple origins; Hammes et al., 2008, 2010a), while the percentage of intact cells provides information on cell viability (Boulos et al., 1999; Falcioni et al., 2008). The latter is especially valuable in chlorinated systems where cell damages are indicative of disinfection efficacy (Ramseier et al., 2011a; Nescerecka et al., 2014).

Additionally, it has been clearly shown in the present study that a change in FCM fingerprints is indicative for a shift in the microbial community composition. A link between community composition and the FCM fingerprints was suggested previously in studies where a shift in community composition was induced in a sample. For example, variations in FCM fingerprints were clearly detected during batch growth of autochthonous bacteria, after mixing of drinking or wastewater samples (Chapter 3; Koch et al., 2013a) or after a change in environmental conditions in bottled water or biogas reactors (e.g. change in temperature, pH or nutrient concentration and type; De Roy et al., 2012; Koch et al., 2013a). However, in our study, the shift in community composition was not only assumed, it was detected and analysed with pyrosequencing. Moreover, this was observed from samples from a full-scale drinking water network in which relatively small changes occur, opposed to laboratory induced changes under controlled conditions. In this way we showed that the shift in FCM fingerprints between the treatment plant and the network samples (Figure 4.4A) was concomitant with a shift in community composition, as determined from the multivariate data analysis of the 454 pyrosequencing results (Figure 4.4B). The direct link between the FCM fingerprints and genetic fingerprints is a major finding for drinking water analysis: it can be concluded in a few minutes from FCM data that a community shift has occurred in a drinking water sample. To our knowledge this is the first study demonstrating the link between FCM fingerprints and pyrosequencing-based genetic fingerprints in real drinking water samples.

4.4.2 Detailed description of community shift using pyrosequencing combined with flow cytometry

454 pyrosequencing is valuable for an in-depth characterization of microbial communities in water, by providing the information on the relative abundance of organisms in water samples. In the present study, the results showed that the dominant phylum was *Proteobacteria,* which was also the case in multiple drinking water systems (Hoefel et al., 2005; Hong et al., 2010; Hwang et al., 2012; Pinto et al., 2012; Lautenschlager et al., 2013; Liu et al., 2013c), although studies have demonstrated that the predominant class (*Alpha*, *Beta*, *Gammaproteobacteria*) differed from one network to another (Mathieu et al., 2009; Hwang et al., 2012; Liu et al., 2013d). Our study showed that even within the same system, water distribution can result in community structure changes. *Proteobacteria* remained prevalent, but the relative abundance was modified and new phyla could

be detected. Also within the *Proteobacteria* phylum, the class composition changed with an increased proportion of *Betaproteobacteria,* as already observed elsewhere (Pinto et al., 2012).

However, pyrosequencing of amplified 16S rRNA gene fragments only provides information on the relative abundance of bacterial groups in terms of absolute cell concentrations (Lin et al., 2014). Thus, combining the relative abundance of each phylum determined by pyrosequencing with the total cell concentration in the water sample measured with FCM was valuable for quantitative description of changes that occurred at the phyla level. Although FCM and pyrosequencing have been used in parallel in other drinking water studies (Liu et al., 2013d; Lautenschlager et al., 2013), the data of the two methods have to our knowledge never been combined before. We showed in this way that quantitative information can be obtained that is not available from the relative abundance data only: the cell concentration increased for some bacterial phyla (e.g. *Proteobacteria*) while it decreased for others (e.g. *Actinobacteria*, Figure 4.5). The cause of this community change is not clear yet, as processes in networks are complex and cannot only be explained by bacterial growth. Such community shifts can have multiple origins, such as attachment of certain species to pipe wall biofilms and detachment of others (Flemming et al., 2002a) or re-suspension of bacteria from sediments (Lehtola et al., 2004). Competition between bacterial species can also result in the growth of specific bacterial populations taking advantage over others (Egli, 2010). Moreover, other organisms such as viruses or protozoa can target specific bacterial species or groups, leading to their decline or even disappearance. Considerably more knowledge on the drinking water distribution pipeline ecology and dynamics is needed and the combination of pyrosequencing data with flow cytometric cell counting will help gaining understanding on microbial dynamics in such systems.

4.4.3 Combining flow cytometry and pyrosequencing data: a promising approach

The approach of combining flow cytometry and pyrosequencing of amplified 16S rRNA gene fragments has clear potential for applications in drinking water quality monitoring in full-scale treatment and distribution systems as well as for advanced studies on distribution pipeline ecology.

Major advances in the understanding of distribution pipeline ecology can be achieved using the combined tools. Because of the ease and rapidity of the FCM method, a large number of samples can be analysed for pre-selection of samples of interest. For example, van Nevel et al. (2013b) described the methodology for measuring at a rate of approximately 1 sample per minute from 96-well plates. The multiple parameters (total cell concentration, percentage of intact cells and fluorescence fingerprints) provide a first set of information on the bacterial community characteristics in the water sample, and can reveal for example areas in the network with excessive bacterial growth or external

contamination (Hammes et al., 2010a; Liu et al., 2013c; Nescerecka et al., 2014). From such a high-throughput screening, a sub-set of selected samples can be further characterised in detail with pyrosequencing. This approach has clear value to investigate fundamental pipeline ecology related phenomena, such as competition processes between different bacterial groups (Egli, 2010), interactions between bulk, sediment and biofilm compartments (Liu et al., 2013a) or associations of specific bacterial groups with others (Wang et al., 2014). Problematic scenarios (e.g. bio-corrosion or excessive growth) might be linked with specific bacterial groups or species that can be identified in this way and could serve as indicator organisms for future monitoring. Although the information obtained nowadays with pyrosequencing is often limited to a list of group names, a constantly increasing database on drinking water bacteria types and their specificities is evolving. In our opinion, the possibility to identify bacterial groups with specific functions (e.g. nitrifiers or methanogens, Gomez-Alvarez, 2013; Wang et al., 2014) or characteristics (e.g. anoxic or anaerobic bacteria, Liu et al., 2013d) will therefore increase manifold in the near future. Such information, when examined quantitatively in combination with FCM cell counts, will lead to the characterization of microbial dynamics in systems with specific features (e.g. systems treating deep ground water vs. surface water, systems distributing water with or without residual disinfectant, systems with specific pipe materials, Hwang et al., 2012; Wang et al., 2014) and improvement of maintenance strategies can be considered.

We can also envision that flow cytometry and pyrosequencing have the potential to become standard tools in the future for drinking water quality monitoring. Flow cytometry has already been proposed as an ideal monitoring tool because of the detection sensitivity of the method, as well as the rapidity and ease of measurement (Hammes et al., 2008; SLMB, 2012). These features might even improve in the future with smaller, automated or even online systems (Hammes et al., 2012; Van Nevel et al., 2013). Pyrosequencing might also be considered in the future for monitoring purposes, as technical advances in the method are expected to lead to smaller sequencers with lower prices, increased rapidity and ease (Cardenas and Tiedje, 2008; Fakruddin and Chowdhury, 2012). Long term systematic monitoring using both methods will lead to the construction of a database on the water microbial characteristics, including cell concentrations and community composition. The naturally present bacterial groups or species in the system can be identified, and the genetic fingerprint of the water can be built in a quantitative way when the pyrosequencing data is combined with the flow cytometric cell concentrations. Moreover, the long term monitoring will reveal variations in the water microbial community characteristics in time and in space, that are inherent to the distribution system (this study; Nescerecka et al., 2014). With such a database, flow cytometry can be used for the rapid detection of events related to uncontrolled bacterial growth or

bacterial intrusion (e.g. bio-corrosion or pipe breakage) that require to take action. The events can be further characterized with pyrosequencing by comparing the bacterial community in the problematic samples with the database.

The combination of flow cytometry and pyrosequencing could also be considered in future for the detection and quantification of specific bacterial groups or species of interest. Because pyrosequencing can screen in one measurement for the total bacterial community structure and composition in water samples, the technique could replace existing culture-based methods that are time consuming and can only target specific bacterial groups or species (e.g. heterotrophic bacteria, *Legionella, Aeromonas*, …), resulting in less effort (Ye and Zhang, 2011). Several improvements of the sequencing method are still needed for such applications. Presently, 16S rRNA gene pyrosequencing does not allow the detection of specific species with certainty, due to the short sequence read lengths (Aw and Rose, 2012; Wang et al., 2014). However, further developments are expected in the coming years with foreseeable improvements with respect to read length allowing the identification of OTUs, possibly down to species level. Also, new sequencers are emerging with higher reads and throughput, and lower cost than 454 pyrosequencing (e.g. Ion Proton or Ilumina; Fakruddin and Chowdhury, 2012; Morey et al., 2013).

4.5 Conclusions

Studies on microbial community characteristics in a full-scale drinking water distribution system with high frequency sampling at two locations (treatment plant effluent and a location in the network) showed that:

- flow cytometry and pyrosequencing enable the detection of events affecting the water microbial community that are not detected with classical methods such as ATP or HPC;
- flow cytometric (FCM) fingerprints can be used as an indicator for shifts in bacterial community composition;
- The combination of data obtained with the two methods provides quantitative information on microbial community composition that cannot be obtained from the methods individually.

The combination of flow cytometry and pyrosequencing is a promising approach for monitoring full-scale drinking water distribution systems and for gaining knowledge on drinking water distribution pipeline microbial dynamics.

SUPPLEMENTARY INFORMATION – Chapter 4

S4.1 Flow cytometric fingerprinting

a. Calculation of relative nucleic acid content

During flow cytometric measurements, fluorescence intensity is recorded for each bacterial cell present in a water sample. Bacterial cells are usually labeled with fluorescent dyes prior to the measurement. When the dye binds specifically to nucleic acids (e.g. SyBr Green I), the recorded fluorescence intensity is dependent on the amount of nucleic acids contained in the bacterial cell. Therefore, the distribution of the green fluorescence measured for all bacteria in a water sample is indicative for the distribution of the nucleic acid content of the bacteria in the sample.
It is possible to calculate an estimation of the geometric average of the green fluorescence using the formula below (Eq. S4.1.1). The obtained value is an estimation of the average nucleic acid content of the bacteria in the water sample, and referred to as relative nucleic acid content. The calculation (Eq. S4.1.1) is based on the classical separation between low and high nucleic acid cells (LNA and HNA) usually observed in aquatic samples and takes into account the distribution of the bacterial cells into the two clusters. The separation between the LNA and HNA clusters is determined using fixed gating as described in Chapter 3.

Eq. (S4.1.1) **Relative nucleic acid content = (Median LNA x %LNA) + (Median HNA x %HNA)**

where Median LNA and Median HNA are the median fluorescence values of the LNA and HNA groups and %LNA and %HNA are the percentages of the LNA and HNA groups concentration compared to the total bacterial cell concentration (LNA+HNA).

b. Fingerprinting approach

For easy interpretation and comparison of fingerprints of different samples, the percentage of HNA cells compared to total cells (%HNA) and the relative nucleic acid content were plotted (Figure 4.4A). Using this approach, the treatment outlet and distribution network samples were separated in two clearly distinct clusters. Morning and afternoon samples were also separated, although with some overlap, for samples of each location.

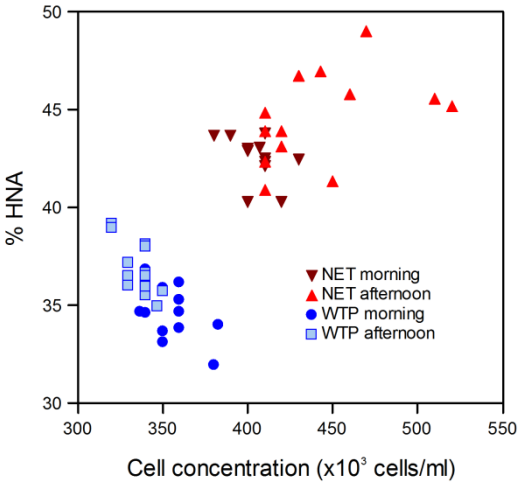


Figure S4.1.1. Alternative fingerprint graph, plotting the total bacterial cell concentration against the percentage of HNA cells (%HNA). Comparison of samples taken in the morning and in the afternoon of the same day, at the treatment outlet (WTP) and at the network location (NET).

In Chapter 3, another fingerprinting approach was proposed, where the percentage of HNA cells was plotted against the total cell concentration measured in the water sample. Figure S4.1.1 shows that, using this plot, the same discrimination was observed between treatment outlet and distribution network samples, as well as morning and afternoon samples. This shows that the results presented in the present paper are independent from the fingerprinting strategy chosen for discrimination of water samples.

S4.2 Bacterial community analysis with 16S rRNA gene pyrosequencing: detailed protocol

Polymerase chain reaction (PCR) was performed for each DNA sample (triplicate reactions) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and then purified using Agencourt Ampure beads (Agencourt Bioscience Corp., Beverly, MA). 454 pyrosequencing was carried out at MR DNA Lab (Shallowater, TX, USA) on the Roche 454 FLX Titanium genome sequencer according to the manufacturer's instructions. Sequence data was processed at MR DNA Lab (Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences <150bp were removed, as well as sequences with ambiguous base calls and with homopolymer runs exceeding 6bp. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI and GreenGenes. Multidimensional scaling (MDS) was performed with the Bray-Curtis coefficient using the R statistical package to ordinate the pyrosequencing OTU data.

S4.3 Overview of routine parameters results

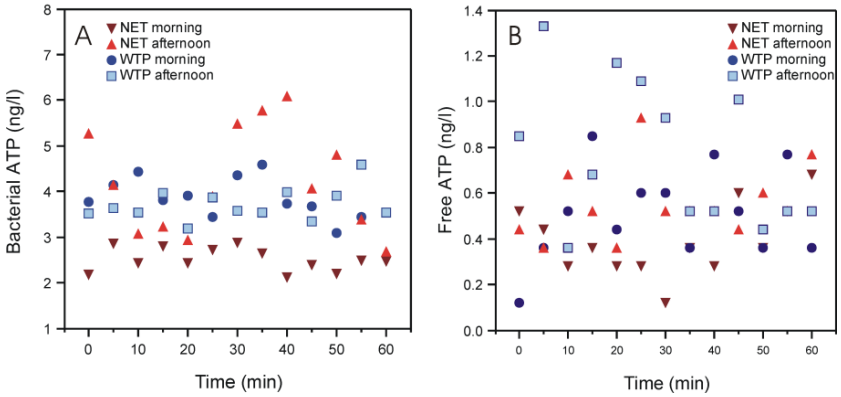


Figure S4.3.1. Variations over 1 h, in the morning (8:00-9:00) and in the afternoon (13:00-14:00), of (A) bacterial and (B) free adenosine tri-phosphate (ATP) of drinking water at the treatment outlet (WTP) and at the distribution network location (NET). Drinking water samples were taken every 5 min during each sampling period.

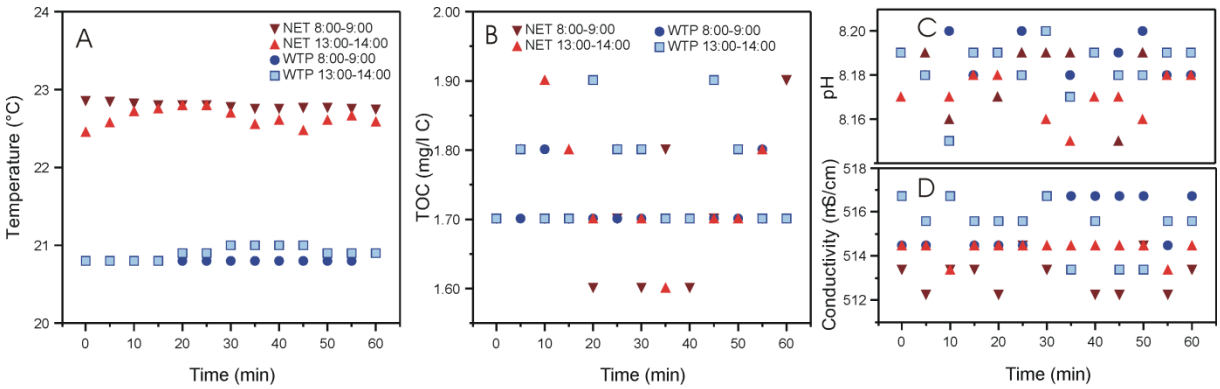


Figure S4.3.2. Overview of routine parameters variations over 1 h, in the morning (8:00-9:00) and in the afternoon (13:00-14:00) of the same day, at the treatment outlet (WTP) and at the network location (NET). (A) water temperature, (B) TOC, (C) pH, and (D) conductivity. Drinking water samples were taken every 5 min during each sampling period.

TOC was measured according to the Dutch standard procedure (NEN-EN-1484) with a TOC analyser (TOC-V CPN, Leverancier Shimadzu). The detection limit was 1 mg L^{-1}.

S4.4 Comparison of fluorescence fingerprints and community composition with time sequence

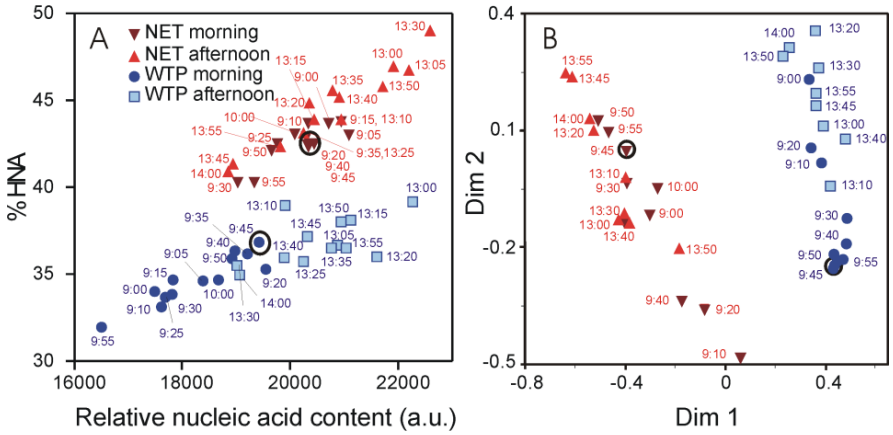
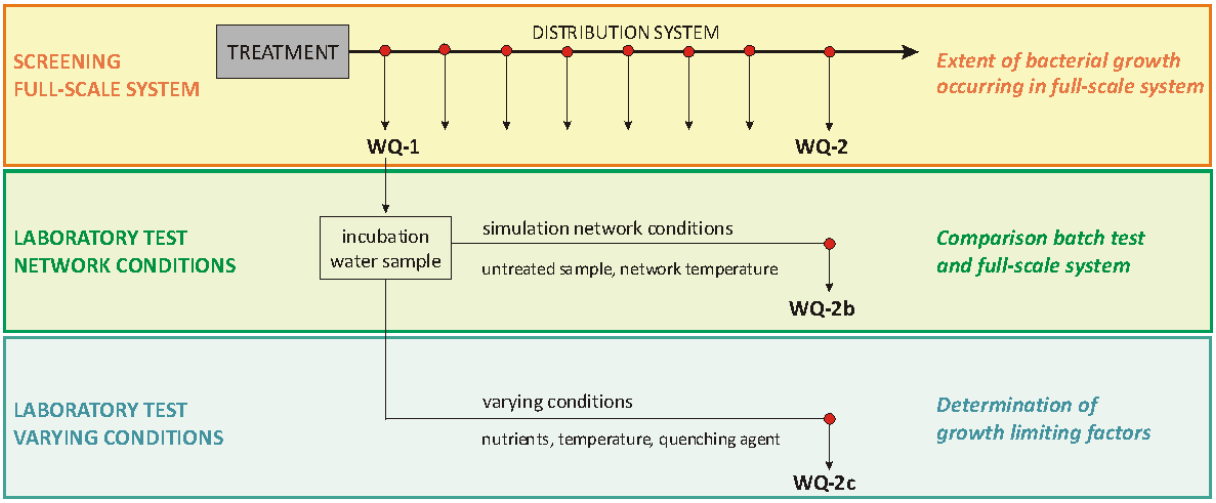


Figure S4.4.1. Comparison of processed (A) flow cytometric fingerprints and (B) genetic fingerprints (molecular data, multidimensional scaling graph) of bacteria in the drinking water samples taken at the treatment outlet (WTP) and at the distribution network location (NET) in the morning (08:00-09:00) and in the afternoon (13:00-14:00). Drinking water samples were taken every 5 min during each sampling period. The circles indicate two samples, taken at 8:45 at both locations, that were studied in detail in Figures 4.4C, 4.4D and 4.5. The numbers indicate the sampling time for each data point.

Chapter 5

A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems



This chapter has been submitted for publication as: E.I. Prest, F. Hammes, S. Kötzsch, M.C.M. van Loosdrecht, J.S. Vrouwenvelder. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems.

Abstract

A systematic approach is presented for the assessment of (i) bacterial growth-controlling factors in drinking water and of (ii) the impact of distribution conditions on the extent of bacterial growth in full-scale distribution systems. The approach combines (i) quantification of changes in autochthonous bacterial cell concentrations in full-scale distribution systems with (ii) laboratory-scale batch bacterial growth-potential tests of drinking water samples under defined conditions. The latter include (i) the simulation of distribution network temperature and residence time and (ii) the step-wise assessment of bacterial growth-controlling factors by additions of e.g. organic and inorganic nutrients or residual disinfectant quenching agents. The growth-potential tests were done by direct-incubation of water samples, without modification of the original bacterial flora, and with flow cytometric quantification of bacterial growth. This method was shown to be reproducible (ca. 4% relative standard deviation on triplicates) and sensitive (detection of bacterial growth down to 5 µg L^{-1} of added assimilable organic carbon). The principle of step-wise assessment of bacterial growth-controlling factors was demonstrated on bottled water, shown to be primarily carbon limited at 133 (±18) × 10^3 cells mL^{-1} and secondarily limited by inorganic nutrients at 5500 (±1700) × 10^3 cells mL^{-1}. The systematic approach was applied to a Dutch full-scale drinking water treatment plant and distribution system. Analysis of the treatment plant effluent showed (1) bacterial growth inhibition as a result of end-point chlorination, (2) organic carbon limitation at 192 (±72) × 10^3 cells mL^{-1} and inorganic nutrient limitation at 375 (±31) × 10^3 cells mL^{-1}. Significantly lower net bacterial growth was measured in the full-scale system (176 (±25) × 10^3 cells mL^{-1}) than after batch growth of the same water under defined direct incubation conditions (294 (±35) × 10^3 cells mL^{-1}), highlighting the effect of drinking water distribution on bacterial growth. The systematic approach described herein provides quantitative information on the effect of drinking water properties and distribution system conditions on biological stability, which can assist water utilities in decision making on treatment or distribution system improvements to better control bacterial growth during water distribution.

5.1 Introduction

Water utilities aim to distribute biologically stable drinking water, i.e. to operate drinking water distribution systems (DWDS) in which no adverse bacterial growth occurs. Uncontrolled growth of bacteria in distribution pipelines can lead to aesthetic, operational and even hygienically-related problems (van der Kooij, 2003). Assessment of the bacterial growth potential of drinking water and identification of microbial growth-controlling factors are therefore required to enable better control of bacterial growth in DWDS.

The bacterial growth potential of drinking water is an experimental quantification of the extent of bacterial growth that water can promote under defined laboratory conditions (e.g. temperature), and depends on (i) all the growth-promoting/limiting compounds (e.g., organic and inorganic nutrient composition), (ii) the presence of growth-inhibiting substances (e.g., residual disinfectant) and on (iii) the autochthonous bacterial community. Organic carbon is often the main growth-controlling compound in drinking water (Joret et al., 1991; Escobar et al., 2001). Hence, conventional bacterial growth assays, such as the assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC) methods either assume organic carbon limitation (van der Kooij et al., 1982; Servais et al., 1989) or incorporate excess inorganic nutrients to ensure organic carbon limitation (Miettinen et al., 1999). However, previous studies have revealed phosphate to be the growth-limiting compound in several drinking waters (Miettinen et al., 1997; Sathasivan et al., 1997; Juhna and Rubulis, 2004).

The current use of conventional bacterial growth assays has three shortcomings. (1) Although growth potential methods are available, systematic investigation of specific bacterial growth limitations in drinking water are nearly never performed, which provides limited understanding of water characteristics. (2) Conventional growth potential methods are essentially used to estimate the concentration of the growth limiting compounds (e.g., AOC) rather than to estimate the bacterial growth. (3) Water transport in full-scale DWDS can alter the water characteristics and subsequently affect bacterial growth. For example, DWDS conditions can promote disinfectant residual decay (LeChevallier et al., 1996; Nescerecka et al., 2014), and the release of organic or inorganic compounds into the water from pipe walls and sediments (Niquette et al., 2000; Zacheus et al., 2001; Lehtola et al., 2004; Bucheli-Witschel et al., 2012), or from the mixing of waters differing in composition (Niquette et al., 2001). Bacteria present in large numbers in pipe and reservoir biofilms and sediments can also compete with bulk water bacteria for available biodegradable nutrients and be released into the drinking water. In addition, varying water residence times and temperatures

(Kerneis et al., 1995; Uhl and Schaule, 2004; Hammes et al., 2010) are likely to modify bacterial growth kinetics in comparison with controlled and standardized batch conditions applied for laboratory growth tests. Consequently, controlled laboratory-scale growth potential tests are not sufficient to predict bacterial growth actually occurring during water distribution. In situ water sampling is needed to provide a picture of the extent of bacterial growth occurring in full-scale DWDS (Niquette et al., 2001; Lautenschlager et al., 2013; Nescerecka et al., 2014).

To address these issues we propose to integrate full-scale DWDS investigations and step-wise laboratory-scale growth potential tests into one systematic approach. For the laboratory tests we adapted the approach used by Gillepsie et al. (2014), which consists of the direct-incubation of drinking water samples without modifying the autochthonous community, and combined it with a step-wise assessment of bacterial growth-controlling factors. In addition, the bacterial growth behaviour of the same drinking water in well-defined laboratory conditions and the full-scale distribution network was directly compared. The objective of this study was to evaluate the integrated approach for (i) quantification of bacterial growth in DWDS, (ii) identification of bacterial growth-controlling factors in drinking water and (iii) evaluation of the effect of full-scale drinking water distribution on the extent of bacterial growth.

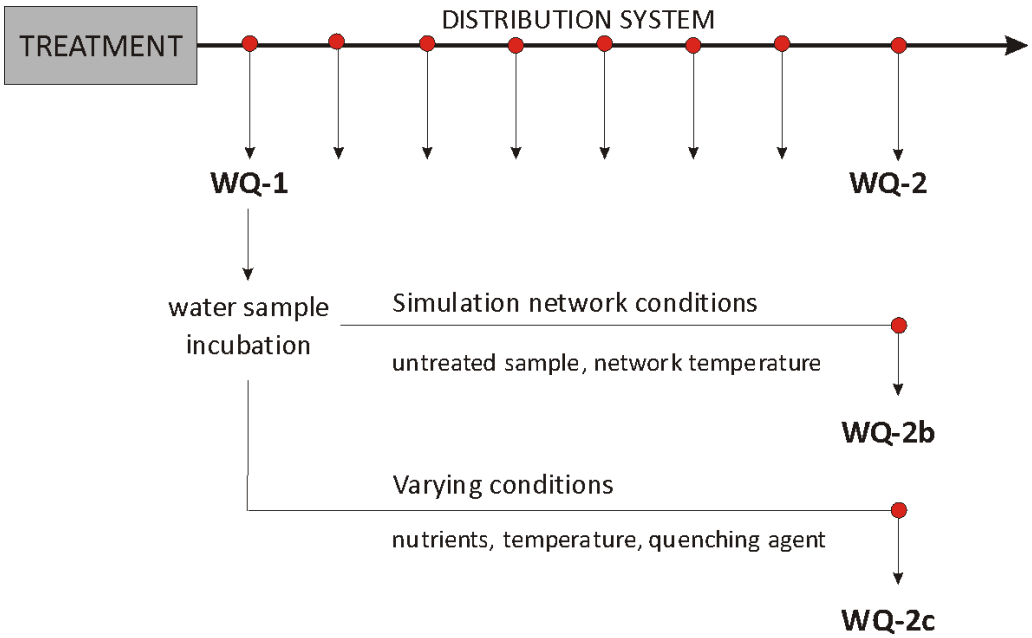


Figure 5.1. Schematic overview of the systematic approach for the evaluation of bacterial growth controlling factors during drinking water distribution. WQ-1: Water quality at the treatment outlet; WQ-2: water quality in the distribution system; WQ-2b,c: water quality after bacterial growth potential tests under controlled laboratory conditions. WQx are compared.

5.2 Description of the systematic approach

Figure 5.1 provides an overview of the proposed three-step approach for determining bacterial growth-controlling factors in drinking water and evaluating the effect of full-scale distribution.

5.2.1 Quantification of changes in bacterial cell concentrations during full scale distribution

As a first step water samples are collected at the drinking water treatment plant effluent, and at full-scale distribution system (DWDS) sampling points with various estimated water residence times, for the quantification of suspended bacterial cells in the DWDS. The total and intact planktonic bacterial cell concentrations are accurately quantified using flow cytometry (FCM) (Lautenschlager et al., 2013; Chapter 3). We argue herein that the difference in bacterial cell concentrations between the treatment plant and the network locations reflects the extent of bacterial growth, and thus biological stability, in the DWDS (Niquette et al., 2001; Lautenschlager et al., 2013; Chapter 4).

5.2.2 Comparison of bacterial growth in laboratory and full-scale conditions

In a second step, laboratory-scale tests are used to quantify bacterial growth in the unmodified treatment plant effluent, which is then compared with the changes detected during full-scale water distribution (5.2.1). The treatment plant effluent sample is incubated as such in AOC-free glassware at the same temperature as the distributed water and bacterial cell concentrations are measured at time intervals representative of the DWDS samples. Differences in the extent of bacterial growth in the laboratory batch test and in the DWDS at comparable residence times and temperature are reflective of the effect of the prevailing distribution conditions on the extent of bacterial growth in the DWDS.

5.2.3 Determination of bacterial growth-controlling factors in the treatment effluent water

The third step is an assessment of bacterial growth-controlling factors in the treatment plant effluent. This is performed with a combination of growth potential tests with varying incubation conditions (Table 5.1). The tests step-wise assess (1) the presence of residual disinfectant, (2) absence of viable bacterial cells, (3) organic carbon limitation, (4) inorganic nutrient limitations, and/or combinations of these. To enable comparison, all growth tests are performed at a fixed sample incubation temperature and incubation time.

Table 5.1. Scheme for comprehensive investigation of growth-limiting factors in drinking water.

Test	Quench*	Inoculum**	Nutrients***	Acetate	Compare with	Investigation
T1	-	-	-	-	-	What is the water growth potential?
T2	+	-	-	-	T1	Is there oxidative toxicity (disinfectant)?
T3	+	+	-	-	T2	Are there live cells?
T4	+	+	+	-	T3	Is a non-carbon compound limiting growth?
T5	+	+	-	+	T3	Is the water carbon limited?
T6	+	+	+	+	T4 and T5	Are there multiple limitations?

*: add only in case of treatments including chemical disinfection
**: add only in case of treatments using chemical disinfection or physical barrier (membrane filtration, e.g. UF, NF, RO).
***: contains all nutrients needed for bacterial growth except carbon. A similar approach can be applied if a non-carbon limitation is detected, by adding individually different compounds (e.g. phosphorous, iron, …).

Figure 5.2 proposes a supportive scheme to (i) select appropriate tests for the studied drinking water system and/or (ii) interpret data obtained from combined tests for the evaluation of risks associated with bacterial growth limitations in a water sample.

5.2.4 Novelty and value

While individual components of this approach have been applied in previous studies, the value of the present work is in the systematic and combined investigations at laboratory and full scales. The combination of laboratory tests and on-site investigations aims at the prediction of scenarios that can affect the water microbial growth properties, causing uncontrolled bacterial growth. This provides a basis for further detailed investigation and/or decision making on treatment or distribution condition improvements, to better control bacterial growth in DWDS.

5.3 Materials and methods

5.3.1 Water samples

Experiments were carried on bottled water (Evian, France) and on drinking water produced at a treatment facility in the Netherlands (Kralingen, NL). At this location, surface water is treated by coagulation, flocculation and sedimentation, followed by ozonation (1.0-1.3 mg L^{-1}, 10 min), dual medium filtration, and granular active carbon filtration (approx. 15 min bed contact time).

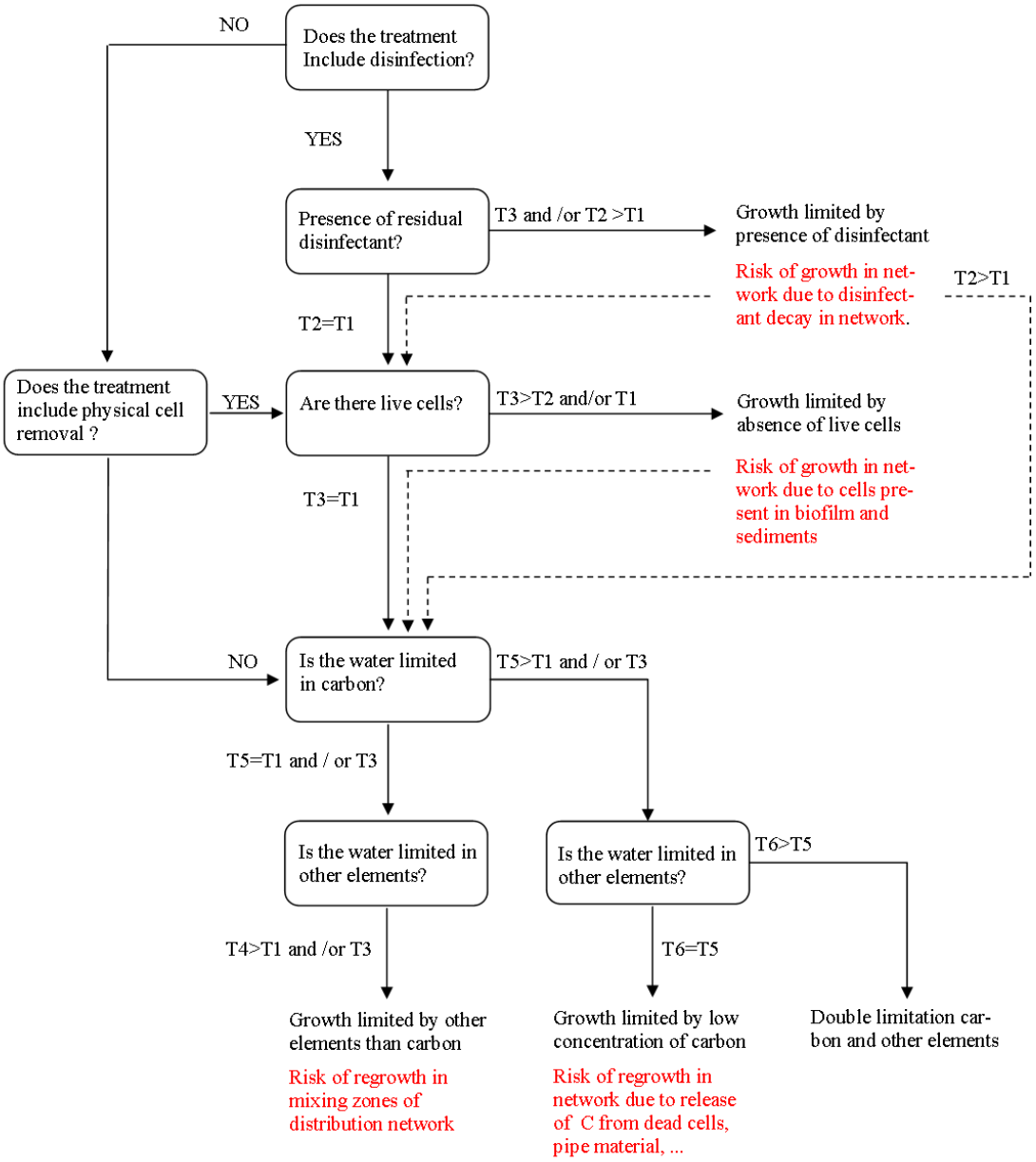


Figure 5.2. Decision making tree for the assessment of growth-limiting factors in a water sample and associated risks for uncontrolled bacterial growth in a full-scale distribution system. Tx refer to the test numbers as indicated in Table 5.1. T1=T2: same extent of growth in both tests; T2>1: extent of growth higher in T2 than in test 1.

Chlorine dioxide (0.1 mg L^{-1}) is added at the end of the treatment and the water is collected in a reservoir before distribution, resulting in a ClO_2 concentration in the water after storage of 0.006 mg L^{-1} on average (Table 5.2).Chlorine dioxide concentrations within the subsequent distribution system are below detection limit (< 0.001 mg L^{-1}) in most cases and were not reported by the water utility. An overview of the drinking water treatment effluent properties is given in Table 5.2.

Table 5.2. Overview of water treatment plant effluent properties. Minimum, maximum and average values of each parameter measured in (N) number of samples over one year period.

Parameter	unit	Min	Max	average	N
Temperature	°C	2.3	22	12.5	416
Dissolved oxygen	mg/l O_2	10.3	19.4	13.9	52
Turbidity	FTU	<0.05	0.36	<0.05	466
pH		7.79	8.32	8.08	468
Conductivity (20 °C)	mS/m	40.5	49.6	44.8	417
UV-extinction, 254 nm	1/m	1.1	2.6	2.1	27
DOC	mg/l C	1.07	1.97	1.61	28
AOC	µg/l ac-C	5.3	43	28	20
Chlorine dioxide	mg/l ClO_2	0	0.034	0.006	93
Carbon dioxide	mg/l CO_2	<1	2.8	1.9	13
Hydrogen carbonate	mg/l HCO_3	106	134	121	13
Chloride	mg/l Cl	50.7	60.2	53.5	13
Sulphate	mg/l SO_4	43	66	52	13
Sodium	mg/l Na	34	42	37	13
Potassium	mg/l K	5	6.5	5.6	13
Calcium	mg/l Ca	43.4	50	46.9	13
Magnesium	mg/l Mg	6.7	7.8	7.2	13
Hardness	mmol/l	1.363	1.538	1.463	13
Ammonium	mg/l NH_4	<0.03	0.05	<0.03	52
Nitrite	mg/l NO_2	<0.01	<0.01	<0.01	13
Nitrate	mg/l NO_3	11.2	14.6	12.8	13
Silicium	mg/l Si	1.25	3	2.1	13
Iron	µg/l Fe	<5	9	<5	36
Manganese	µg/l Mn	<5	<5	<5	4
Aluminium	µg/l Al	<10	<10	<10	13
Antimony	µg/l Sb	<1	<1	<1	4
Arsenic	µg/l As	<1	<1	<1	4
Barium	µg/l Ba	14	22	18	4
Boron	mg/l B	0.042	0.049	0.046	4
Cadmium	µg/l Cd	<0.05	<0.05	<0.05	4
Chromium	µg/l Cr	<1	<1	<1	4
Mercury	µg/l Hg	<0.03	<0.03	<0.03	4
Nickel	µg/l Ni	1	2	2	4
Selenium	µg/l Se	<1	<1	<1	4
Strontium	µg/l Sr	140	160	150	4
Bromide	mg/l Br	0.07	0.111	0.087	13
Fluoride	mg/l F	0.19	0.27	0.22	13
Cyanide	µg/l CN	<0.5	<0.5	<0.5	13
Bromate	µg/l BrO_3	2.3	6.8	3.6	26
Chlorate	µg/l ClO_3	<40	57	<40	13

5.3.2 *Flow cytometric (FCM) measurements*

FCM total cell concentration (TCC) was measured as proposed in the standardized Swiss guideline for drinking water analysis (SMLB, 2012; Chapter 3). In short, samples (500 µL) were pre-heated to 35 °C (5 minutes) and then stained with 10 µL mL^{-1} SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), and incubated in the dark for 10 minutes at 35 °C before FCM measurement. For the assessment of intact bacterial cell concentrations (ICC), a working solution containing SYBR® Green I (1:100 dilution in DMSO; Molecular Probes) and propidium iodide (0.3 mM) was used for bacterial staining following the same protocol as described above. FCM measurements were performed using a BD Accuri C6® instrument (BD Accuri cytometers, Belgium) equipped with a 50 mW laser emitting at a fixed wavelength of 488 nm. The FCM is equipped with volumetric counting hardware, calibrated to measure the number of particles in 50 µL of a 500 µL sample. Measurements were performed at pre-set flow rate of 35 µL min^{-1}. A threshold value of 700 a.u. was applied on the green fluorescence channel (FL1). Triplicate FCM measurements had a relative standard deviation below 5%.

5.3.3 AOC-free material and experiments

All glassware used for water sampling and growth potential tests was prepared as described previously to make it AOC-free (Greenberg et al., 1993; Hammes and Egli, 2005). In short, the glassware was soaked in acid overnight (HCl, 0.2N) and subsequently rinsed with ultrapure water (mQ, Biocel), air dried and heated at 550 °C for 6 hours. The caps to cover the incubation glass vials (40 mL) and sampling bottles were Teflon coated and were soaked in a 10% sodium persulfate solution at 60 °C for at least 1 hour, rinsed with ultrapure water and air-dried. All glassware cleaning procedures, preparation of nutrient solutions and growth potential tests have been performed in a clean laboratory environment, with reduced input of volatile organic compounds in the air. During growth potential tests, pipet tips were rinsed 10 times with ultrapure water before pipetting water from vials for FCM measurements, to prevent AOC from the pipet tip plastic to be released in the sample.

5.3.4 Preparation of stock solutions

All stock solutions of quenching agent (sodium nitrite), organic carbon (sodium acetate), and inorganic nutrients were prepared with ultrapure water in AOC-free glass bottles. Final concentrations of quenching agent and organic carbon in water samples are given in the relevant sections below. The acidified inorganic nutrient stock solution was adapted from Ihssen and Egli (2004) and contained 1.28 g L^{-1} $Na_2HPO_4.2H_2O$, 0.3 g L^{-1} KH_2PO_4 and 1.77 g L^{-1} $(NH_4)_2SO_4$, as well as the

following trace elements : 80 mg L^{-1} $CaCO_3$, 11 mg L^{-1} $MnCl_2.4H_2O$, 1.5 mg L^{-1} $CuSO_4.5H_2O$, 1.3 mg L^{-1} $CoCl_2.6H_2O$, 4 mg L^{-1} ZnO, 1.2 mg L^{-1} H_3BO_3, 134 mg L^{-1} $MgCl_2.6H_2O$, 10 mg L^{-1} $NaMoO_4.2H_2O$, and 30 mg L^{-1} EDTA $Na_4.2H_2O$. A solution containing 2.7 g L^{-1} $FeCl_3.6H_2O$ was prepared separately. To ensure carbon limitation during a growth potential experiment, 680 µL of the inorganic nutrient stock solution and 10 µL of the iron stock solution were added to 20 mL water sample.

5.3.5 Direct incubation method for the assessment of growth potential: proof-of-principle

Control experiments were done with bottled mineral water (Evian, France) for the evaluation of the direct water sample incubation method for bacterial growth potential tests. This bottled water was specifically selected, as it contains low organic carbon concentrations, a range of inorganic nutrients and an indigenous community of viable bacteria (Berney et al., 2008; Marcussen et al., 2013).

5.3.5.1 Evaluation of the direct incubation growth potential method

Bottled water was transferred into AOC-free glass vials. Sodium acetate was added to triplicate vials to obtain final carbon concentrations of 0, 5, 10, 15, 20 and 25 µg-C L^{-1}. Inorganic nutrients were added as described in section 5.3.4. All vials were incubated in the dark at 30 °C for 3 days, and the initial and final total cell concentrations were measured with FCM (section 5.3.1). Measurements were not performed after longer incubation times, as previous studies have shown that stationary phase is reached after 3 days for bacterial communities growing in bottled water at 30 °C (Hammes and Egli, 2005). The extent of bacterial growth, or net bacterial growth was calculated from the increase in total cell concentration between start and end of the water sample incubation.

5.3.5.2 Assessment of bacterial growth-controlling factor(s) in bottled water

Bottled water was transferred into AOC-free glass vials and treated with or without the addition of organic carbon, inorganic nutrients, and/or quenching agent (Table 5.1). Organic carbon (acetate) was added to triplicate vials to obtain a final concentration of 1 mg-C L^{-1} and ensure that organic carbon was not the limiting compound. Inorganic nutrients were added as described in section 5.3.4. The bottled water did not contain any disinfectant residual, but in order to verify that the quenching agent solution does not promote additional bacterial growth (e.g., from contamination), quenching agent was added in part of the glass vials at a final concentration of 5 mmol L^{-1} $NaNO_2$. No inoculum was added in this experiment, as the bottled water already contains viable bacterial cells and no growth-inhibiting substance. The samples were thereafter incubated in the dark at 30 °C for 3 days, and the initial and final total bacterial cell concentrations were measured with FCM.

5.3.6 Application of the systematic approach to a full-scale drinking water distribution system

5.3.6.1 Quantification of bacterial growth within the distribution system

Quantification of actual bacterial growth in the full-scale distribution system was performed by collecting water samples at locations with varying residence times (0, 24, 48-72 and 120 h). However, as the available tools to estimate residence time provide only rough estimations, sampling of the "same water" at both locations could not be accurately achieved, and all samples were taken on the same day. For each estimated residence time, three to five water samples were taken at different locations in the drinking water distribution network. The samples were collected in HD-PE bottles containing 2 mL L^{-1} of a mixed solution of sodium thiosulfate solution (20 g L^{-1}) and of nitrilotriacetic acid (25 g L^{-1}), as routinely applied for microbial water sampling in the Netherlands. The water temperature in the network samples was 11.6 ± 1.8 °C. The samples were transported on melting ice in a cooling box and kept at 4 °C until analysis. FCM measurements were performed in less than 24 h after sampling for the determination of total and intact bacterial cell concentrations.

5.3.6.2 Evaluation of growth potential of unmodified treatment effluent samples

For the assessment of the growth potential of the treatment plant effluent, a water sample was taken in a 250 mL AOC-free glass bottle, after the reservoir at the end of the treatment train. The water was transferred into triplicate AOC-free glass vials within 2 h of sampling. The vials were thereafter incubated in the dark, without further treatment, for 5 days at 12 °C, and FCM measurements were performed at start and after 1, 2, 3, and 5 days of incubation for a comparison with bacterial growth occurring in the full-scale distribution system.

5.3.6.3 Assessment of bacterial growth-controlling factors in the treatment plant effluent

Identification of bacterial growth-controlling factors was performed on water collected at the full-scale treatment plant after the chlorine dioxide addition point (before the reservoir), to ensure the presence of disinfectant and to evaluate the potential of the laboratory growth potential method to detect the impact of residual disinfectant on bacterial growth potential. A water sample was collected in a glass bottle without addition of quenching agent to study the effect of chlorine dioxide on bacterial growth. Another sample was collected in a separate bottle and quenched with sodium nitrite (5 mmol L^{-1}). An additional sample was taken before the addition of chlorine dioxide (i.e. granular active carbon filtrate) to be used as inoculum. All samples were collected in AOC-free glass bottles. The water without quenching agent addition was transferred into triplicate AOC-free glass

vials, within 2 h of sampling, without further treatment (Test 1 in Table 5.1). The water sample containing the quenching agent was also transferred into glass vials that were treated with the addition of bacterial inoculum, organic carbon and/or inorganic nutrients, according to Table 5.1 (tests 2 to 6). Inoculum (i.e. GAC filtrate containing 125×10^3 cells mL^{-1} as determined with FCM) was added in order to add 10^4 bacterial cells mL^{-1} to the individual vials. Acetate was added to triplicate vials to obtain a final concentration of 500 µg-C L^{-1}. Inorganic nutrients were added as described in section 5.3.4. All treatments were done in triplicate vials, which were incubated in the dark at 30 °C for 10 days. The total cell concentration in each vial was measured with FCM at the start and after 3, 7 and 10 days of incubation, to check for stationary phase.

Table 5.3. Overview of investigations with corresponding water sampling locations and experiment descriptions. WTP: water treatment plant effluent.

Investigation	Water type and/or sampling location	Experiment description	Growth potential incubation conditions	Section	Figure
Direct incubation for the assessment of growth potential: proof of principle					
Evaluation of the direct incubation growth potential method	Bottled water	Growth potential tests with varying acetate concentrations	30 °C, 3 days Addition of acetate at varying concentrations	5.4.1	5.3
Assessment of growth-limiting factor(s) in bottled water	Bottled water	Growth potential tests with additions of quenching agent, nutrients and carbon solutions	30 °C, 3 days Additions according to Table 5.1	5.4.1	5.4
Application of the systematic approach to a full-scale distribution system					
Quantification of changes in planktonic bacterial cell concentration during water distribution	Full-scale system, various locations	Total and intact cell concentration measurements in water samples taken at various residence times	No growth potential tests	5.4.2.1	5.5
Evaluation of growth potential of WTP effluent water	WTP effluent water after reservoir	Growth potential test without addition, incubated at full-scale system water temperature	12 °C, 5 days No addition	5.4.2.2	5.6
Assessment of bacterial growth-limiting factor(s) in water	WTP effluent water before reservoir	Growth potential tests with additions of quenching agent, inoculum, nutrients and carbon solutions	30 °C, 10 days Additions according to Table 5.1	5.4.2.3	5.7

Table 5.3 provides an overview of the drinking water types and sampling locations of the drinking water samples collected for each experiment, as well as the incubation conditions for the bacterial growth potential tests.

5.4 Results

5.4.1 Direct incubation for the assessment of growth potential: proof-of-principle

Growth potential control experiments with bottled water showed that direct incubation of water samples without modification of the autochthonous bacterial community provides a sensitive and repeatable assessment of the extent of bacterial growth that a water sample can support (Figure 5.3). The extent of bacterial growth (net growth) was proportional to the carbon content in the water when no other nutrients were limiting and concentrations steps as low as 5 µg L^{-1} could be distinguished. A numerical yield of 1.4 (±0.2) × 10^7 cells (µg Ac-C)$^{-1}$ was measured, which is in the upper range of previous observations (van der Kooij, 2002; Ross et al., 2013). The results were obtained with good repeatability, with less than 4% error on average on triplicate samples.

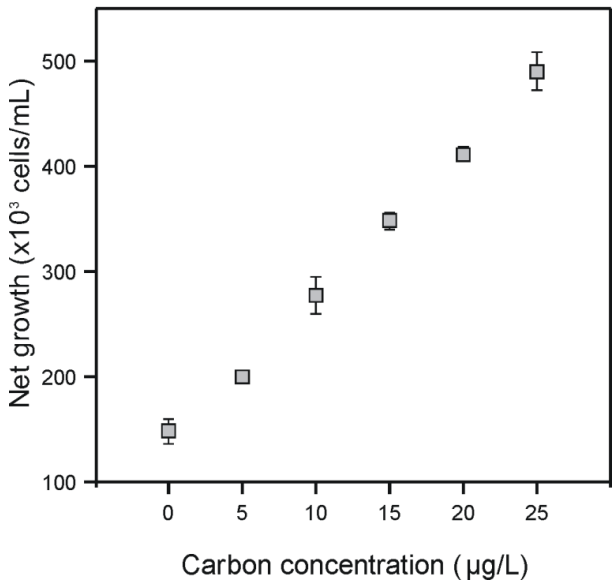


Figure 5.3: Relationship between carbon concentration and bacterial growth in bottled water. Acetate was added to bottled water at varying concentrations, and each sample was incubated in triplicate AOC-free glass vials in the dark at 30°C. The bacterial cell concentration in each vial was measured after 3 days using flow cytometry. The net growth is the increase in bacterial cell concentration between the start and end of the incubation period. The error bars indicate the error on triplicate samples.

The step-wise approach proposed in Table 5.1 was further used to identify bacterial growth-controlling compounds in the bottled water samples. Figure 5.4 shows that this particular bottled water without any additives supported bacterial growth up to 133 (±18) × 10^3 cells mL^{-1} (corresponding to approximately 9.8 µg Ac-C L^{-1} equivalent; based on the yield determined above). Quenching reagent addition did not significantly affect the extent of bacterial growth in the case of this water that did not contain residual disinfectant. The addition of excess inorganic nutrients had a negligible effect on growth (170 (±10) × 10^3 cells mL^{-1}), compared to the addition of 1 mg L^{-1} carbon (5500 (±1700) × 10^3 cells mL^{-1}), clearly indicating that the primary bacterial growth-limiting compound was organic carbon. However, the bacterial cell concentration obtained with the addition of acetate-carbon was lower than expected from the introduced carbon concentration (c.a. 1.4 ×10^7 cells mL^{-1}; Figure 5.3), suggesting a possible secondary limitation. This was confirmed by significantly higher cell concentrations (1.1 (±0.34) × 10^6 cells mL^{-1}) in the samples where both acetate-carbon (1 mg L^{-1}) and excess inorganic nutrients were added. In summary, these control experiments with the direct incubation method showed sensitive quantification (as low as 5 µg-C L^{-1}) of proportional bacterial growth, and the identification of a primary organic carbon limitation of growth at 1.7 × 10^5 cells mL^{-1} and a secondary inorganic nutrient limitation of growth at 5.5 × 10^6 cells mL^{-1}.

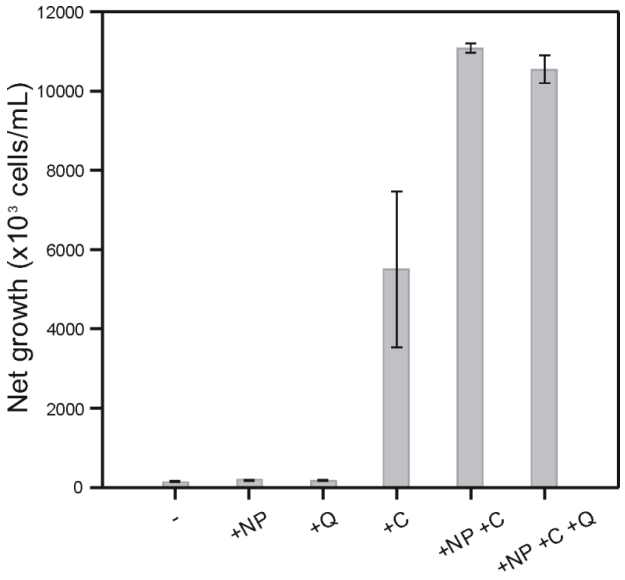


Figure 5.4: Investigation of bacterial growth-limiting factor(s) in bottled water. The water sample was treated with different solutions, as summarized in Table 5.1. The net growth is the increase in cell concentration between the start and end of the incubation period (3 days, 30°C), as measured by flow cytometry. The error bars indicate the error on triplicate samples for each condition. (-) No addition; (+Q) addition of quenching agent; (+NP) addition of inorganic nutrient solution; (+C) addition of organic carbon.

5.4.2 Application of the systematic approach to a full-scale distribution system

The combination of full-scale sample investigation and laboratory-scale growth potential tests described in section 5.2 (Figures 5.1 and 5.2, Table 5.1) was applied to samples from a Dutch full-scale drinking water distribution system (DWDS).

5.4.2.1 Quantification of changes in planktonic bacterial cell concentration during water distribution

FCM analysis of samples taken at the treatment plant effluent and at different locations in the DWDS enabled the detection of changes in cell concentrations during water distribution (Figure 5.5). The water sampled at the treatment plant effluent contained 117 (±2) × 10^3 cells mL^{-1}, with 38 (±2) % of intact bacterial cells. This low percentage of intact cells is attributed to the addition of chlorine dioxide after the final treatment step, before storage in the clear water reservoir. Significantly higher total cell concentrations (TCC) (147 (±18) × 10^3 cells mL^{-1}) were measured in DWDS samples with approximately 24 h residence time (t=2.92, p=0.02 based on independent samples t-tests). TCC at longer residence times in the distribution network ranged from 165 × 10^3 to 176 × 10^3 cells mL^{-1} and was not significantly different from the 24 h samples. Notably, the TCC increase after 24 h residence time in the DWDS was mainly due to an increase in intact cell concentration (ICC) (from 45 (±1) × 10^3 cells mL^{-1} to 65 (±15) × 10^3 cells mL^{-1}), with the percentage ICC consequently increasing up to 44 (±5) %.

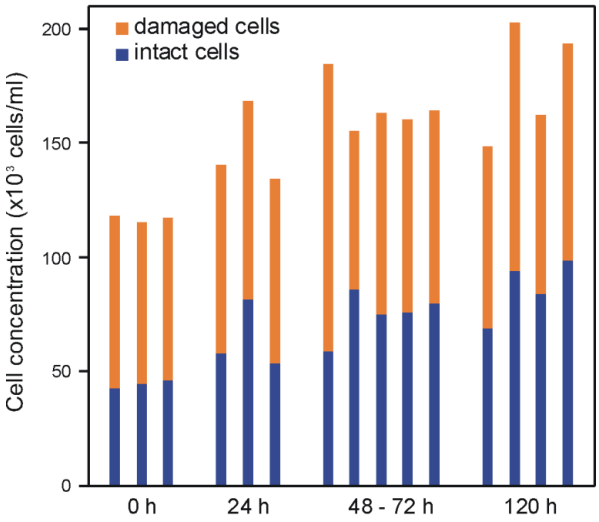


Figure 5.5. Investigation of bacterial growth in a Dutch full-scale drinking water distribution system. Total, intact and damaged cell concentrations of water samples taken at various residence times from the network.

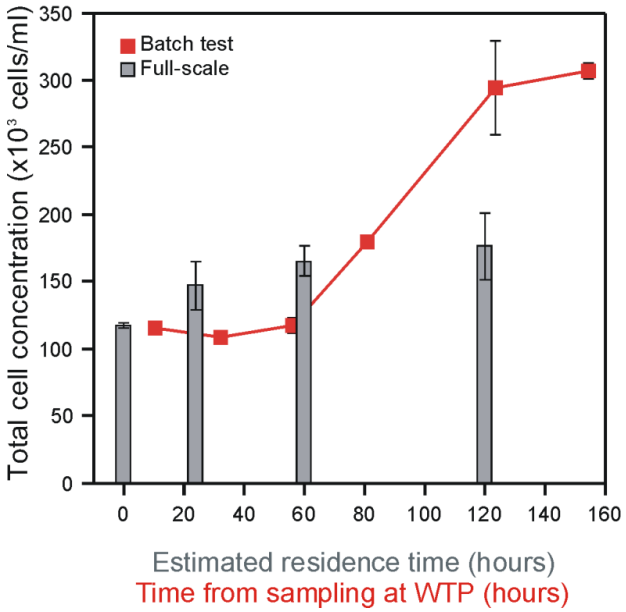


Figure 5.6. Comparison of the total bacterial cell concentrations measured in water samples taken from a Dutch full-scale distribution system at different residence times (histograms) and the bacterial growth observed in the water treatment plant (WTP) effluent incubated in triplicate in the laboratory at network temperature (12°C) (growth curve). The errors bars on the growth curve indicate the standard deviation on triplicate samples. Three to five samples were taken in the full-scale system for each residence time range (0 hours = WTP effluent water, 24 hours, 48 to 72 hours, 120 hours). The histogram error bars indicate the standard deviation on the samples taken at each residence time range.

5.4.2.2 Comparison of data from the full-scale system and laboratory tests

Bacterial growth in the treatment plant effluent incubated without any modifications under temperature and time conditions similar to the DWDS (12 °C; 24–120 h) showed discrepancies compared to the increase in bacterial cell concentrations in the full-scale DWDS (Figure 5.6). In the batch growth tests, a lag phase was observed up to 60 h, which was not observed in the full-scale samples. Comparable TCC values were measured in the batch tests and DWDS after approximately 80 h incubation. However, at longer residence times (120 h), significantly higher cell concentrations were recorded in the batch test (294 (±35) × 10^3 cells mL^{-1}) than in the full-scale system (176 (±25) × 10^3 cells mL^{-1}). The differences in cell concentrations measured in the laboratory scale batch growth tests and in the full-scale DWDS show that the distribution conditions affect bacterial growth in the pipelines.

5.4.2.3 Assessment of bacterial growth-controlling factors in the treatment plant effluent

Multiple growth limitations were identified and quantified in the treatment plant effluent samples assessed with a series of step-wise growth potential tests (Figure 5.7). Firstly, a decrease in TCC (-116 (±6) × 10^3 cells mL^{-1}) was observed in the absence of quenching agent addition, suggesting cell death

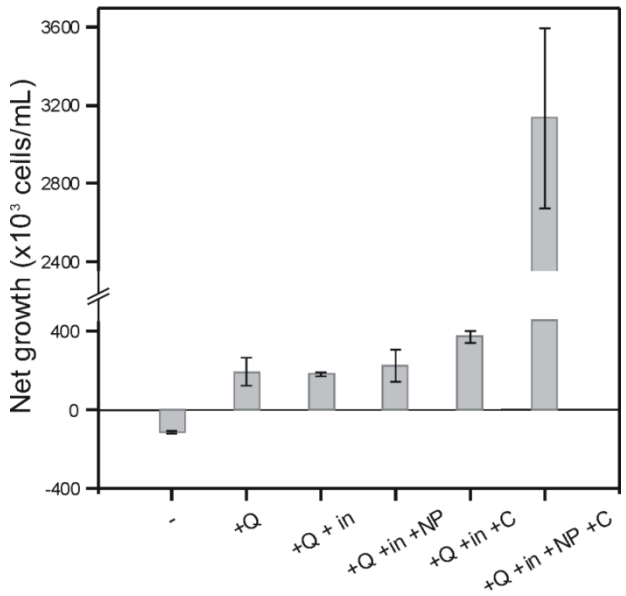


Figure 5.7. Investigation of the growth-limiting factor(s) in the treatment effluent water of a Dutch full-scale drinking water treatment plant, sampled after the chlorine dioxide addition point. The water sample was treated with different solutions, as summarized in Table 5.1. The net growth is the increase in cell concentration between the start and end of the incubation period (10 days, 30°C), as measured by flow cytometry. The error bars indicate the error on triplicate samples for each condition. (-) No addition; (+Q) addition of quenching agent; (+in) addition of inoculum; (+NP) addition of inorganic nutrient solution; (+C) addition of organic carbon. Note the y-axis break.

caused by residual disinfectant. However, when the disinfectant was quenched, bacterial growth occurred (net growth of 192 (±72) × 10^3 cells mL^{-1}). The extent of bacterial growth was not significantly affected by the addition of a viable bacterial inoculum or by the addition of inorganic nutrients. This suggests that viable bacteria were initially present in the water sample despite the disinfectant and that the primary bacterial growth-limiting compound was not an inorganic nutrient. The latter observation was supported by the addition of excess organic carbon (0.5 mg-C L^{-1}) that resulted in slight but significantly higher net growth (375 (±31) ×10^3 cells mL^{-1}; t=4.08 and p=0.008), showing that the water sample was primarily limited in organic carbon. However, the obtained cell concentrations were considerably lower than expected from the addition of 0.5 mg-C L^{-1}, suggesting a possible secondary limitation. This was confirmed by the addition of both organic carbon (0.5 mg-C L^{-1}) and excess inorganic nutrients, which resulted in significantly higher cell concentrations (net growth of 3.1 (±0.5) × 10^6 cells mL^{-1}). Overall, the set of bacterial growth tests revealed: (1) bacterial growth inhibition by residual chlorine dioxide; (2) primary growth limitation by organic carbon at ca. 1.9 x 10^5 cells/mL; and (3) secondary growth limitation by inorganic nutrients at ca. 3.8 x 10^5 cells/mL.

5.5 Discussion

We propose and demonstrate here a systematic approach to (i) identify and quantify bacterial growth-controlling factors in drinking water and (ii) evaluate the impact of distribution conditions on the extent of bacterial growth in full-scale drinking water distribution systems (DWDS) (Figures 5.1 and 5.2). Laboratory-scale growth potential tests were performed by direct incubation of water samples, thus measuring the response of the autochthonous community to varying conditions (incubation temperature and time, chemical additions; Figure 5.1, Table 5.1). This direct incubation growth potential method was shown to be accurate and sensitive (Figure 5.3), and suitable for the identification of the growth-controlling factors in water (Figures 5.4 and 5.7). When applied to a full-scale DWDS, the comprehensive systematic approach showed that changes in bacterial cell concentrations during distribution (Figure 5.5) were always lower but not strictly in accordance to the predictions of growth potential tests (Figure 5.6), highlighting the influence of the distribution conditions on the bacterial growth in the DWDS. Interestingly, the growth potential tests revealed multiple limitations (disinfectant residuals, organic carbon and inorganic nutrients) in these specific samples (Figure 5.7).

5.5.1 Direct incubation of water samples for the evaluation of bacterial growth potential

5.5.1.1 Advantages of the direct incubation method

The direct incubation growth potential method is a straightforward, simplified version of existing growth potential tests using indigenous bacterial communities (Werner and Hambsch, 1986; Hammes and Egli, 2005) and an improvement of the method recently proposed by Gillepsie and co-workers (2014). It is based on the incubation of water samples, without modification of the autochthonous community with steps such as filtration (Hammes and Egli, 2005) or pasteurization (van der Kooij, 1982). The reduction of these sample handling steps is a major advantage compared to existing growth potential tests such as AOC or BDOC assays, limiting both labour and risk of sample contamination with external nutrients (Ross et al., 2013). Our method differs from the approach suggested by Gillespie and co-workers (2014) in that we used specifically prepared AOC-free glassware, instead of plastic containers for water incubation, to avoid potential contamination (Niquette et al., 2000; van der Kooij and Veenendaal, 2001; Bucheli-Witschel et al., 2012). In the direct growth potential method, the growth of the autochthonous bacteria is studied rather than pure culture(s). The bacterial community is grown in its own environment and is therefore likely the most adapted community to consume the maximum amount of available nutrients, therefore

revealing the water's maximum growth potential. Drinking water bacterial communities are typically very diverse and contain up to 30 different phyla, 1500 operational taxonomic units (OTUs) (Pinto et al., 2012; Liu et al., 2014; Lin et al., 2014; Wang et al., 2014; Chapter 4), and likely organisms already adapted to the specific water environment. The method was shown to be reproducible and sensitive, with the detection of bacterial growth from carbon concentration down to 5 µg-C L^{-1} (Figure 5.3), which is comparable with other methods (between 1 µg-C L^{-1}, van der Kooij et al., 1982, and 10 µg-C L^{-1}, Hammes and Egli, 2005). The easy handling of the direct incubation growth potential method offers the possibility to be applied on regular basis by water utilities at lower costs for water characterization.

5.5.1.2 Assessment of growth potential vs. nutrient content

In the proposed growth potential method, we evaluate the maximum extent of growth that the autochthonous bacterial community can reach in the water, rather than estimating the concentration of specific compounds (e.g., assimilable organic carbon) used for growth. Bacterial growth has been shown to be proportional to the concentration of easily assimilable organic carbon compounds (e.g. acetate), both in the cases of pure bacterial strains (van der Kooij et al., 1982; van der Kooij and Hijnen, 1985) and in the case of mixed bacterial communities (Werner and Hambsch, 1986; Hammes and Egli, 2005; Figure 5.3 in this study), as long as all other elements required for growth are available in excess. Thus, net-growth can in theory be converted to organic carbon (equivalent) concentrations using measured or estimated growth yields (van der Kooij and Hijnen, 1985; Hammes and Egli, 2005, Ross et al., 2013). However, the conversion can only be accurate when single bacterial species and a single substrate are used, as yields vary between substrates and organisms (Kaplan et al., 1993; van der Kooij, 2002). Moreover, the linearity between the extent of bacterial growth and organic carbon concentration would not apply if the AOC content cannot be used entirely by bacteria due to limitation in another element such as phosphate (this study; Miettinen et al., 1997). Conversion to carbon concentrations would then be erroneous if no other element is added in the growth test (Miettinen et al., 1999; Chow, 2009; Ihssen and Egli, 2004), and would lead to a misinterpretation of the water growth properties. Hence we chose to express the growth potential results as the actually measured bacterial cell concentrations (cells mL^{-1}), thus avoiding potentially erroneous conversions of bacterial cell concentrations to e.g. carbon concentrations (van der Kooij et al., 1982; Hammes and Egli, 2005). The absence of bacterial growth in such tests is a straightforward indicator of a biological stable water with its given characteristics at the sampling location and time, and under the incubation conditions. However, changing conditions during water distribution might induce instability in the same water (discussed below).

5.5.2 Identification of bacterial growth-controlling factors in water

Adapting the growth potential method with straightforward chemical additions is a simple procedure for the identification of bacterial growth-controlling factors in water. Until now, most drinking water studies that include growth potential assays focused only on organic carbon limitation (van der Kooij et al., 1992; Escobar et al., 2001; Niquette et al., 2001). Here we argue for a comprehensive and systematic assessment of growth-controlling compounds in water and their effect on the growth potential. The addition of quenching agent, bacterial inoculum, organic carbon and/or inorganic nutrients needed for bacterial growth revealed multiple growth-limiting factors (Figures 5.4 and 5.7). This information is particularly relevant for practice by shedding light on scenarios that can convert apparently stable water into unstable water with uncontrolled bacterial growth, as described in Figure 5.2. For example, bacterial growth in the Dutch drinking water described in the present study was shown to be inhibited by the presence of disinfectant residuals, which would inevitably decay in the distribution system and thus would leave room for bacterial growth (results of the test T2>T1 in Figure 5.2). Similarly, a phosphate-limited water could be subject to promote unexpected bacterial growth if additional phosphate is brought to the water via the mixing of different water types, cell death or the re-suspension of sediments (Niquette et al., 2001; Zacheus et al., 2001; Lehtola et al., 2004). Organic carbon leaching from pipe materials can also provide bacterial growth-promoting nutrients in carbon-limited waters (Niquette et al., 2000; van der Kooij and Veenendaal, 2001; Bucheli-Witschel et al., 2012), but would not alter growth if e.g. phosphate is limited. In this regard the double nutrient limitation observed in the presently studied water (T4>T3, T5>T3 and T6>T5 in Figure 5.2) is a particularly efficient barrier against uncontrolled and unpredicted bacterial growth in the distribution system.

Table 5.1 proposes basic tests for rapid evaluation of the bacterial growth-promoting properties of water. A primary limitation in organic carbon and a secondary limitation in inorganic nutrients have been detected in both bottled water and the full-scale water treatment effluent (Figures 5.4 and 5.7). The individual compound responsible for the secondary limitation was not identified in this study, but can easily be done through addition of individual inorganic compounds instead of the combined inorganic nutrients solution. Based on the nutrient requirements of bacteria we propose the order of additions to be as follows: N, P, Fe, and trace elements. Consequently, improvements to drinking water treatment can be considered to further reduce concentrations of identified growth-limiting nutrient(s) in water and thus reduce the overall growth potential of the water. In addition, the water sample incubation temperature can be easily adapted to quantify the extent of bacterial growth that can result from temperature fluctuations in distribution systems (Niquette et al., 2001; Hammes et al., 2010), or from increased water temperatures at household levels (Lipphaus et al., 2014). The

incubation time of the growth test is another straightforward variable to evaluate the risks for bacterial growth-related problems that can rise in distribution areas with high residence time, particularly dead-end zones or household taps, where residence times can be much longer than what is commonly found in DWDS. Overnight stagnation especially has been shown to lead to increased bacterial cell numbers (Lautenschlager et al., 2010). Such systematic understanding of bacterial growth-controlling factors in drinking water can provide a solid basis for the improvement of water treatment processes, to lower the growth potential of the water, and/or for the improvement of distribution conditions to avoid uncontrolled microbial growth in full-scale DWDS.

5.5.3 Combining laboratory and full-scale investigations

Based on the present study, we argue that laboratory and full-scale investigations are complementary and should always be conducted in parallel to link predicative growth potential measurements with actual changes in the DWDS. Laboratory-scale tests are applied to investigate the water bacterial growth potential under controlled conditions and predict potentially problematic situations during full-scale water distribution (Figures 5.2, 5.6 and 5.7), and are useful for optimization of treatment and/or distribution conditions. However, growth potential assays are relatively straightforward planktonic batch growth experiments under defined conditions. Full scale DWDS in turn are extremely complex with variable conditions, and the assessment of bacterial growth in such systems can only be achieved by direct investigation in the system (Niquette et al., 2001; Lautenschlager et al., 2013; Liu et al., 2014). This enables the detection of changes in the microbial water characteristics (Chapter 4) and the identification of so-called “hot-spot” areas within the same network with bacterial growth concern (Lautenschlager et al., 2013; Nescerecka, 2014). Hence, the effect of drinking water distribution conditions on bacterial growth should be evaluated by comparing the full scale actual measurements and laboratory scale predictive data. The present study was the first to directly compare the extent of bacterial growth in the same water under laboratory and full-scale conditions. We observed faster planktonic cell concentration increase in the DWDS compared to the laboratory scale tests, but lower final cell concentrations at approximately 120 h (Figures 5.5 and 5.6). We argue here that the increase in DWDS planktonic cells is a direct consequence of growth, either directly in the planktonic phase or in the biofilms with subsequent detachment. The faster increase in DWDS cell concentrations is attributed to a rapid depletion of residual disinfectant in the network, as well as the presence of viable bacteria in distribution network biofilms. The lower final growth in the network at long residence times (120 h) (Figure 5.6) is attributed to various factors, such as the predation by protozoa or viruses (Sibille, 1998; van Lieverloo et al., 2002) and/or the competition between bulk water and biofilm bacteria for the

available nutrients. Arguably, a significant proportion of the available nutrients would be consumed by sediment and biofilm bacteria, as more than 95% of the total biomass in distribution pipelines recorded to be in the biofilm and sediment phases (Zacheus et al., 2001; Flemming et al., 2002; Liu et al., 2014). Investigation of the specific growth-controlling factors within the distribution system can be considered, based on hypothesis formulated after comparison between growth tests and full-scale investigations.

5.6 Conclusions

A systematic approach combining full scale investigations with laboratory scale growth potential tests is proposed for (i) the assessment of bacterial growth-controlling factors in drinking water and (ii) the evaluation of drinking water distribution on the extent of bacterial growth in full-scale distribution systems.

Controlled bacterial growth potential tests of bottled water showed that:

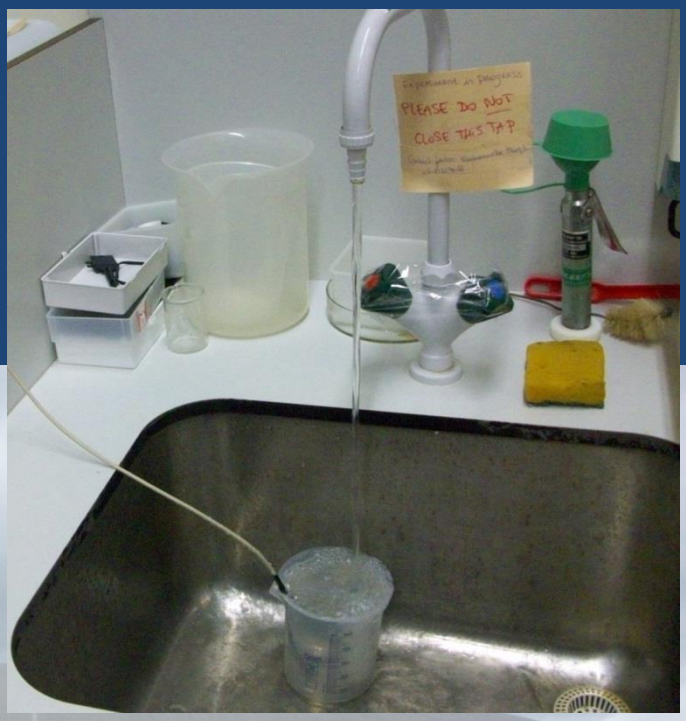
- direct incubation of water samples with the unmodified autochtonous bacterial community provides a straightforward quantification of the water growth potential.
- bacterial growth in bottled water was limited by carbon at 133 (±18) × 10^3 cells mL^{-1} and by inorganic nutrients at 5500 (±1700) × 10^3 cells mL^{-1}.

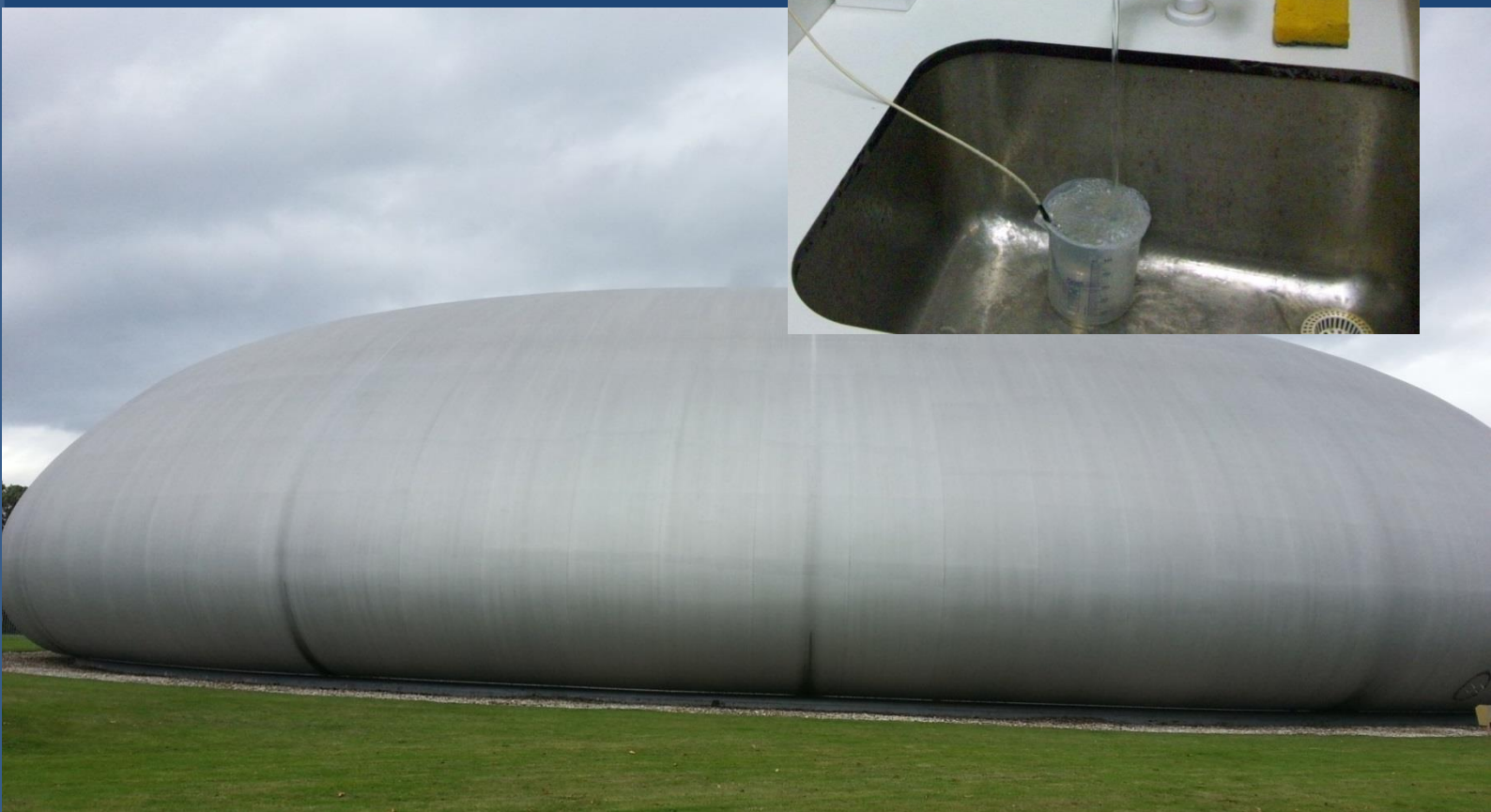
Application of the systematic approach to a full-scale drinking water distribution system showed that:

- bacterial growth in drinking water samples was (1) inhibited by the presence of disinfectant residual, (2) primarily limited by organic carbon up to 192 (±72) × 10^3 cells mL^{-1}, (3) and secondary limited by inorganic nutrients at 375 (±31) × 10^3 cells mL^{-1}.
- faster increase but lower final total cell concentrations occurred in the full-scale drinking water distribution system than in laboratory bacterial growth potential tests.

The systematic approach provides the basis for understanding bacterial growth-controlling factors in drinking water distribution systems and optimisation of water treatment and distribution conditions.

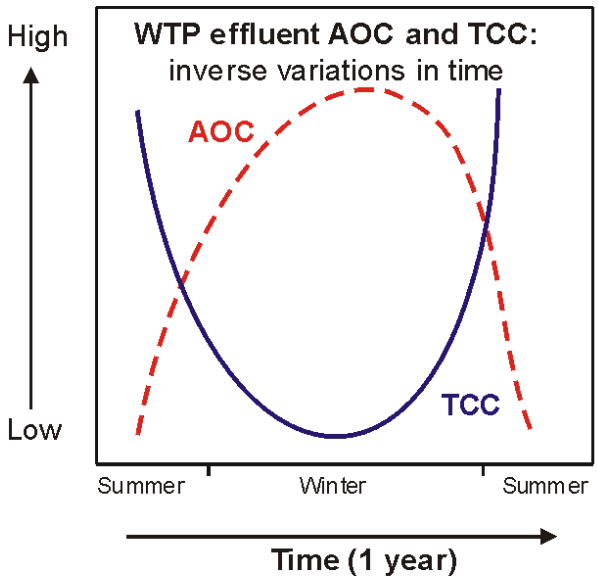
FULL-SCALE STUDIES



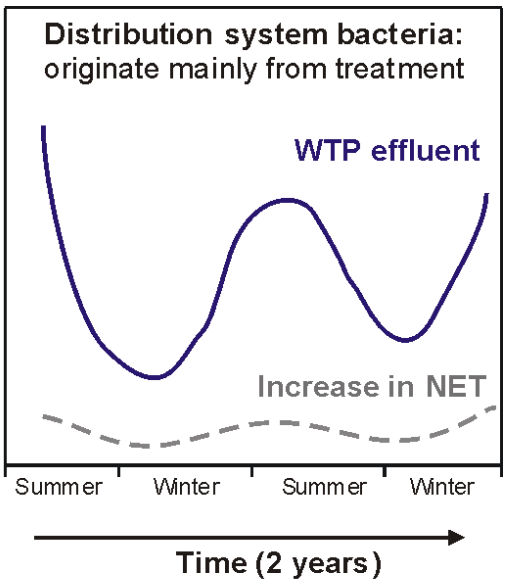


Chapter 6

Long-term bacterial dynamics in a full-scale drinking water distribution system



AOC: assimilable organic carbon
TCC: total bacterial cell concentration



WTP: water treatment plant
NET: distribution network

This chapter has been submitted for publication as: E.I. Prest, D.G. Weissbrodt, F. Hammes, M.C.M. van Loosdrecht, J.S. Vrouwenvelder. Long-term bacterial dynamics in a full-scale drinking water distribution system.

Abstract

Temporal dynamics in bacterial community characteristics were investigated during a two-year drinking water monitoring campaign in a full-scale distribution system operating without detectable disinfectant residual. A total of 360 water samples were collected on a biweekly basis at the water treatment plant (WTP) effluent and at one fixed location in the drinking water distribution system (DWDS). The samples were analysed for heterotrophic plate counts (HPC), *Aeromonas* plate counts, adenosine-tri-phosphate (ATP) concentrations, and flow cytometric (FCM) total and intact cell counts (TCC, ICC), water temperature, pH, conductivity, total organic carbon (TOC) and assimilable organic carbon (AOC). Multivariate analysis of the large dataset was performed to explore correlative trends between microbial and environmental parameters. The WTP effluent displayed considerable seasonal variations in TCC (from 90×10^3 cells mL^{-1} in winter time up to 455×10^3 cells mL^{-1} in summer time) and in bacterial ATP concentrations (<0.1 – 3.6 ng L^{-1}), which were congruent with water temperature variations. These fluctuations were not detected with HPC and *Aeromonas* counts. The water in the network was predominantly influenced by the characteristics of the WTP effluent. The increase in ICC between the WTP effluent and the network sampling location was small (34×10^3 cells mL^{-1} on average) compared to seasonal fluctuations in ICC in the WTP effluent. Interestingly, the extent of bacterial growth in the DWDS was inversely correlated to AOC concentrations in the WTP effluent (Pearson's correlation factor r=-0.35), and positively correlated with water temperature (r=0.49). Collecting a large dataset at high frequency over two years enabled the characterization of previously undocumented seasonal dynamics in the DWDS. Moreover, high-resolution FCM data enabled prediction of bacterial cell concentrations at specific water temperatures and time of year. The study highlights the need to systematically assess temporal fluctuations in parallel to spatial dynamics for individual DWDS.

6.1 Introduction

Drinking water distribution can lead to aesthetic water quality deterioration, such as bad taste, malodourous or coloured water, to operational problems such as bio-corrosion and/or fouling of water installations. Water quality changes are usually attributed to microbial processes in the distribution pipelines, including the growth of benign autochthonous bacteria and/or re-suspension or detachment of bacterial cells from pipe wall sediments and/or biofilms into the bulk water. Though the involved organisms are not necessarily hygienically relevant, water quality deterioration is of concern for water utilities, as it is a major cause of customer complaints and maintenance costs (Polychronopolous et al., 2003; Vreeburg et al., 2004). Numerous studies have shown that drinking water distribution results in spatial changes in the autochthonous microbial communities, i.e. in the type, amounts and proportions of bacteria present in water, as well as their activity and viability states (LeChevallier et al., 1987; Prévost et al., 1998; Nescerecka et al., 2014; Pinto et al., 2014). Short-term temporal variations in bacterial cell concentrations have also been measured on daily and weekly bases in the drinking water distribution system (DWDS) and at the consumer's tap (Besmer et al., 2014; Nescerecka et al., 2014; Chapter 7). On longer time-scales, bacterial quantification in large-scale DWDS have revealed temporal changes in coliforms (LeChevallier et al., 1996), heterotrophic plate counts (HPC) (Maul et al., 1985a; Francisque et al., 2009), bacterial cell counts (Prévost et al., 1998; Lautenschlager et al., 2013), adenosine-tri-phosphate (ATP) concentrations (van der Wielen and van der Kooij, 2010), and bacterial community composition (Pinto et al., 2014). Numerous factors could be the cause of seasonal variations in the microbial quality of drinking water, and include fluctuations in raw water quality, treatment efficiency, and water temperature. Better understanding of microbial dynamics during drinking water production and distribution is therefore needed to produce and maintain good water quality up to the consumer's tap.

Earlier studies of drinking water microbiology were predominantly based on HPC measurements (Maul et al., 1985a; Francisque et al., 2009). However, this approach has been shown to be inappropriate for the enumeration of autochthonous bacterial communities, as only a minute fraction of drinking water bacteria are able to grow on conventional growth-media (Hoefel et al., 2003). Flow cytometry (FCM) has been proposed as an alternative microbial monitoring method for rapid enumeration of the total number of bacterial cells, evaluation of cell viability, and fingerprinting of bacterial communities in water samples (Hammes et al., 2008; Berney et al., 2008; Chapter 3). Adenosine-tri-phosphate (ATP) measurement has also been suggested as a useful method to evaluate the viability of bulk water bacteria (van der Wielen and van der Kooij, 2010), particularly complementary to FCM (Berney et al., 2008; Hammes et al., 2010a; Vital et al., 2012a; Liu

et al., 2013c; Nescerecka et al., 2014). In addition, high-throughput sequencing methods, such as 454-pyrosequencing, Illumina, or Ion-torrent sequencing, provide high resolution information on bacterial community composition and structure (Mathieu et al., 2009; McCoy and van Briesen, 2012; Hwang et al., 2012; Pinto et al., 2014; Roeselers et al., 2015). A complete description of bacterial communities can only be obtained using a combination of such methods (Lautenschlager et al., 2013; Chapter 4), and microbial data should ideally be complemented by environmental and abiotic parameters. A few long-term microbial monitoring campaigns in DWDS have been performed with FCM, ATP or sequencing-based methods individually. However, the techniques were generally not combined and/or were applied without a link with abiotic parameters (Hwang et al., 2012; Mc Coy and VanBriesen, 2012; Pinto et al., 2014). In addition, available long-term studies were performed at low water sampling frequency and/or without investigation of the temporal dynamics (van der Wielen and van der Kooij, 2010; Hammes et al., 2010b; Lautenschlager et al., 2013; Roeselers et al., 2015). To date, there is no study available on long-term and detailed investigation of bacterial dynamics in full-scale DWDS using a combination of new methods together with conventional drinking water quality parameters.

In this study, temporal dynamics in water quality in a full-scale system distributing drinking water without detectable disinfectant residual was specifically studied by monitoring (i) a wide range of conventional microbial, organic, inorganic, and environmental parameters together with ATP and FCM (ii) over a 2-year period with (iii) high frequency sampling on a biweekly basis. The studied system is representative for water utilities treating surface water with extensive processes including ozonation and biofiltration, and distributing water without disinfectant residual. The objectives of this study were (i) to assess long-term temporal variations in the bacterial community abundance and viability at the outlet of the drinking water treatment plant and at one location in the distribution system, and (ii) to investigate the environmental factors responsible for these variations.

6.2 Materials and methods

6.2.1 Study location

Water sampling was performed at a Dutch full-scale drinking water treatment plant (Rotterdam area, The Netherlands) and in the corresponding distribution system (DWDS). The study location was selected for its representativeness for full-scale surface water treatment and well-maintained DWDS in industrialised countries, and for the availability of earlier datasets on the system (Chapter 4). The annual drinking water production was 40 x 10^6 m^3 per year. Surface water was treated at this

location by coagulation, flocculation and sedimentation followed by ozonation, dual medium filtration, and granular active carbon filtration. Final disinfection (0.1 mg L^{-1} chlorine dioxide) was applied at the inlet of a large storage reservoir where the water was collected before distribution. The concentration of chlorine dioxide in the reservoir effluent was below the minimum detection limit (<0.001 mg L^{-1}). The water was thus distributed in a DWDS operating without detectable residual disinfectant.

6.2.2 Water sampling

Drinking water samples were taken at the outlet of the water treatment plant (WTP) and at one location in the distribution network (NET) for the study of long-term temporal bacterial dynamics at both locations. The NET sampling site was selected based on an earlier study that showed significant differences in the water characteristics between this location and the WTP in the summer period (Chapter 4). Water samples were collected at the two locations every 2 weeks for a total period of 2 years. On every sampling day, 4 water samples were taken at each location at 30 minutes intervals (09:00, 09:30, 10:00 and 10:30), in order to be able to identify potential outliers and unexpected events. In total, 184 samples were taken per location. For practical reasons, the analyses of one or more parameters were not performed for a limited number of samples. The relative standard error on the flow cytometric results of the four samples was less than 15 % in the majority of sampling days, and outliers were removed when this value was exceeded. The residence time of the water in the DWDS at the NET location is of approximately 2 days, but was not taken into account in the sampling scheme, similarly to the sampling strategy applied in our preliminary study (Chapter 4). Available methods to estimate residence time provide only rough estimations, and sampling of the same water fraction at both locations could actually not be accurately achieved.

The water sampling taps at both locations were running continuously during the entire study period. On each sampling day and time, the water temperature was measured and water was collected in separate bottles for each parameter to be measured, i.e. FCM total and intact cell concentrations and fluorescence fingerprints, adenosine tri-phosphate concentration (ATP), heterotrophic plate counts (HPC), total organic carbon (TOC), pH and conductivity. For comparison to other microbial parameters, *Aeromonas* counts were measured during the last 6 sampling months, since it is commonly analysed in the Netherlands as an indicator for bacterial growth in DWDS. Additionally, the water utility provided data on concentrations of assimilable organic carbon (AOC) measured during the two-year period in the WTP effluent on independent sampling days than for the other parameters.

High-density polyethylene (HD-PE) plastic bottles (Identipack BV, NL) containing 2 mL L^{-1} of a mixed solution of sodium thiosulfate (20 g L^{-1}) and of nitrilotriacetic acid (25 g L^{-1}) were used to collect water for microbial analysis of ATP and HPC, and for FCM analysis, as routinely used by accredited laboratories for drinking water analysis in the Netherlands. Drinking water samples were collected in polyethylene terephthalate (PET) bottles without headspace (Identipack BV, the Netherlands) for pH and conductivity analysis and in glass bottles (Identipack BV, the Netherlands) containing sulphuric acid (8 mol L^{-1}, 0.2 mL in 100 mL bottle) for TOC analysis. The water samples were transported on ice, stored at 4 °C until analysis, and processed within 24 h after sampling.

6.2.3 Conventional parameters (HPC, ATP, Aeromonas*, AOC, TOC, pH, and electrical conductivity)*

HPC was measured by Aqualab Zuid (Werkendam, NL), according to the Dutch standard procedure (NEN-EN-ISO 6222, 1999). In short, 2 mL of the water sample were transferred to a sterile Petri-dish and mixed with 20 mL of yeast extract agar. The agar was kept at 44 °C before plating. The samples were incubated at 22 °C for 3 days. ATP was measured by Het Waterlaboratorium (Haarlem, NL), as described previously by Magic-Knevez and van der Kooij (2004). The ATP measurement is based on the emission of light resulting from the reaction between the ATP molecule and a luciferin/luciferase reagent (LuminATE, Celsis). For total ATP determination, ATP was first released from suspended microbial cells with nucleotide-releasing buffer (LuminEX, Celsis), while this step was not performed for the assessment of free ATP. The intensity of the emitted light was measured using a luminometer (Celsis AdvanceTM) that was calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure given by the reagent manufacturer. The detection limit of the method was 1 ng ATP L^{-1}. Bacterial ATP concentrations were calculated by subtracting free ATP from total ATP concentrations. *Aeromonas*, AOC, TOC, pH and conductivity were measured by Aqualab Zuid (Werkendam, NL), according to Dutch standard procedures. TOC was measured on a Shimadzu TOC analyser (type TOC-V CPN). *Aeromonas* measurements were performed by filtration (0.45 µm, cellulose nitrate filter) of 100 mL of water samples and plate incubation at 30 °C on a selective Ampicillin-Dextrin agar (Dutch guideline NEN 6263). AOC concentrations were determined following the procedure proposed by van der Kooij (1982) and according to the Dutch guideline NEN 6271. In short, pure bacterial strains P17 and NOX were inoculated in pasteurized water samples which were incubated in AOC-free glassware at 15 °C. Bacterial growth was assessed by plate counting on Lab-Lemco agar incubated for 2 days at 25 °C. The maximal growth was converted to organic carbon concentrations using specific yield values of the used organisms.

6.2.4 Flow cytometry (FCM)

The protocol described in chapters 3 and 4 was applied for water sample staining, FCM measurements and data analysis. Samples (500 µL) were pre-heated to 35 °C for 5 minutes, stained with fluorescent dyes and incubated in the dark for 10 minutes at 35 °C before measurement. For the determination of total bacterial cell concentrations (TCC), the samples were stained with 10 µL mL^{-1} of SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), while 10 µL mL^{-1} of a working solution containing a mixture of SYBR® Green I (1:100 dilution in DMSO; Molecular Probes) and propidium iodide (0.3 mM) was used for the assessment of intact bacterial cell concentrations (ICC). FCM measurements were 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. The FCM is equipped with volumetric counting hardware, calibrated to measure the number of particles in a 50 µL volume fraction of a 500 µL sample. Measurements were performed at a pre-set flow rate of 35 µL min^{-1}. A threshold value of 700 a.u. was applied on the green fluorescence channel (FL1). Bacterial signals were selected on the BD Accuri CFlow® software using electronic gating on density plots of green fluorescence (FL1; 533 nm), and red fluorescence (FL3; >670 nm). FCM fluorescence "fingerprints" of the bacterial community were visualized as green fluorescence histograms (Chapter 3). The analysis of fingerprints is detailed in supplementary information.

6.2.5 Multivariate numerical analyses of correlative trends

Computational multivariate analyses were conducted in R with dedicated packages (R development Core Team, 2008) according to the numerical ecology framework proposed by Weissbrodt et al. (2014) and applied in Besmer et al. (2014), following Borcard et al. (2011).

The simplified numerical methodology adapted here comprised the following sequential steps in a pragmatic computational approach (Figures S6.1.1 to S6.1.5, Supplementary information): (***i***) concatenation of all microbial and environmental datasets in one single squared matrix with consideration of same measurement dates and/or using linear interpolation to fill in gaps in temporal datasets, and importation in R; (***ii***) panel plots of dynamics in individual measured variables over time in order to delineate global trends; (***iii***) pair-wise x-y plots between all measured variables for preliminary visual observation of apparent linear and monotonic correlations; (***iv***) self-contained correlation matrices following Pearson's and Spearman's approaches for delineating linear and monotonic correlative trends between variables, respectively; (***v***) normalization and standardization of the datasets of different dimensions (FCM and environmental variables) by scaling into zero mean and unit variance using the "decostand" function; (***vi***) separate computation of Pearson's correlation

and Spearman's rank-order correlation coefficients between all variables, complemented by p-values at 95% confidence level in order to assess the significance of correlations; (***vii***) graphing and hierarchical clustering using the Ward algorithm of measured variables displaying analogous correlation patterns with all other variables in heat maps. This step enables a straightforward representation of the gradients in positive and inverse Pearson's correlations and Spearman's rank-order correlations between parameters and rapid identification of major correlative trends.

This numerical methodology was applied on datasets collected from the effluent of the WTP and from the sampling location in the NET. In the latter case, the changes in microbial parameters (ΔNET) between the WTP and NET were considered for multivariate numerical computation.

6.3 Results

Temporal variations in drinking water planktonic bacterial communities were investigated over 2 years at the treatment plant (WTP) effluent and at one location (NET) of a full-scale drinking water distribution system (DWDS) operating without detectable disinfectant residual. Table 6.1 provides an overview of the data presented in this paper.

Table 6.1. Overview of data collected during two-year monitoring of drinking water in a full-scale non-chlorinated distribution system, at the water treatment plant (WTP) effluent and at one location in the distribution network (NET).

Data set	Studied location	Information	Section	Figure
Variations in microbial parameters at two locations over a two-year period	WTP effluent, and one NET location	Seasonal variations in: TCC, ICC, and temperature bacterial ATP, HPC and *Aeromonas*	6.3.1	 6.1 6.2
WTP effluent characterization	WTP effluent	 Relationship between microbial and environmental parameters Effect water treatment	6.3.2 6.3.2.1 6.3.2.2	 6.3, 6.4, 6.5 6.5, 6.6
Characterization of change during water distribution	NET, compared to WTP	Increase in microbial parameters: Temporal variations Relationship with environmental parameters	6.3.3	 6.7 6.8

WTP: water treatment plant; NET: distribution network; TCC and ICC: total and intact bacterial cell concentrations; ATP: Adenosine-tri-phosphate; HPC: heterotrophic plate counts; FCM: flow cytometry.

6.3.1 Variations in microbial parameters at two locations over a two-year period

Clear and reproducible seasonal trends were observed in the microbial characteristics of the WTP effluent, which in turn determined the characteristics of the water in the NET (Figures 6.1 and 6.2). Large seasonal variations in total and intact bacterial cell concentrations (TCC and ICC) were measured at the WTP effluent (Figure 6.1). The highest concentrations were recorded in the summer periods concurrent with the highest water temperatures, and inverse trends were observed in winter periods. The TCC ranged from 90×10^3 to 455×10^3 cells mL^{-1}, of which only 26 ± 8 % were intact. Seasonal variations were also recorded in bacterial ATP concentrations in the WTP effluent (ranging between <0.1 and 3.6 ng L^{-1}; Figure 6.2). However, no trend was observed in heterotrophic plate counts (HPC), which remained below 5 CFU mL^{-1}, except for 32 seemingly random measurements (out of 176 data points). *Aeromonas,* commonly analysed in the Netherlands as a bacterial growth indicator, was measured in the last 6 months of the study (January – June 2014) that covered a wide range of water temperatures (6.5 - 18.5 °C). Similarly to HPC, the *Aeromonas* counts remained below 2 CFU 100 mL^{-1} in the WTP effluent. Low values for the percentage ICC, HPC and *Aeromonas* are attributed to the addition of chlorine dioxide before the storage reservoir at the end of the treatment train, although disinfectant residuals were not detectable after the reservoir.

Only minor changes in the drinking water microbial parameters were recorded at the NET location compared to the WTP effluent (Figures 6.1 and 6.2). A change in TCC could be observed between the two locations over the two-year study period, which was significant ($P<0.0001$ based on a paired t-test) but not systematic and relatively small (22% increase on average over the two years). However, increased ICC values (43×10^3 to 214×10^3 cells mL^{-1}) and bacterial ATP concentrations (0.7 to 4.5 ng L^{-1}) were consistently recorded (Figures 6.1B and 6.2A), with a significant ($P<0.0001$) and clear increase in ICC (71% on average) in comparison to the TCC increase. Changes were also detected in FCM fluorescence fingerprints based on ICC (Figure S6.2.1, Supplementary information). Similarly, *Aeromonas* counts increased between the two locations (from 22 to 428 CFU 100 mL^{-1}) along the 5 months monitored (Figure 6.2C), with the highest values being recorded at warmest water temperatures (15-18 °C). An increase in HPC values between the two locations was only detected in the summer periods (May - October of each year, Figure 6.2B), with values reaching up to 125 CFU mL^{-1}. Overall, the data shows that the major seasonal changes in TCC and ICC detected in the NET result from changes at the WTP, but that minor but detectable bacterial growth occurred during water distribution.

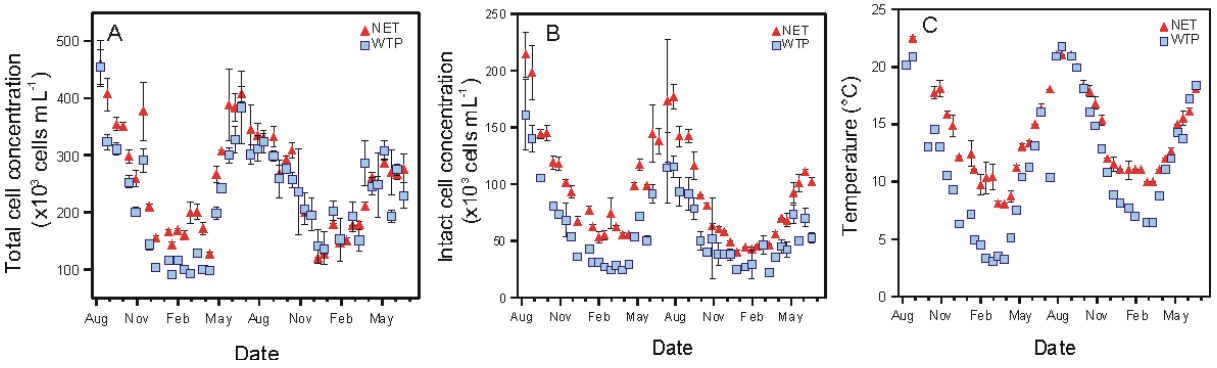


Figure 6.1. Variations in time over two years (August 2012 - June 2014) of (A) total bacterial cell concentration, (B) intact bacterial cell concentration and (C) water temperature at the water treatment plant (WTP) outlet and at one location in the network (NET). Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

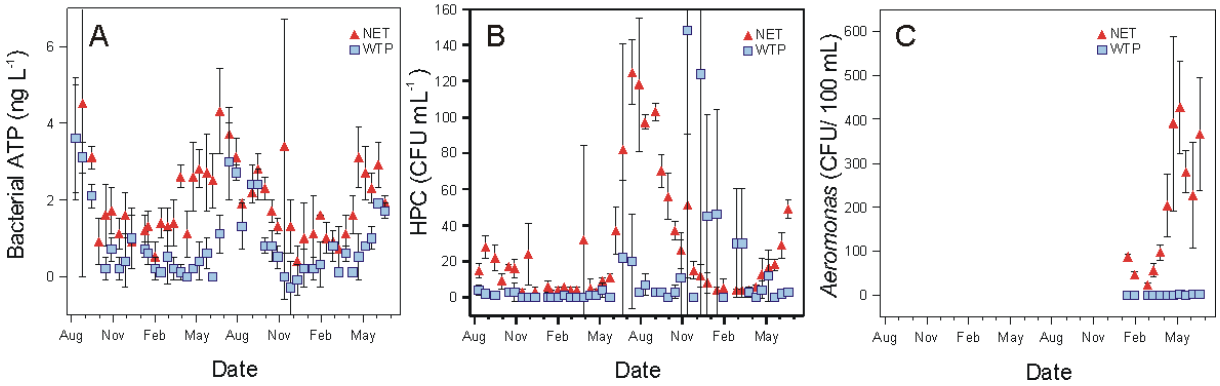


Figure 6.2. Variations in time over two years (August 2012 - June 2014) of (A) bacterial adenosine tri-phosphate (ATP), (B) heterotrophic plate counts (HPC), and (C) *Aeromonas* at the water treatment plant (WTP) outlet and at one location in the network (NET). *Aeromonas* was measured starting from January 2014, i.e. during the last 6 months of the investigation period. Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

6.3.2 WTP effluent characterization

6.3.2.1 Relationship between microbial and environmental parameters

Multivariate numerical analysis of the WTP effluent dataset suggested that water temperature had a strong linear relationship with microbial characteristics (Figure 6.3). A significant, strong, and positive linear correlation was found between water temperature and TCC ($p<0.001$; Pearson's correlation coefficient $r=0.78$; $N=176$), ICC ($p<0.001$; $r=0.75$; $N=173$) and bacterial ATP concentrations ($p<0.001$; $r=0.69$; $N=175$). The linear correlation between water temperature and HPC was not significant, though a weak but significant positive monotonic correlation ($p<0.001$; $r=0.37$; $N=175$) was found between the two parameters using a Spearman's test, which suggests a stronger influence of water temperature on HPC at higher temperatures (Figure S6.3, Supplementary information). This weak

correlation is probably due to the fact that extremely low HPC values were found in the WTP effluent (82 % of the HPC values were below 5 CFU mL^{-1}).

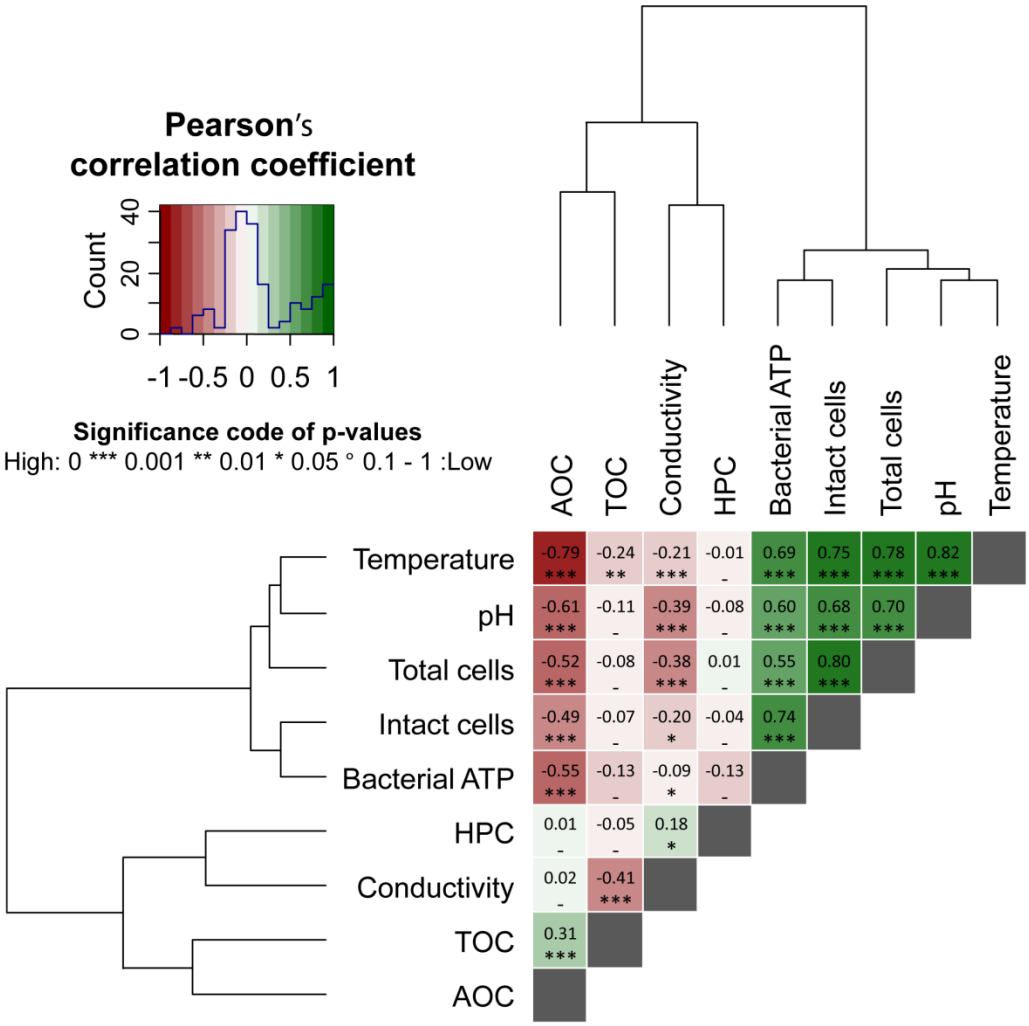


Figure 6.3. Heatmap of pair-wise Pearson's correlation coefficients (*i.e.* linear trends) computed between the microbial parameters (namely total cell concentration, intact cell concentration, bacterial ATP, and heterotrophic plate counts (HPC)), and the environmental parameters (namely temperature, pH, electrical conductivity, total organic carbon (TOC), and assimilable organic carbon (AOC)) measured in the effluent of the water treatment plant (2-year dataset, 184 water samples in total). Hierarchical clustering using the Ward's algorithm was first applied to reorder all parameters in clusters according to their correlation patterns as displayed by the dendrograms. The values and directions of the correlation coefficients are displayed according to the color key, *i.e.* positive correlations as green gradients from 0 to 1 and inverse correlations as red gradients from 0 to −1. Similar analysis of monotonic trends (*i.e.* Spearman's rank-order correlations) between microbial and environmental parameters is available in Figure S6.3 in supplementary information. This approach is analogous to the one developed and used in Weissbrodt *et al.* (2014) and Besmer *et al.* (2014).

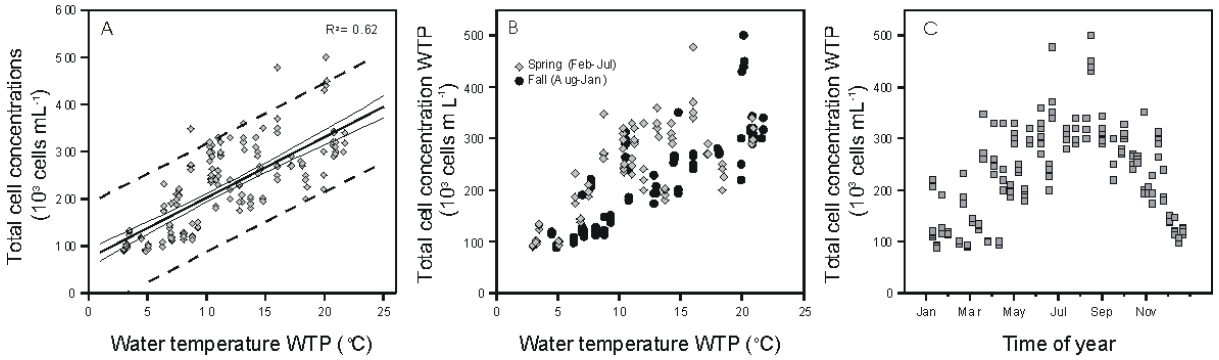


Figure 6.4. Relationship between water temperature and total bacterial cell concentration in the samples collected from the water treatment plant (WTP) effluent over the two years of investigation (August 2012 – June 2014). (A) 95% confidence interval (thin lines) and the 95% prediction interval (dotted lines). (B) Discrimination between spring and fall samples. (C) Classification of water samples based on sampling time of year.

Figure 6.4A shows the overall linear correlation between TCC and water temperature in the WTP effluent (N=168), suggesting that temperature can be used to predict TCC values. However, a careful inspection of the data showed that the samples taken during the cooling period of fall (August – January) and the warming period of spring (February – July) displayed dissimilar correlations with temperature (Figure 6.4B, 6.4C), suggesting that temperature was not the only driving factor for the TCC changes in the WTP effluent. Possible secondary influencing factors for the TCC seasonal variations include abiotic factors. TCC (N=176) was significantly and positively correlated to pH ($p<0.001$, $r=0.70$), and inversely correlated with conductivity ($p<0.001$, $r=-0.30$) and concentration of assimilable organic carbon (AOC) ($p<0.001$, $r=-0.52$), the three parameters also displaying seasonal variations (Figure S6.4, Supplementary information; Figure 6.5A).

6.3.2.2 Effect of water treatment on drinking water characteristics

The water treatment processes applied at the studied location shaped the bacterial community abundance and viability in the treated drinking water. FCM measurements performed in the effluent of each treatment step showed that the vast majority of bacteria was removed during the ozonation step (Figure 6.6), as previously observed in other systems (Hammes et al., 2010b; Vital et al., 2012a). The TCC in the WTP effluent was therefore fully controlled by the microbial dynamics in the subsequent treatment steps. Bacterial activity and proliferation in the biological filters (rapid sand filter and granular active carbon filter) are dependent on e.g. the water temperature and on the availability in organic and inorganic nutrients. Dynamics in the nutrient composition and concentrations in the ozonated influent of the biological filters would need further investigation. However, large variations in water temperature were indeed recorded and most likely partly responsible for seasonal variations in bacterial cell concentrations in the effluent of the WTP.

Bacterial viability was greatly influenced by the addition of chlorine dioxide, prior storage in the reservoir, as the percentage of intact cells decreased from 86 % after the GAC down to 14% after the reservoir. This dramatic decrease in the percentage of intact cells is attributed to the contact time of chlorine dioxide with the bacterial cells in the reservoir.

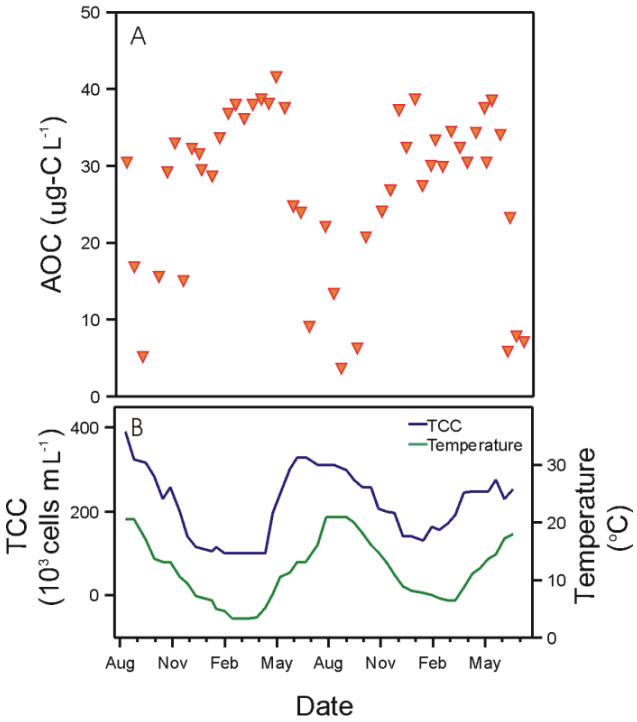


Figure 6.5. Variations in time over two years (August 2012 - June 2014) of (A) assimilable organic carbon (AOC) in the water treatment plant (WTP) effluent, compared to (B) variations in total bacterial cell concentration (TCC) and water temperature in the WTP effluent (data presented in Figure 6.1).

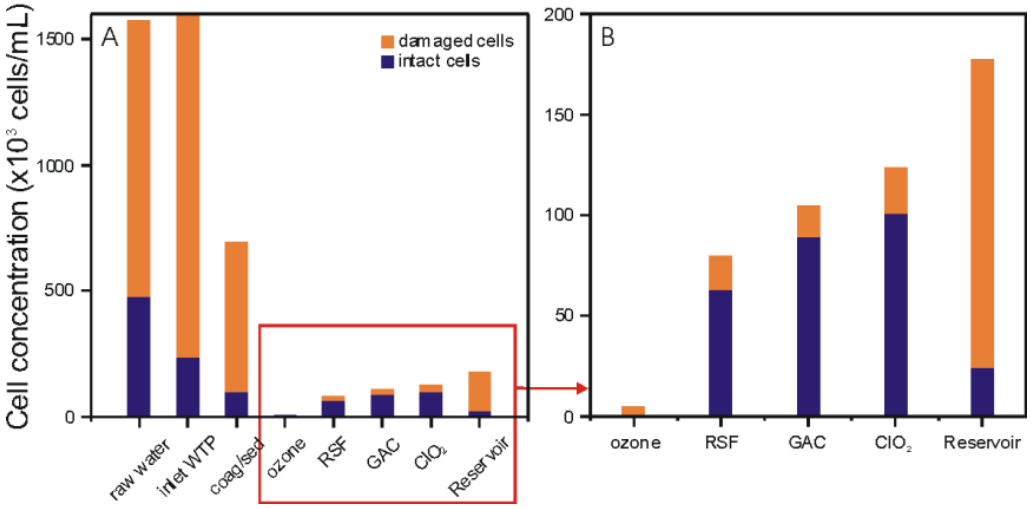


Figure 6.6. Flow cytometry analysis of water samples taken after each treatment step (sampling on a single day at the water treatment plant; November 2012). (A) Total, intact and damaged bacterial cell concentrations, (B) cell concentrations after the ozonation treatment step (enlarged from A). Legend: WTP: water treatment plant; coag/sed: coagulation and sedimentation; RSF: rapid sand filtration; GAC: granular active carbon filtration; ClO_2: chlorine dioxide addition.

Water temperature in the WTP effluent was strongly and inversely correlated to AOC concentrations ($p<0.001$; $r=-0.79$; $N=168$) (Figure 6.3), with AOC displaying an inverse seasonal trend compared to the water temperature and bacterial cell concentrations over the two-year period (Figure 6.5). The lowest AOC concentrations (3.5 µg Ac-C eq. L^{-1}) were recorded at highest water temperatures and highest bacterial cell concentrations, and vice-versa, with highest AOC concentrations reaching 41.4 µg Ac-C eq. L^{-1} in winter (Figure 6.5). The high AOC and low TCC values in winter times in the WTP effluent can be attributed to a low bacterial activity in the biofilters, due to low water temperatures, that would result in a low AOC removal capacity (Fonseca et al., 2001; Hammes et al., 2006). Overall, both the bacterial abundance and the nutrient content of the WTP effluent were shaped by the combination of ozonation and biofiltration treatment steps, in which water temperature apparently played a major role, while bacterial viability was controlled by chlorine dioxide addition and residence time into the storage reservoir.

6.3.3 Characterization of microbiological changes during water distribution

The extent of change in microbial parameters between the WTP effluent and the NET varied over the year and followed a predictable trend similar to the water temperature but an unexpected inverse correlation to the AOC concentration in the WTP effluent. Figure 6.7 shows that the ICC increase between the two locations ranged between 11 × 10^3 and 58 × 10^3 cells mL^{-1}, the highest increase being recorded in the warm periods. Similar observations were made for HPC, which increased significantly (up to 105 CFU mL^{-1}) in the summer periods, and for *Aeromonas* counts (increase up to 427 CFU / 100 mL at warm temperatures).

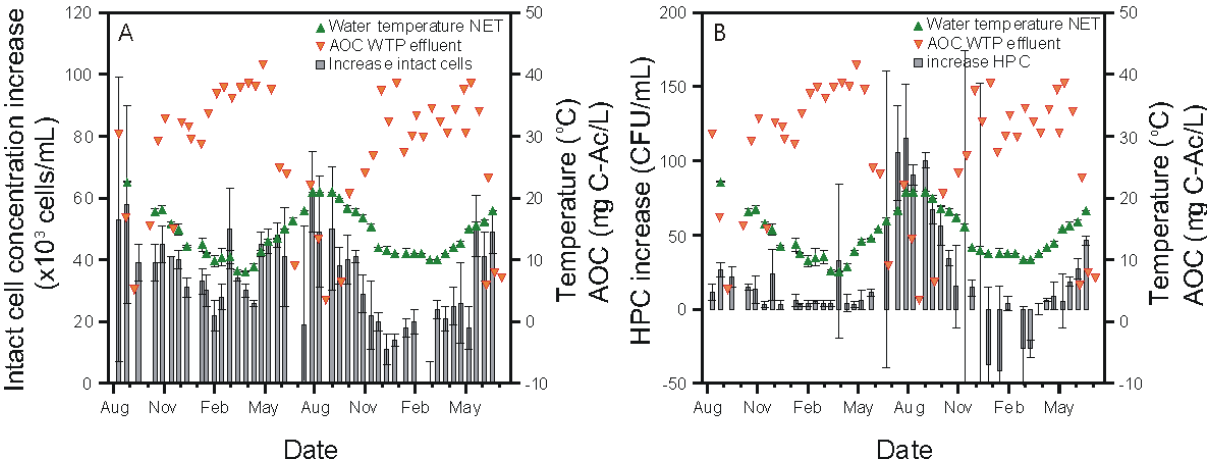


Figure 6.7. Variations in time over two years (August 2012 - June 2014) of the assimilable organic carbon (AOC) concentration in the treatment plant effluent (WTP) and the network (NET) water temperature, compared to (A) the intact cell concentration increase and (B) the HPC increase between the WTP effluent and the NET location. Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

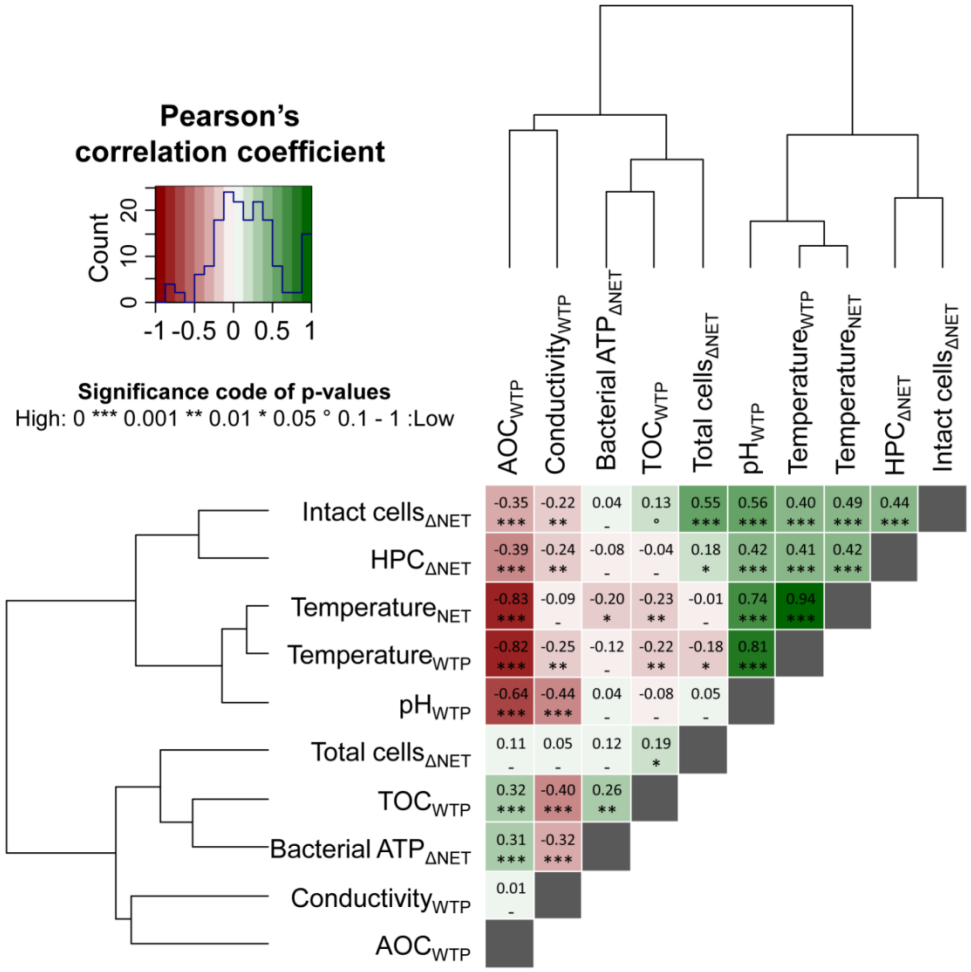


Figure 6.8. Heatmap of pair-wise Pearson's correlation coefficients (*i.e.* linear trends) computed between the changes in microbial parameters (namely total cell concentration, intact cell concentration, bacterial ATP, and heterotrophic plate counts (HPC)) occurring in the distribution network (ΔNET) between the water treatment plant (WTP) effluent and the sampling location in the network (NET), and environmental parameters (namely temperature, pH, electrical conductivity, total organic carbon (TOC), and assimilable organic carbon (AOC)) in the WTP effluent and/or at the NET location (2-year dataset, 184 water samples in total). Hierarchical clustering using the Ward's algorithm was first applied to reorder all parameters in clusters according to their correlation patterns as displayed by the dendrograms. The values and directions of the correlation coefficients are displayed according to the color key, *i.e.* positive correlations as green gradients from 0 to 1 and inverse correlations as red gradients from 0 to −1. Similar analysis of monotonic trends (*i.e.* Spearman's rank-order correlations) between microbial and environmental parameters is available in Figure S6.6 in supplementary information. This approach is analogous to the one developed and used in Weissbrodt *et al.* (2014) and Besmer *et al.* (2014).

Water temperature was significantly and positively correlated to the change in intact cell concentration ($p<0.001$; $r=0.49$; $N=168$) and with the change in HPC ($p<0.001$; $r=0.42$; $N=173$) (Figure 6.8). The increase in *Aeromonas* counts also displayed a strong relationship with water temperature (Figure S6.5, Supplementary information). No or only small HPC increases (below 10 CFU mL^{-1}) between the two sampling locations were recorded at low water temperatures, and the increase in bacterial ATP did not display seasonal trends. Overall, the strongest bacterial growth, as assessed by FCM, HPC, and *Aeromonas* analyses, was measured on the days with highest water temperatures,

which corresponded to the lowest AOC concentrations in the WTP effluent. Although this result seems counter intuitive, it suggests that the AOC concentration in the WTP effluent was not the only bacterial growth-controlling factor at this specific NET sampling location. This argument is supported by a significant but inverse and weak linear correlation between the concentration of AOC in the WTP effluent and the increase in intact cells ($p<0.001$; $r=-0.35$; $N=168$) or the increase in HPC ($p<0.001$; $r=-0.39$; $N=173$) during water distribution (Figure 6.8).

6.4 Discussion

Two-year monitoring of drinking water planktonic bacterial communities was performed at the water treatment plant (WTP) effluent and one location (NET) in a Dutch full-scale drinking water distribution system (DWDS), operating without detectable residual disinfectant. Considerable seasonal variations in total (TCC) and intact (ICC) bacterial cell concentrations and bacterial ATP concentrations were detected in the WTP effluent, while no change was measured in HPC and *Aeromonas* (Figures 6.1 and 6.2). The TCC in the WTP effluent was governed by microbial dynamics in the biofiltration treatment steps, and clearly correlated to water temperature (Figures 6.3, 6.4 and 6.6). Increases in microbial parameters during disinfectant-free water distribution were measurable, but small compared to the variations observed in the WTP effluent (Figures 6.1, 6.2 and 6.9). Bacterial growth in the DWDS was correlated with water temperature and but not with the concentration of AOC in the WTP effluent (Figures 6.7 and 6.8).

6.4.1 Strategy for characterization of full-scale drinking water treatment and distribution systems

Careful design of sampling/monitoring strategies is crucial for an accurate characterization of microbial community dynamics in full-scale DWDS, and it is evident that both spatial and temporal variations should be considered. Several studies have shown spatial variations in microbial communities during water distribution (Maul et al., 1985a; LeChevallier et al., 1996; Nescerecka et al., 2014; Pinto et al., 2014). A striking example has been given by Nescerecka and co-workers (2014), who have sampled and analysed water from 50 network locations in the chlorinated DWDS of Riga (Latvia) and measured FCM TCC up to one log unit higher than in the water leaving the WTP. Such spatial changes can be due to a loss of residual disinfectant (Maul et al., 1985a; LeChevallier et al., 1996; Wang et al., 2014), to the release of growth-promoting substances from pipe materials (Bucheli-Witschel et al., 2012; Niquette et al., 2000; van der Kooij and Veenendaal, 2001), and/or to long residence times in the DWDS (Maul et al., 1985a; Pinto et al., 2014; Wang et al., 2014).

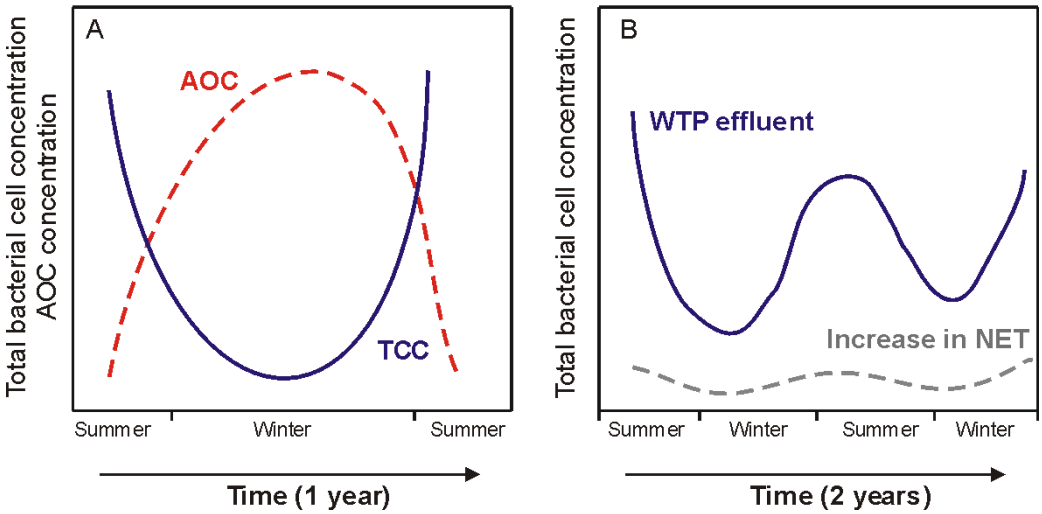


Figure 6.9. Conceptual summary of observations based on two-year monitoring of drinking water quality at the effluent of a full-scale water treatment plant (WTP) and at one location in the distribution network (NET) operating without disinfectant residual. (A) Assimilable organic carbon (AOC) concentration and total bacterial cell concentration (TCC) display inverse variations over one year in the WTP effluent. (B) Large seasonal variations are observed in the TCC in the WTP effluent and the variations are repeatable over two years. The increase in TCC between the WTP and the NET sampling locations (i.e. increase in NET) is minor compared to the variations in TCC in the WTP effluent.

Spatial dynamics are thus best understood by sampling at numerous, dedicated sites from various distribution zones, differing in residence time, residual disinfectant concentrations, or pipe materials (Maul et al., 1985b; Nescerecka et al., 2014; Pinto et al., 2014). The present study focussed on temporal dynamics and thus was limited to only two locations. However, it was clear that spatial changes between the WTP and NET were minor, and that the water in the DWDS was mainly reflecting the water microbial characteristics leaving the WTP (Figure 6.1 and 6.9). Similar observations have been made in Dutch and Swiss facilities treating ground or surface water and distributing water without detectable disinfectant residuals (Vital et al., 2012a; Lautenschlager et al., 2013), in which bacterial cells concentrations in the range of 10^5 cells mL^{-1} and ATP concentrations in the range of 12 ng L^{-1} remained stable during water distribution. The bacterial community composition analysed by pyrosequencing were also very similar in the WTP effluent and in the DWDS in these non-chlorinated DWDS (Lautenschlager et al., 2013; Roeselers et al., 2015), as well as in an American DWDS operating with residual disinfectants (Pinto et al., 2014). Evidently it is critical to always include the WTP effluent in the sampling scheme when spatial and temporal dynamics are investigated.

Pinto et al. (2014) showed that in some DWDS spatial changes in microbial community composition were minor compared to temporal changes. Short-term temporal variations (daily - weekly) in bacterial cell concentrations and/or community composition were reported in a full-scale DWDS

(Nescerecka et al., 2014; Chapter 7) and at a consumer's tap (Besmer et al., 2014). Such variations are attributed to varying water consumption, resulting in changes in water flow velocities and residence times in the system. Increased flow velocities would cause the detachment of bacterial cells from biofilms and the re-suspension of sediments into the bulk water and therefore increase the concentration of suspended cells (Lehtola et al., 2004). The short-term variations are therefore crucial to consider when establishing sampling strategies, as different results would be obtained from a same DWDS location at different sampling moments. For long-term temporal variations (months-to-years), previous studies showed different trends in the microbial community abundance during water distribution for different seasons or water temperatures (Maul et al., 1985a; LeChevallier et al., 1996; Prévost et al., 1998; van der Wielen and van der Kooij, 2010). In the present study, large variations were observed in the WTP effluent in TCC, ICC and ATP concentrations, with cell concentrations being up to 5 times higher in the summer than in the winter period (Figures 6.1, 6.2 and 6.9). Such seasonal variations were potentially caused by changes in the nutrient composition in the raw water (e.g. due to increased rainfalls or algal blooms in the case of surface waters; Ledesma et al., 2012; Herzsprung et al., 2012), or by changes in treatment efficiency (e.g. reduced biological activity in biofilters at low water temperatures; Fonseca et al., 2001; Hammes et al., 2006), and would need further investigation. It is clear that a one-off or infrequent grab sampling approach would specifically not be representative for other times of the year. Temporal dynamics can only be described by a long-term and high-frequency sampling strategy.

In summary, understanding microbial dynamics in a full-scale DWDS requires:

(i) **Assessment of spatial dynamics**, by sampling the WTP effluent, and several locations in the DWDS (see for example Lautenschlager et al., 2013, Nescerecka et al., 2014, and Pinto et al., 2014). The DWDS locations should be carefully selected in collaboration with water utilities based on the features of the DWDS zones (hydraulic residence time, residual disinfectant, pipe material).

(ii) **Assessment of short-term temporal dynamics**, by sampling with high frequency over short, daily and weekly scales (Besmer et al., 2014; Nescerecka et al., 2014; Chapter 7), at the WTP effluent and selected locations in the DWDS.

(iii) **Assessment of long-term temporal dynamics,** over periods of several years (this study; Pinto et al., 2014), by sampling drinking water at the WTP effluent and at fixed selected locations in the DWDS on regular time intervals, with high frequency. It is crucial to sample at fixed day and time to avoid additional effects of short-term dynamics.

6.4.2 A combination of parameters for a complete description of bacterial community in water

Our study highlights that a combination of bioanalytical methods was essential for a complete description of the microbial dynamics in water, as suggested elsewhere (Lautenschlager et al., 2013; Nescerecka et al., 2014; Chapter 4). FCM TCC were indicative for bacterial cells produced in biological filters (Figure 6.6), independently from the effect of chlorine dioxide added thereafter. On the other hand, ICC provided insight on the disinfection effect of chlorine dioxide on the bacteria (Ramseier et al., 2011; Gagnon et al., 2004). While 80 to 85 % of the cells were intact after the biofiltration steps (Figure 6.6), ICC represented only 27 ± 11 % on average of the TCC in the water after chlorine dioxide addition and storage in the clear water reservoir, in which the disinfectant was fully consumed down to below the minimum detection limit (<0.001 mg L^{-1}). Cell membrane integrity is, however, not a guaranty for cell viability. The latter can be assessed by bacterial ATP concentrations, which were as low as 0.9 ng L^{-1} on average over the two years in the WTP effluent. It was estimated that on average 15 % of the intact cells in the WTP effluent were active, even after the addition of chlorine dioxide, by converting the bacterial ATP concentrations to active cell concentrations, using the conversion factor of 1.75×10^{-10} nmol ATP/cell proposed by Hammes et al. (2010a). However, bacterial viability in the WTP effluent was not detected by the cultivation-based measurement of heterotrophic bacteria (i.e. cultivable HPC bacteria) and *Aeromonas*. The extremely low counts of both types of organisms in the WTP effluent over the two-year sampling period (Figure 6.2) can be attributed to the sensitivity to chlorine dioxide of *Aeromonas* (Medema et al., 1991) and cultivable HPC bacteria, that possibly enter a “viable but non culturable” state (Lisle et al., 1998; Hoefel et al., 2005; Lenz et al., 2010). In the disinfectant-free DWDS, however, significant increase in *Aeromonas* counts (Figure 6.2C and Figure S6.5 in supplementary information) and in HPC counts in the case of warm temperatures (above 15 °C; Figure 6.7B) were indicative for bacterial growth. Overall, the study showed that the HPC and *Aeromonas* parameters are meaningful under conditions that notably lead to changes such as high temperatures and absence of residual disinfectant. However, the cultivation-based methods were less sensitive than the FCM and ATP methods, particularly in the presence of disinfectant residuals. Moreover, analytical results from HPC and *Aeromonas* measurements are only obtained after a minimum of two days, which is significantly longer than the time required form FCM and ATP analysis (in the range of minutes). HPC and *Aeromonas* monitoring is nevertheless useful for comparison with temporal archives (feed-forward control), while rapid and sensitive measurements such as FCM and ATP ones are necessary for rapid corrective actions (feed-back measures).

In addition to these bacterial quantification methods, the value of 16S rRNA gene based fingerprinting (e.g. DGGE or T-RFLP) and amplicon sequencing methods (e.g 454-pyrosequencing or

Illumina) has also previously been demonstrated for the study of microbial community composition and structure in water during treatment (Pinto et al., 2012; Wang et al., 2013; Roeselers et al., 2015) and distribution (Hwang et al., 2012; Wang et al., 2014; McCoy and vanBriesen, 2012; Pinto et al., 2014). In the present study we also used 16S rRNA gene-based amplicon-sequencing to characterize the bacterial community composition in all samples. Due to spatial limitations, this information will be presented in detail in a separate manuscript.

In summary, investigating and understanding autochthonous bacterial community dynamics in drinking water systems requires the following methods:

(i) **FCM for the enumeration of total and intact bacterial cells** in the WTP effluent water and for rapid and sensitive detection of changes in cell concentrations during water distribution (Vital et al., 2012a; Lautenschlager et al., 2013). The data analysis can be complemented by FCM fingerprints as a rapid indicator for changes in bacterial community composition or structure (Koch et al., 2013; Chapter 4).

(ii) **ATP as a complementary indicator for bacterial viability and activity** (Vital et al., 2012a; Nescerecka et al., 2014). ATP alone is less sensitive than FCM and did not allow for the detection of changes at cold temperatures. However, the method provided complementary information to the intact bacterial cell concentrations, particularly in the case of the presence of disinfectant residuals (this study).

(iii) **16S rRNA-gene based sequencing methods** for a detailed description of microbial community composition and structure, and an in-depth understanding and description of microbial dynamics (Pinto et al., 2014; Wang et al., 2014).

6.4.3 Microbial dynamics are system-specific

Each drinking water system is unique in the combination of its raw water, treatment, and distribution features (LeChevallier et al., 1996), which affect microbial dynamics during drinking water treatment and distribution. The influencing factors include raw water characteristics (chemical composition, organic and inorganic nutrients), treatment design and efficiency (Prévost et al., 1998; Hammes et al., 2008; Pinto et al., 2012), residual disinfection implementation (or not) and disinfectant type (LeChevallier et al., 1996; Wang et al., 2014), water temperature ranges (LeChevallier et al., 1996; van der Wielen and van der Kooij, 2010), distribution pipeline materials (Niquette et al., 2000; van der Kooij and Veenendaal, 2001), and residence times (Maul et al., 1985a; Pinto et al., 2014; Wang et al., 2014). Prévost et al. (1998) and van der Wielen and van der Kooij (2010) have highlighted that analogous water systems display different trends in bacterial dynamics during water distribution,

when treatment strategies (e.g. biological filtration vs. conventional treatments) or compositions of the effluents of water treatment plants (e.g. AOC concentrations) differ. The present study showed that large seasonal variations occurred in the microbial characteristics of drinking water (Figures 6.1, 6.2 and 6.9) produced from surface water with an extensive treatment scheme including ozonation followed by biological filtration, and distributed with final disinfection but without detectable residual disinfectant. In contrast, Hammes et al. (2010b) have observed no significant temporal changes in TCC either in the WTP effluent or the DWDS of a Swiss infrastructure with similar water treatment steps. The different behaviour in microbial responses in the WTP effluent can partially be attributed to the fact that the water temperature variations observed from 3.9 to 7.1 °C in the Swiss WTP were very limited compared to the ones observed from 3.0 to 21.7 °C in the Dutch treatment utility. The latter can have considerable impact on bacterial growth in the biofilters. Nevertheless, the temperatures varied significantly (3.6 - 17.7 °C) in the Swiss DWDS but these variations did not influence the cell concentrations in the system, which contradicts the observations in the Dutch DWDS. In summary, microbial dynamics in water treatment and DWDS should be considered as site-dependent, and each system needs to be studied in detail. It is recommended to apply a standard approach and unified methods, as detailed in the previous sections, for direct comparison between studies, and to evaluate if the observation made in the present system can be generalized.

6.4.4 Long-term assessment of natural fluctuations in DWDS

To reveal natural variations in drinking water quality for a specific treatment and distribution system, LeChevallier and co-workers (1996) advised to collect large datasets of monitoring results for routine environmental parameters such as pH, temperature, free-chlorine residuals, and for coliforms, HPC, and AOC tests. In the present study, the approach has been extended to parameters such as ATP and FCM TCC and ICC, and large data sets (360 samples) were collected over a two-year period. The complete dataset clearly revealed that the considerable variations in TCC and ICC over the measuring period (Figure 6.1) were natural seasonal fluctuations for this particular WTP and DWDS. The data can be used to estimate a range of predictable values for a defined period of time (e.g. winter vs. summer; Figure 6.4). Moreover, long-term measurements of TCC, ICC, HPC and *Aeromonas* showed that increased risk for uncontrolled bacterial growth during water distribution is expected at warm temperatures (Figure 6.7 and Figure S6.5, Supplementary information). In this framework, the value of numerical ecology methods was demonstrated for rationalization of large analytical datasets collected through high-frequency water monitoring, in order to highlight the main correlative trends between microbial, environmental, and engineering variables (Weissbrodt et al., 2014; Besmer et al. 2014; this study). The approach of long-term, high frequency monitoring and the definition of

predictable ranges of values could be applied to any parameter of interest, and would be particularly useful for newly introduced parameters, such as FCM, that are not related to any guideline value and for which reference data are lacking. In this respect one could envision in future the use of continuous on-line microbial monitoring (Besmer et al., 2014), as already routinely performed for several abiotic process parameters.

6.4.5 Long-term assessment of variations in microbial parameters for in-depth understanding of bacterial dynamics in DWDS

Understanding microbial dynamics during drinking water treatment and distribution requires identification of the bacterial growth-limiting or growth-promoting parameters in a specific DWDS. For this purpose, it is necessary to relate microbial parameters and their temporal and spatial variations with varying environmental conditions. In this study, specific attention was given to water temperature and to assimilable organic carbon (AOC) concentration in the WTP effluent (Figures 6.7 and 6.9). Water temperature is indeed a major factor in the bacterial growth kinetics (Madigan and Martinko, 2006), whereas AOC is often depicted as the main growth-controlling nutrient in drinking water (van der Kooij and Hijnen, 1985; LeChevallier et al., 1991; Escobar et al., 2001). We showed that AOC was varying seasonally with an opposite trend as water temperature and TCC (Figure 6.5), which was partially attributed to the treatment applied at this specific facility. Additionally, the results suggest that AOC concentration in the WTP effluent was not the only controlling factor for bacterial growth at the studied network sampling location in this particular DWDS (Figure 6.7 and 6.8). In fact, the highest bacterial growth was recorded at times where the lowest AOC concentrations were measured in the WTP effluent, which were below 10 µg Ac-C eq. L^{-1}. The latter value is commonly used to qualify a drinking water as biological stable in systems without detectable residual disinfectant (van der Kooij, 1992; 2000). Possible alternative controlling factors include inorganic nutrients such as phosphate, which was reported by the water utility to be always below detection limit and could not be further investigated. However, one cannot exclude the hypothesis that the available AOC in the water was not fully consumed at the NET sampling site, given the short residence time of approximately 2 days of the water until the NET sampling point. In addition, bacterial growth rates and yield on AOC in drinking water are likely influenced by water temperature (Kovářová et al., 1996; Vital et al., 2012b), which would impact the AOC consumption kinetics in winter and in summer.

One should note that bacterial growth-limitations in a given DWDS are likely to change over a year in conjunction with the seasonal variations in microbial and chemical compositions of the WTP effluent

and water temperature, such as displayed in this study. Pinto et al. (2014) have recently shown that different environmental parameters such as pH and concentrations in ammonium, phosphate, sulphate and TOC are responsible for the changes in amplicon-sequencing profiles of bacterial community structures over a year. In DWDS where residual disinfection is applied to control bacterial growth, the residual concentration is also an obvious parameter to consider. The disinfectant decay is likely to be faster at warmer water temperatures, higher bacterial cell concentrations, and/or higher organic carbon concentrations. This results in DWDS areas with low residual concentrations of disinfectant at specific times of the year that can be subjected to increased bacterial growth (Maul et al., 1985a; Volk and Joret, 1994; Prévost et al., 1998; Francisque et al., 2009).

6.5 Conclusions

Two-year and high-frequency drinking water monitoring in a full-scale DWDS operating without residual disinfectant, using a combination of conventional and new microbial analytical methods led to the following key scientific conclusions, and recommendations for the practice:

- Large seasonal variations occurred in bacterial cell concentrations and viability of the drinking water analysed at the effluent of the treatment facility. Assessment of temporal dynamics of microbial parameters is a key approach toward improved understanding of full-scale water systems.
- Microbial characteristics of drinking water in the DWDS were highly dependent on the characteristics of the water leaving the treatment facility; only minor changes occurred during distribution. Investigation of spatial microbial dynamics in DWDS should always include detailed characterization of the WTP effluent.
- Bacterial growth occurring during distribution up to the specific network location was not controlled by AOC concentrations in the WTP effluent. Likely not only one single parameter can be considered as controlling factor of microbial growth in DWDS. Growth-controlling parameters are expected to change in time in a specific system. Future research should therefore specifically target mechanisms that drive microbial dynamics in DWDS.
- The collection of a large dataset by long-term and high-frequency monitoring of water quality parameters sustained the assessment of natural fluctuations in the system as well as the determination of a predictable range of data at specific water temperatures and/or time.

SUPPLEMENTARY INFORMATION – Chapter 6

S6.1 Numerical ecology methodology applied to the 2 year datasets collected from the effluent of the water treatment plant (WTP)

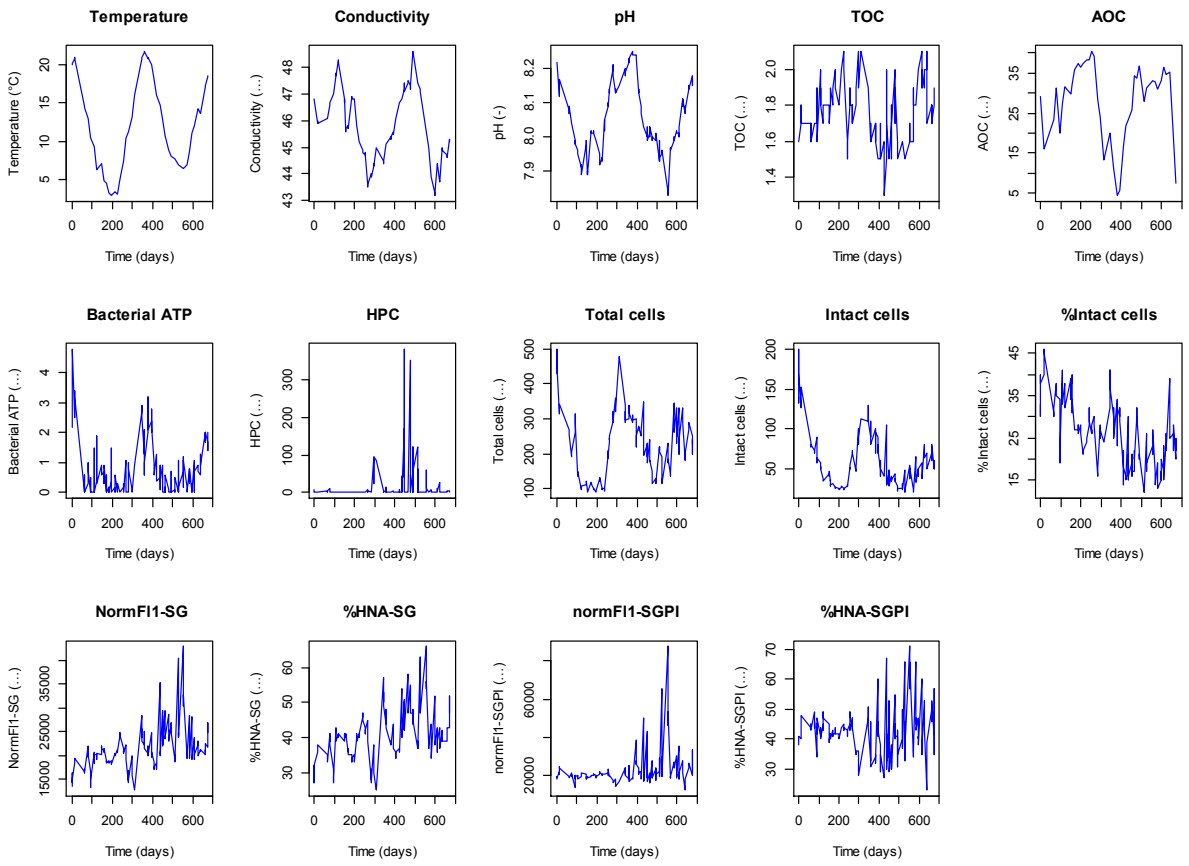


Figure S6.1.1. Panel plot of dynamics in individual measured variables over time in order to delineate global trends (concatenated 2 year WTP datasets, 184 water samples in total).

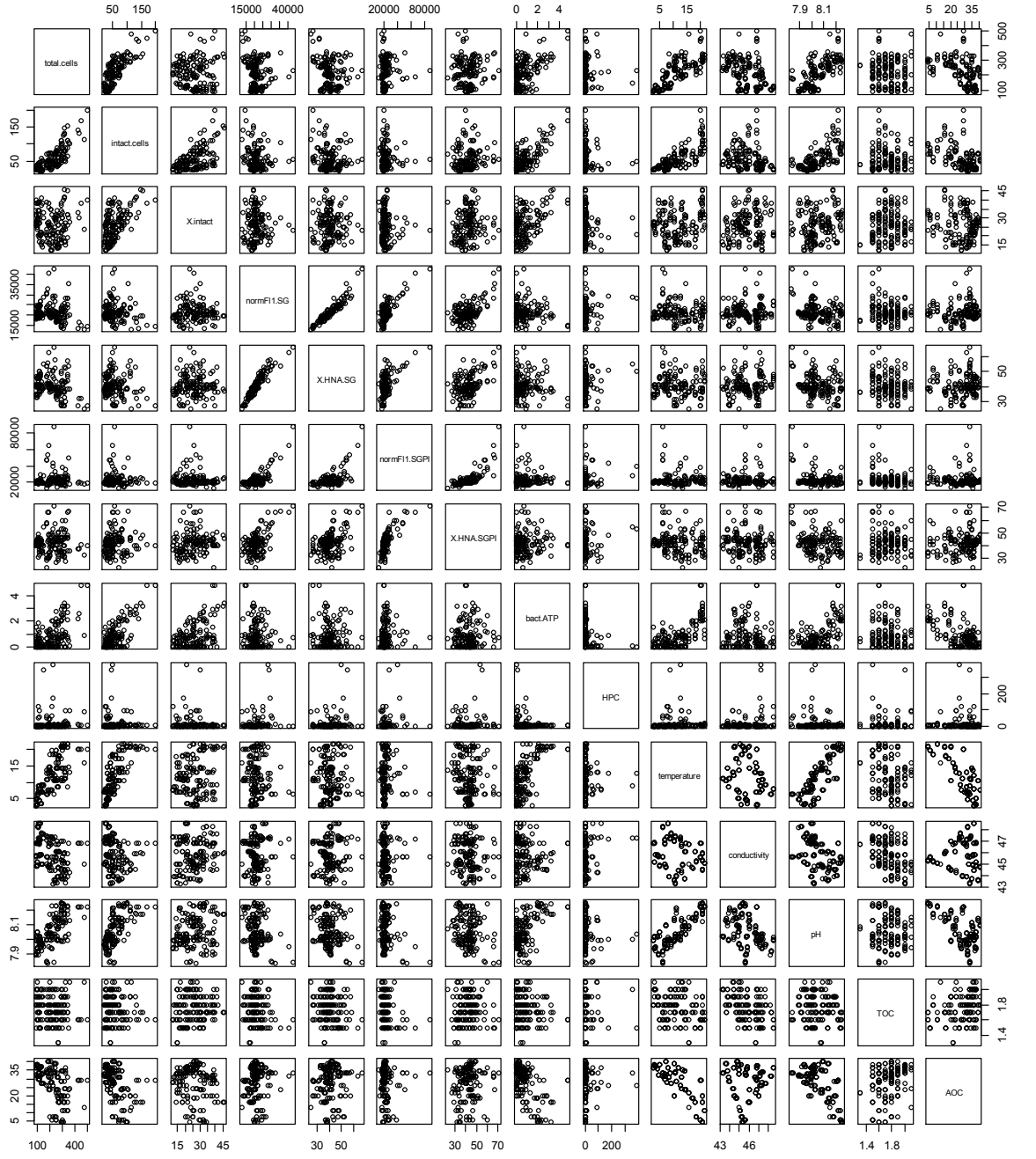


Figure S6.1.2. Pair-wise x-y plots between all measured variables for preliminary visual observation of apparent linear and monotonic correlations (concatenated 2 year WTP datasets, 184 water samples in total). The meaning of abbreviations are given in Table S6.1.

Table S6.1. Overview of abbreviations in use in Figures S1b to S1e.

Total cells	Total cell concentration, as determined with flow cytometry following bacterial staining with Sybr Green I only
Intact cells	Intact cell concentration, as determined with flow cytometry following bacterial staining with a mixture of Sybr Green I and propidium iodide
X.intact	Percentage of intact cells compared to total cells
NormFl1.SG	Normalized green fluorescence as determined with flow cytometry following bacterial staining with Sybr Green I only (Chapter 4)
X.HNA.SG	Percentage of high nucleic acid cells compared to total cells, as determined with flow cytometry following bacterial staining with Sybr Green I only (Chapter 4)
NormFl1.SGPI	Normalized green fluorescence as determined with flow cytometry following bacterial staining with a mixture of Sybr Green I and propidium iodide (Chapter 4)
X.HNA.SGPI	Percentage of high nucleic acid cells compared to total cells, as determined with flow cytometry following bacterial staining with a mixture of Sybr Green I and propidium iodide (Chapter 4)
Bact.ATP	Bacterial ATP
HPC	Heterotrophic plate count
TOC	Total organic carbon concentration
AOC	Assimilable organic carbon concentration
WTP	Refers to samples taken at the water treatment plant effluent
NET	Refers to samples taken at the distribution network sampling location
ΔNET	Refers to changes measured between water treatment plant effluent and distribution network sampling location

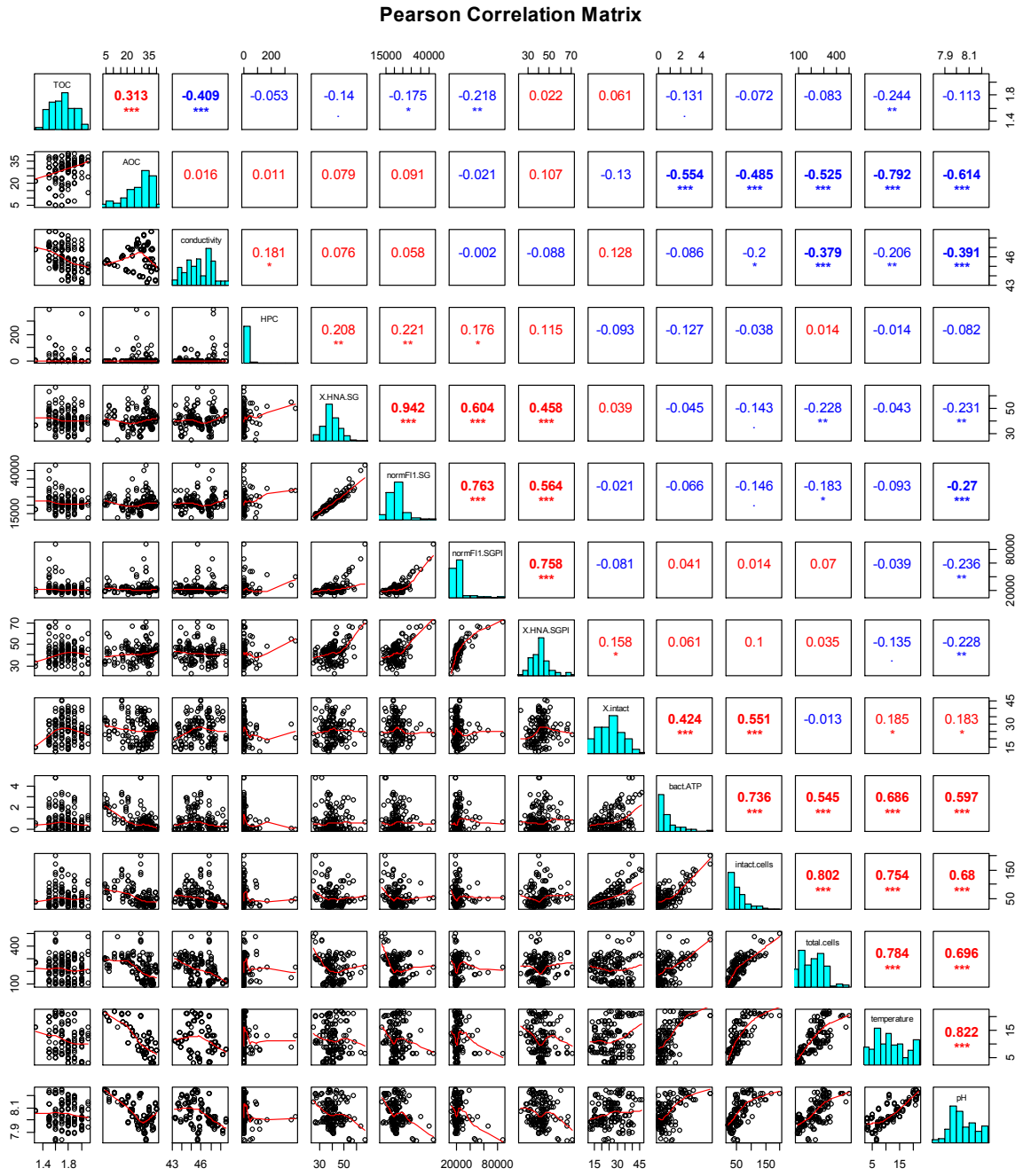


Figure S6.1.3. Self-contained Pearson's correlation matrix used to delineate linear correlative trends between variables (concatenated 2 year WTP datasets, 184 water samples in total). The upper section of the matrix displays Pearson's correlation coefficient, that indicate positive (red) or negative (blue) correlative trends.

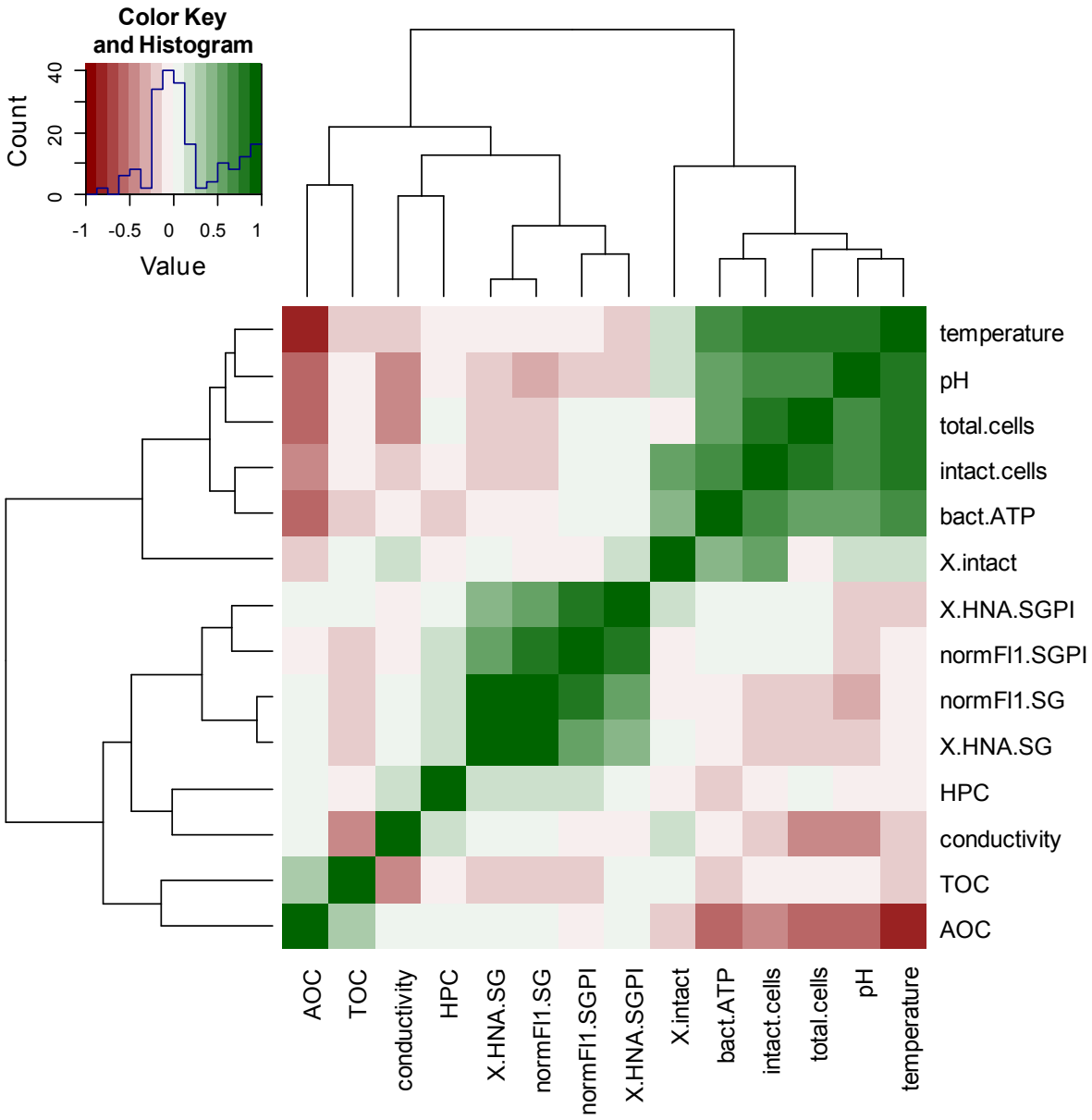


Figure S6.1.4. Graphing and hierarchical clustering using the Ward's algorithm of measured variables displaying analogous correlation patterns with all other variables. The heat map provides a straightforward representation of the gradients in positive and inverse Pearson's correlations (*i.e.* linear trends) between variables toward rapid identification of major correlative trends (concatenated 2 year WTP datasets).

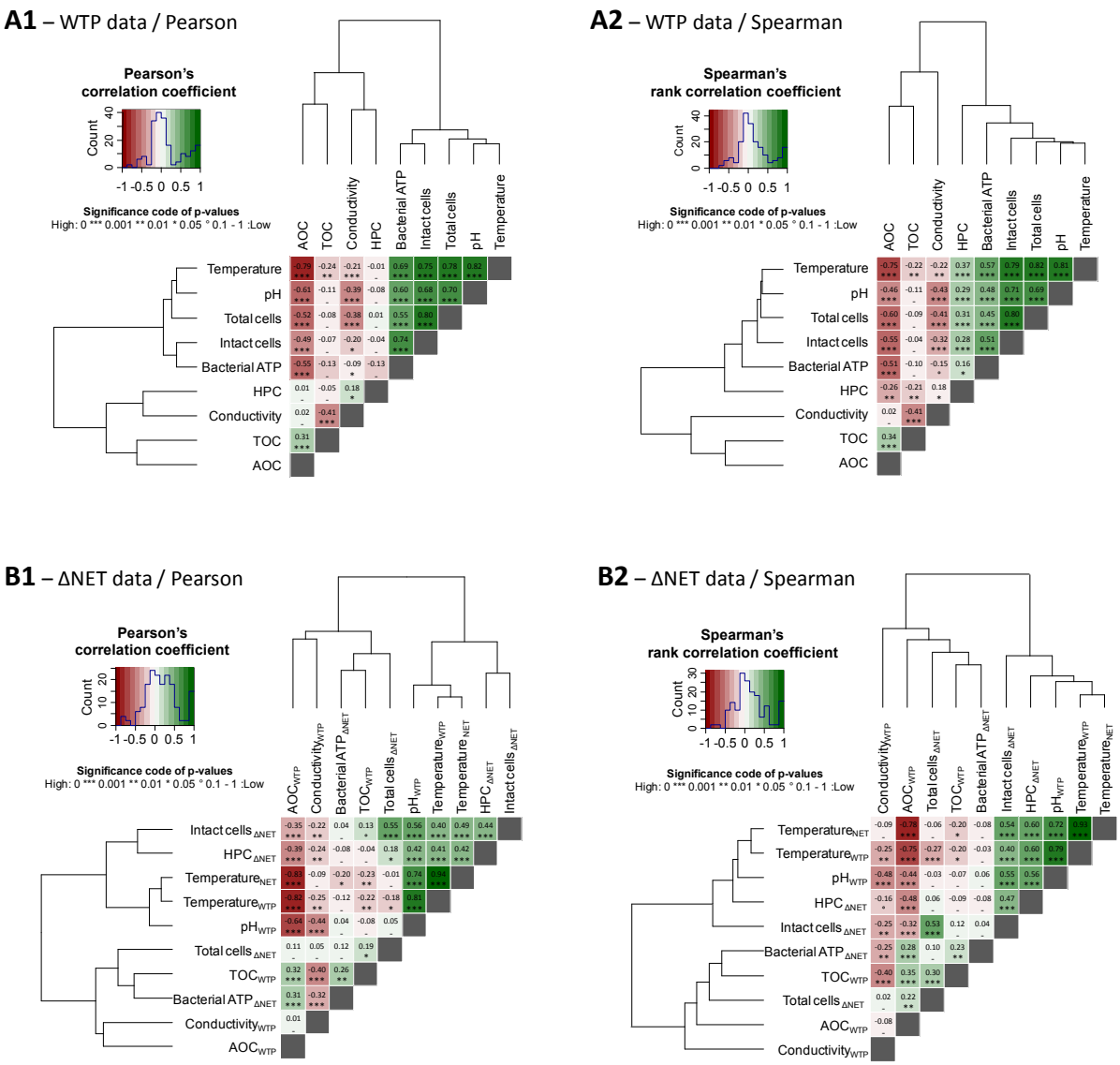


Figure S6.1.5. Comparison of the half heat maps of Pearson's correlation (*i.e.* linear trends) and Spearman's rank-order correlations (*i.e.* monotonic trends) between measured variables originating from the datasets collected from the effluent of the water treatment plant (WTP, panel A1-2) and from the changes in the water distribution network (ΔNET, panel B1-2), over the 2-year water analysis campaign. In the latter dataset, the absolute value of the water temperature in the network ($temperature_{NET}$) was considered.

S6.2 Analysis of flow cytometric fluorescence fingerprints

Fluorescence fingerprints of drinking water samples typically display two clusters differentiated by fluorescence intensity, corresponding to bacteria with low (LNA) and high (HNA) nucleic acid content. LNA and HNA clusters were separated using fixed electronic gates and quantification and straightforward comparison of fingerprints from different water samples was made using the percentage of HNA cells, compared to total cells (Chapter 3).

Flow cytometric (FCM) fluorescence fingerprints are indicative of the bacterial community composition in drinking water samples (Chapter 4). The fingerprints obtained based on intact cell enumeration conducted using staining with a mixture of SYBR Green I and propidium iodide were examined, but neither revealed variations in time in the percentage of HNA cells at both locations nor displayed clear seasonal trends (Figure S6.2a). However, a clear shift in bacterial community composition occurred during water distribution, with a consistent and significant ($P<0.0001$) increase in the percentage of high nucleic acid (HNA) bacterial cells between the two locations (increase from 41.5 to 50.9 % on average). The fingerprints obtained from total cell enumeration, conducted using staining with SYBR Green I only, did not display seasonal variations at the WTP effluent nor in the network (Figure S6.2b). Significant ($P=0.0006$ based on a paired t-test) but very limited change in the percentage of HNA cells (from 40.3 to 30.9 % on average over the two years) was revealed between the two locations. This can be due to measurement sensitivity, as the water samples contained a large amount of damaged bacterial cells (70 % on average), probably hampering the detection of small changes occurring within the intact fraction of bacterial cells. The temporal and spatial variations in bacterial community composition will be further examined in depth in a separate self-contained article on high-throughput investigation of microbial community dynamics using 16S rRNA gene-based amplicon-sequencing analysis.

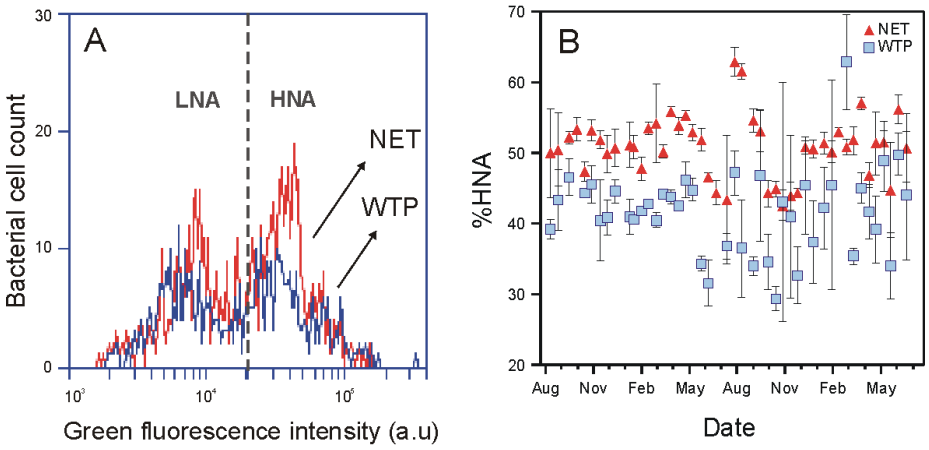


Figure S6.2.1. Bacterial community analysis using flow cytometric fingerprints based on intact cell measurements (bacterial staining with SYBR Green I and propidium iodide). (A) Comparison of unprocessed fingerprints (green fluorescence distribution) of drinking water samples taken at the water treatment plant (WTP) effluent and at one location in the network (NET) on the same day. (B) Variations in time over two years (August 2012 - June 2014) of percentage of high nucleic acid bacterial cells (HNA) at the two locations. Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

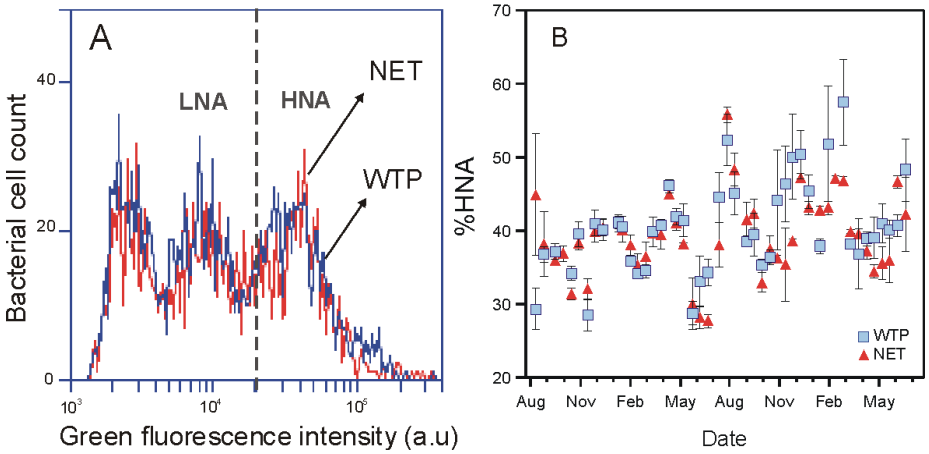


Figure S6.2.2. Bacterial community analysis using flow cytometry (FCM) fingerprints based on total cell measurements (bacterial staining with SYBR Green I only). (A) Comparison of unprocessed fingerprints (green fluorescence distribution) of drinking water samples collected at the water treatment plant (WTP) effluent and in the water distribution network (NET) on the same day. (B) Temporal variations over two years (August 2012 - June 2014) of the percentage of high nucleic acid bacterial cells (HNA) at the two sampling locations. Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

S6.3 Additional information on long-term monitoring study on a full-scale drinking water distribution system

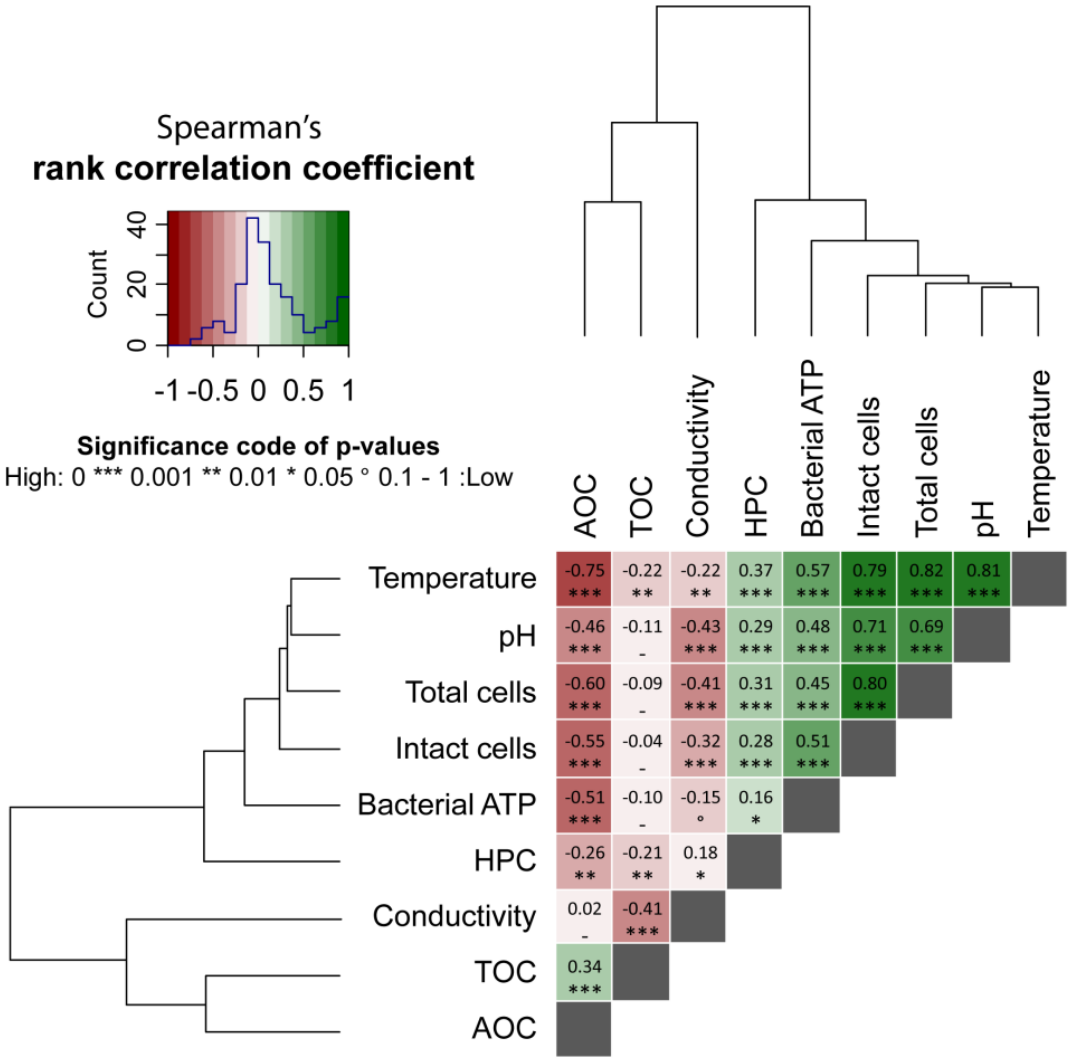


Figure S6.3. Heatmap of pair-wise Spearman's correlation coefficients (*i.e.* monotonic trends) computed between the microbial parameters (namely total cell concentration, intact cell concentration, bacterial ATP, and heterotrophic plate counts (HPC)), and the environmental parameters (namely temperature, pH, electrical conductivity, total organic carbon (TOC), and assimilable organic carbon (AOC)) measured in the effluent of the water treatment plant (2-year dataset, 184 water samples in total). Hierarchical clustering using the Ward's algorithm was first applied to reorder all parameters in clusters according to their correlation patterns as displayed by the dendrograms. The values and directions of the correlation coefficients are displayed according to the color key, *i.e.* positive correlations as green gradients from 0 to 1 and inverse correlations as red gradients from 0 to −1. This approach is analogous to the one developed and used in Weissbrodt *et al.* (2014) and Besmer *et al.* (2014).

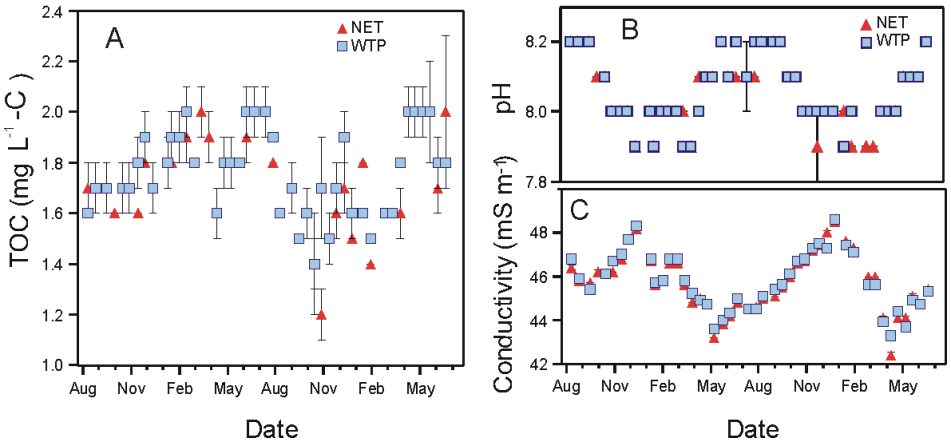


Figure S6.4. Temporal variations over two years (August 2012 - June 2014) in the (A) concentration of total organic carbon (TOC), (B) pH, and (C) electrical conductivity of the drinking water at the outlet of the water treatment plant (WTP) and at one location in the water distribution network (NET). Error bars indicate the standard deviation on four samples taken at the same location over a 2 h period.

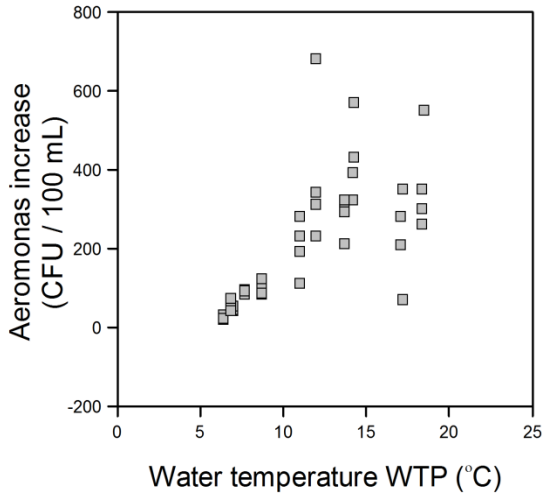


Figure S6.5. Relationship between the temperature of drinking water at the outlet of the water treatment plant (WTP) and the increase in the counts of *Aeromonas*, between samples taken at the WTP effluent and at the sampling location in the water distribution network during the two year investigation period (August 2012 – June 2014).

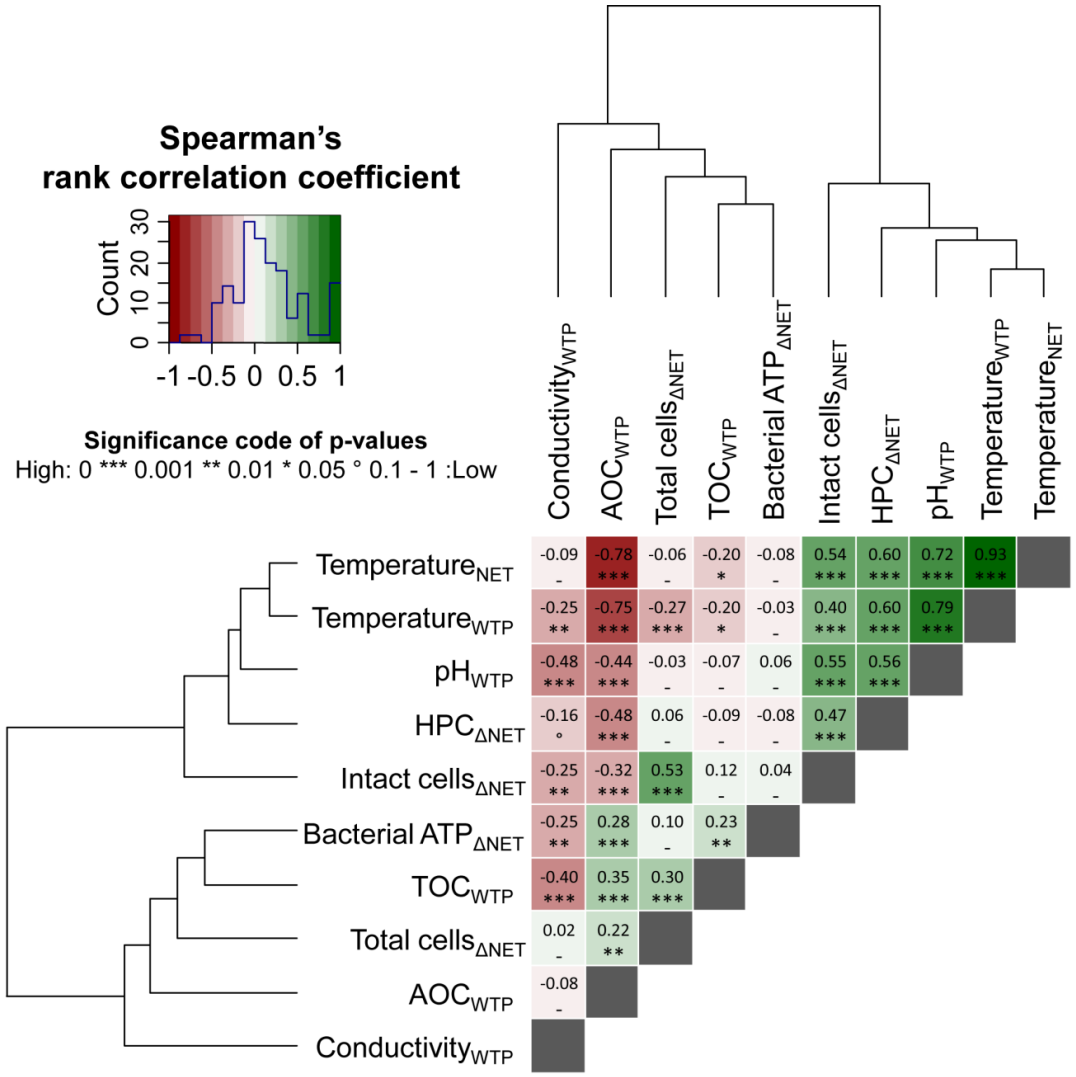
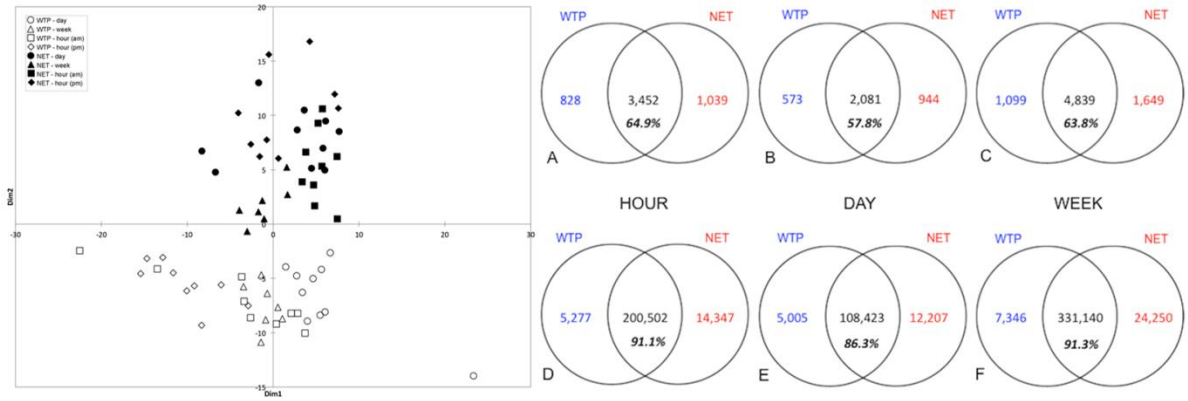


Figure S6.6. Heatmap of pair-wise Spearman's correlation coefficients (*i.e.* linear trends) computed between the changes in microbial parameters (namely total cell concentration, intact cell concentration, bacterial ATP, and heterotrophic plate counts (HPC)) occurring in the distribution network (ΔNET) between the water treatment plant (WTP) effluent and the sampling location in the network (NET), and environmental parameters (namely temperature, pH, electrical conductivity, total organic carbon (TOC), and assimilable organic carbon (AOC)) in the WTP effluent and/or at the NET location (2-year dataset, 184 water samples in total). Hierarchical clustering using the Ward's algorithm was first applied to reorder all parameters in clusters according to their correlation patterns as displayed by the dendrograms. The values and directions of the correlation coefficients are displayed according to the color key, *i.e.* positive correlations as green gradients from 0 to 1 and inverse correlations as red gradients from 0 to −1. This approach is analogous to the one developed and used in Weissbrodt *et al.* (2014) and Besmer *et al.* (2014).

Chapter 7

Dynamics of bacterial communities before and after distribution in a full-scale drinking water network



This chapter has been published as: J. El-Chakhtoura, E.I. Prest, P. Saikaly, M.C.M. van Loosdrecht, F. Hammes, J.S. Vrouwenvelder (2015). Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. *Water Research* 74, 180-190.

Emmanuelle Prest has contributed to the study design and organization, water sampling, flow cytometric analysis and flow cytometric data interpretation. Joline El-Chakthouta (KAUST) has contributed to sequencing sample preparation, sequencing data analysis and interpretation. The manuscript has been written by Joline El-Chakhtoura.

Abstract

Understanding the biological stability of drinking water distribution systems is imperative in the framework of process control and risk management. The objective of this research was to examine the dynamics of the bacterial community during drinking water distribution at high temporal resolution. Water samples (156 in total) were collected over short time-scales (minutes/ hours/ days) from the outlet of a treatment plant and a location in its corresponding distribution network. The drinking water is treated by biofiltration and disinfectant residuals are absent during distribution. The community was analyzed by 16S rRNA gene pyrosequencing and flow cytometry as well as conventional, culture-based methods. Despite a random dramatic event (detected with pyrosequencing and flow cytometry but not with plate counts), the bacterial community profile at the two locations did not vary significantly over time. A diverse core microbiome was shared between the two locations (58–65% of the taxa and 86–91% of the sequences) and found to be dependent on the treatment strategy. The bacterial community structure changed during distribution, with greater richness detected in the network and phyla such as *Acidobacteria* and *Gemmatimonadetes* becoming abundant. The rare taxa displayed the highest dynamicity, causing the major change during water distribution. This change did not have hygienic implications and is contingent on the sensitivity of the applied methods. The concept of biological stability therefore needs to be revised. Biostability is generally desired in drinking water guidelines but may be difficult to achieve in large-scale complex distribution systems that are inherently dynamic.

7.1 Introduction

Drinking water distribution systems (DWDSs) serve as a vital network for transporting clean, safe, palatable and ideally biologically stable water. These systems are complex, governed by variable natural and operational conditions that influence the indigenous microbial communities which thrive in the water, biofilm and sediments. Monitoring water quality during distribution and establishing appropriate remedial actions are therefore imperative in the framework of process control and risk management (Smeets et al., 2010). Biological stability is generally defined as the inability of water (and/or pipe material) to promote microbial growth, and many guidelines have been proposed for its evaluation, linked to conventional parameters such as assimilable organic carbon (AOC), biofilm formation rate and heterotrophic plate counts (Liu et al., 2013a; van der Kooij, 2000). Since these methods are mostly indicative and often inaccurate, recent studies have recommended more advanced, sensitive tools such as pyrosequencing and flow cytometry that can provide in-depth qualitative and quantitative data on microbial cell concentrations and community structure variations (Lautenschlager et al., 2013; Liu et al., 2013a; Chapter 4).

To deliver biologically stable water to end users water utilities normally apply a final oxidative disinfection step (primarily chlorination) and maintain a sufficient disinfectant residual in the network to suppress microbial growth (LeChevallier et al., 1993). Chlorine however has instigated customer complaints concerning taste and odor and has been linked with harmful by-products such as trihalomethanes (Rook, 1976). Chlorination can inhibit certain microorganisms while selecting for opportunistic pathogens that are relatively chlorine-resistant such as *Mycobacterium avium* (Ingerson-Mahar and Reid, 2012). Moreover, disinfectant residuals can react with particles, organics and pipe material releasing AOC that can be consumed by microorganisms, contributing to biological instability (Polanska et al., 2005; Ramseier et al., 2011b). As an alternative, high-quality, biologically stable water can be produced by limiting organic carbon and other growth-supporting nutrients during treatment (van der Kooij, 2000) and this is often carried out by benign microbial communities colonizing biological filters.

Previous studies have examined spatial and temporal variations in the microbial water quality, in both chlorinated and non-chlorinated DWDSs. Distribution effects were mainly determined based on sampling a few locations in the network (Lautenschlager et al., 2013; Liu et al., 2014; McCoy and VanBriesen, 2014) while temporal studies focused primarily on monthly or seasonal variations (Hu et al., 1999; Pinto et al., 2014; Revetta et al., 2010). Most studies reported long-term effects to be more significant than spatial variations, although distribution network samples were rarely compared to

the original treatment plant samples. Studies investigating in-depth short-term dynamics are scarce but important for establishing the specific trend characteristic of a particular DWDS, allowing deviations from that trend to be easily recognized and investigated.

In this study, a full-scale, well-maintained drinking water distribution system was sampled intensively over short time-scales (minutes/ hours/ days) and across two locations (156 samples in total), and analyzed using advanced techniques like 16S rRNA gene pyrosequencing and flow cytometry as well as conventional, culture-based methods like plate counts. The objective of the research was to (i) examine bacterial dynamics at high temporal resolution and (ii) determine the impact of distribution on the water microbiology, in an effort to evaluate the biological stability of systems that apply biofiltration treatment and distribute water without disinfectant residuals.

7.2 Materials and methods

7.2.1 Sampling scheme

The research was conducted on a drinking water treatment plant in The Netherlands and its corresponding distribution network. The plant treats surface water from the Meuse River by coagulation, flocculation and sedimentation followed by ozonation, rapid dual-medium filtration and granular activated carbon filtration. The filtrate is dosed with chlorine dioxide to a concentration of 0.1 mg ClO_2 L^{-1} before being pumped into a storage reservoir located at the plant. The effluent of this reservoir that is distributed contains no detectable disinfectant residual. The drinking water production rate of the plant is 4×10^7 m^3 $year^{-1}$ (Chapter 4) and it is the sole source of water for this distribution network (no mixing). The pipes in this network are made of cemented steel and (at some locations) PVC. Bulk water samples were collected simultaneously from the outlet of the water treatment plant (WTP) and from one location in the distribution network, both from a continuously running tap to avoid unwanted stagnation influences. The hydraulic residence time was estimated by the local water utility to be 2 days. The research comprised three parts; hour, day and week studies and an overview of these studies is shown in Table 7.1. In the (i) hour study, samples were taken from the two locations every five minutes for one hour, done in the morning (8 – 9 am) and afternoon (1 – 2 pm) of the same day, and resulting in 52 samples. In the (ii) day study, samples were taken every hour for an entire day (48 samples). In the (iii) week study, samples were taken four times a day (at 8 am, 11 am, 2 pm and 4 pm) for one week, resulting in 56 samples. The sampling was conducted in August 2012 when the water temperature was 20.9 $\pm$ 0.1 °C at the plant outlet and 22.7 $\pm$ 0.1 °C at the network location.

Table 7.1. Overview of three studies examining short-term bacterial dynamics of drinking water at the treatment plant outlet and distribution network location.

Study set	Sampling frequency	No. of samples	Pyrosequencing samples
HOUR	5 min	52	36
DAY	1 h	48	20
WEEK	4 x / day	56	56

Depending on the subsequent analysis, samples were collected in specific, separate bottles as described in Chapter 4. All samples were transported on ice to the laboratory, stored at 4 °C and processed within 24 h.

7.2.2 Chemical and microbial analysis

Every drinking water sample was analyzed for temperature, conductivity, pH, total organic carbon (TOC), heterotrophic plate counts (HPC), adenosine triphosphate (ATP) and flow cytometric (FCM) bacterial cell counts. Temperature was measured directly on site. Conductivity, pH, TOC and HPC were analyzed by Aqualab Zuid (Werkendam, NL). TOC was measured according to a Dutch standard procedure (NEN-EN 1484). The HPC method was performed with yeast extract agar and the plates were incubated at 22 °C for 3 days (Dutch procedure NEN-EN-ISO 6222). ATP analysis was carried out by Het Waterlaboratorium (Haarlem, NL) as described previously by Magic-Knezev and van der Kooij (2004). A pre-calibrated luminometer (Celsis Advance TM230) was used to measure the intensity of the emitted light. The detection limit of the method was 1 ng ATP L^{-1}. Unlike free ATP measurements, a nucleotide-releasing buffer step was added for total ATP analysis. Bacterial ATP concentrations were calculated by subtracting free ATP from total ATP concentrations. Measurement of total bacterial cell concentrations with flow cytometry was done according to the standardized protocol proposed in Chapter 3. Briefly, samples were pre-heated to 35 °C for 5 min, stained with 10 µL mL^{-1} SYBR Green I (Molecular Probes, Eugene, OR, USA) and then incubated in the dark at 35 °C for 10 min. An Accuri C6 flow cytometer equipped with a 50 mW laser was used for the analysis.

7.2.3 Bacterial community analysis: pyrosequencing

A selection of 112 drinking water samples was processed for bacterial community analysis with 16S rRNA gene pyrosequencing. For the hour study, 36 out of 52 samples were processed (sample time 0, 10, 20, 30, 40, 45, 50, 55 and 60 min on both locations; randomly selected). For the day study, 20 out of 48 samples were processed (sample time 8, 10, 11, 14, 19, 21, 23, 1, 4 and 8 o'clock on both locations; selected based on FCM results). For the week study, all 56 samples were processed and the four individual samples taken per day were pooled for further analyses. Each 2 L sample was filtered

through a 0.2 µm-pore-size Isopore membrane filter within 5 h of sampling, using sterile (autoclaved) filtration units (Millipore, Billerica, MA, USA). The filters were stored at –20 °C until processing.

Genomic DNA was extracted from the collected biomass using the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. Bacterial 16S rRNA genes were amplified with the bacteria-specific forward primer 515F (5'-LinkerA-Barcode-GTGYCAGCMGCCGCGGTA-3') and reverse primer 909R (5'-LinkerB-CCCCGYCAATTCMTTTRAGT-3'). A single-step 28-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) was performed for each DNA sample (triplicate reactions) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s; 53 °C for 40 s and 72 °C for 1 min; after which a final elongation step at 72 °C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and then purified using Agencourt AMPure beads (Agencourt Bioscience Corp., Beverly, MA, USA).

Pyrosequencing was carried out at MR DNA Lab (Shallowater, TX, USA) on the Roche 454 FLX Titanium genome sequen cer according to the manufacturer's instructions. Sequence data was processed at MR DNA Lab. In summary, sequences were depleted of barcodes and primers, then sequences <150 bp were removed, as well as sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI and Greengenes.

7.2.4 Statistical analysis

Statistical methods were applied to assess the similarity in bacterial community structure among samples. Weighted multidimensional scaling (MDS) was performed with the Bray–Curtis matrix using the R statistical package to ordinate the OTU data (samples with similar community structure cluster together, taking into account the relative abundance of each OTU). Temporal variation of total (3% divergence clustering), general (top 10 most abundant) and rare (<0.01% average abundance) taxa was also analyzed by nonmetric MDS ordination (NCSS 8 statistical software). Analysis of similarity (ANOSIM) was used to examine the statistical significance of differences among samples using the Bray–Curtis measure of similarity (vegan package within R), where the *R* statistic value ranges between 0 (complete similarity) and 1 (complete separation).

7.3 Results

7.3.1 Overall variation

A large number of samples was collected at high frequency from the water treatment plant outlet and one distribution network location in three sets: hour, day and week. To depict overall variation, all samples from the three studies were combined in one MDS plot shown in Figure 7.1. Treatment plant and distribution network samples clustered separately indicating different bacterial community structures for the two locations and this difference between the locations was confirmed with ANOSIM (p = 0.0001; R = 0.8617). Over time at the individual locations, a similar bacterial community structure was found. For a better understanding of short-term dynamics, results from the day and week studies will be presented below. Results from the hour study have been shown elsewhere (Chapter 4) and will not be presented in detail herein.

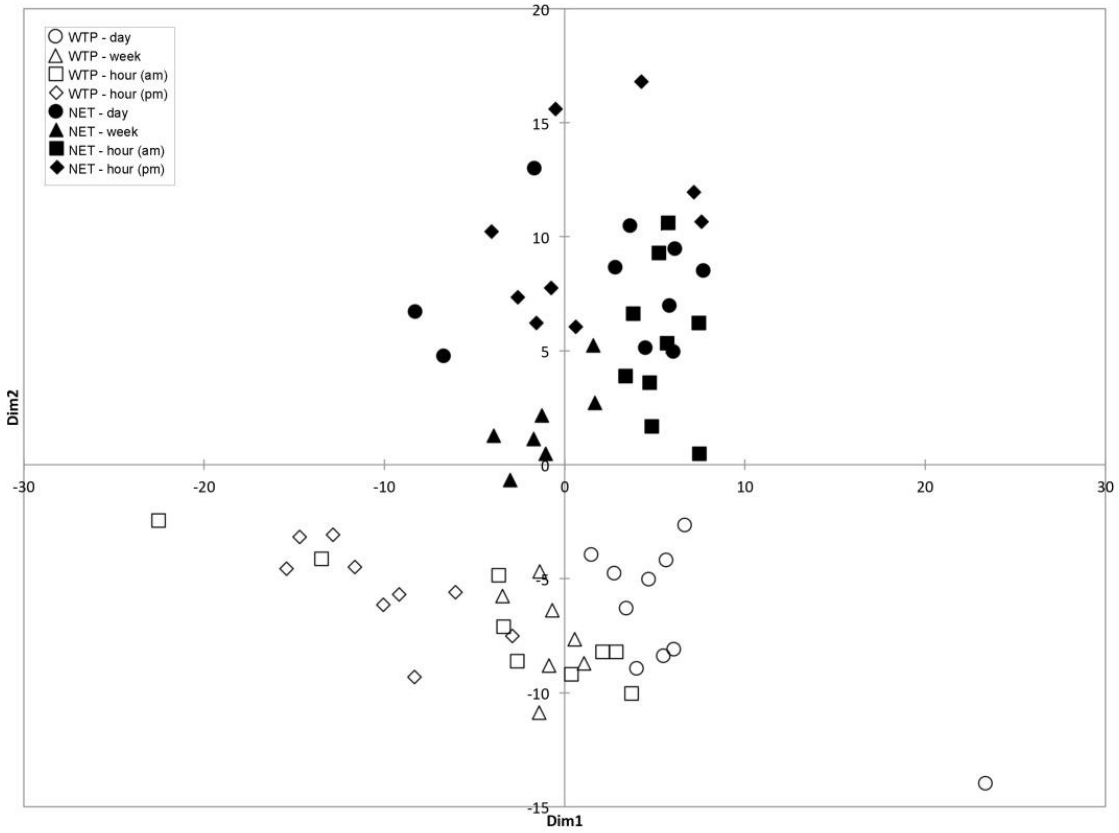


Figure 7.1. Weighted multidimensional scaling (MDS) plot for water treatment plant outlet (WTP) and distribution network location (NET) drinking water samples collected from the hour (morning; am – afternoon; pm), day and week studies. Each symbol represents an individual sample. The bigger the distance between samples, the bigger the difference in microbial community structure.

7.3.2 Day study

A closer look at the day study samples is shown in Figure 7.2. Two separate clusters were observed for the treatment plant and distribution network samples, indicating different bacterial community structures at the two locations. The network samples clustered more closely (Figure 7.2), although two outliers were found at 10 am and 11 am, while the plant samples contained one outlier at 2 pm. Statistically significant differences between the two locations were found with ANOSIM (p = 0.0001; R = 0.9231).

Total cell concentrations showed small (7.5%) variation at the WTP with an average of 3.8×10^5 cells mL^{-1} (Figure 7.3A). In the network samples a dramatic increase in the total bacterial cell concentration was detected between 9 and 11 am (from 420×10^3 to 1440×10^3 cells mL^{-1}), correlating with the community outliers detected with pyrosequencing. After 11 am the cell concentration decreased gradually down to the WTP values, with little (5.9%) variation after 5 pm.

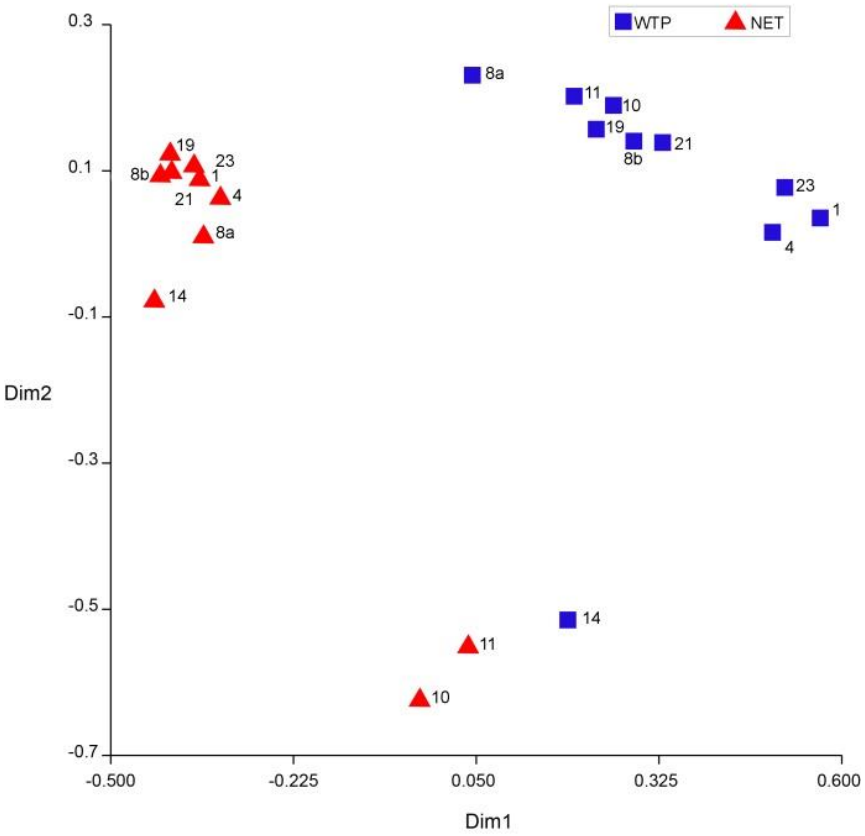


Figure 7.2. Weighted multidimensional scaling (MDS) plot for water treatment plant outlet (WTP) and distribution network location (NET) samples collected during the day study. Each symbol represents a specific sampling time, from 8 am (8a) until 8 am the next day (8b).

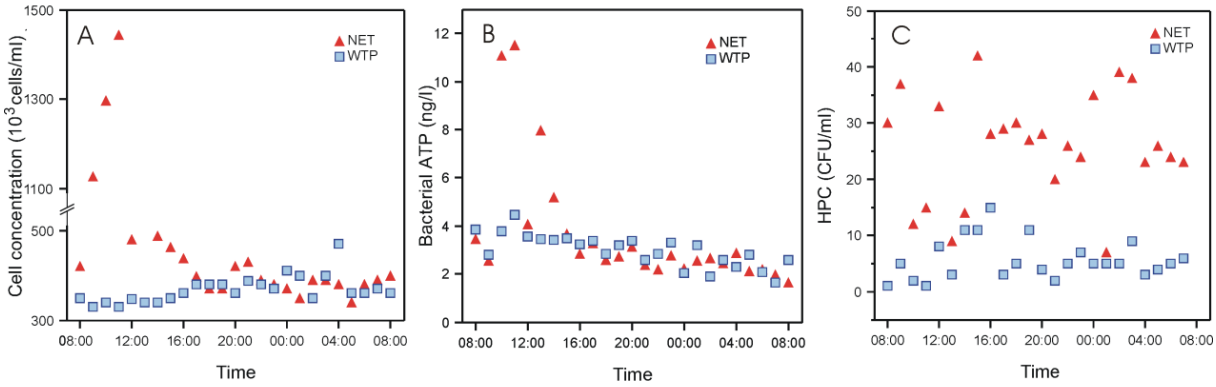


Figure 7.3. Evolution over one day of (A) flow cytometric bacterial cell concentrations, (B) bacterial ATP concentrations and (C) heterotrophic plate counts of samples collected from the water treatment plant outlet (WTP) and distribution network location (NET).

While free ATP values were below the detection limit in most samples (<1 ng L^{-1}), bacterial ATP variations revealed a trend similar to FCM results (Figure 7.3B). A 20% variation was observed at the WTP (average of 3 ng L^{-1}). In the network, a significant increase in ATP was detected between 9 and 11 am (from 2.5 to 11.5 ng L^{-1}), followed by a decrease reaching WTP values after 3 pm (13% variation thereafter). With respect to plate counts, higher values were continuously detected at the network location (average of 26 CFU mL^{-1} versus 6 CFU mL^{-1} at the treatment plant), but this method did not detect the peak in bacterial numbers during the morning (Figure 7.3C). All other methods (pyrosequencing, FCM and ATP) independently showed and thus confirmed an unusual dramatic event.

Pyrosequencing revealed that the drinking water at the plant outlet and in the distribution network harbored a diverse bacterial community (Figure 7.4). At phylum level classification, bacteria with a relative abundance below 1% across all samples were grouped together under the “other” category. After this percentage cutoff, 9 phyla (including one candidate phylum) were identified at the plant and exhibited relative stability over 24 h (Figure 7.4A). *Proteobacteria* dominated the community with a relative abundance of 58.3 ± 6.7 %. The remaining phyla were *Planctomycetes* (12.2 ± 2.6 %), *Bacteroidetes* (8.3 ± 2.1 %), *Actinobacteria* (5.3 ± 1.6 %), *Chloroflexi* (3.8 ± 0.9 %), *Cyanobacteria* (2.5 ± 1.2 %), *Nitrospirae* (2.3 ± 0.6 %), *Firmicutes* (2.0 ± 1.0 %) and GN02 (1.1 ± 0.4 %). *Proteobacteria* subclasses were detected in the following order of decreasing abundance (Figure 7.4B): *Betaproteobacteria* (19.6 ± 4.7 %), *Deltaproteobacteria* (15.8 ± 3.9 %), *Alphaproteobacteria* (11.8 ± 2.9 %) and *Gammaproteobacteria* (11.0 ± 7.5 %). The outlier observed with MDS analysis at 2 pm (Figure 7.2) was characterized by the highest abundance of *Gammaproteobacteria* (32.2%). With respect to the network, a bacterial community composition similar to the WTP effluent was found but with varying proportions (Figures 7.4C and 7.4D).

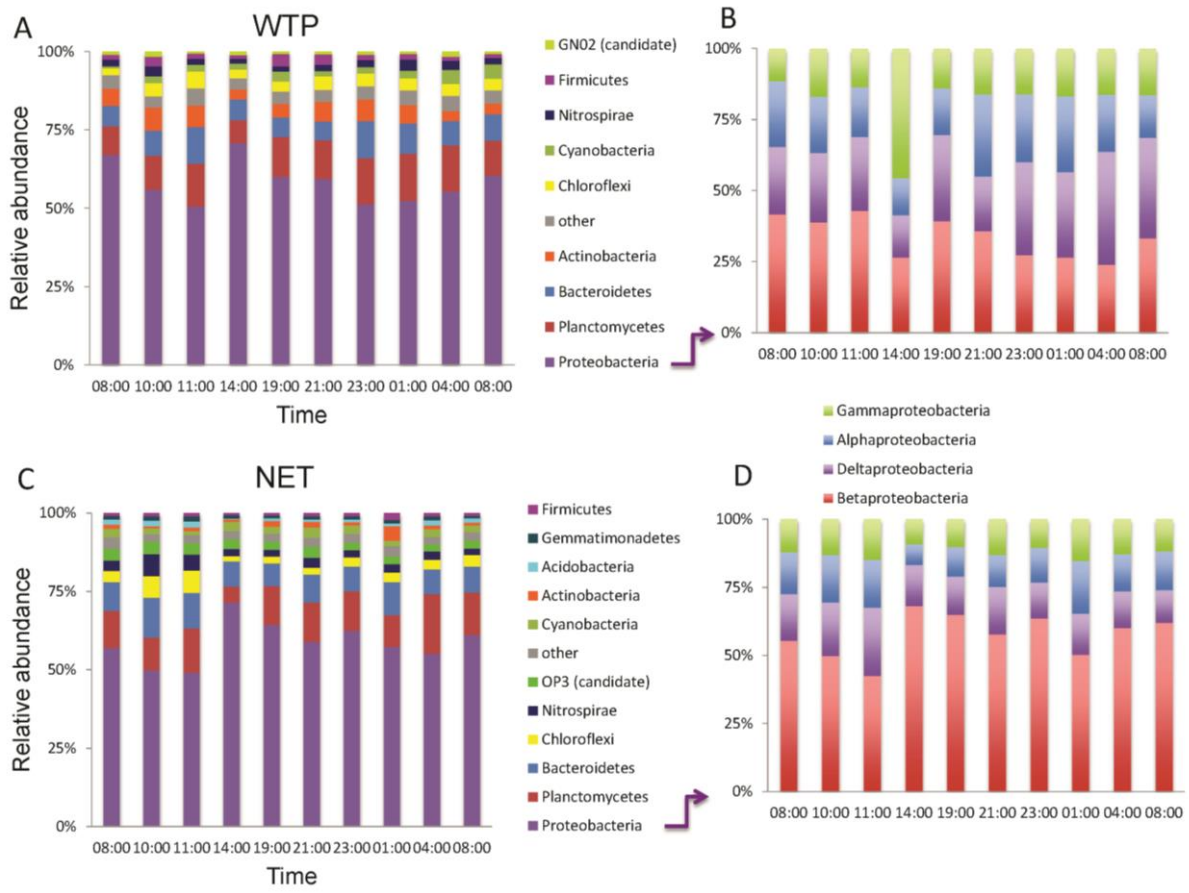


Figure 7.4. Relative abundance of bacterial phyla (A and C) and Proteobacteria classes (B and D) in samples collected from the water treatment plant outlet (WTP) and distribution network location (NET) over one day.

Additionally, 3 new phyla appeared as abundant members (OP3, *Acidobacteria* and *Gemmatimonadetes*) compared to the treatment plant samples, while the GN02 candidate phylum became a rare group (<1% abundance). The relative abundance of each phylum was (Figure 7.4C): *Proteobacteria*; 58.6 ± 6.7 %, *Planctomycetes*; 12.2 ± 3.5 %, *Bacteroidetes*; 9.2 ± 1.8 %, *Chloroflexi*; 3.6 ± 1.9 %, *Nitrospirae*; 3.3 ± 1.6 %, OP3; 3.1 ± 0.6 %, *Cyanobacteria*; 2.4 ± 0.6 %, *Actinobacteria*; 1.5 ± 1.1 %, *Acidobacteria*; 1.2 ± 0.5 %, *Gemmatimonadetes*; 1.1 ± 0.3 % and *Firmicutes*; 1.1 ± 0.4 %. At the class level (Figure 7.4D), an increase in the relative abundance of *Betaproteobacteria* was observed (34.0 ± 8.2 %) coupled with a decrease in the other groups (*Deltaproteobacteria*; 9.4 ± 1.5 %, *Alphaproteobacteria*; 8.1 ± 1.5 % and *Gammaproteobacteria*; 7.1 ± 0.7 %). The outliers observed with other analyses at 10 and 11 am in the distribution network (Figures 7.2 and 7.3) had a different bacterial community structure (lower relative abundance of *Betaproteobacteria* and higher relative abundance of *Chloroflexi* and *Nitrospirae*) as compared to the other network samples that exhibited a relatively stable community over 24 h.

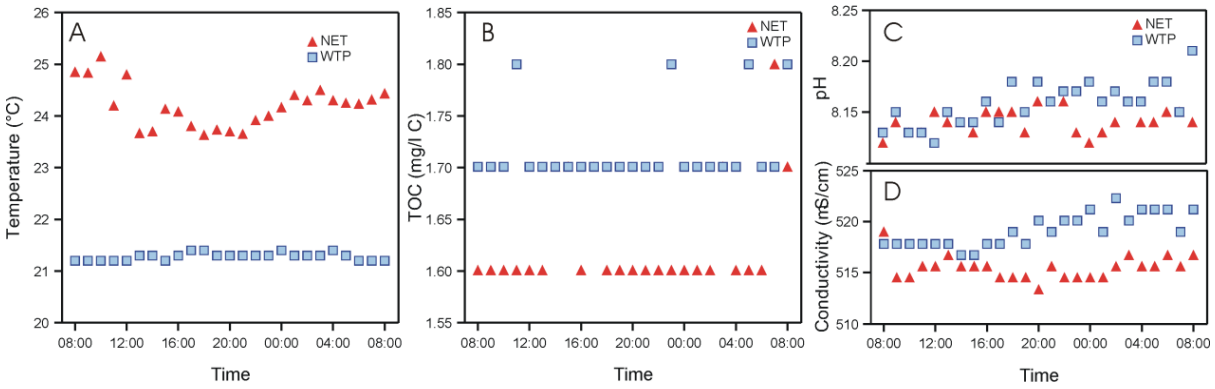


Figure 7.5. Evolution over one day of (A) temperature, (B) total organic carbon concentrations, (C) pH and (D) conductivity of drinking water samples collected from the water treatment plant outlet (WTP) and distribution network location (NET).

Physico-chemical parameters are shown in Figure 7.5. Water temperature increased during distribution (from 21.3 to 24.2 °C on average), but was constant over 24 h, with <2% variation at both locations (Fig. 5A). TOC concentrations were slightly (0.1 mg C L^{-1} difference) but consistently lower in the network, and also stable during the day with 2–3% variation at both locations (Fig. 5B). Stable pH and conductivity values were measured at both locations over time (<1% variation), with slightly lower conductivity values (4 μS cm^{-1} difference on average) detected in the network (Fig. 5C,D).

7.3.3 Week study

Weighted MDS analysis of the week study samples showed an ordination similar to the day study, with two distinct clusters representing the treatment plant and distribution network bacterial communities (Figure S7.1). The WTP community appeared less stable than the network community (larger distance between samples). ANOSIM confirmed a statistically significant and strong difference between the WTP and network community structure (p = 0.0002; R = 1), while temporal variations within the two locations individually were significant but small (p = 0.0001; R = 0.3423 for the WTP and p = 0.0007; R = 0.2715 for the network). Total cell concentrations were rather stable at the two locations with ~7% variation at the WTP and ~10% variation in the network (Figure S7.2A). Higher cell concentrations were continuously detected in the network (400 × 10^3 cells mL^{-1} on average versus 330 × 10^3 cells mL^{-1} for the WTP effluent) and lower cell concentrations were measured at both locations during the weekend with a surge observed on Monday (from 310 × 10^3 to 360 × 10^3 cells mL^{-1} at the WTP and from 380 × 10^3 to 450 × 10^3 cells mL^{-1} in the network). Bacterial ATP variations over time were not significant and slightly lower values were found in the network (Figure S7.2B), close to the detection limit however. Plate counts varied over time and were higher in the network

(Figure S7.2C). This culture-based method showed high (up to 100%) variation during the same day (four measurements).

Pyrosequencing showed a bacterial community composition very similar to the day study, with the same groups of bacteria identified (Figure 7.6). Temporal stability was observed at the plant over the week (Fig. 6A) with the following phyla: *Proteobacteria* (55.4 ± 2.7 %), *Planctomycetes* (15.6 ± 1.6 %), *Bacteroidetes* (8.4 ± 1.0 %), *Cyanobacteria* (4.2 ± 1.1 %), *Chloroflexi* (3.9 ± 0.5 %), *Actinobacteria* (3.6 ± 0.5 %), *Nitrospirae* (2.7 ± 0.4 %) and *Firmicutes* (1.3 ± 0.2 %). *Proteobacteria* subclasses were detected in the following order of decreasing abundance (Fig. 6B): *Betaproteobacteria* (19.9 ± 3.4 %), *Deltaproteobacteria* (14.3 ± 1.4 %), *Alphaproteobacteria* (11.8 ± 1.1 %) and *Gammaproteobacteria* (9.3 ± 2.0 %). With respect to the network, a similar community was found in different proportions (Fig. 6C,D) with OP3, *Acidobacteria* and *Gemmatimonadetes* also becoming abundant groups.

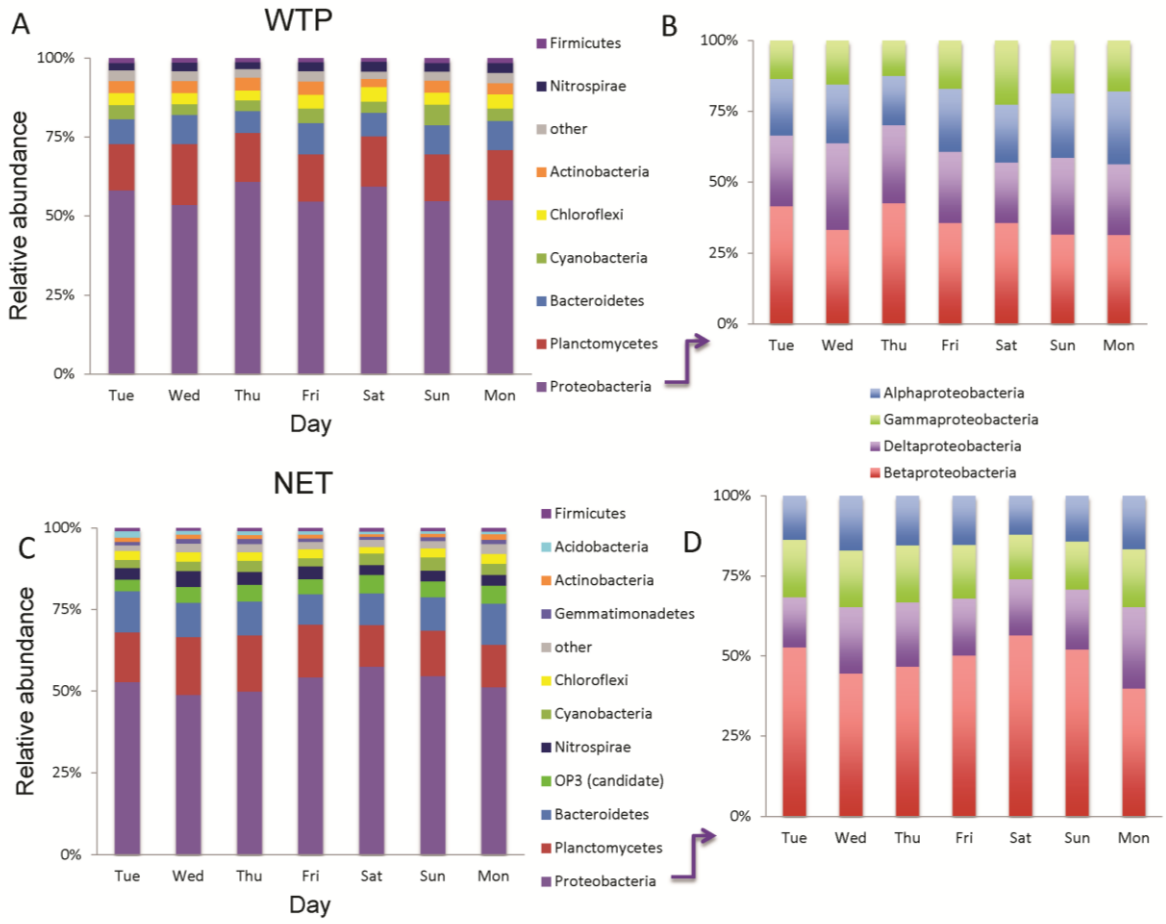


Figure 7.6. Relative abundance of bacterial phyla (A and C) and Proteobacteria classes (B and D) in samples collected from the water treatment plant outlet (WTP) and distribution network location (NET) over one week.

The relative abundance of each phylum was (Fig. 7.6C): *Proteobacteria*; 52.5 ± 3.0 %, *Planctomycetes*; 15.1 ± 2.0 %, *Bacteroidetes*; 10.7 ± 1.3 %, OP3; 4.9 ± 0.7 %, *Nitrospirae*; 3.7 ± 0.6 %, *Cyanobacteria*; 3.2 ± 0.5 %, *Chloroflexi*; 2.7 ± 0.3 %, *Gemmatimonadetes*; 1.3 ± 0.2 %, *Actinobacteria*; 1.2 ± 0.3 %, *Acidobacteria*; 1.1 ± 0.4 % and *Firmicutes*; 0.9 ± 0.1 %. At the class level, a stable bacterial community was also observed over time (Fig. 6D). Compared to the WTP, *Betaproteobacteria* increased in relative abundance (25.8 ± 4.2 %) while the other classes decreased (although the Gamma group became more abundant than the Alpha group): *Deltaproteobacteria*; 10.2 ± 1.4 %, *Gammaproteobacteria*; 8.7 ± 0.5 % and *Alphaproteobacteria*; 7.8 ± 0.6 %.

Temporal dynamics of the total, general (or dominant), and rare bacterial taxa (defined in section 7.2.4) are shown in Figure 7.7. Total and general taxa displayed similar dynamics (for both the treatment plant and distribution network). In the network, taxa representing Friday, Saturday and Sunday were adjacent on the plot. Considering the wide distribution of the rare taxa in the ordination and their distant position with respect to the other taxa (especially for the network), these taxa were the most dynamic.

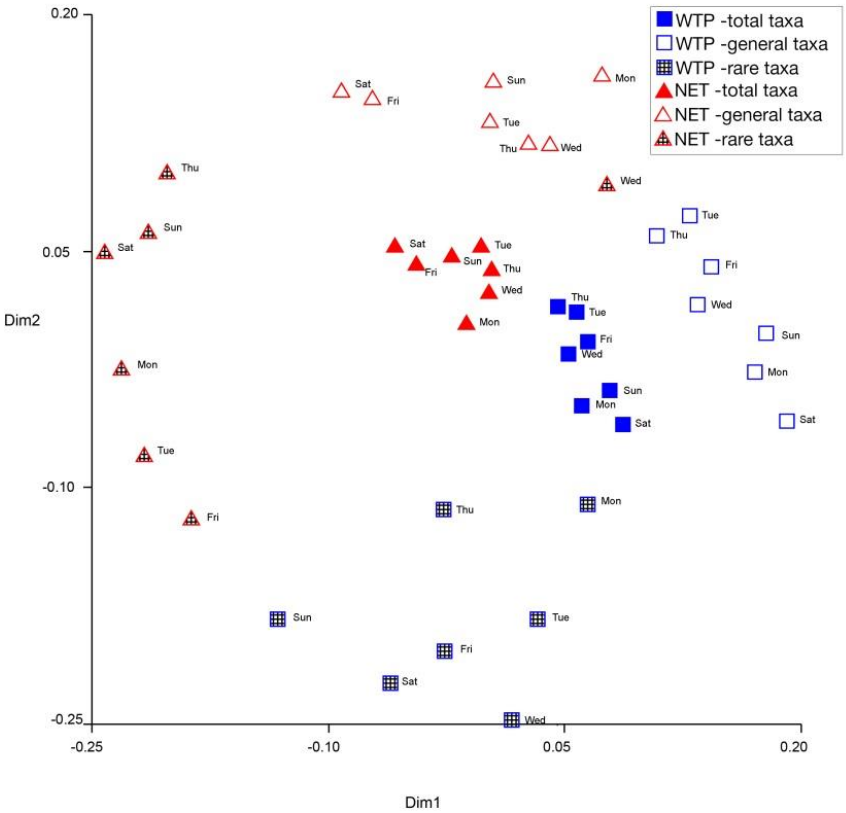


Figure 7.7. Ordination of nonmetric multidimensional scaling (MDS) based on operational taxonomic units (OTUs) (3% divergence) for total, general and rare taxa obtained from the water treatment plant outlet (WTP) and distribution network location (NET) samples collected over one week.

Regarding physico-chemical parameters, water temperature was higher in the network (1.6 °C difference on average) and decreased over the week at both locations (Figure S7.3A). TOC concentrations were very similar at both locations and stable during the week with 3–4% variation (Figure S7.3B). Similar pH and conductivity values were measured at both locations with <1% variation over the week (Figures S7.3C and D).

7.3.4 Core microbiome

OTUs and sequences characteristic of the plant outlet and/or network samples are shown in Figure 7.8, generated based on a normalization zone of ~6700 sequences per sample. In the three studies the network harbored a larger number of unique OTUs and sequences, thus showing greater bacterial richness. On the other hand, 57.8–64.9 % of the OTUs comprising 86.3–91.3 % of the reads were found at both sites. This large fraction of common taxa constitutes the core microbiome for this drinking water distribution system.

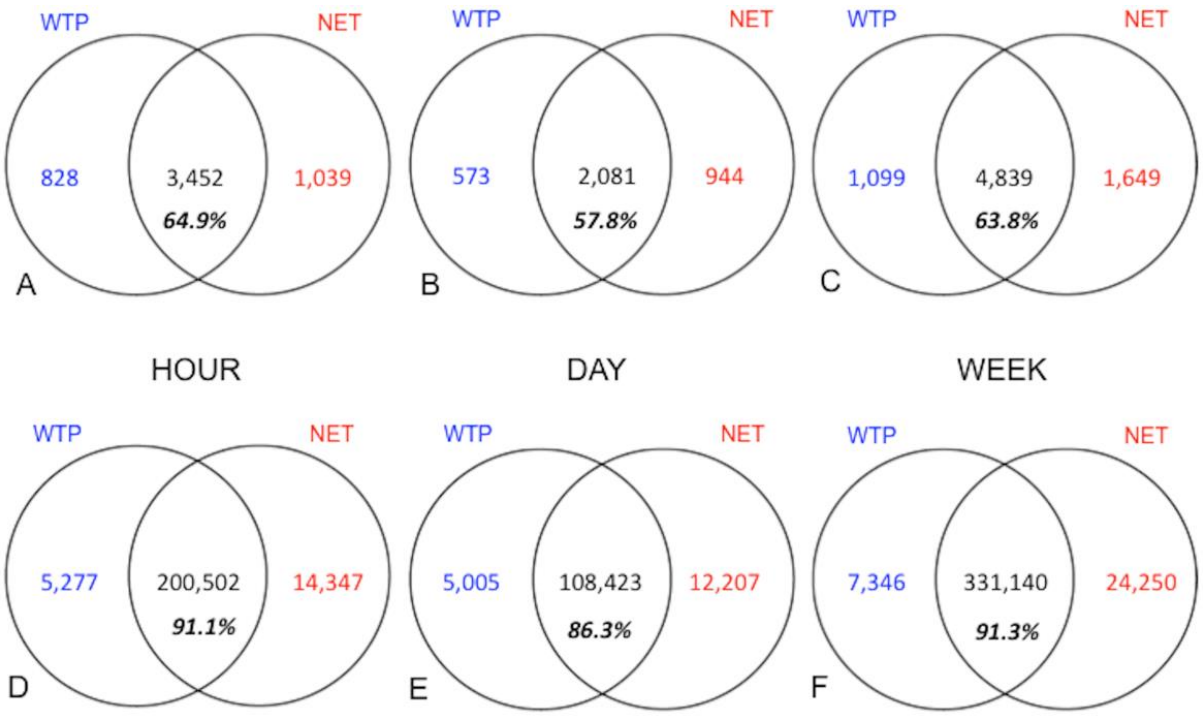


Figure 7.8. Venn diagrams showing number of operational taxonomic units (OTUs) (A, B and C) and sequences (D, E and F) in the water treatment plant outlet (WTP), distribution network location (NET) and both (shared fraction) for the hour (A and D), day (B and E) and week (C and F) studies. The shared fraction is also expressed as a percentage (%).

7.4 Discussion

7.4.1 Temporal variations: insignificant at plant and network locations

The microbial community at the two individual sampling locations was stable over time. With respect to the plant effluent, FCM total bacterial cell count variations were less than 10% and bacterial activity (ATP measurements) did not fluctuate significantly either (Figs. 3 and S2). With the exception of one outlier (Figs. 2 and 4), pyrosequencing revealed a similar bacterial community composition at all sampling times with a small change in the proportion of each phylotype (<7%). One reason for this relative stability could be high flow rates near the plant that hinder water stagnation, sedimentation and other disrupting processes (Nescerecka et al., 2014). A second justification involves appropriate treatment applying biofiltration which is likely to have exerted a stabilizing force on the bacterial communities in the produced water. This was also shown in a study by Pinto and colleagues (2012) where postfiltration samples were largely decoupled from the temporal effects observed in prefiltration samples. At the network location, flow cytometric temporal variations were also less than 10%, excluding the dramatic event that was observed during the 24 h sampling when a notable increase in bacterial cell concentration was measured at 10 and 11 am (also detected with ATP and pyrosequencing analyses but not with HPC) (Figs. 2, 3, 4 and S2). The tap in this study was located in a university laboratory and thus the event cannot be justified by local water consumption patterns as most people arrive at 8–9 am. An event early in the morning or around lunchtime when water usage changes would have been more plausible (Besmer et al., 2014). The reason behind an increased abundance of nitrifiers and *Chloroflexi* in these two particular samples is unknown. These two groups of bacteria have been associated with loose deposits (Liu et al., 2014). An inexplicable hydraulic event may have occurred causing a disturbance in suspended solids and loose deposits, leading to increased cell concentrations and the appearance of these bacteria in the bulk water phase. It should be pointed out that the hour measurements where repeated in the morning of 7 additional days and analyzed with flow cytometry (data not shown). The morning peak was not detected again and variations were less than 8% on all days, showing that the detected peak was incidental. Another discrepancy detected was the weekday/weekend pattern (Figs. 7, S1 and S2A). Samples collected during the weekend clustered together in MDS plots and had lower cell counts. This could be linked with water consumption routines, highlighting the influence of hydraulic conditions on bacterial communities in tap water. Nevertheless, disregarding the morning event observed in the network, temporal variations in the water microbiology were generally not substantial at both sites. It should be highlighted that this water was distributed in the network without disinfectant residuals.

7.4.2 Distribution effect: change linked with rare taxa

A change in bulk water microbiology was found during distribution. Significantly higher FCM total cell concentrations were measured at the network location (403×10^3 cells mL^{-1} on average) compared to the plant outlet (347×10^3 cells mL^{-1} on average) for the three studies combined (hour, day and week). This increase in cell number would be associated with a conversion of 5.6 µg AOC L^{-1} (Hammes et al., 2006). A significant change in the community structure was also found with pyrosequencing and statistical analyses. Some taxa present at the plant disappeared in the network (Fig. 8). This could be due to cell lysis by indigenous, resistant viruses and/or protozoa (if present) (Berry et al., 2006) or simply due to the stressful, oligotrophic environment of DWDSs where some species have a competitive advantage over others. Chlorine dioxide disinfection at the plant may also have caused the slow disintegration of these microorganisms in the network despite the absence of disinfectant residuals in the produced drinking water. More importantly, planktonic bacteria represent a small fraction of the microbial load established in a DWDS, whereby bacterial activity can be heavily associated with pipe biofilms and loose deposits (Liu et al., 2014). These habitats are inherently dynamic and during water distribution some species may have become trapped in biofilms or sediments resulting in their “disappearance” from the water phase.

Compared to the plant effluent, the network was characterized by greater bacterial richness (Fig. 8) and promoted the growth of some phylotypes that were not abundant at the plant (Figs. 4 and 6). These rare taxa constituted a diverse, small fraction of the community (3–4%) and exhibited the highest dynamicity (Fig. 7). Likely these microorganisms are highly oligotrophic, however their role in DWDSs is not described in the literature yet and the specific environmental or hydraulic conditions that favored their growth are unknown. While pH and conductivity did not vary during distribution a small rise in temperature was detected (Figs. 5 and S3). Temperature changes can greatly affect microbial processes and metabolism. For instance higher bacterial abundance and richness in drinking water during the summer season has been previously reported (McCoy and VanBriesen, 2014; Pinto et al., 2014). The increased water temperature, combined with the absence of disinfectant residuals, could have induced the growth of rare taxa. Furthermore, the continuously running tap may have affected water hydraulics causing the detachment of some cells from biofilms and/or the resuspension of sediment-associated bacteria, hence their manifestation in the water phase. Pipe material also may have affected the microbial community of this system. The main piping originating from the treatment plant is cemented steel while in the university town PVC is used. It is possible that this conversion in pipe material had affected the bacterial community composition and

corresponding ecosystem balance, with e.g. the different materials releasing chemicals that would act as substrates for some bacteria and as inhibitors for others (Ingerson-Mahar and Reid, 2012).

7.4.3 Core microbiome: diverse and persistent

Despite the change detected during distribution a substantial shared microbiome was found between the produced and distributed water (Fig. 8). The bacterial phyla detected were characteristic of DWDSs, particularly those that apply a similar treatment strategy. *Proteobacteria* found in most freshwater environments dominated and the remaining phylotypes have been found in drinking water treated by biofiltration and distributed without disinfectant residuals, i.e. applying the "starvation versus suppression" approach common in European countries (Hammes et al., 2010a; Smeets et al., 2009). A similar bacterial community composition has been reported in drinking water systems that employ this strategy despite using groundwater as a source (Liu et al., 2014; Martiny et al., 2005), thus supporting the finding that the biofilter both seeds and shapes the water microbiome (Lautenschlager et al., 2014; Pinto et al., 2012). The core microbiome in this study was diverse and persistent with time and location, and variations happened mainly with the rare phylotypes. *Betaproteobacteria* have been found more frequently in non-chlorinated DWDSs (Emtiazi et al., 2004; Lautenschlager et al., 2013) and this could explain their increased abundance during distribution. *Nitrospirae* have been found in the absence of chlorinated nitrogen compounds, originating from sand filter biofilms and surviving starvation periods (Martiny et al., 2005). The precise conditions that promoted the growth of other less abundant groups such as *Acidobacteria* and *Gemmatimonadetes* are unknown, although these two phyla were linked with similar warm temperatures (20–25 °C) in a study by Pinto and colleagues (2014).

7.4.4 Biological stability: accepting change

"Biologically stable water does not promote the growth of microorganisms during its distribution" (Rittmann and Snoeyink, 1984). There are no guidelines that delineate the concept of biological stability in terms of microbial community composition (identity), structure (relative abundance) or cell number. A substantial baseline microbiome was established for this DWDS and temporal variations were insignificant. A change however in the bacterial community structure was observed during distribution, driven mainly by the rare taxa. Further research is needed to understand the function of these rare phylotypes and their impact on the overall ecology. Given the stringent regulations enforced in the Netherlands and the high quality of the supplied drinking water, this change or "biological instability" detected during distribution did not have hygienic implications– it is

therefore the norm and contingent on the sensitivity of the applied analytical methods (pyrosequencing and FCM). When a core microbiome is established for a DWDS, deviations can be accepted as long as the water quality is not compromised from a public health perspective. Biostability is generally desired in drinking water guidelines but it is not an absolute measure of safety and may be difficult to achieve in large-scale complex distribution systems that are inherently dynamic. The concept of biological stability needs to be revised and quantified, allowing a certain degree of change in microbiology.

7.5 Conclusions

This is the first study that applies a combination of methods to examine drinking water bacterial dynamics at high temporal resolution in a full-scale drinking water distribution system. The main findings were:

- The bacterial community profile at the treatment plant and distribution network locations did not vary significantly over time (hour/ day/ week).
- A substantial core microbiome was shared between the produced and distributed water, shaped by the treatment strategy i.e. biofiltration and absence of disinfectant residuals.
- The bacterial community structure changed during distribution, with greater bacterial richness detected in the network.
- The rare taxa exhibited the highest dynamicity causing the major change detected during water distribution.
- Conventional microbial analysis techniques were not adequate to measure changes in the bacterial community.
- With the advance of more sensitive microbial analysis techniques, the concept of biological stability needs to be revised.

SUPPLEMENTARY INFORMATION – Chapter 7

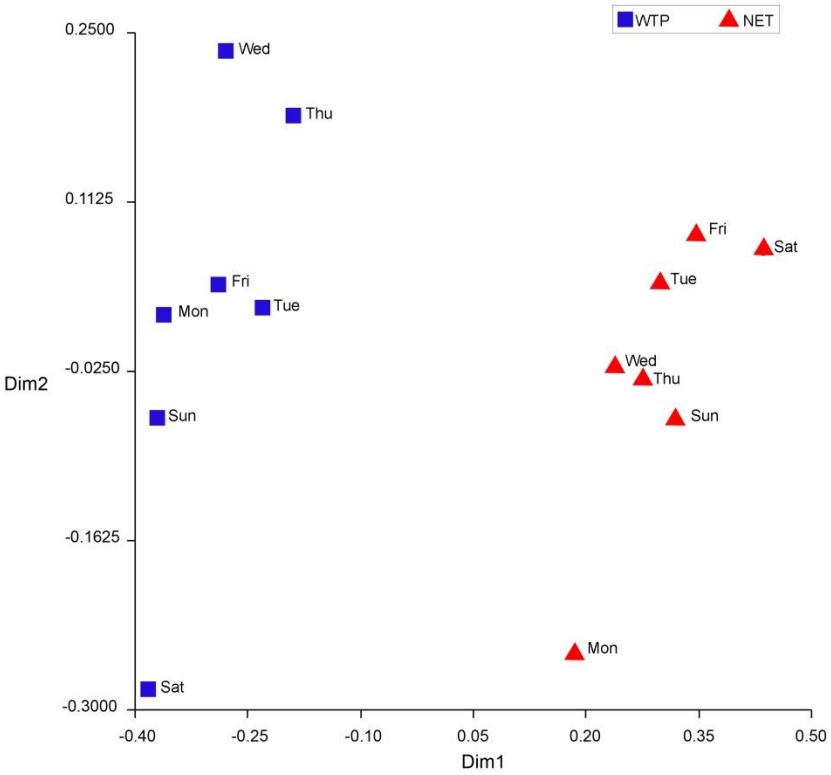


Figure S7.1. Weighted multidimensional scaling (MDS) plot for water treatment plant outlet (WTP) and distribution network location (NET) samples collected from the one week study.

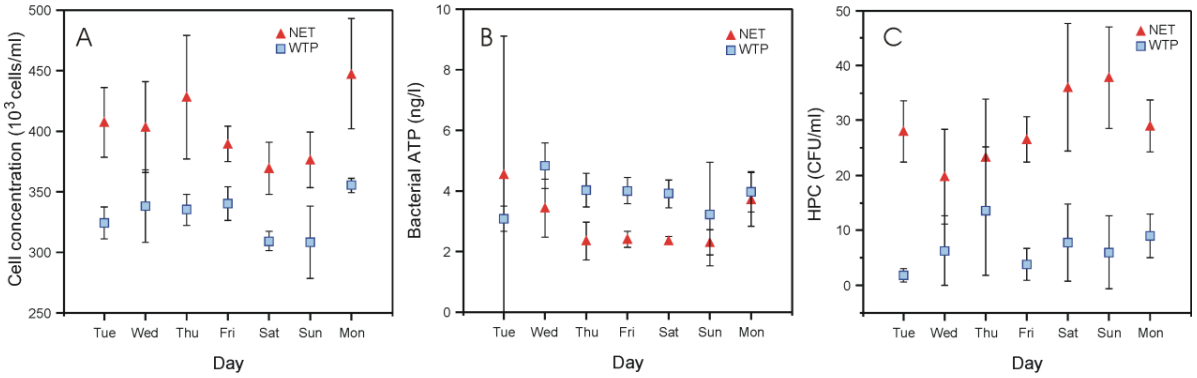


Figure S7.2. Evolution over one week of (A) flow cytometric cell concentrations, (B) bacterial ATP concentrations and (C) heterotrophic plate counts of samples collected from the water treatment plant outlet (WTP) and distribution network location (NET).

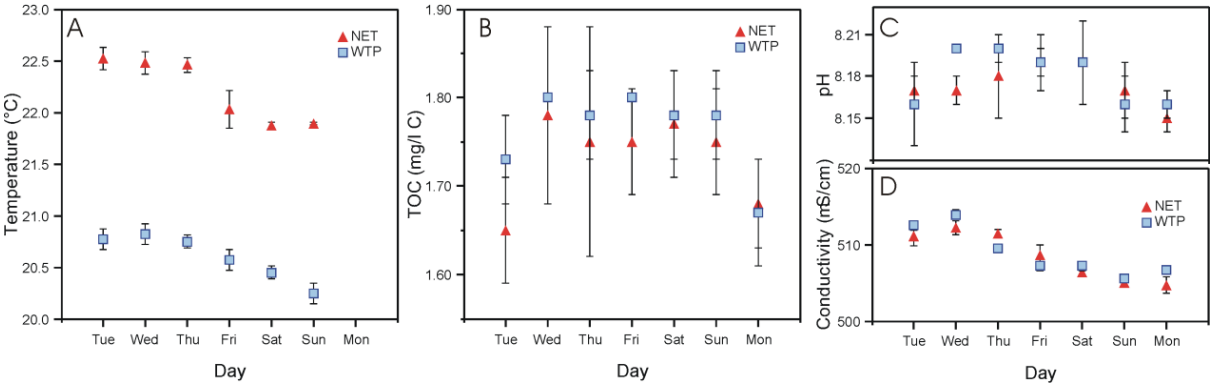
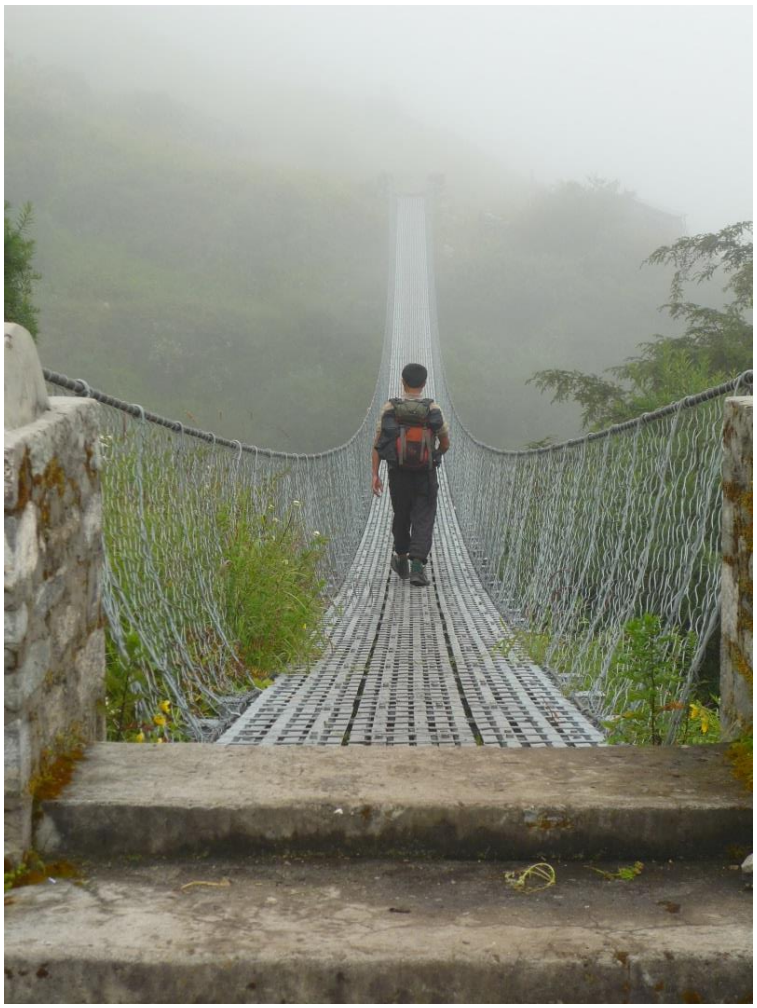


Figure S7.3. Evolution over one week of (A) temperature, (B) total organic carbon concentrations, (C) pH and (D) conductivity of samples collected from the water treatment plant outlet (WTP) and distribution network location (NET).

OUTLOOK

Chapter 8

Conclusions and future perspectives



8.1 General conclusions

In this thesis, new methodological approaches were investigated for the assessment of biological stability and study of bacterial dynamics in drinking water distribution systems. A new set of analytical methods was tested, integrated into a simple approach, and applied to a full-scale drinking water distribution system in the Netherlands.

Analytical method testing for the characterization and study of bacterial communities in drinking water showed the following:

- A standardized flow cytometric (FCM) method enabled sensitive detection of changes in drinking water bacterial communities, based on bacterial cell concentrations and fluorescence fingerprints, as analysed with a simple approach (Chapter 3).
- Changes in FCM fluorescence fingerprints were rapid indicators for changes in bacterial community composition (Chapter 4).
- The combination of FCM and high-throughput 16S rRNA gene sequencing methods such as 454 pyrosequencing was adequate to provide a detailed description of drinking water bacterial communities (Chapter 4).
- A straightforward growth potential method was useful for bacterial growth potential evaluation and assessment of growth limitations in drinking water (Chapter 5).

Application of the developed methods for studies at a full-scale treatment facility producing drinking water from surface water in the Netherlands and its associated distribution system transporting water without detectable residual disinfectant, showed that:

- Changes in bacterial community characteristics (abundance, viability, and/or community composition) were systematically recorded during drinking water distribution using FCM and high-throughput sequencing methods, while no change was detected with conventional cultivation-based methods (Chapters 4, 5, 6 and 7).
- Large seasonal variations in bacterial cell concentrations occurred at the drinking water treatment effluent, and these strongly affected the water quality in the distribution system (Chapter 6).
- Produced drinking water was subject to multiple bacterial growth limitations in organic carbon and inorganic nutrients (Chapter 5).
- Full-scale drinking water distribution conditions have a significant impact on bacterial abundance in the distribution system, compared to the growth potential of the produced drinking water (Chapter 5).

From the above-mentioned observations, it was concluded that the combination of controlled laboratory-scale growth tests and on-site measurements of microbial water quality is useful for investigation of bacterial growth-controlling factors in drinking water and during water distribution. In addition, it was clearly demonstrated that both spatial and temporal on-site investigations are essential for the study of microbial dynamics in drinking water distribution systems.

8.2 Proposed approach for the study of bacterial dynamics in drinking water distribution systems

Achieving biological stability requires that (i) biological stable water is produced and (ii) is distributed in conditions that do not promote uncontrolled changes in the microbial community, until the point of consumption (Chapter 2). Consequently, and based on the results presented in this thesis, a comprehensive, integrated approach is proposed for the study of bacterial dynamics in drinking water distribution systems, requiring the use of both predictive methods in controlled laboratory tests for the assessment of water bacterial growth-promoting properties, and analytical methods for direct on-site spatial and temporal monitoring of bacterial communities (Figure 8.1). Most likely bacterial dynamics are specific to each and every system, as the combination of raw water characteristics, applied treatments, distribution system properties (size, pipe material) and distribution conditions (temperature, hydraulic conditions) are unique. As a consequence, detailed investigations are required at each and every site, which can be performed with the comprehensive toolbox proposed below. However, parts of the comprehensive approach can be selected depending on the application, for e.g. regular water monitoring, collection of information towards targeted improvement of treatment or distribution conditions, or in-depth research on bacterial dynamics in drinking water distribution systems, as detailed in section 8.3.

8.2.1 In-situ *assessment of spatial and temporal bacterial dynamics in distribution systems*

Designing an *in-situ* sampling strategy is a crucial step for assessing biological stability. The sampling strategy should cover different aspects of microbial quality variations in drinking water distribution systems:

- Assess spatial variations in bulk water quality in distance during water distribution, due to either intrinsic treated water growth potential and/or influences of distribution conditions. The sampling scheme should therefore include the treatment effluent and various locations in the distribution system. Nescerecka et al. (2014) described a randomised sampling approach for so-called hot-spot detection. As alternative, directed sampling can be considered based on hydraulic

models/calculations, tracer tests, and/or pressure zones, to select distribution areas of interest (e.g., based on residence time in the system or distance to the treatment plant; Lautenschlager et al., 2013; Van de Wielen and van der Kooij, 2010; Liu et al., 2014).

- Assess temporal variations in bulk water quality in the treatment effluent and at selected locations in the distribution system. Temporal variations should be assessed on both short (hour-to-week) time-scales to detect diurnal patterns and events (Besmer et al., 2014; Nescerecka et al., 2014; Chapters 4 and 7) and long (multi-year) time-scales to detect seasonal changes (Pinto et al., 2014; Chapter 6).
- Spatial and temporal investigations of biofilm and sediments should ideally also be included in the design of studies on microbial dynamics in full-scale drinking water distribution systems. These two phases are, however, more difficult to sample and therefore not ideal for high frequency monitoring. Sediment sampling can be achieved by distribution networks flushing (Liu et al., 2014). Biofilm sampling can only be performed following pipe extraction from the system (Wingender and Flemming, 2004), e.g. during a pipe replacement by water utilities. Alternatively, biofilm traps/ coupons/ reactors can be directly connected to the system on long-term basis for representative biofilm formation (Servais et al., 1995; Wingender and Flemming, 2004).

The analysis of the water quality should be performed with a combination of methods to analyse microbial and environmental parameters. The following combination of methods is suggested:

- Assessment of bacterial abundance with FCM (Hammes et al., 2010; Lautenschlager et al., 2013);
- Assessment of bacterial activity/viability with FCM combined with viability-targeted fluorescent dye and complemented with adenosine tri-phosphate (ATP) measurements (Vital et al., 2012a; Nescerecka et al., 2014);
- Detection of basic shifts in bacterial community composition by the analysis of a large number of samples with FCMc fingerprints (Chapter 4);
- In-depth analysis of bacterial community composition in selected samples with high-throughput 16S rRNA-gene sequencing methods (e.g. pyrosequencing or Ilumina) (Pinto et al., 2014; Roeselers et al. 2015).
- Analysis of environmental parameters, including temperature, pH, conductivity, concentrations of biodegradable organic (e.g. assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC)) and inorganic nutrients (e.g. phosphate, sulfure and nitrogen-based compounds such as ammonium and sulfate, and metallic compounds such as iron). AOC and BDOC tests in this regard are not used as bacterial growth predictive methods but rather as measurements of the organic content of the water, useful for interpretation of data collected with other analysis.

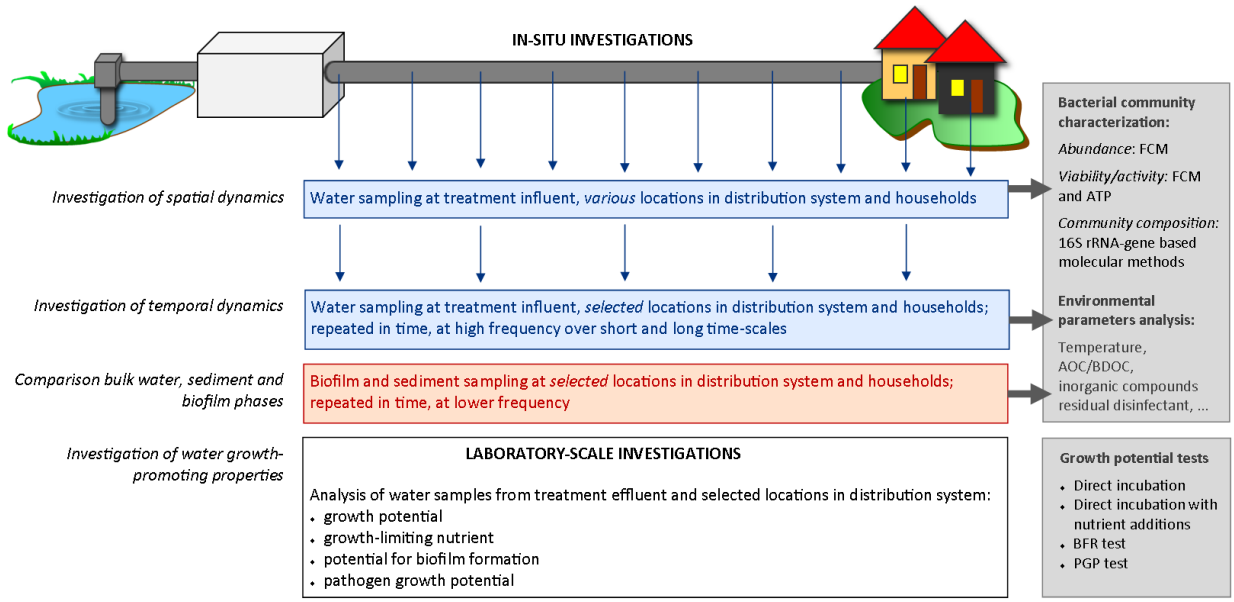


Figure 8.1. Suggested approach and methods for studying biological stability in drinking water distribution systems.

8.2.2 Predictive methods for the evaluation of potential for biological stability

Predictive methods are useful to characterize water and to provide supportive information for decision-making of water treatment and/or distribution conditions improvements. The tests should be applied to the treatment effluent and on a selection of distribution locations to evaluate both the properties of the treated water and how these are affected by distribution conditions. Depending on the question addressed, the laboratory tests can include:

- Evaluation of inherent bacterial growth potential of drinking water samples, by direct, untreated incubation of water samples under controlled laboratory conditions (Chapter 5). Incubation conditions can be adapted to be similar to those encountered in distribution systems with low water temperature (e.g., 12 °C) and possibly long residence times (e.g., 10 days).
- Identification of bacterial growth-limiting nutrients in water, by adding a selection of nutrients prior incubation of water samples in controlled laboratory conditions (Chapter 5);
- Evaluation of bacterial growth promoting properties of water for biofilm formation, using e.g. the BFR method (van der Kooij et al., 1994);
- Evaluation of growth-promoting properties of pathogenic organisms, alone or in competition with the indigenous bacterial community (Vital et al., 2008; 2012b);
- Evaluation of bacterial growth-controlling properties of materials in contact with water (e.g. Bucheli-Witschel et al., 2012).

8.3 Applications

The proposed tests can be applied individually or combined depending on the targeted information. Below are listed a number of applications.

8.3.1 Drinking water quality monitoring

Routine drinking water monitoring is essential for water quality surveillance. A number of technical, chemical, and environmental parameters (e.g. pH, conductivity, temperature, water flow velocity) are presently used for water quality monitoring in real time with on-line apparatus or smart metering technologies. However, monitoring of water microbial characteristics has remained conservative with the use of cultivation methods for both general water quality (HPC) and hygienically relevant organisms. The disadvantages of cultivation-based methods have been discussed in Chapters 1 and 2, but essentially, the results are only obtained few days after sampling, which does not allow for rapid detection of system failure (e.g. pipe leakage or break) and for immediate corrective actions. In this regard, *flow cytometry* is an excellent candidate for general microbial water quality monitoring, as the method provides results within 15 minutes (Hammes et al., 2008; Chapter 3). The Swiss legislation has already taken a major step forward compared to other European countries, by implementing FCM as a routine analytical method in the Swiss drinking water guidelines (SLMB, 2011). Routine FCM measurements could be best complimented by the assessment of *total and free ATP concentration*s for viability assessment, which are already routinely applied by water utilities in the Netherlands. As on-line flow cytometric technologies are emerging (Besmer et al., 2014), one could envision in future the real-time monitoring of total and intact bacterial cell counts at treatment plant effluents and several locations in drinking water distribution systems. In case of detection of "abnormal change", continuous control with flow cytometry and ATP should be complimented with more extensive methods, such as *high-throughput sequencing*, or screening for specific pathogenic organisms. Technical improvements of sequencing technologies are expected in future, with lower costs and higher read lengths and throughput, possibly enabling the identification of OTUs down to species levels. Recently, a sequencer of the size of a USB stick, providing long sequencing reads within 6 h has been developed and used for bacterial identification (MinION; Kilianski et al., 2015; Ashton et al., 2015). One could envision in future the use of such device for rapid detection of pathogenic organisms in drinking water.

One key advantage of monitoring microbial parameters on a high-frequency basis is the sensitivity for change detection in drinking water quality or characteristics. Any change in chemical/

environmental property of water would result in a change in the bacterial community characteristics (Chapter 2). This change could be detected sensitively by flow cytometry, ATP or high-throughput sequencing methods, while other methods for measuring chemical properties of water would not be sufficiently sensitive. As an example, an increase in organic components in the water of 1 µg C/L would result in an increase of 10^4 cells/mL. Flow cytometry enables the counting of bacterial cells down to concentrations of 100 cells/mL. Theoretically, this implies that current FCM would enable to quantify bacterial growth occurring from the consumption of 0.01 µg C/L. In comparison, detection limits of AOC methods are usually in the range of 1 to 10 µg C/L, while the current analytical techniques for DOC measurements have higher detection limits (from 10 µg C/L in the case of high quality apparatus).

The current essential bottleneck in the application of flow cytometry as a standard monitoring method is the lack of guideline values for bacterial cell concentrations in drinking water (Chapters 2 and 6). Establishment of guideline values would require the description of inherent variations (e.g., daily or seasonal) to each drinking water distribution system under normal conditions, by collecting large microbial water quality data in space and time. Such an approach would provide a baseline for the detection of abnormal changes (LeChevallier et al., 1996; Besmer et al., 2014; Chapter 6), and for establishing the degree of acceptable change specific to a given distribution network. Key questions at this stage are whether guideline values should be (i) system-specific or applicable to all systems and (ii) established as absolute cell concentration values or as e.g. percentage of change, as dramatically different cell concentrations can be found in various drinking waters (from 10^3 to 10^6 cells/mL).

8.3.2 Improvement of drinking water production and distribution condition

The combined results obtained from *in-situ* and laboratory-scale analyses provide a basis for water treatment and distribution conditions evaluation (Chapter 5). The extent of bacterial growth that water can support in bulk water and biofilms can be assessed using the *direct incubation growth potential test* proposed in Chapter 5 and the biofilm formation rate test (BFR; van der Kooij et al., 1994). The direct incubation growth potential test is also useful for identification of bacterial growth-limiting compounds in water. The gathered information from these tests provide solid basis for choosing adequate treatment(s) to reduce the bacterial growth potential of water, by decreasing the concentration of identified growth-limiting compound(s). Besides, the effect of changing operation conditions of specific treatment (e.g. contact time within biofilters) or of implementation of new treatment steps on the bacterial growth potential of water can be evaluated using the same

methods. However, decisions should also be based on results from on-site measurements to evaluate the extent of growth actually occurring within the specific system, based on flow cytometric and ATP measurements. As an example, bacterial cell concentrations measured in water distributed in a full-scale drinking water distribution system were lower than in the same water after incubation in controlled laboratory conditions at similar water temperature and residence time (Chapter 5). Implementation of more extensive treatments might not be needed in this case.

8.3.3 In-depth research for new insights in the biological stability concept

The comprehensive approach and large set of methods proposed in Figure 8.1 can be applied to unravel the knowledge gaps on microbial dynamics in drinking water treatment and distribution systems. Below are listed key research questions to address in future research.

(i) Are bacterial limitations in inorganic nutrients frequent in drinking water?

There is evidence that bacterial growth in drinking water can be limited by other elements than organic carbon (Miettinen et al., 1997; Chapters 5 and 6). However, the focus of past research has essentially remained on organic carbon limitations. Systematic determination of growth limitations in drinking water at various sites is needed to determine whether inorganic nutrients limitations are frequent in drinking water. Moreover, it is unclear whether a shift in limiting nutrient can occur during water distribution, i.e. due to nutrient release from pipe material, sediments or biofilms, or to mixing of waters from different treatment facilities.

(ii) What is the role of biofilms and sediments in maintaining water quality in distribution systems?

Significant differences in bacterial growth in controlled experiments and full-scale distribution systems (Chapter 5) could be partially explained by the presence of biofilms and sediments in distribution networks. Essential questions need to be addressed related to the role of biofilms and sediments in drinking water distribution systems: To which extent are biofilms and sediments contributing to the bacterial community composition in the bulk water, through detachment or re-suspension? Do biofilms and sediments contribute to the consumption of nutrients and how does this affect the growth potential of water in the distribution system? Do protozoa, viruses and other organisms in biofilms and sediments play a major role in controlling bacterial growth in drinking water distribution systems?

(iii) What is the importance of autotrophic growth in drinking water distribution systems?

Arguably, autotrophic growth should remain negligible in comparison with heterotrophic growth, due to lower yields of autotrophs. However, their occurrence has been reported in various drinking

water distribution systems, and have often been related to aesthetic or operational problems (e.g. bio-corrosion, altered taste and odour; Chapter 2). Specific data is lacking on the contribution of autotrophs on the total biomass production and bacterial growth in drinking water distribution systems.

(iv) Is it needed and/or possible to fully control bacterial community characteristics in drinking water at the treatment stage and at any time?

Evidence has been given that bacterial abundance (Chapter 6) and community composition (Pinto et al., 2014) in drinking water can vary significantly with seasons. The causes for such seasonal variations in bacterial communities can be multiple and include variations in raw water quality and/or in treatment performances. It is however not clear to which extent seasonal changes affect long term bacterial colonization of drinking water distribution systems and have consequence on drinking water quality. One remaining question is whether production of drinking water of exact same quality all over the year would be useful and whether this could be achieved by improved water treatments.

Drinking water characteristics and bacterial dynamics during water distribution will likely be affected by expected evolving treatment technologies and changing environmental conditions such as: improvement of water treatments and/or change in water treatment types, towards e.g. membrane filtration technologies such as nanofiltration and reverse osmosis; changes in global water consumption; introduction of water re-use cycles; global warming. Good understanding of bacterial dynamics in drinking water production and distribution systems would enable the implementation of strategies to maintain good water quality up to the consumer's tap in future, by (i) directed drinking water monitoring, (ii) effective corrective actions and (iii) funded decisions for the optimization of water treatment production and/or distribution conditions to better control bacterial growth in drinking water distribution systems.

References

A

Abu-Shkara, F., Neeman, I., Sheinman, R., and Armon, R. (1998) The effect of fatty acid alteration in coliform bacteria on disinfection resistance and/or adaptation. Water Sci. Technol. 38(12), 133-139.

Allen, M.J., Edberg, S.C. and Reasoner, D.J. (2004) Heterotrophic plate count bacteria- what is their significance in drinking water? Int. J. Food Microbiol. 92(3), 265-274.

Allion, A., Lassiaz, S., Peguet, L., Boillot, P., Jacques, S., Peultier, J. and Bonnet, M.C. (2011) A long term study on biofilm development in drinking water distribution system: comparison of stainless steel grades with commonly used materials. Revue de Métallurgie 108(4), 259-268.

Anonymous (1998) Council Directive 93/83/EC of 3 November 1998 on the quality of water intended for human consumption. The council of the European Union (ed.), pp. L330/332-L330/354.

Ashton, P.M., Nair, S., Dallman, T., Rubino, S., Rabsch, W., Mwaigwisya, S., Wain, J. and O'Grady, J. (2015) MinIon nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nat. Biotech. 33(3), 296-300.

Aw, T.G. and Rose, J.B. (2012). Detection of pathogens in water: from phylochips to qPCR to pyrosequencing. Curr. Opin. Biotechnol. 23 (3), 422-430.

B

Barbeau, B., Gauthier, V., Julienne, K., and Carriere, A. (2005) Dead-end flushing of a distribution system: Short and long-term effects on water quality. J. Water SRT - AQUA 54(6), 371-383.

Batté, M., Féliers, C., Servais, P., Gauthier, V., Joret, J.C. and Block, J.C. (2006) Coliforms and other microbial indicators occurrence in water and biofilm in full-scale distribution systems. Water Sci. Technol. 54(3), 41.

Bernarde, M.A., Snow, W.B, Olivieri, V.P., and Davidson, B. (1967) Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl. Microbiol. 15(2), 257-265.

Berney, M., Vital, M., Hulshoff, I., Weilenmann, H.U., Egli, T. and Hammes, F. (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res. 42(14), 4010-4018.

Berry, D., Xi, C. and Raskin, L. (2006) Microbial ecology of drinking water distribution systems. Curr. Opin. Biotechnol. 17(3), 297-302.

Besmer, M.D., Weissbrodt, D.G., Kratochvil, B.E., Sigrist, J.A., Weyland, M.S. and Hammes, F. (2014) The feasibility of automated online flow cytometry for in-situ monitoring of microbial dynamics in aquatic ecosystems. Front. Microbiol. 5, 265.

Bichai, F., Payment, P. and Barbeau, B. (2008) Protection of waterborne pathogens by higher organisms in drinking water: a review. Can. J. Microbiol. 54(7), 509-524.

Block, J.C., Haudidier, K., Paquin, J.L., Miazga, J., and Levi, Y. (1993) Biofilm accumulation in drinking water distribution systems. Biofouling 6, 333-343.

Boddy, L., Wilkins, M.F., and Morris, C.W. (2001) Pattern Recognition in Flow Cytometry. Cytometry 44, 195-209.

Boe-Hansen, R., Albrechtsen, H.J., Arvin, E., and Jørgensen, C. (2002a) Bulk water phase and biofilm growth in drinking water at low nutrient conditions. Water Res. 36, 4477-4486.

Boe-Hansen, R., Albrechtsen, H.J., Arvin, E., and Jørgensen, C. (2002b) Dynamics of biofilm formation in a model drinking water distribution system. J. Water SRT -AQUA 51(7), 399-406.

Bolto, B., Dixon, D., Eldridge, R., King, S., and Linge, K. (2002) Removal of natural organic matter by ion exchange. Water Res. 36, 5057-5065.

Bombach, P., Hübschmann, T., Fetzer, I., Kleinsteuber, S., Geyer, R., Harms, H., and Müller, S. (2010) Resolution of natural microbial community dynamics by community fingerprinting, flow cytometry, and trend interpretation analysis. Adv. Biochem. Eng. Biotechnol. 124, 151-181.

Borcard, D., Gillet, F.O., and Legendre, P. (2011) Numerical Ecology with R. New York; London: Springer.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardin, R. (1999). LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37, 77-86.

Bouvier, T., Del Giorgio, P.A., and Gasol, J.M. (2007). A comparative study of the cytometric characteristics of high and low nucleic-acid bacterioplankton cells from different aquatic ecosystems. Environ. Microbiol. 9 (8), 2050-2066.

British standards 6920 (2000) Suitability of non-metallic products for use in contact with water intended for human consumption with regard to their effect on the quality of water - methods of test - growth of aquatic microorganisms test.

Bucheli-Witschel, M., Kötzsch, S., Darr, S., Widler, R. and Egli, T. (2012) A new method to assess the influence of migration from polymeric materials on the biostability of drinking water. Water Res. 46(13), 4246-4260.

Buitenwerf-van der Molen (2007). De cholera-epidemie van 1866. In God van vooruitgang, de popularisering van het modern-theologische gedachtegoed in Nederland (1857-1880). ISBN 978-90-6550-956-7.

Burke, V., Robinson, J., Gracey, M., Peterson, D., and Partridge, K. (1984) Isolation of Aeromonas hydrophila from a metropolitan water supply: seasonal correlation with clinical isolates. Appl. Environ. Microbiol. 48(2), 361-366.

C

Camper, A.K. (2004) Involvement of humic substances in regrowth. Int. J. Food Microbiol. 92(3), 355-364.

Campos, C., and Harmant, Ph. (2002) Assessing the impact of dissolved organic carbon changes on disinfectant stability in a distribution system. Water Sci. Technol. 2(3), 251-257.

Cardenas, E. and Tiedje, J.M. (2008) New tools for discovering and characterizing microbial diversity. Curr. Opin. Biotechnol. 19 (6), 544-549.

Cerrato, J.M., Falkinham, J.O., 3rd, Dietrich, A.M., Knocke, W.R., McKinney, C.W. and Pruden, A. (2010) Manganese-oxidizing and -reducing microorganisms isolated from biofilms in chlorinated drinking water systems. Water Res. 44(13), 3935-3945.

Chiao, T.H., Clancy, T.M., Pinto, A., Xi, C. and Raskin, L. (2014) Differential resistance of drinking water bacterial populations to monochloramine disinfection. Environ. Sci. Technol. 48(7), 4038-4047.

Chow, C.W.K., Kuntke, P., Fabris, R. and Drikas, M. (2009). Organic characterisation tools for distribution system management. Water Sci. Technol. 9(1), 1.

Christensen, S.C., Nissen, E., Arvin, E. and Albrechtsen, H.J. (2011) Distribution of Asellus aquaticus and microinvertebrates in a non-chlorinated drinking water supply system--effects of pipe material and sedimentation. Water Res. 45(10), 3215-3224.

Colbourne, J.S., and Brown, D.A. (1979) Dissolved oxygen utilization as an indicator of total microbial activity on non-metallic materials in contact with potable water. J. Appl. Bacteriol. 47, 223-231.

Cook, D., Morran, J., Mobius, W., and Drikas, M. (2014) Impact of water quality change in the river Murray on monochloramine decay. (Austr. Water Assoc. J.) Water, 41, 2, 110-116

D

de Moel P.J., Verberk, J.Q.J.C., and van Dijk, J.C. (2006). Drinking water - Principles and practices. World Scientific Publishing, Hackensack, USA.

De Roy, K., Clement, L., Thas, O., Wang, Y. and Boon, N. (2012) Flow cytometry for fast microbial community fingerprinting. Water Res. 46(3), 907-919.

De Roy, K., Marzorati, M., Negroni, A., Thas, O., Balloi, A., Fava, F., Verstraete, W., Daffonchio, D. and Boon, N. (2013) Environmental conditions and community evenness determine the outcome of biological invasion. Nat. Commun. 4, 1383.

de Vet, W.W., Dinkla, I.J., Muyzer, G., Rietveld, L.C. and van Loosdrecht, M.C. (2009) Molecular characterization of microbial populations in groundwater sources and sand filters for drinking water production. Water Res. 43(1), 182-194.

D'Onofrio, A., Crawford, J.M., Stewart, E.J., Witt, K., Gavrish, E., Epstein, S., Clardy, J. and Lewis, K. (2010) Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem. Biol. 17(3), 254-264.

Douterelo, I., Boxall, J.B., Deines, P., Sekar, R., Fish, K.E. and Biggs, C.A. (2014) Methodological approaches for studying the microbial ecology of drinking water distribution systems. Water Res. 65, 134-156.

Dukan, S., Levi, Y., Piriou, P., Guyon, F., and Villon, P. (1996) Dynamic modelling of bacterial growth in drinking water networks. Water Res. 30(9), 1991-2002.

E

Egli, T. (2010) How to live at very low substrate concentration. Water Res. 44(17), 4826-4837.

El-Chakhtoura, J., Prest, E., Saikaly, P., van Loosdrecht, M., Hammes, F. and Vrouwenvelder, H. (2015) Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. Water Res. 74C, 180-190.

Emde, K.M.E., Smith, D.W., and Facey, R. (1992) Initial investigation of microbially influenced corrosion (MIC) in a low temperature water distribution system. Water Res. 26(2), 169-175.

Emtiazi, F., Schwartz, T., Marten, S.M., Krolla-Sidenstein and P., Obst, U. (2004) Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. Water Res. 38 (5), 1197–1206.

Epstein, S.S. (2013) The phenomenon of microbial uncultivability. Curr. Opin. Microbiol. 16(5), 636-642.

Escobar, I.C., and Randall, A.A. (2001a) Assimilable organic carbon (AOC) and biodegradable dissolved organic carbon (BDOC): complementary measurements. Water Res. 35(18), 4444-4454.

Escobar, I.C., Randall, A.A., and Taylor, J.S. (2001b) Bacterial Growth in Distribution Systems: Effect of Assimilable Organic Carbon and Biodegradable Dissolved Organic Carbon. Environ. Sci. Technol. 35(17), 3442-3447.

F

Fakruddin and Abhijit Chowdhury (2012) Pyrosequencing - An Alternative to Traditional Sanger Sequencing. Am. J. Biochem. Biotechnol. 8 (1), 14-20.

Falcioni, T., Papa, S. and Gasol, J.M. (2008) Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. Appl. Environ. Microbiol. 74 (6), 1767-1779.

Fass, S., Block, J.-C., Boualam, M., Gauthier, V., Gatel, D., Cavard, J., Benabdallah, S., and Lahoussine, V. (2003) Release of organic matter in a discontinuously chlorinated drinking water network. Water Res. 37, 493-500.

Flemming, H.C., Percival, S.L., and Walker, J.T. (2002a) Contamination potential of biofilms in water distribution systems. Water Sci. Technol. 2(1), 271-280.

Flemming, H.C. (2002b) Biofouling in water systems--cases, causes and countermeasures. Appl. Microbiol. Biotechnol. 59(6), 629-640.

Flemming, H.C. and Wingender, J. (2010) The biofilm matrix. Nat. Rev. Microbiol. 8(9), 623-633.

Flemming, H.-C., Bendinger, B., Exner, M., Gebel, J., Kistemann, T., Schaule, G., Szewzyk, U., and Wingender, J. (2014) The last meters before the tap: where drinking water quality is at risk. In: Microbial growth in drinking water supplies - Problems, causes, control and research needs. van der Kooij, D. and van der Wielen P.W.J. (ed.), pp. 207-238, IWA Publishing, London.

Fonseca, A.C., Summers, R.S., and Hernandez, M.T. (2001) Comparative Measurements of Microbial Activity in Drinking Water Biofilters. Water Res. 35(16), 3817-3824.

Francisque, A., Rodriguez, M.J., Miranda-Moreno, L.F., Sadiq, R. and Proulx, F. (2009) Modeling of heterotrophic bacteria counts in a water distribution system. Water Res. 43(4), 1075-1087.

Fredrickson, A.G., and Stephanopoulos, G. (1981) Microbial competition. Science 213, 972-979.

Frias, J., Ribas, F., and Lucena, F. (1995) Comparison of methods for the measurement of biodegradable organic carbon and assimilable organic carbon in water. Water Res. 29(12), 2785-2788.

G

Gagnon, G.A., O'Leary K.C., Volk, C.J., Chauret, C., Stover, L., and Andrews, R.C. (2004) Comparative Analysis of Chlorine Dioxide, Free Chlorine, and Chloramines on Bacterial Water Quality in Model Distribution Systems. J. Environ. Eng. 130(11), 1269-1279.

Gasol, J.M., Zweifel, U.L., Peters, F., Fuhrman, J.A., and Hagstrom, A. (1999) Significance of Size and Nucleic Acid Content Heterogeneity as Measured by Flow Cytometry in Natural Planktonic Bacteria. Appl. Environ. Microbiol. 65 (10), 4475-4483.

Gasol, J.M., and Del Giorgio, P.A. (2000). Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci. Mar. 64 (2), 197-224.

Gauthier, V., Gérard, B., Portal, J.-M., Block, J.-C., and Gatel, D. (1999) Organic matter as loose deposits in a drinking water distribution system. Water Res. 33(4), 1014-1026.

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Weir, P., Juskowiak, K., Jefferson, B., Jarvis, P. and Nocker, A. (2014) Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry. Water Res. 65, 224-234.

Gomes, I.B., Simoes, M. and Simoes, L.C. (2014) An overview on the reactors to study drinking water biofilms. Water Res. 62, 63-87.

Gomez-Alvarez, V., Schrantz, K.A., Pressman, J.G., Speitel, G.E. and Wahman, D.G. (2013) Pyrosequencing Analysis of Bench-Scale Nitrifying Biofilters Removing Trihalomethanes. Environ. Eng. Sci. 30(9), 582-588.

Gottschal, J.C., de Vries, S., and Kuenen, J.G. (1979) Competition between the falcutatively chemolithotrophic Thiobacillus A2, an obligately chemolithotrophic Thiobacillus and a heterotrophic Spirillum for inorganic and organic substrates. Arch. Microbiol. 121, 241-249.

Greenberg, A. E., Clesceri, L. S., and Eaton, A. D. (1993) Eds. Standard Methods for the Examination of Water and Wastewater, 18th ed.; American Public Health Association: Washington, D.C.

Grefte, A., Dignum, M., Baghoth, S.A., Cornelissen, E.R. and Rietveld, L.C. (2011) Improving the biological stability of drinking water by ion exchange. Water Sci. Technol.: Water Supply 11(1), 107.

Günther, S., Hubschmann, T., Rudolf, M., Eschenhagen, M., Roske, I., Harms, H., and Müller, S. (2008) Fixation procedures for flow cytometric analysis of environmental bacteria. J. Microbiol. Methods 75 (1), 127-134.

H

Haddix, P.L., Shaw, N.J. and LeChevallier, M.W. (2004) Characterization of Bioluminescent Derivatives of Assimilable Organic Carbon Test Bacteria. Appl. Environ. Microbiol. 70(2), 850-854.

Hambsch, B., Werner, P., and Frimmel, F.H. (1992) Bakterienvermehrung in aufbereiteten Wässern verschiedener Herkunft. Acta Hydrochmica at Hydrobiologica 20(1), 9-14.

Hammes, F., and Egli, T. (2005) New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. Environ. Sci. Technol. 39(9), 3289-3294.

Hammes, F., Salhi, E., Köster, O., Kaiser, H.P., Egli, T. and von Gunten, U. (2006) Mechanistic and kinetic evaluation of organic disinfection by-product and assimilable organic carbon (AOC) formation during the ozonation of drinking water. Water Res. 40(12), 2275-2286.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O. and Egli, T. (2008) Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res. 42(1-2), 269-277.

Hammes, F., Berger, C., Köster, O. and Egli, T. (2010a) Assessing biological stability of drinking water without disinfectant residuals in a full-scale water supply system. J. Water SRT -AQUA 59(1), 31-40.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y. and Egli, T. (2010b) Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. Water Res. 44(13), 3915-3923.

Hammes, F., and Egli, T. (2010c) Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. Anal. Bioanal. Chem. 397 (3), 1083-1095.

Hammes, F., Vital, M., and Egli, T. (2010d) Critical evaluation of the volumetric "bottle effect" on microbial batch growth. Appl. Environ. Microbiol. 76 (4), 1278-1281.

Hammes, F., Berney, M. and Egli, T. (2011) Cultivation-independent assessment of bacterial viability. Adv. Biochem. Eng. Biotechnol. 124, 123-150.

Hammes, F., Broger, T., Weilenmann, H.U., Vital, M., Helbing, J., Bosshart, U., Huber, P., Odermatt, R.P. and Sonnleitner, B. (2012) Development and laboratory-scale testing of a fully automated online flow cytometer for drinking water analysis. Cytometry A 81(6), 508-516.

Hansen, S.R., and Hubbell, S.P. (1980) Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science 207, 1491-1493.

Hedegaard, M.J. and Albrechtsen, H.J. (2014) Microbial pesticide removal in rapid sand filters for drinking water treatment--potential and kinetics. Water Res. 48, 71-81.

Heijman, S.G.J., van Paassen, A.M., van der Meer, W.G.J. and Hopman, R. (1999) Adsorptive removal of natural organic matter during drinking water treatment. Water Sci. Technol. 40(9), 183-190.

Henne, K., Kahlisch, L., Draheim, J., Brettar, I., and Höfle, M.G. (2008) Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Sci. Technol.: Water Supply 8 (5), 527.

Henne, K., Kahlisch, L., Brettar, I. and Höfle, M.G. (2012) Analysis of structure and composition of bacterial core communities in mature drinking water biofilms and bulk water of a citywide network in Germany. Appl. Environ. Microbiol. 78(10), 3530-3538.

Herzsprung, P., von Tumpling, W., Hertkorn, N., Harir, M., Buttner, O., Bravidor, J., Friese, K. and Schmitt-Kopplin, P. (2012) Variations of DOM quality in inflows of a drinking water reservoir: linking of van Krevelen diagrams with EEMF spectra by rank correlation. Environ. Sci. Technol. 46(10), 5511-5518.

Hijnen, W.A.M., and van der Kooij, D. (1993) AOC removal and accumulation of bacteria in experimental sand filters. Revue des sciences de l'eau 5, 17-32.

Ho, L., Braun, K., Fabris, R., Hoefel, D., Morran, J., Monis, P., and Drikas, M. (2012) Comparison of drinking water treatment process streams for optimal bacteriological water quality. Water Res. 46 (12), 3934-3942.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., and Saint, C.P. (2003). Enumeration of water-borne bacteria using viability assays and flow cytometry: a comparison to culture-based techniques. J. Microbiol. Methods 55 (3), 585-597.

Hoefel, D., Monis, P.T., Grooby, W.L., Andrews, S. and Saint, C.P. (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. J. Appl. Microbiol. 99(1), 175-186.

Hong, P.Y., Hwang, C., Ling, F., Andersen, G.L., LeChevallier, M.W., and Liu, W.T. (2010) Pyrosequencing analysis of bacterial biofilm communities in water meters of a drinking water distribution system. Appl. Environ. Microbiol. 76 (16), 5631-5635.

Hu, J.Y., Wang, Z.S., Ng, W.J., and Ong, S.L. (1999) The effect of water treatment processes on biological stability of potable water. Water Res. 33(11), 2587-2592.

Hügler, M., Bockle, K., Eberhagen, I., Thelen, K., Beimfohr, C. and Hambsch, B. (2011) Development and validation of a FISH-based method for the detection and quantification of E. coli and coliform bacteria in water samples. Water Sci. Technol. 64(7), 1435-1442.

Huisman, L., and Wood, W.E. (1974) Slow sand filtration. World Health Organization. ISBN 9241540370

Hwang, C., Ling, F., Andersen, G.L., LeChevallier, M.W. and Liu, W.T. (2012) Microbial community dynamics of an urban drinking water distribution system subjected to phases of chloramination and chlorination treatments. Appl. Environ. Microbiol. 78(22), 7856-7865.

I, J

Ihssen, J. and Egli, T. (2004) Specific growth rate and not cell density controls the general stress response in Escherichia coli. Microbiology 150(Pt 6), 1637-1648.

Ingerson-Mahar, M. and Reid, A. (2012) MIP: The microbiology of the water distribution system. American Academy of Microbiology, Boulder, CO.

Joret, J.C., and Levi, Y. (1986) Méthode rapide d'évaluation du carbone éliminable des eaux par voie biologique. Trib. Cebedeau 39, 3-9.

Joret, J.C., Levi, Y., Dupin, T., and Gibert, M. (1988) Rapid method for estimating bioeliminable carbon in water. Ann. Meeting, Am. Wat. Wks Assoc., Orlando, Fla.

Joret, J.C., Levi, Y., and Volk, C. (1991) Biodegradable dissolved organic carbon (BDOC) content of drinking water and potential regrowth of bacteria. Water Sci. Technol. 24(2), 95-101.

K

Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296, 1127-1129.

Kalmbach, S., Manz, W., and Szewzyk, U. (1997) Dynamics of biofilm formation formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. FEMS Microbiol. Ecol. 22, 265-279.

Kaplan, L.A., Bott, T.L., and Reasoner, D.J., 1993. Evaluation and simplification of the assimilable organic carbon nutrient bioassay for bacterial growth in drinking water. Appl. Environ. Microbiol. 59(5), 1532-1539.

Karl, D.M. (1980) Cellular nucleotide measurements and applications in microbial ecology. Microb. Reviews 44, 739-796.

Kerneis, A., Nakache, F., Deguin, A, and Feinberg, M. (1995) The effects of water residence time on the biological quality in a distribution network. Water Res. 29(7), 1719-1727.

Kilianski, A., Haas, J.L., Corriveau, E.J., Liem, A.T., Willis, K.L., Kadavy, D., Rosenzweig, C.N., and Minot, S.S. (2015) Bacterial and viral identification and differentiation by amplicon sequencing on the MinION nanopore sequencer. GigaScience 4:12. Doi: 10.1186/s13742-015-0051-z.

Knochel, S. (1991) Chlorine resistance of motile Aeromonas spp. Water Sci. Technol. 24(2), 327-330.

Koch, C., Fetzer, I., Harms, H., and Müller, S. (2013a). CHIC-an automated approach for the detection of dynamic variations in complex microbial communities. Cytometry A 83 (6), 561-567.

Koch, C., Fetzer, I., Schmidt, T., Harms, H., and Müller, S. (2013b) Monitoring Functions in Managed Microbial Systems by Cytometric Bar Coding. Environ. Sci. Technol. 47 (3), 1753-1760.

Koch, C., Günther, S., Desta, A.F., Hübschmann, T. and Müller, S. (2013c) Cytometric fingerprinting for analyzing microbial intracommunity structure variation and identifying subcommunity function. Nat. Protoc. 8 (1), 190-202.

Kötzsch, S., and Egli, T. (2013) Kunststoffe in Kontakt mit Trinkwasser. Aqua & Gas 44(3), 44-52.

Kovářová, K., Zehnder, A.J.B., and Egli, T. (1996) Temperature-dependent growth kinetcis of Escherixhia coli ML 30 in Glucose-limited continuous culture. J. Bacteriology 178(15), 4530-4539.

Kruithof, J.C., Flomer, H.C., Nederlof, M.M., and van Hoof, S.C.J.M. (2001) Development of a membrane integrity monitoring strategy for the UF/RO Heemskerk drinking water treatment plant. Water Sci. Technol.: Water Supply 1(5), 261-271.

L

Långmark, J., Storey, M.V., Ashbolt, N.J., and Stentröm, T.A. (2005) Biofilms in an urban water distribution system: measurement of biofilm biomass, pathogens and pathogen persistance within the Greated Stockholm area, Sweden. Water Sci. Technol. 52(8), 181-189.

Lautenschlager, K., Boon, N., Wang, Y., Egli, T. and Hammes, F. (2010) Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. Water Res. 44(17), 4868-4877.

Lautenschlager, K. (2011). Origin, function and stability of microbial communities in non-chlorinated public drinking water. Dissertation for obtaining the degree of Doctor at the ETH Zürich, nr. 19950.

Lautenschlager, K., Hwang, C., Liu, W.T., Boon, N., Koster, O., Vrouwenvelder, H., Egli, T. and Hammes, F. (2013) A microbiology-based multi-parametric approach towards assessing biological stability in drinking water distribution networks. Water Res. 47(9), 3015-3025.

Lautenschlager, K., Hwang, C., Ling, F., Liu, W.T., Boon, N., Koster, O., Egli, T. and Hammes, F. (2014) Abundance and composition of indigenous bacterial communities in a multi-step biofiltration-based drinking water treatment plant. Water Res. 62, 40-52.

Lebaron, P., Parthuisot, N., and Catala, P. (1998) Comparison of Blue Nucleic Acid Dyes for Flow Cytometric Enumeration of Bacteria in Aquatic Systems. Appl. Environ. Microbiol. 65 (5), 1725-1730.

Lebaron, P., Servais, P., Agogue, H., Courties, C., and Joux, F. (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Appl. Environ. Microbiol. 67 (4), 1775-1782.

Lebaron, P., Servais, P., Baudoux, A.C., Bourrain, M., Courties, C., and Parthuisot, N. (2002) Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry. Aquat. Microb. Ecol. 28, 131-140.

LeChevallier, M.W., Babcock, T. M., and Lee, R. G. (1987) Examination and Characterization of Distribution System Biofilms. Appl. Environ. Microbiol. 53(12), 2714-2724.

LeChevallier, M.W., Cawthon, C.D., and Lee, R.G. (1988) Mechanisms of bacterial survival in chlorinated drinking water. Water Sci. Technol. 20(11-12), 145-151.

LeChevallier, M.W., Schulz, W., and Lee, R.G. (1991) Bacterial Nutrients in Drinking Water. Appl. Environ. Microbiol. 57(3), 857-862.

LeChevallier, M.W., Becker, W.C., Schorr, P., and Lee, R.G. (1992) AOC reduction by biologically active filtration. Revue des sciences de l'eau 5, 113-142.

LeChevallier, M.W., Shaw, N.E., Kaplan, L.A., and Bott, T.L. (1993) Development of a rapid assimilable organic carbon method for water. Appl. Environ. Microbiol. 59(5), 1526-1531.

LeChevallier, M.W., Welch, N. J., and Smith, D. B. (1996) Full-scale Studies of Factors Related to Coliform Regrowth in Drinking Water. Appl. Environ. Microbiol. 67(7), 2201-2211.

Ledesma, J.L., Kohler, S.J. and Futter, M.N. (2012) Long-term dynamics of dissolved organic carbon: implications for drinking water supply. Sci Total Environ 432, 1-11.

Lee, S.H., O'Connor, J.T., and Banerji, S.K. (1980) Biologically mediated corrosion and its effects on water quality in distribution systems. J. Am. Wat. Wks Assoc. 72(11), 636-645.

Lehtola, M.J., Nissinen, T.K., Miettinen, I.T., Martikainen, P.J., and Vartiainen, T. (2004) Removal of soft deposits from the distribution system improves the drinking water quality. Water Res. 38 (3), 601-610.

Lehtola, M.J., Laxander, M., Miettinen, I.T., Hirvonen, A., Vartiainen, T. and Martikainen, P.J. (2006) The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Water Res. 40(11), 2151-2160.

Lenz, J., Linke, S., Gemein, S., Exner, M. and Gebel, J. (2010) Verification of the efficiency of chemical disinfection and sanitation measures in in-building distribution systems. Int. J. Hyg. Environ. Health 213(3), 198-203.

Levin, R.B., Epstein, P.R., Ford, T.E., Harrington, W., Olson, E., and Reichard, E.G. (2002) US drinking water challenges in the twenty-first century. Environ. Health Perspectives 110(1), 43-52.

Li, W.K.W., Jellett, J.F., and Dickie, P.M. (1995) DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. Limnol. Oceanogr. 40 (8), 1485-1495.

Liikanen, R., Miettinen, I., and Laukkanen, R. (2003) Selection of NF membrane to improve quality of chemically treated surface water. Water Res. 37, 864-872.

Lin, W., Yu, Z., Chen, X., Liu, R., and Zhang, H. (2013) Molecular characterization of natural biofilms from household taps with different materials: PVC, stainless steel, and cast iron in drinking water distribution system. Appl. Microbiol. Biotechnol. 97 (18), 8393-8401.

Lin, W., Yu, Z., Zhang, H. and Thompson, I.P. (2014) Diversity and dynamics of microbial communities at each step of treatment plant for potable water generation. Water Res. 52, 218-230.

Lin, X., McKinley, J., Resch, C.T., Kaluzny, R., Lauber, C.L., Fredrickson, J., Knight, R. and Konopka, A. (2012) Spatial and temporal dynamics of the microbial community in the Hanford unconfined aquifer. ISME J. 6(9), 1665-1676.

Lipphaus, P., Hammes, F., Kotzsch, S., Green, J., Gillespie, S. and Nocker, A. (2014) Microbiological tap water profile of a medium-sized building and effect of water stagnation. Environ. Technol. 35(5-8), 620-628.

Lipponen, M.T.T., Suutari, M.H., and Martikainen, P.J. (2002) Occurence of nitrifying bacteria and nitrification in Finnish drinking water distribution systems. Water Res. 36, 4319-4329.

Lisle, J.T., Pyle, B.H., Broadway, S.C., and McFeters, G.A. (1998) Rapid Enumeration of Active Bacteria in Water After Disinfection. SAE Technical Papers, 28th International Conference on Environmental Systems. 13-16 July 1998.

Liu, G., Verberk, J.Q. and Van Dijk, J.C. (2013a) Bacteriology of drinking water distribution systems: an integral and multidimensional review. Appl. Microbiol. Biotechnol. 97(21), 9265-9276.

Liu, G., Lut, M.C., Verberk, J.Q. and Van Dijk, J.C. (2013b) A comparison of additional treatment processes to limit particle accumulation and microbial growth during drinking water distribution. Water Res. 47(8), 2719-2728.

Liu, G., Van der Mark, E.J., Verberk, J.Q., and Van Dijk, J.C. (2013c). Flow cytometry total cell counts: a field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. Biomed. Res. Int. 2013, 595872.

Liu, G., Ling, F.Q., Magic-Knezev, A., Liu, W.T., Verberk, J.Q., and Van Dijk, J.C. (2013d). Quantification and identification of particle-associated bacteria in unchlorinated drinking water from three treatment plants by cultivation-independent methods. Water Res. 47 (10), 3523-3533.

Liu, G., Bakker, G.L., Li, S., Vreeburg, J.H., Verberk, J.Q., Medema, G.J., Liu, W.T. and Van Dijk, J.C. (2014) Pyrosequencing reveals bacterial communities in unchlorinated drinking water distribution system: an integral study of bulk water, suspended solids, loose deposits, and pipe wall biofilm. Environ. Sci. Technol. 48(10), 5467-5476.

Longnecker, K., Sherr, B.F., and Sherr, E.B. (2005) Activity and phylogenetic diversity of bacterial cells with high and low nucleic acid content and electron transport system activity in an upwelling ecosystem. Appl. Environ. Microbiol. 71 (12), 7737-7749.

Lucena, F., Frias, J. and Ribas, F. (1990) A new dynamic approach to the determination of biodegradable dissolved organic carbon in water. Environ. Technol. 12(4), 343-347.

M

Magic-Knezev, A., and van der Kooij, D. (2004) Optimization and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. Water Res. 38, 3971-3979.

Mallevialle, J., Odendall, P.E., and Wiesner, M.R. (1996) Water treatment membrane processes. McGraw-Hill, New York. ISBN 0-07-001559-7.

Manuel, C.M., Nunes, O.C. and Melo, L.F. (2010) Unsteady state flow and stagnation in distribution systems affect the biological stability of drinking water. Biofouling 26(2), 129-139.

Marcussen, H., Holm, P.E. and Hansen, H.C.B. (2013). Composition, Flavor, Chemical Foodsafety, and Consumer Preferences of Bottled Water. Comprehensive Reviews in Food Science and Food Safety 12(4), 333-352.

Martiny, A.C., Jørgensen, T.M., Albrechtsen, H.J., Arvin, E. and Molin, S. (2003) Long-Term Succession of Structure and Diversity of a Biofilm Formed in a Model Drinking Water Distribution System. Appl. Environ. Microbiol. 69(11), 6899-6907, A., Vepsalainen, M. and Sillanpaa, M. (2010) Natural organic matter removal by coagulation during drinking water treatment: a review. Adv. Colloid Interface Sci. 159(2), 189-197.

Martiny, A.C., Albrechtsen, H.J., Arvin, E. and Molin, S. (2005) Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with Nitrospira spp. Appl. Environ.l Microbiol. 71(12), 8611–8617.

Mathieu, L., Bouteleux, C., Fass, S., Angel, E. and Block, J.C. (2009) Reversible shift in the alpha-, beta- and gamma-proteobacteria populations of drinking water biofilms during discontinuous chlorination. Water Res. 43(14), 3375-3386.

Maul, A., El-Shaarawi, A. H., and Block, J.-C. (1985a) Heterotrophic bacteria in water distribution systems. I. Spatial and temporal variations. Sci. Total Environ. 44, 201-214.

Maul, A., El-Shaarawi, A. H., and Block, J.-C. (1985b) Heterotrophic Bacteria in Water Distribution Systems. II. Sampling Design for Monitoring. Sci. Total Environ. 44, 215-224.

McCoy, S.T. and VanBriesen, J.M. (2012) Temporal Variability of Bacterial Diversity in a Chlorinated Drinking Water Distribution System. J. Environ. Eng. 138(7), 786-795.

McCoy, S.T. and VanBriesen, J.M. (2014) Comparing spatial and temporal diversity of bacteria in a chlorinated drinking water distribution system. Environ. Eng. Sci. 31(1), 32–41.

Medema, G.J., Wondergem, E., van Dijk-Looyaard, A.M., and Havelaar, A.H. (1991) Effectivity of chlorine dioxide to control Aeromonas in drinking water distribution systems. Water Sci. Technol. 24(2), 325-326.

Meylan, S., Hammes, F., Traber, J., Salhi, E., von Gunten, U. and Pronk, W. (2007) Permeability of low molecular weight organics through nanofiltration membranes. Water Res. 41(17), 3968-3976.

Miettinen, I.T., Vartiainen, T., and Martikainen, P.J. (1997) Phosphorous and bacterial growth in drinking water. Appl. Environ. Microbiol. 63(8), 3242-3245.

Miettinen, I.T., Vartiainen, T., and Martikainen, P.J. (1999) Determination of assimilable organic carbon in humus-rich drinking waters. Water Res. 33(10), 2277-2282.

Morey, M., Fernandez-Marmiesse, A., Castineiras, D., Fraga, J.M., Couce, M.L. and Cocho, J.A. (2013) A glimpse into past, present, and future DNA sequencing. Mol. Genet. Metab. 110 (1-2), 3-24.

Morton, S.C., Zhang, Y. and Edwards, M.A. (2005) Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. Water Res. 39(13), 2883-2892.

Muela, A., Orruño, M., Alonso, M.L., Pazos, M., Arana, I., Alonso, R.M., Jiménez, R.M., Garaizabal, I., Maguregui, M.I. and Barcina, I. (2011) Microbiological parameters as an additional tool to improve wastewater treatment plant monitoring. Ecological Indicators 11 (2), 431-437.

Müller, S., Ullrich, S., Losche, A., Loffhagen, N., and Babel, W. (2000) Flow cytometric techniques to characterise physiological states of Acinetobacter calcoaceticus. J. Microbiol. Methods 40, 67-77.

Müller, S., Harms, H., and Bley, T. (2010) Origin and analysis of microbial population heterogeneity in bioprocesses. Curr. Opin. Biotechnol. 21 (1), 100-113.

Münster, U. (1993) Concentrations and fluxes of organic carbon substrates in the aquatic environment. Antonie Van Leeuwenhoek 63, 243–274.

N, O

Nescerecka, A., Rubulis, J., Vital, M., Juhna, T., and Hammes, F. (2014) Biological instability in a chlorinated drinking water distribution network. PLoS ONE 9(5).

Niquette, P., Servais, P., and Savoir, R. (2000) Impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system. Water Res. 34(6), 1952-1956.

Niquette, P., Servais, P., and Savoir, R. (2001) Bacterial dynamics in the drinking water distribution system of Brussels. Water Res. 35(3), 675-682.

Norton, C.D., and LeChevallier, M.W. (2000) A pilot study of bacteriological population changes through potable water treatment and distribution. Appl. Environ. Microbiol. 66(1), 268-276.

Okabe, S., and Watanabe, Y. (2002) Biofilm formation potentials in drinking waters treated by different advanced treatment processes. Water Sci. Technol. 2(4), 97-104.

Okuda, T., Uehara, Y., Tsai, T.-Y., Nakai, S., Akiba, M., Nishijima, W. and Okada, M. (2009) Production of assimilable organic carbon (AOC) from bacteria and picophytoplankton by chlorination. Water Sci. Technol. 9(3), 337.

Oliver, J.D. (2005) The viable but nonculturable state in bacteria. J. Microbiol. 43, 93-100.

ÖNorm B 5018-1 (2002) Prüfung der Verkeimungsneigung von Trinkwasserrohren. Teil 1: Prüfverfahren, Teil 2: Bewertung. Normungsinstitut, Ö. (ed), 1020 Wien.

P

Park, S.K. and Hu, J.Y. (2010) Assessment of the extent of bacterial growth in reverse osmosis system for improving drinking water quality. J. Environ. Sci. Health A: Tox. Hazard Subst. Environ. Eng. 45(8), 968-977.

Pepper, I.L., Rusin, P., Quintanar, D.R., Haney, C., Josephson, K.L. and Gerba, C.P. (2004) Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. Int. J. Food Microbiol. 92(3), 289-295.

Phe, M.H., Dossot, M., Guilloteau, H., and Block, J.C. (2005) Nucleic acid fluorochromes and flow cytometry prove useful in assessing the effect of chlorination on drinking water bacteria. Water Res. 39 (15), 3618-3628.

Pinto, A.J., Xi, C. and Raskin, L. (2012) Bacterial community structure in the drinking water microbiome is governed by filtration processes. Environ. Sci. Technol. 46(16), 8851-8859.

Pinto, A.J., Schroeder, J., Lunn, M., Sloan, W. and Raskin, L. (2014) Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome. MBio 5(3), e01135-01114.

Polanska, M., Huysman, K. and van Keer, C. (2005) Investigation of assimilable organic carbon (AOC) in flemish drinking water. Water Res. 39(11), 2259–2266.

Polychronopolous, M., Dudley, K., Ryan, G., and Hearn, J. (2003) Investigation of factors contributing to dirty water events in reticulation systems and evaluation of flushing methods to remove deposited particles. Water Sci. Technol. 3(1-2), 295-306.

Pomati, F., Jokela, J., Simona, M., Veronesi, M., and Ibelings, B.W. (2011) An automated platform for phytoplankton ecology and aquatic ecosystem monitoring. Environ. Sci. Technol. 45 (22), 9658-9665.

Prévost, M., Rompré, A., Coallier, J., Servais, P., Laurent, P., Clément, B., and Lafrance, P. (1998) Suspended bacterial biomass and activity in full-scale drinking water distribution systems: impact of water treatment. Water Res. 32(5), 1393-1406.

Proctor, C.R. and Hammes, F. (2015) Drinking water microbiology-from measurement to management. Curr. Opin. Biotechnol. 33C, 87-94.

R

R Development Core Team (2008) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing Available online at: http://cran.r-project.org/

Ramette, A. (2007) Multivariate analyses in microbial ecology. FEMS Microbiol. Ecol. 62 (2), 142-160.

Ramseier, M.K., von Gunten, U., Freihofer, P. and Hammes, F. (2011a) Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate. Water Res. 45(3), 1490-1500.

Ramseier, M.K., Peter, A., Traber, J. and von Gunten, U. (2011b) Formation of assimilable organic carbon during oxidation of natural waters with ozone, chlorine dioxide, chlorine, permanganate, and ferrate. Water Res. 45(5), 2002-2010.

Read, S., Marzorati, M., Guimaraes, B.C. and Boon, N. (2011) Microbial Resource Management revisited: successful parameters and new concepts. Appl. Microbiol. Biotechnol. 90(3), 861-871.

Reckhow, D.A., Singer, P.C., and Malcolm, R.L. (1990) Chlorination of humic materials: byproduct formation and chemical interpretations. Environ. Sci. Technol. 24(11), 1655-1664.

Regulation EC 1935/2004 (2004) Regulation of the European Parliament and of the Council of 27 October 2004 on Materials and Articles Intended to Come into Contact with Food and Repealing Directives 80/590/EEC and 89/109/EEC.

Regulation EU 10/2011 (2011) Commission Regulation of 14 January 2011 on Plastic Materials and Articles Intended to Come into Contact with Food.

Revetta, R.P., Pemberton, A., Lamendella, R., Iker, B. and Santo Domingo, J.W. (2010) Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. Water Res. 44(5), 1353-1360.

Revetta, R.P., Gomez-Alvarez, V., Gerke, T.L., Curioso, C., Santo Domingo, J.W. and Ashbolt, N.J. (2013) Establishment and early succession of bacterial communities in monochloramine-treated drinking water biofilms. FEMS Microbiol. Ecol. 86(3), 404-414.

Ribas, F., Frias, J., and Lucena, F. (1991) A new dynamic method for the rapid determination of the biodegradable dissolved organic carbon in drinking water. J. Appl. Bacteriol. 71, 371-378.

Rittmann, B.E., and Snoeyink, V.L. (1984) Achieving biologically stable drinking water. J. Am. Wat. Wks Ass. 76, 106-114.

Rittmann, B.E., and Huck, P.M. (1989) Biological treatment of public water supplies. Critical Reviews in Environmental control, 19 (2), 119-184.

Robertson, B.R., and Button, D.K. (1989) Characterizing Aquatic Bacteria According to Population Cell Size, and Apparent DNA Content by Flow Cytometry. Cytometry 10, 70-76.

Roeder, R.S., Heeg, K., Tarne, P., Benolken, J.K., Schaule, G., Bendinger, B., Flemming, H.-C., and Szewzyk, U. (2010) Influence of Materials, Water Qualities and Disinfection Methods on the Drinking Water Biofilm Community. Wat. Practice & Technol. 5(4), 1-12.

Roeselers, G., Coolen, J., van der Wielen, P.W., Jaspers, M.C., Atsma, A., de Graaf, B. and Schuren, F. (2015) Microbial biogeography of drinking water: patterns in phylogenetic diversity across space and time. Environ Microbiol. In press.

Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., and Laurent, P. (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J. Microbiol. Methods 49, 31-54.

Ronaghi, M. (2001) Pyrosequencing Sheds Light on DNA Sequencing. Genome Res. 11, 3-11.

Rook, J.J. (1976) Haloforms in drinking-water. J. Am. Water Wks Assoc. 68(3), 168–172.

Ross, P.S., Hammes, F., Dignum, M., Magic-Knezev, A., Hambsch, B. and Rietveld, L.C. (2013) A comparative study of three different assimilable organic carbon (AOC) methods: results of a round-robin test. Water Sci. Technol. 13(4), 1024.

Rossman, L.A., Clark, R.M., and Grayman, W.M. (1994) Modeling chlorine residuals in drinking-water distribution systems. J. Environ. Eng. 120(4), 803-820.

S

Sack, E.L., van der Wielen, P.W. and van der Kooij, D. (2010) Utilization of oligo- and polysaccharides at microgram-per-litre levels in freshwater by Flavobacterium johnsoniae. J. Appl. Microbiol. 108(4), 1430-1440.

Sarathy, S. and Mohseni, M. (2009) The fate of natural organic matter during UV/H2O2advanced oxidation of drinking waterA paper submitted to the Journal of Environmental Engineering and Science. Canadian J. Civil Eng. 36(1), 160-169.

Sartory, D.P. (2004) Heterotrophic plate count monitoring of treated drinking water in the UK: a useful operational tool. Int. J. Food. Microbiol. 92(3), 297-306.

Sathasivan, A., Ohgaki, S., Yamamoto, K., and Kamiko, N. (1997) Role of inorganic phosphorous in controlling regrowth in water distribution system. Water Sci. Technol. 35(8), 37-44.

Schets, F.M., Nobel, P.J., Strating, S., Mooijman, K.A., Engels. G.B., and Brouwer, A. (2002) EU drinking water directive reference methods for enumeration of total coliforms and Escherichia coli compared with alternative methods. Letters in Appl. Microbiol. 34, 227-231.

Schmidt, W., Hambsch, B., and Petzoldt, H. (1998) Classification of algogenic organic matter concerning its contribution to the bacterial regrowth potential and by-products formation. Water Sci. Technol. 37(2), 91-96.

Scott, B.A. and Pepper, I.L. (2010) Water distribution systems as living ecosystems: impact on taste and odor. J. Environ. Sci. Health A: Tox. Hazard Subst. Environ. Eng. 45(7), 890-900.

Servais, P., Billen, G., and Hascoët, M.-C. (1987) Determination of the biodegradable fraction of dissolved organic matter in waters. Water Res. 21(4), 445-450.

Servais, P., Anzil, A., and Ventresque, C. (1989) Simple method for determination of biodegradable dissolved organic carbon in water. Appl. Environ. Microbiol. 55(10), 2732-2734.

Servais, P., Billen, G., Laurent, P., Levi, Y. and Randon, G. (1992) Studies of BDOC and bacterial dynamics in the drinking water distribution system of the Northern Parisian suburbs. Revue des sciences de l'eau 5, 69.

Servais, P., Billen, G., and Bouillot, P. (1994) Biological colonization of granular activated carbon filters in drinking-water treatment. J. Environ. Eng. 120, 888-899.

Servais, P., Laurent, P., and Randon, G. (1995) Comparison of the bacterial dynamics in various french distribution systems. J. Water SRT-AQUA 44(1), 10-17.

Servais, P., Casamayor, E.O., Courties, C., Catala, P., Parthuisot, N., and Lebaron, P. (2003) Activity and diversity of bacterial cells with high and low nucleic acid content. Aquat. Microb. Ecol. 33, 41-51.

Seth, A., Bachmann, R., Boxall, J., Saul, A., and Edyvean, R. (2004) Characterization of materials causing discoloration in potable water systems. Water Sci. Technol. 49(2), 27-32.

Sherr, E.B., and Sherr, B.F. (2002) Significance of predation by protists in aquatic microbial food webs. Antonie van Leeuwenhoek 81, 293-308.

Sibille, I., Mathieu, L., Paquin, J.L., Gatel, D., and Block, J.-C. (1997) Microbial characteristics of a distribution system fed with nanofiltered drinking water. Water Res. 31(9), 2318-2326.

Sibille, I. (1998a) Stabilité biologique des réseaux de distribution d'eau potable. Année Biol. 78, 117-161.

Sibille, I., Sime-Ngando, T., Mathieu, L., and Block, J.-C. (1998b) Protozoan bacterivory and and Escherichia coli survival in drinking water distribution systems. Appl. Environ Microbiol. 64(1), 197-202.

Skjevrak, I., Due, A., Gjerstad, K.O. and Herikstad, H. (2003) Volatile organic components migrating from plastic pipes (HDPE, PEX and PVC) into drinking water. Water Res. 37(8), 1912-1920.

SLMB (2012) Determining the total cell count and ratios of high and low nucleic acid content cells in freshwater using flow cytometry. Analysis method 333.1, The Swiss Food Book (Schweizerische Lebensmittelbuch). Federal Office of Public Health, Switzerland.

Sly, L.I., Hodgkinson, M.C., and Arunpairojana, V. (1990) Deposition of manganese in a drinking water distribution system. Appl. Environ. Microbiol. 56(3), 628-639.

Smeets, P.W.M.H., Medema, G.J., and van Dijk, J.C. (2009) The Dutch secret: how to provide safe drinking water without chlorine in the Netherlands. Drink. Water Eng. Sci. 2, 1-14.

Smeets, P.W.M.H., Rietveld, L.C., van Dijk, J.C. and Medema, G.J. (2010) Practical applications of quantitative microbial risk assessment (QMRA) for water safety plans. Water Sci. Technol. 61(6), 1561–1568.

Srinivasan, S. and Harrington, G.W. (2007) Biostability analysis for drinking water distribution systems. Water Res. 41(10), 2127-2138.

Srinivasan, R. and Sorial, G.A. (2011) Treatment of taste and odor causing compounds 2-methyl isoborneol and geosmin in drinking water: A critical review. J. Environ. Sci. 23(1), 1-13.

Staley, J.T., and Konopka, A. (1985) Measurement of in situ activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. Ann. Review Microbiol. 39, 321-346.

States, S.J., Conley, L.F., Ceraso, M., Stephenson, T.E., Wolford, R.S., Wadowsky, R.M., McNamara, A.M., and Yee, R.B. (1985) Effects of Metals on Legionella Pneumophila Growth in Drinking Water Plumbing Systems. Appl. Environ. Microbiol. 50(5), 1149-1154.

Sun, H., Shi, B., Lytle, D.A., Bai, Y. and Wang, D. (2014) Formation and release behavior of iron corrosion products under the influence of bacterial communities in a simulated water distribution system. Environ. Sci. Process Impacts 16(3), 576-585.

Szewzyk, U., Szewzyk, R., Manz, W., and Schleifer, K.-H. (2000) Microbiological safety of drinking water. Ann. Review Microbiol. 54, 81-127.

T, U

Tallon, P., Magajna, B., Lofranco, C., and Leung, K.T. (2005) Microbial indicators of faecal contamination in water: a current perspective. Water, Air and Soil Pollution 166, 139-166.

Thomas, J.M., and Ashbolt, N.J. (2011) Do free-living Amoebae in treated drinking water systems present an emerging health risk? Environ. Sci. Technol. 45, 860-869.

Torvinen, E., Suomalainen, S., Lehtola, M.J., Miettinen, I.T., Zacheus, O., Paulin, L., Katila, M.L. and Martikainen, P.J. (2004) Mycobacteria in Water and Loose Deposits of Drinking Water Distribution Systems in Finland. Appl. Environ. Microbiol. 70(4), 1973-1981.

Tsai, Y.P. (2005) Impact of flow velocity on the dynamic behaviour of biofilm bacteria. Biofouling 21 (5-6), 267-277.

Uhl, W. and Schaule, G. (2004) Establishment of HPC(R2A) for regrowth control in non-chlorinated distribution systems. Int. J. Food Microbiol. 92(3), 317-325.

Unicef and World Health Organization (2012) Progress on drinking water and sanitation: 2012 update.

United Nations General Assembly (July 2010) Resolution A/RES/64/292.

Urano, K., Wada, H., and Takemasa, T. (1983) Empirical rate equation for trihalomethane formation with chlorination of humic substances in water. Water Res. 17(12), 1797-1802.

V

van der Kooij, D., Visser, A., and Hijnen, W.A.M. (1980) Growth of Aeromonas hydrophila at low concentrations of substrates added to tap water. Appl. Environ. Microbiol. 39, 1198-1204.

van der Kooij, D., Visser, A., and Hijnen, W.A.M. (1982) Determining the concentration of easily assimilable organic carbon in drinking water. J. Am. Water Works Assoc. 74, 540-545.

van der Kooij, D., and Hijnen, W.A.M. (1985a) Regrowth of bacteria on assimilable organic carbon in drinking water. J. Fr. Hydrol. 16, 201-218.

van der Kooij, D., and Hijnen, W.A.M. (1985b) Determination of the concentration of Maltose- and starch- like compounds in drinking water by growth measurements with a well-defined strain of a Flavobacterium species. App. Environ. Microbiol. 49(4), 765-771.

van der Kooij, D. (1987) The effect of treatment on assimilable organic carbon in drinking water. P.M. Huck and P. Toft, T.o.d.w.f.o.c. (ed), pp. 317-328, Pergamon Press, Edmonton, Canada.

van der Kooij, D., Hijnen, W.A.M, and Kruithof, J.C. (1989) The effects of ozonation, biological filtration and distribution on the concentration of easily assimilable organic carbon (AOC) in drinking water. Ozone Sci. Eng. 11, 297-311.

van der Kooij, D. (1990) Drinking water microbiology, progress and recent developements. McFeters, G.A. (ed), pp. 57-87, Springer-Verlag, New York.

van der Kooij, D. (1992) Assimilable Organic Carbon as an Indicator of Bacterial Regrowth. J. Am. Water Works Assoc. 84(2), 57-65.

van der Kooij, D., and Veenendaal, H.R. (1994) Assessment of the biofilm formation potential of synthetic materials in contact with drinking water during distribution, pp. 1395-1407, Miami, Florida 1993.

van der Kooij, D., Vrouwenvelder, H.S., and Veenendaal, H.R. (1995) Kinetic aspects of biofilm formation on surfaces exposed to drinking water. Water Sci. Technol. 32(8), 61-65.

van der Kooij, D. (2000) Biological Stability: A Multidimensional Quality Aspect of Treated Water. Water Air Soil Pollut. 123, 25-34.

van der Kooij, D., and Veenendaal, H.R. (2001) Biomass production potential of materials in contact with drinking water: method and practical importance. Water Sci. Technol. 1(3), 39-45.

van der Kooij, D. (2002) Encyclopedia of environmental microbiology. Bitton, G. (ed), pp. 312-327, John Wiley & Sons, New York.

van der Kooij, D. (2003) Managing regrowth in drinking water distribution systems. In Heterotrophic pate counts and drinking water safety. Bartman, J., Cotruvo, J., Exner, M., Fricker, C., Glasmacher, A (ed), pp. 199-232, IWA Publishing, London, UK.

van der Wende, E., Characklis, W.G., and Smith, D.B. (1989) Biofilms and bacterial drinking water quality. Water Res. 23(10), 1313-1322.

van der Wielen, P.W.J.J., Voost, S., and van der Kooij, D. (2009) Ammonia-oxidizing bacteria and archea in groundwater treatment and drinking water distribution systems. Appl. Environ. Microbiol. 75(14), 4687-4695.

van der Wielen, P.W. and van der Kooij, D. (2010) Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Res. 44(17), 4860-4867.

van der Wielen, P.W., and van der Kooij, D. (2013) Nontuberculous Mycobacteria, Fungi, and Opportunistic Pathogens in Unchlorinated Drinking Water in the Netherlands. Appl. Environ. Microbiol. 79(3), 825-834.

van Lieverloo, J.H.M., van der Kooij, D., and Hoogenboezem, W. (2002a) Encyclopedia of Environmental Microbiology. Bitton, G.e. (ed), pp. 1718-1733, John Wiley & Sons, New York.

van Lieverloo, J.H.M., Mesman, G.A.M., Nobel, P.J., and Kroesbergen, J. (2002b) Hygiënecode drinkwater; opslag, transport en distributie. BTO report 2001.175 Kiwa, Nieuwegein, the Netherlands.

van Nevel, S., De Roy, K. and Boon, N. (2013a) Bacterial invasion potential in water is determined by nutrient availability and the indigenous community. FEMS Microbiol. Ecol. 85(3), 593-603.

van Nevel, S., Koetzsch, S., Weilenmann, H.U., Boon, N., and Hammes, F. (2013b) Routine bacterial analysis with automated flow cytometry. J Microbiol Methods 94 (2), 73-76.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., and Attfield, P.V. (2000) Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunological Methods 243, 191-210.

Velten, S., Hammes, F., Boller, M. and Egli, T. (2007) Rapid and direct estimation of active biomass on granular activated carbon through adenosine tri-phosphate (ATP) determination. Water Res. 41(9), 1973-1983.

Vila-Costa, M., Gasol, J.M., Sharma, S., and Moran, M.A. (2012) Community analysis of high- and low-nucleic acid-containing bacteria in NW Mediterranean coastal waters using 16S rDNA pyrosequencing. Environ. Microbiol. 14 (6), 1390-1402.

Vital, M., Fuchslin, H.P., Hammes, F. and Egli, T. (2007) Growth of Vibrio cholerae O1 Ogawa Eltor in freshwater. Microbiol. 153(Pt 7), 1993-2001.

Vital, M., Hammes, F. and Egli, T. (2008) Escherichia coli O157 can grow in natural freshwater at low carbon concentrations. Environ. Microbiol. 10(9), 2387-2396.

Vital, M., Stucki, D., Egli, T. and Hammes, F. (2010) Evaluating the growth potential of pathogenic bacteria in water. Appl. Environ. Microbiol. 76(19), 6477-6484.

Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L. and Hammes, F. (2012a) Flow cytometry and adenosine tri-phosphate analysis: alternative possibilities to evaluate major

bacteriological changes in drinking water treatment and distribution systems. Water Res. 46(15), 4665-4676.

Vital, M., Hammes, F. and Egli, T. (2012b) Competition of Escherichia coli O157 with a drinking water bacterial community at low nutrient concentrations. Water Res. 46(19), 6279-6290.

Vives-Rego, J., Lebaron, P., and Nebe-von Caron, G. (2000) Current and future applications of flow cytometry in aquatic microbiology. FEMS microbiol. reviews 24, 429-448.

Volk, C. and Joret, J.C.C. (1994) Paramètres prédictifs de l'apparition des coliformes dans les réseaux de distribution d'eau d'alimentation. Revue des sciences de l'eau 7(2), 131-152.

Volk, C.J., and LeChevallier, M.W. (1999) Impacts of the reduction of nutrients levels on bacterial water quality in distribution systems. Appl. Environ. Microbiol. 65(11), 4957-4966.

Vreeburg, J.H.G., Schaap, P.G., and van Dijk, J.C. (2004) Particles in the drinking water system: from source to discolouration. Water Sci. Technol. 4(5), 431-438.

Vreeburg, J. (2007a) Discolouration in drinking water systems: a particular approach. Dissertation for obtaining the degree of Doctor at the Delft university of Technology. ISBN 978-90-74741-91-0.

Vreeburg, J.H. and Boxall, J.B. (2007b) Discolouration in potable water distribution systems: a review. Water Res. 41(3), 519-529.

Vreeburg, J.H., Schippers, D., Verberk, J.Q. and van Dijk, J.C. (2008) Impact of particles on sediment accumulation in a drinking water distribution system. Water Res. 42(16), 4233-4242.

Vreeburg, J.H.G., Blokker, E.J.M., Horst, P. and van Dijk, J.C. (2009) Velocity-based self-cleaning residential drinking water distribution systems. Water Sci. Technol. 9(6), 635.

Vrouwenvelder, H.S., Beerendonk, E.F., Verdouw, J., and van der Kooij, D. (2004) Removal of AOC and NOM from water with nanofiltration, pp. 117-122, IWA publishing, London, UK.

W

Wakelin, S., Page, D., Dillon, P., Pavelic, P., Abell, G.C.J., Gregg, A.L., Brodie, E., DeSantis, T.Z., Goldfarb, K.C., and Anderson, G. (2011) Microbial community structure of a slow sand filter schmutzdecke: a phylogenetic snapshot based on rRNA sequence analysis. Water Sci.Technol.: Water Supply 11 (4), 426.

Wang, H., Edwards, M.A., Falkinham, J.O., 3rd and Pruden, A. (2013a) Probiotic approach to pathogen control in premise plumbing systems? A review. Environ. Sci. Technol. 47(18), 10117-10128.

Wang, H., Pryor, M.A., Edwards, M.A., Falkinham, J.O., 3rd and Pruden, A. (2013b) Effect of GAC pre-treatment and disinfectant on microbial community structure and opportunistic pathogen occurrence. Water Res. 47(15), 5760-5772.

Wang, H., Masters, S., Edwards, M.A., Falkinham, J.O., 3rd and Pruden, A. (2014) Effect of disinfectant, water age, and pipe materials on bacterial and eukaryotic community structure in drinking water biofilm. Environ. Sci. Technol. 48(3), 1426-1435.

Wang, Y., Hammes, F., and Egli, T. (2008) The impact of industrial-scale cartridge filtration on the native microbial communities from groundwater. Water Res. 42 (16), 4319-4326.

Wang, Y., Hammes, F., Boon, N., Chami, M., and Egli, T. (2009) Isolation and characterization of low nucleic acid (LNA)-content bacteria. ISME J. 3 (8), 889-902.

Wang, Y., Hammes, F., De Roy, K., Verstraete, W., and Boon, N. (2010) Past, present and future applications of flow cytometry in aquatic microbiology. Trends Biotechnol. 28 (8), 416-424.

Waterleidingbesluit (2001) Besluit van 9 januari 2001 tot wijziging van het Waterleidingbesluit in verband met de richtlijn betreffende de kwaliteit van voor menselijke consumptie bestemd water. Staatsblad van het Koninkrijk der Nederlanden, 31.

Weissbrodt, D.G., Shani, N. and Holliger, C. (2014) Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. FEMS Microbiol. Ecol. 88(3), 579-595.

Werner, P. (1984) Untersuchung zur Substrateigenschaft organischer Wasserinhaltsstoffe bei der Trinkwasseraufbereitung. Zentralblatt für Bakteriologie, Mikrobiologie and Hygiene I(Abt. Orig. B, 180), 46-61.

Werner, P., and Hambsch, B. (1986). Investigation of the growth of bacteria in drinking water. Water supply 4(3), 227-232.

Wingender, J., and Flemming, H.-C. (2004) Contamination potential of drinking water distribution network biofilms. Water Sci. Technol. 49(11), 277-286.

Wingender, J. and Flemming, H.C. (2011) Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214(6), 417-423.

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W. and Boon, N. (2009) Initial community evenness favours functionality under selective stress. Nature 458(7238), 623-626.

Wolfe, R.L., Lieu, N.I., Izaguirre, G., and Means, E.G. (1990) Ammonia-oxidizing bacteria in a chloraminated distribution system: seasonal occurrence, distribution, and disinfection resistance. Appl. Environ. Microbiol. 56(2), 451-462.

Wong, S., Hanna, J.V., King, S., Carroll, T.J., Eldridge, R.J., Dixon, D.R. and Bolto, B.A. (2002) Fractionation of natural organic matter in drinking water and characterization by 13C cross polarization Magic-angle spinning NMR spectroscopy and size exclusion chromatography. Environ. Sci. Technol. 36, 3497-3503.

World Health Organization, W.H.O (2006) Guidelines for drinking water quality: incorporating first addendum. Vol. 1, Recommendations. 3rd ed., Geneva.

Y, Z

Ye, L. and Zhang, T. (2011) Pathogenic bacteria in sewage treatment plants as revealed by 454 pyrosequencing. Environ. Sci. Technol. 45 (17), 7173-7179.

Yin, Q., Fu, B., Li, B., Shi, X., Inagaki, F., and Zhang, X.-H. (2013) Spatial variations in microbial community composition in surface seawater from the ultra-oligotrophic center to rim of the south pacific gyre. PLoS ONE 8(2), e55148. doi:10.1371/journal.pone.0055148.

Yu, J., Kim, D. and Lee, T. (2010) Microbial diversity in biofilms on water distribution pipes of different materials. Water Sci. Technol. 61(1), 163-171.

Zacheus, O.M., Lehtola, M.J., Korhonen, L.K., and Martikainen, P.J. (2001) Soft deposits, the key site for microbial growth in drinking water distribution networks. Water Res. 35(7), 1757-1765.

Zipper, H., Brunner, H., Bernhagen, J., and Vitzthum, F. (2004) Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic Acids Res. 32 (12), e103.

List of abbreviations

ANOSIM: analysis of similarity

AOC: assimilable organic carbon

ATP: adenosine tri-phosphate

a.u.: arbitrary unit

BDOC: biodegradable dissolved organic carbon

BFP: biofilm formation potential

BFR: biofilm formation rate

BOM: biodegradable organic matter

BPP: biomass production potential

CFU: colony forming unit

DGGE: denaturing gradient gel electrophoresis

DOC: dissolved organic carbon

DWDS: drinking water distribution system

EPS: extracellular polymeric substances

FCM: flow cytometer / flow cytometry

FISH: fluorescence in situ hybridization

HD-PE: high-density polyethylene

HNA: high nucleic acid bacterial cells

HPC: heterotrophic plate counts

ICC: intact bacterial cell concentration

IEX: ion exchange

LNA: low nucleic acid bacterial cells

MDS: multidimensional scaling

NET: distribution network

NF: nanoflitration

NOM: natural organic matter

OTU: operational taxonomic unit

PCR: polymerase chain reaction

PET: polyethylene terephthalate

RO: reverse osmosis

RSD: relative standard deviation

TCC: total bacterial cell concentration

TOC: total organic carbon

T-RFLP: terminal restriction fragment length polymorphism

WQ: water quality

WTP: water treatment plant

%HNA: percentage of high nucleic acid content bacterial cells

About the author

Emmanuelle Prest was born on 5th of May 1986 in Paris, France. From 2004, she followed preparatory classes (Lycée Condorcet, Paris) for competitive entrance examination to the French institutes of science and technology, and subsequently studied at the National Graduate School of Chemistry (french "Grande Ecole") in Montpellier, France. In 2010, she obtained a Master's degree in Chemistry (diplôme d'ingénieur), with a specialization in Environmental Chemistry. She completed her master project at Wetsus, centre of excellence for sustainable water technology in Leeuwarden, The Netherlands, in collaboration with the University of Copenhagen, Denmark. Her Master thesis titled "A direct in-situ non-desctructive method to study biofouling of spiral wound membrane systems" was awarded the *Professor Marcel Mulder encouragement Prize 2010.* After graduating, she worked for 6 months at Biaqua, a start-up company located in Delft, specialized in the development of biologically based solutions for control of biofouling of membranes in use for drinking water treatment. She started her phD studies in May 2011 at the Delft University of Technology, in the Environmental Biotechnology group, under the supervision of Hans Vrouwenvelder and Mark van Loosdrecht. The research was conducted in close collaboration with Eawag, Swiss Federal Institute of Aquatic Science and Technology, under the co-supervision of Frederik Hammes, and with the King Abdullah University of Science and Technology (KAUST). The research focussed on biological stability in drinking water distribution systems.

List of publications and contributions

Peer-reviewed publications

El-Chakthoura J., Prest E.I., Saikaly P.E., van Loosdrecht M.C.M, Hammes F., Vrouwenvelder J.S., 2015. Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. Water Research 74, 180-190.

Prest E.I., El-Chakhtoura J., Hammes F., Saikaly P.E., van Loosdrecht M.C.M, Vrouwenvelder J.S., 2014. Combining flow cytometry and 16S rRNA gene pyrosequencing: a promising approach for drinking water monitoring and characterization. Water Research 63, 179-189.

Prest E.I., Hammes F., Kötzsch S., van Loosdrecht M.C.M, Vrouwenvelder J.S., 2013. Monitoring microbial changes in drinking water systems using a fast and reproducible flow cytometric method. Water Research 47 (19), 7131-7142.

Prest E.I., Staal M., Kühl M., van Loosdrecht M.C.M., Vrouwenvelder J.S., 2012. Quantitative measurement and visualization of biofilm O2 consumption rates in membrane filtration systems. Journal of membrane science 392-393, 66-75.

Staal M., Prest E.I., Vrouwenvelder J.S., Rickelt L.F., Kühl M., 2011. A simple optode based method for imaging O2 distribution and dynamics in tap water biofilms. Water research 45 (16), 5027-5037.

Manuscripts under review

Prest E.I., Weissbrodt D.G., Hammes F., van Loosdrecht M.C.M, Vrouwenvelder J.S. Long term bacterial dynamics in a full-scale drinking water distribution system. Under review.

Prest E.I., Hammes F., Kötzsch S., van Loosdrecht M.C.M, Vrouwenvelder J.S. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems. Under review.

Prest E.I., Hammes F., van Loosdrecht M.C.M, Vrouwenvelder J.S. Biological stability of drinking water: controlling factors, definitions and methods. Under review.

Oral and poster communications at international conferences

IWA conference: Biofilms in drinking water systems, from treatment to tap, Arosa, Switzerland, 2015.
Oral presentation: *Long-term evaluation of biological stability in a full-scale drinking water distribution system*

American Water Works Association: Water quality technology conference, New Orleans, USA, 2014.
Oral presentation: *Quantitative monitoring of biological stability in drinking water distribution systems.*
Poster presentation: *Long-term variations in drinking water microbiology in a Dutch full-scale distribution system.*

2nd International Conference on Water Research, Singapore, Singapore, 2013.
Oral presentation: *Rapid characterization and detection of changes in drinking water bacterial communities.*
Poster presentation: *Tracking changes in microbiological water quality during distribution of drinking water: monitoring and evaluation of biological stability.*

Wetsus Congress, Leeuwarden, 2013.

Oral presentation: *Influence and treatment and distribution on biological stability of drinking water.*

Prizes

Poster award at the TU-Delft Process Technology Institute annual event, 2013: *Tracking changes in microbiological water quality during distribution of drinking water: monitoring and evaluation of biological stability.*

Master thesis awarded by the Professor Marcel Mulder encouragement Prize 2010: *A direct in-situ non-desctructive method to study biofouling of spiral wound membrane systems.*

Acknowledgements

About 5 years ago, I was offered the opportunity for a PhD study which seemed perfectly fitted for me. At that time I was up for any position but a PhD, and it took me 6 months to convince myself to enroll for it. Now that I have completed this adventure, I do not regret this decision in any way. I have worked with incredibly inspiring persons, met many people from diverse specialties, backgrounds and nationalities. I would like to thank them all to have made this experience unique.

My very first thoughts go to my promotors and co-promotor Hans Vrouwenvelder, Mark van Loosdrecht and Frederik Hammes. Their unalterable enthusiasm, constant support and unlimited trust have given me the energy to complete this thesis. Hans, I could never thank you enough the tremendous help you provided me with, for any matter and at any time. The importance you give to the well-being of people you work with has given me an invaluable feeling of both freedom and safety. I have learned a lot from you in these four years, especially in terms of working style, and this will definitely help me in future. Frederik, this thesis enormously gained from your valuable input. I am more than grateful for your reactivity and for the countless time you have spent in providing detailed comments for every experiment design and manuscript. I enjoyed very much the numerous and inspiring discussions we had, and learned a lot from your sharp and critical way of thinking. Mark, discussions with you were always inspiring, and encouraged me to look at things with different perspectives. I admire your ability to make yourself available, even on short notice and despite your busy agenda, and to take time for informal discussions. From these I have often gathered good tips of all kinds, ranging from design of experiments to great ideas for travel destinations.

I would like to acknowledge Evides Waterbedrijf for financial and technical support of the studies, as well as fruitful discussions. Particularly, I would like to thank Henk Ketelaars, Sjack van Agtmaal and Markus Flick for encouraging this research and for recognizing the time required for writing the manuscripts and thesis; Rinnert Schurer and Trudy Suylen for inspiring discussions and collaboration; and Jan Bahlman for efficient coordination of water sampling and analysis and friendly communication.

I am also grateful to the committee members Jean-Claude Block, Ameet Pinto, Gertjan Euverink and Jan-Peter van der Hoek for their evaluation of this thesis and their comments.

This work has involved the sampling and analysis of over 700 water samples. This would not have been possible without the commitment of and effective coordination with Jan Bahlman (Evides) and many others: Marco Vos and Ramon Imamdi (Het Waterlaboratorium, HWL); Ed van der Mark (Dunea); and all sample takers and technicians from Evides, AqualabZuid and Het Waterlaboratorium. Besides, I was lucky to supervise two enthusiastic and hard-working students, Elodie Loubineaud and Fezeka Sotywambe, who have performed a large number of analysis and experiments, and have brought colorful attitudes in our research group. At the university, I have found great practical and material help from Sjaak Lispet who is able to find a smart solution to any problem, and from Ben Abbas and Udo van Dongen. Robert van Tuijll has also been most helpful with receiving and sending packages rapidly. Finally, part of the data presented in this thesis has been the result of joint efforts with Joline El-Chakhtoura, who has performed all the molecular work. Working with Joline has resulted in more than fruitful collaboration, and I am happy to count her as a dear friend.

My work led me to meet many people from different institutions, and I would like to thank them for interesting interactions, discussions and/or collaborations: Maria Kennedy, Sergio Salinas, Loreen Villacorte, Jan Schippers, Yuli Ekowati (UNESCO-IHE); Walter van der Meer, Marjolein Peters, Gang Liu, Peter Wessels, Jos Dusseldorp (TU-Delft, CiTG); Stefan Koetzsch, Thomas Egli, Michael Besmer, Caitlin Proctor, Alina Nescerecka (Eawag); David Weissbrodt, Cristian Picioreanu (TU-Delft, EBT); Pascal Saikaly, Gary Amy, Amber Siddiqui, Rodrigo Valladares Linares, Szilard Bucs, Abelziz Belila, Nadia Farhat (KAUST); Sam van Nevel (U-Gent); Geo Bakker (Vitens); Marco Dignum (Waternet).

I also had the chance to be hosted for a few months by Eawag and HWL. I have found at both institutions extremely friendly welcome, and practical help that have made my stays very enjoyable. For this I would like to thank from Eawag: Hans-Ueli Weilenman, for his energetic, friendly and enthusiastic help, but also Stefan Koetzsch and my office-mates Karin, Johannes and Ruedi, as well as Collette, Zoe, and many others. From HWL: Aleksandra Magic-Knezev, Ruud Steen and Roland Tschumie, as well as the entire HWL laboratory-team with whom I spent most of my four months at HWL.

The EBT group is made of various personalities and nationalities that altogether create a very friendly and enriching atmosphere. In four years time, some leave and others arrive, and I have been lucky to share offices with Leonie, Helena J., Shiva, Salah, Marco, Florence, Oezlem, Olga, Eveline, Matthijs, Bart, Danny, Bruno and Khaled, who have created a cheerful environment. A special thank you to Maaike and Eveline for pleasant times spent together, and to many others for their friendly attitudes: Monica, Emma, Jelmer, Gerben, Peter, Tommaso, Andrea, Robbert, Cristian, Helena M.G., Ben, Udo,

Mitchell, Yang, Mario, Julio, Simon, Marissa, David, and Yuemei. Many thanks to Matthijs, Danny, Maaike, Gerben and Eveline for translating my summary and propositions in Dutch, for their help with the design of the thesis cover and for their support prior and during the printing process.

Living far away from one's family and friends is sometimes difficult, but I had the inestimable luck of living nearby my dear Femke de Vries, who has been acting altogether like a friend, a sister and a mother, and offered me an unlimited and warm welcome in her life. I also found the strength of living abroad for the past 5 years, thanks to the unalterable and true friendship of my dearest friends from basic and high schools Sacha, Gaiane, Manue, Sonia, Morgane, Adrien, and Sloane. Besides, I had the chance to meet in the Netherlands extremely friendly persons with whom I could share joyful times: Elisabetta, Eda, Alessandro, Mounir and Arvind; Kevin and Tanya; my climbing mates Laura, Judith, and Evelinde; and finally the music enthusiasts, Eva-Maria and Susana.

Above all, the love and support of my parents and brother have been key success factors in my PhD adventure. I admire the efforts my parents have constantly made to understand what I have been working on, as far it was from their own specialties. Despite the distance, I feel surrounded by their love, which always gives me strength and confidence to pursue what I initiate.

Finally, I could not list here all the things Gaël should be acknowledged for. But surely for his helpful advices and for his patience in dealing with my preoccupation with the studies and my varying moods; and above all, for all we have shared and is still to share.