

DISS. ETH Nr. 19950

**Origin, function and stability of microbial communities in non-chlorinated  
public drinking water**

ABHANDLUNG  
zur Erlangung des Titels

DOKTORIN DER WISSENSCHAFTEN  
der  
ETH ZÜRICH

vorgelegt von

**Karin Lautenschlager**  
Dipl. Biologin, Universität Konstanz

geboren am 18. November 1980 in Mühlacker  
Deutschland

Angenommen auf Antrag von  
Prof. Dr. Thomas Egli, Referent  
Prof. Dr. Josef Zeyer, Korreferent  
Prof. Dr. Jean Claude-Block, Korreferent  
Dr. Frederik Hammes, Korreferent

2011



**.Table of contents**

|   |      |
|---|------|
| Abbreviations   | i`   |
| Zusammenfassung   | iii` |
| Summary   | vii` |
| 1. General introduction   | 1    |
| 2. Abundance, composition and function of indigenous bacterial communities in a full-scale drinking water treatment plant                       | 21   |
| 3. Influence of filter age and operation on the biomass distribution, activity and microbial community composition of covered slow sand filters | 49   |
| 4. A multi-parameter approach towards assessing biological stability in drinking water distribution networks                                    | 75   |
| 5. Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition                       | 97   |
| 6. Conclusions and Outlook  | 123  |
| References  | 137  |
| Curriculum vitae  | 155  |



**Abbreviations**

|           |   |
|-----------|---|
| AOC       | assimilable organic carbon                        |
| ATP       | adenosine tris-phosphate                          |
| BDOC      | biodegradable organic carbon                      |
| CARD-FISH | catalyzed reporter deposition                     |
| CFDA      | carbofluorescein diacetate                        |
| CFU       | colony forming units                              |
| DGGE      | denaturing gradient gel electrophoresis           |
| DOC       | dissolved organic carbon                          |
| EBCT      | empty bed contact time                            |
| EPS       | exopolymeric substances                           |
| FCM       | flow cytometry                                    |
| FISH      | fluorescent in situ hybridization                 |
| GAC       | granular activated carbon                         |
| HNA       | high nucleic acid                                 |
| HPC       | heterotrophic plate count                         |
| LC-OCD    | liquid chromatography organic carbon detection    |
| LNA       | low nucleic acid                                  |
| MUB       | methylumbelliferone                               |
| PI        | propidium iodide                                  |
| RSF       | rapid sand filter                                 |
| SSC       | side scatter                                      |
| SSCP      | single strand conformation polymorphism           |
| SSF       | slow sand filter                                  |
| TCC       | total cell concentration                          |
| TOC       | total organic carbon                              |
| T-RFLP    | terminal restriction fragment length polymorphism |



## Zusammenfassung

Oberflächengewässer wird in den meisten europäischen Ländern aufbereitet, bevor es als Trinkwasser verteilt wird. Während der Trinkwasseraufbereitung wird das Wasser desinfiziert, und Partikel, Mikroverunreinigungen und organische Kohlenstoffe werden entfernt. Der Abbau von organischen Kohlenstoffverbindungen erfolgt durch Mikroorganismen in verschiedenen Biofiltrationsstufen. Dies führt zu mikrobiellen Wachstum in diesen Filtern und dadurch auch zu einem Anstieg der Mikroorganismen im Wasser. Nach der Aufbereitung beträgt die Konzentration der Mikroorganismen im Trinkwasser  $10^3$ - $10^5$  Zellen/ml falls keine Enddesinfektion erfolgt. In einigen europäischen Ländern (z. B. Schweiz, Österreich, Niederlande, Deutschland) wird Trinkwasser ohne die Zugabe von Desinfektionsmitteln verteilt, wobei mikrobielles Wachstum durch die geringe Nährstoffkonzentration (meist Kohlenstoffverbindungen) im Wasser verhindert werden soll. Ziel dieser Arbeit war es herauszufinden (1) welche Faktoren die Biomasse und deren Zusammensetzung in den Biofiltern der Trinkwasseraufbereitung und letztendlich auch in unserem Trinkwasser beeinflussen (2) ob sich die mikrobielle Zusammensetzung und Zellkonzentration während der Trinkwasserverteilung im Netzwerk und in Hausinstallationen verändert, bis das Wasser den Endverbraucher erreicht. Um diese Fragestellungen genauer zu untersuchen wurden Proben vor und nach jedem Aufbereitungsschritt der Trinkwasseraufbereitungsanlage der Stadt Zürich, dem zugehörigen Trinkwassernetzwerk sowie von verschiedenen Haushalten genommen und analysiert. Ausserdem wurde die mikrobielle Zusammensetzung der Biofilter in der Aufbereitungsanlage untersucht. In dieser Trinkwasseraufbereitungsanlage wird Seewasser aus 40 m Tiefe ozoniert (dient u.a. der Desinfektion), bevor es über Schnellsandfiltration, Zwischenozonierung und darauffolgende Aktivkohlefiltration und Langsamsandfiltration aufgereinigt wird. Danach wird das Wasser mit einer mikrobiellen Zellkonzentration von  $10^5$  Zellen/ml ohne die Zugabe eines Desinfektionsmittels verteilt.

Zuerst wurde die komplexe mikrobielle Zusammensetzung von drei verschiedenen Biofiltern dieser Anlage (Schnellsandfilter, Aktivkohlefilter und Langsamsandfilter) und ihrer Filtrate analysiert. Auf allen Biofiltern waren mikrobielle Zellkonzentrationen im Bereich von  $1.9 - 5 \times 10^{15}$  Zellen/m<sup>3</sup> vorhanden. Durch die detaillierte Analyse des Kohlenstoffabbaus im Verlauf der Trinkwasseraufbereitung wurde gezeigt, dass dieser Abbau eine essentielle Funktion dieser Biomasse ist. Die Analyse der mikrobiellen Zusammensetzung mit 454 Pyrosequenzierung zeigte, dass ähnliche mikrobielle Gruppen (hauptsächlich *Proteobacteria*, *Planctomycetes*, *Acidobacteria*, *Bacteroidetes*, *Nitrospira* und eine Gruppe nicht klassifizierbarer Bakterien) in allen Filtern und

auch den Filtraten vorhanden waren. Dies zeigte, dass die Filterbiomasse direkt die mikrobielle Zusammensetzung des aufbereiteten Trinkwassers beeinflusst, welches in dieser Anlage ohne Enddesinfektion oder Netzschutz verteilt wird.

Zudem wurden zwölf parallele Langsandsandfilter dieser Anlage, die sich erheblich in ihrem Alter und ihrer Betriebsweise unterschieden analysiert. Dabei wurde der Einfluss dieser Faktoren auf die Biomassekonzentration, Aktivität und mikrobielle Zusammensetzung untersucht. Adenosine triphosphat (ATP) Messungen zeigten, dass die Biomasse innerhalb der ersten fünf Jahre mit dem Filteralter anstieg. In Filtern zwischen 10 und 24 Jahren war die Biomasse konstant und wurde hauptsächlich durch das Abtragen der oberen Schicht der Filter beeinflusst. Eine hohe Korrelation von ATP Konzentration und extrazellulärer Enzymaktivitäten deutete auf einen direkten Zusammenhang der Biomassemenge und ihrem Potential organischen Kohlenstoff abzubauen hin. Die mikrobielle Zusammensetzung war sehr ähnlich in allen 12 Langsandsandfiltern und wurde kaum durch das Filteralter und das Abtragen der Filter beeinflusst. Dies deutete darauf hin, dass die Wasserqualität des Langsandsandfilterzuflusses, als konstantester Faktor die mikrobielle Zusammensetzung in diesem letzten Aufbereitungsschritt bestimmte.

Die biologische Stabilität des Wassers wurde im zugehörigen Netzwerk anhand von Wasserproben mit unterschiedlicher Aufenthaltszeit (0-52 h) über einen Zeitraum von vier Monaten analysiert. An denselben Probenahmestellen wurden zwei Jahre später nochmals Proben genommen. Bemerkenswert stabile Gesamtzellkonzentrationen von  $1.0 (\pm 0.15) \times 10^5$  Zellen/ml wurden in allen Wasserproben vom Reservoir bis in weite Teile des Verteilnetzes mit Durchflusszytometrie gemessen. Diese Stabilität wurde auch durch Messungen der ATP Konzentrationen, aeroben mesophilen Keimzahlen, gelösten organischen Kohlenstoffe und assimilierbaren organischen Kohlenstoffe festgestellt. Die mikrobielle Zusammensetzung, die mit denaturierender gradienten Gelelektrophorese (DGGE) und 454 Pyrosequenzierung analysiert wurde, zeigte mehr als 80 % Ähnlichkeit an diesen Probenahmestellen und während des Zeitraums von zwei Jahren. An den zwei Probennahmestellen mit der längsten Aufenthaltszeit des Wassers wurde ein leichter aber signifikanter Anstieg der Gesamtzellkonzentration auf  $1.3 \times 10^5$  Zellen/ml beobachtet. Dieser Anstieg war auch in einer klaren Veränderung der mikrobiellen Zusammensetzung zu sehen, vor allem stieg das Vorkommen von Bakterien der Familie der *Comamonadaceae* von 2 auf 60 % an. Diese Resultate zeigen, dass bei der Analyse der biologischen Stabilität in Trinkwasserverteilsystemen Messungen der Gesamtzellkonzentration mit der Analyse der mikrobiellen Zusammensetzung ergänzt werden können.

Im Gegensatz zu der hohen biologischen Stabilität des Wassers im Trinkwassernetzwerk, führte Stagnation über Nacht in zwölf verschiedenen Haushalten der Stadt Dübendorf zu einem erheblichen Anstieg der mikrobiellen Gesamtzellkonzentration (2-3 fach), ATP (2-18-fach) und aeroben mesophilen Keimzahl (4-580-fach). Ausserdem führte dieser Anstieg zu einer Änderung der mikrobiellen Zusammensetzung. Die Zellen waren in stagnierten Wasserproben grösser und wiesen eine höhere Aktivität (ATP/Zelle) auf als die gespülten Proben, was auf mikrobielles Wachstum hindeutete. Nach 5 minütigen spülen des Hahns ging die Gesamtzellkonzentration, ATP und aerobe mesophile Keimzahl sowie die Wassertemperatur auf dieselben Werte wie in den Netzwerkproben zurück. Obwohl das hygienische Risiko in dieser Studie nicht analysiert wurde, macht sie die Notwendigkeit für gute Prüfmethode für Materialien, die in Hausinstallationen verwendet werden deutlich. Allerdings reicht meist auch schon ein kurzes Spülen des Hahns vor Gebrauch um das Risiko mikrobiell belastetes Wasser zu erhalten zu verringern.

Die gewonnenen Resultate dieser Doktorarbeit geben einen ersten detaillierten Einblick in die mikrobielle Ökologie eines nicht-chlorierten Trinkwassersystems. Ein besseres ökologisches Verständnis der Biofiltrationsprozesse kann in Zukunft zur Optimierung und dem Design von Trinkwasseraufbereitungsanlagen beitragen. Ausserdem wurde mit neuauftkommenden Methoden die biologische Stabilität im Trinkwassersystem analysiert und die gewonnen Daten können als Vergleichswerte für die Beurteilung der biologischen Stabilität anderer Trinkwassersysteme herbeigezogen werden.



## Summary

In most European countries drinking water is treated before distribution. During drinking water treatment the water is disinfected, and particles, micropollutants and organic carbon compounds are removed. Microorganisms degrade organic compounds during different biofiltration steps, what leads to microbial growth in these filters and also to an increase of microorganisms in the water. After treatment, the concentration of microorganisms in drinking water is in the range of  $10^3$ - $10^5$  cells/ml in case of no final disinfection step. In some European countries, namely Switzerland, Austria, The Netherlands and Germany, drinking water is distributed without the addition of a residual disinfectant. In such systems, microbial growth shall be limited by the low nutrient concentrations (predominantly organic carbon compounds) in the water. The objectives of this thesis were to (1) determine the factors influencing the microbial community composition of the biofilters in the drinking water treatment plant and of the final drinking water; (2) study whether the microbial community composition changes during drinking water distribution in the network or during stagnation in house installations until the water reaches the consumer. To study these questions, samples were taken and analyzed before and after each treatment step of the drinking water treatment plant of the city of Zürich, the adherent distribution network as well as separate household taps. In addition, the microbial community composition of the biofilters was studied. In this drinking water treatment plant lake water from 40 m depths is pre-ozonated (also for disinfection) before being treated by rapid sand filtration, intermediated ozonation, granular active carbon filtration and slow sand filtration. Afterwards the water is distributed with  $10^5$  cells/ml without the addition of a residual disinfectant.

Firstly, the complex microbial community composition of three different biofilter types (rapid sand filters, granular activated carbon and slow sand filters) and their respective effluents in this full-scale multi-step treatment plant was analyzed. Detailed analyses of organic carbon degradation measured with liquid chromatography organic carbon detection underpinned biodegradation as an essential function of the biofilter biomass. This biomass was present in concentrations in the range of  $1.9 - 5 \times 10^{15}$  cells/m<sup>3</sup>. The analysis of the microbial community composition with 454 pyrosequencing showed that similar microbial taxa (predominantly *Proteobacteria*, *Planctomycetes*, *Acidobacteria*, *Bacterioidetes*, *Nitrospira* and a group of unclassified bacteria) were present in all filters and their effluents. This highlighted the direct influence of the filter biomass on the microbial community composition of the final drinking water, which is in this case distributed without final disinfection and without the addition of a residual disinfectant.

In addition twelve parallel slow sand filters of the same drinking water treatment plant, differing considerably with respect to filter age and the time since the last scraping were analyzed. The impact of these factors on the biomass concentration, activity and microbial community composition was studied. Adenosine tri-phosphate (ATP) measurements showed that the biomass increased with filter age during the first five years of filter operation. In filters aged between 10 and 24 years the biomass remained constant and was mostly influenced by scraping. A direct link between the amount of biomass and the potential for organic carbon degradation was demonstrated by a strong correlation of extracellular enzyme activities with ATP concentrations. The microbial community composition was very similar in the 12 SSFs and hardly any influence of filter age and scraping was found. This suggested that the SSF water inflow quality as the most constant factor determined the microbial community composition in this last treatment step.

The biological stability in water samples with varied retention times (0-52 h) of the network was analyzed for a period of four months, with repetition two years later. Remarkably stable total cell concentrations (TCC) of  $1.0 (\pm 0.15) \times 10^5$  cells/ml in water samples from the reservoir and throughout most of the distribution network were measured with flow cytometry. This stability was also observed in the concentrations of adenosine tri-phosphate (ATP), heterotrophic plate counts (HPC), dissolved organic carbon (DOC) and assimilable organic carbon (AOC). The microbial communities analyzed with denaturing gradient gel electrophoresis (DGGE) and 454 pyrosequencing showed more than 80 % similarity at these sampling locations and during the time period of two years. At two sampling locations with the longest water retention times, a slight but significant increase in the TCC to  $1.3 \times 10^5$  cells/ml was observed. This increase was reflected by a clear shift in the microbial community profiles and specifically by an increase from 2 to 60 % in the relative abundance of members belonging to the family of *Comamonadaceae*. These findings suggest that planktonic cell enumeration can be complemented with microbial community analysis when assessing biological stability in drinking water distribution systems.

In contrast to the high biological stability of the water in the drinking water network, overnight stagnation in twelve different household taps lead to a considerable increase in cell concentrations measured by flow cytometry (2-3 fold) in all water samples after stagnation. This increase was also observed in adenosine tri-phosphate (ATP) concentrations (2-18 fold) and heterotrophic plate counts (4-580 fold). In addition the change was accompanied by a change in the microbial composition after stagnation. An observed increase in cell biovolume and ATP-per-cell concentrations furthermore suggests that the increase in cell concentrations was due to microbial growth. After five minutes flushing of the taps, cell concentrations and water temperature decreased

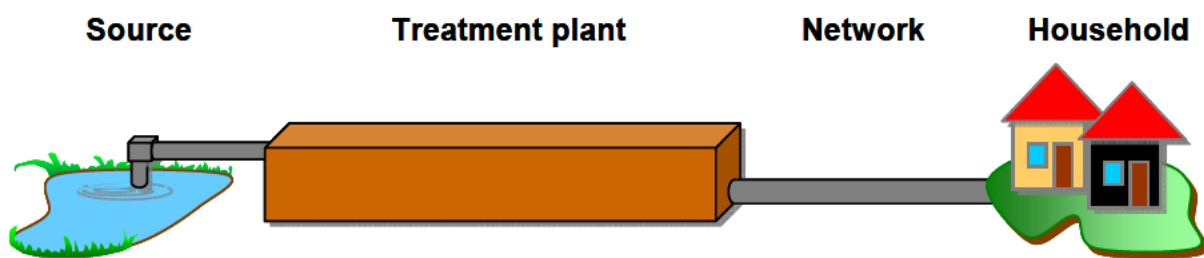
to the level generally found in the drinking water network. While hygienic risk was not directly assessed, it emphasizes the need for the development of good material validation methods for in-house water installations. However, a simple mitigation strategy would be a short flushing of taps prior to use.

The results of this thesis provide a first detailed insight into the microbial ecology of a non-chlorinated drinking water system. An improved ecological understanding of biofiltration processes will contribute positively to future optimization and design of drinking water treatment plants. In addition the biological stability in a drinking water system was analyzed with new emerging methods and the obtained data can be used as reference data for judging biological stability in other drinking water systems.



## 1. General introduction

Non-chlorinated drinking water contains microbial cell concentrations in the range of  $10^3$ - $10^5$  cells/ml (Hammes et al., 2010a; Siebel et al., 2008). These microbes are indigenous bacteria naturally occurring in oligotrophic environments. In drinking water treatment plants, microorganisms play an important role during biofiltration, however, it has to be considered that they are also present in the final drinking water that is distributed. In this thesis, the microbial community composition and its relevance was analyzed from the raw water, via the drinking water treatment and distribution network to the consumer's tap (Fig. 1).



**Fig. 1.** Simplified scheme showing the path of the drinking water from the raw water to the consumer's tap.

### 1. Current drinking water standards

The history of methods currently in use for drinking water monitoring goes back to the 19<sup>th</sup> century when Robert Koch described a new method to count microorganisms from air, soil and water on nutrient media solidified with gelatine (Koch, 1883). Based on Koch's work two methods developed, which are still in use for drinking water monitoring worldwide: (1) The heterotrophic plate count (HPC) as an indicator for the general microbiological water quality and (2) the detection of indicator organisms for fecal contamination such as *Escherichia coli* or *Enterococci* (Koch 1883; Schardinger, 1892). These methods are based on the cultivation of microbial cells present in a water sample on solid nutrient plates. Microbes that are able to grow on such nutrient agar plates form colonies and can be counted after some incubation time, usually after 1-10 days (Reasoner and Geldreich, 1985; SLMB, 2000). The guidelines for monitoring the microbiological drinking water quality are based until today on these two methods, which have been already in use for more than 100 years with only slight modifications (OECD 2003; WHO 2005). Generally, the HPC should be below 20 - 500 colony forming units (CFU) per milliliter in drinking water (varies depending on

country, place of sampling, type of water and method used), and no fecal indicator should be detectable in 100 - 250 ml of sample (Table 1). However, the methods have some severe limitations: Firstly, the HPC method depends on the cultivability of the microbial cells, which is influenced by various factors, including the type of cultivation media or incubation temperature, most of which we presently understand little (Sartory, 2004; van der Kooij, 2003). Secondly, long incubation times are necessary, for example (1) the faecal indicator bacterium *E. coli* can only be detected after 18-24 hours and (2) the result for the HPC is only obtained after 3-10 days, depending on the method used (Table 1). However, the development of fluorescent methods for the staining of cellular components, such as nucleic acids enabled us to stain bacteria and enumerate them by fluorescent microscopy, or more rapidly by flow cytometry (Hobbie et al., 1977; Hammes and Egli, 2005). Today, we know that the total amount of bacteria is considerably underestimated by the HPC; between  $10^3$  and  $10^6$  microbial cells/ml are found in drinking water, from which only a small fraction of 0.001 - 2 % are detected by the HPC (Hammes et al., 2010a; Hoefel et al., 2003; Staley and Konopka, 1985). However, to monitor treatment processes (e.g., disinfection), or changes that might occur in the microbial quality during distribution, a parameter is necessary that comprises the whole microbial community. The HPC method has the disadvantages mentioned above, is not regarded as hygienically relevant, and is not anymore recommended by the WHO as drinking water quality parameter (WHO, 2008). Nevertheless, there is no alternative microbiological parameter proposed to assess for example process performance during drinking water treatment or distribution. As a result the HPC is still used for these purposes. Consequently, there is a need for new methods to assess drinking water quality, the effectiveness of drinking water treatment and changes that might occur in the microbial water quality during drinking water treatment and distribution.

**Table 1.** Drinking water standards for microbiological parameters for drinking water (EDI, 2009; Council Directive 98/83/EC of November, 1998).

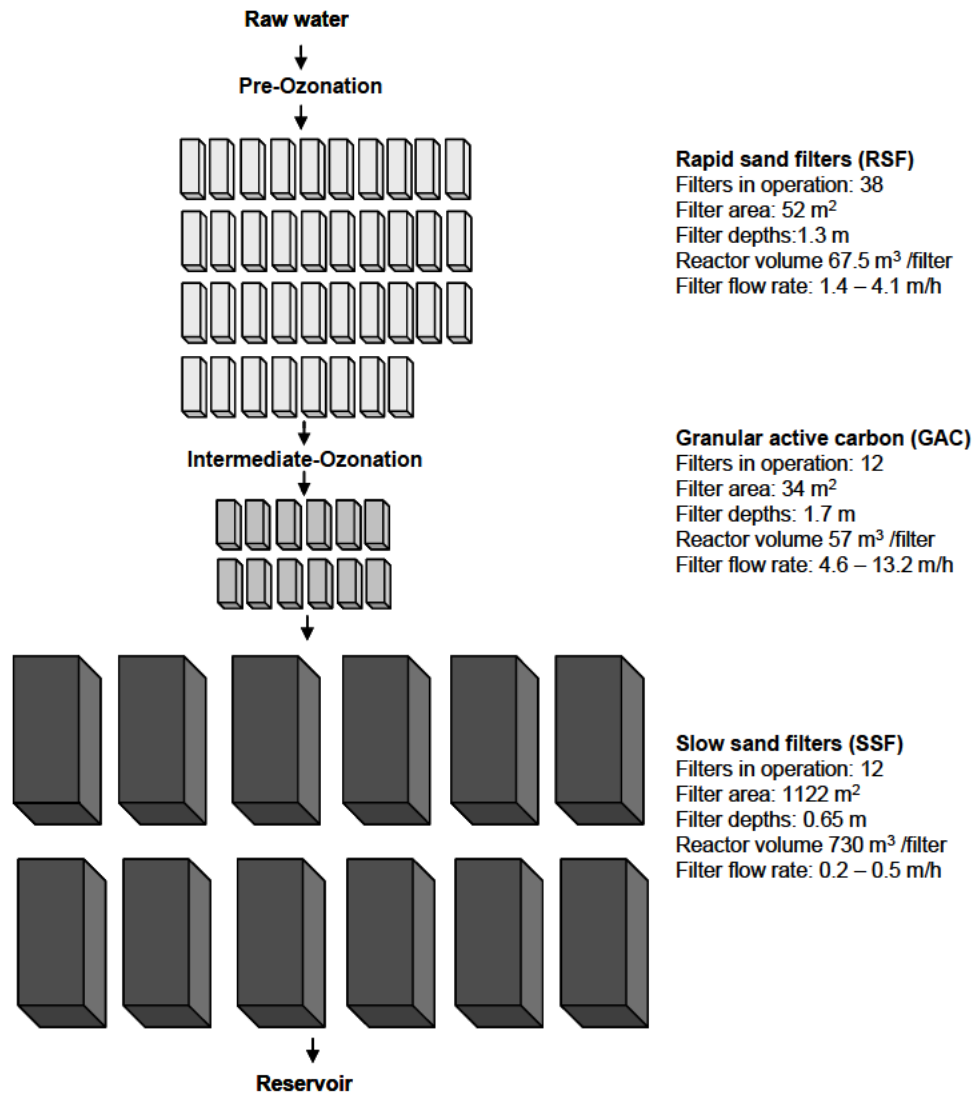
| Bacteria  | Guideline Switzerland   | Guideline EU                            |
|---|---|---|
| <i>Escherichia coli</i>                             | not detectable/100 ml   | not detectable/250 ml                   |
| <i>Enterococci</i>                                  | not detectable/100 ml   | not detectable/250 ml                   |
| Mesophilic aerobic bacteria (determined by the HPC) | < 20 CFU/ml after water treatment<br>< 100 CFU/ml at water catchment for untreated water<br>< 300 CFU/ml in the distribution system | 100 CFU/ml at 22°C<br>20 CFU/ml at 37°C |
| <i>Pseudomonas aeruginosa</i>                       | not detectable/100 ml, only for water filled into containers and for mineral water sources  | not detectable/250 ml                   |

## 2. Drinking water treatment

The goal of drinking water treatment is to produce for the consumer chemically and hygienically safe water, which is free from colour, taste and odour. Depending on water quality and water type (ground, spring or surface water) a treatment is necessary before distribution. Particularly in Europe, surface waters are treated, whereby the type of treatment differs between the countries and also within the countries (Rittmann and Huck, 1989). Generally the water is disinfected, and particles, turbidity, micropollutants and dissolved organic carbon (DOC) are removed (Servais et al., 2005). The removal of DOC is particularly important, because this reduces microbial growth during drinking water distribution and at the same time -if used- it reduces the demand for chlorine and thus the formation of undesirable chlorination by-products (Sedlak and Gunten, 2011). DOC, particles and micropollutants are removed during biofiltration steps. The key of each biofiltration step is an accumulation of a sufficiently large mass of microorganisms on the support material, which degrade organic compounds. Different kinds of biological filters, such as rapid sand filters (RSF), granular activated carbon (GAC) filters and slow sand filters (SSF) are in use (Table 2). These filters vary in many aspects, such as the filter material, the flow rate and filter cleaning (Table 2). For example, RSF and GAC are regularly backwashed to prevent filter clogging, whereas SSF are not backwashed and a *Schmutzdecke* develops at the filter top. Particularly this layer is meant to be most important for the removal of colloidal and dissolved biodegradable material (Huisman et al., 1974). Filter clogging is prevented by occasional scraping of the *Schmutzdecke* (Huisman et al., 1974). SSFs are advantageous because of their mechanical simplicity, but have fallen out of use in many places because of their high surface-area requirements (Rittmann and Huck, 1989). Biofiltration steps are usually combined with disinfection steps, such as ultraviolet light (UV-C) irradiation, chlorination or ozonation (Table 2). Ozonation leads to the oxidation of micropollutants and also easily biodegradable organic carbon compounds are generated from DOC. Consequently, ozonation has to be followed by a biofiltration step and in many treatment plants one to three filtration steps (filter types vary) are combined with up to two disinfection steps. (Servais et al., 2005). A typical multi-step treatment process with many parallel filters in operation is illustrated in Fig. 2.

**Table 2.** Different methods in use for drinking water treatment and their purpose (according to Rittmann and Huck, 1989; Servais et al., 2005)

| Treatment   | Purpose   | Characteristics  |
|---|---|--|
| <b>Rapid sand filter (RSF)</b>                                    | Removal of particles, DOC, $\text{Fe}^{2+}$ and $\text{Mn}^{2+}$ , oxidation of $\text{NH}_4^+$ | Upper layer: Pumic stone (1.5 - 2.5 mm grain size)<br>Lower layer: Sand (0.7-1.2 mm grain size). Are usually cleaned by backwashing.<br>Flow rates: 5-20 m/h   |
| <b>Granular activated carbon filter (GAC)</b>                     | Removal of DOC, micropollutants, $\text{NO}_3^-$ and $\text{NO}_2^-$                            | Activated carbon filters become biological activated carbon filters (BAC) after their adsorption capacity is exhausted (i.e. after 3-6 months).<br>Cleaning: Backwashing<br>Flow rates: 5-20 m/h                           |
| <b>Slow sand filter (SSF)</b>                                     | Removal of particles and DOC, pathogen removal  | Small sand grains (0.15 - 1 mm particle size), removals are attributed to the <i>Schmutzdecke</i> , which forms at the top of the filter. Cleaning: Scraping of 50 to 75 mm medium off the top.<br>Flow rates: 0.1-0.3 m/h |
| <b>Ground passage e.g. river bank filtration, dune filtration</b> | Removal of DOC, $\text{Fe}^{2+}$ and P  | Biological activity is a major part of the treatment, also filtration, adsorption and chemical reactions occur.  |
| <b>Membrane filtration</b>  | Removal of turbidity, precursors and bacteria   |  |
| <b>UVC (254 nm)</b>   | Disinfection  | Damage of DNA, short contact time needed   |
| <b>Ozonation (<math>\text{O}_3</math>)</b>                        | Disinfection, oxidation of micropollutants, oxidation of DOC, Colour removal                    | Pre-ozonation often is recommended to make the distributed water more biologically stable. Oxidation of DOC leads to the formation of easily degradable organic compounds.   |
| <b>Chlorination/Chloramination</b>                                | Disinfection  | Toxic disinfection by-products can be formed.  |



**Fig. 2.** Scheme of the drinking water treatment plant “Lengg” in the city of Zürich, showing parallel and subsequent filters relative to each other. Sizes of the filters are scaled relative to each other.

### 3. Factors influencing the microbial community composition during drinking water treatment

The biofilters in a drinking water treatment plant are densely colonized with bacteria. On GAC filters and SSFs, biomass concentrations in the range of  $10^{15}$ - $10^{16}$  cells/m<sup>3</sup> were reported previously (Velten et al., 2007; Magic-Knezev and van der Kooij., 2004; Mauclaire et al., 2004). These microorganisms are responsible for the organic carbon removal in the biofilters (Rittmann and Huck, 1989). However, the origin of these microbes and the factors that determine their composition in the filters are hardly known. One must assume that the carbon composition of the water which serves as the food source should influence the microbial community composition. There is also some evidence from well-controlled pilot studies suggesting that the water quality might influence the microbial community composition in biofilters (Fonesca et al., 2001; Moll et al., 1998; Li et al.,

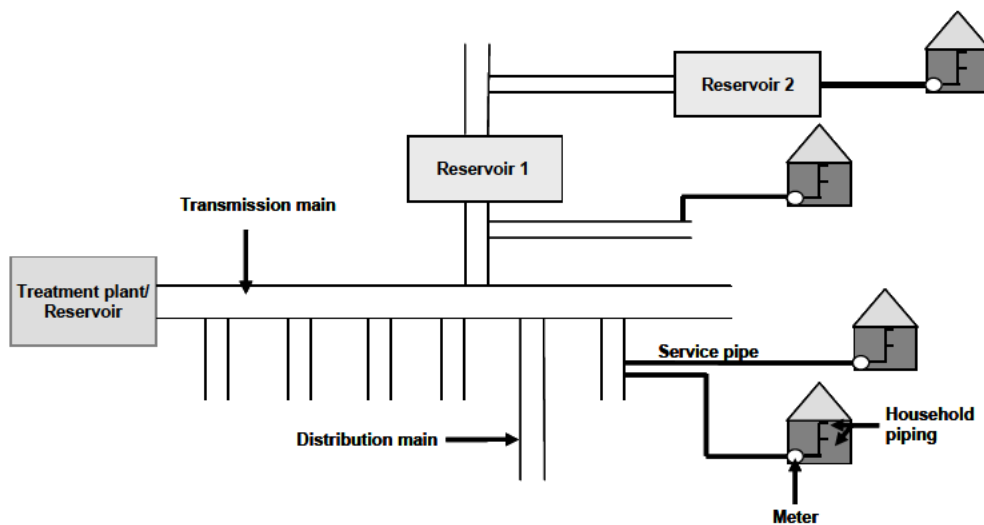
2010). However, in full-scale drinking water treatment plants, various operational factors, such as the filter age, flow rate or filter material are expected to influence microbial community composition. Studying a full-scale drinking water treatment plant might give an indication of the most important factor influencing the microbial community composition. For example, many parallel filters receive the same water source, but vary in their filter age, operation conditions (e.g., the cleaning process), or filter flow rate. In case the microbial communities on filters at the same treatment step are mainly influenced by the water quality, there should be only little variation in the microbial community composition between parallel filters. In contrast, microbial communities should differ between the subsequent filtration steps of a multi-step treatment process, due to a change in the nutrient composition of the water at each treatment step. Particularly ozonation steps lead to the formation of easily biodegradable organic carbon compounds and consequently to a change in the nutrient quality (von Gunten, 2003a; Volk and LeChevallier, 2002; Hoigné, 1998). To study parallel filters in combination with the whole treatment train could give further information about factors influencing the microbial community composition and its functioning in a full-scale drinking water treatment plant. In addition, the microbial community composition on the biofilters can directly influence the microbial community composition of the final drinking water in case the water is distributed without disinfection (Hammes et al., 2010a). Knowing the origin of the microbial communities in the biofilters and the factors influencing them, might then also give information about the origin of the microbial communities in the final drinking water.

#### ***4. Drinking water distribution***

##### ***4.1. Drinking water networks***

Drinking water distribution systems are built to deliver the drinking water to the consumers. The systems are generally complex and comprise pipes of varying sizes, such as transmission mains (305-4270 mm), distribution mains (75-254 mm) and service pipes (20-50 mm), before the water enters household installation systems (Fig. 3). The surface of these pipes is colonized with bacteria that form a biofilm and can be released into the water phase (Block, 1992). Storage and reservoirs are provided for fluctuations in water demand and stabilizing pressure in distribution systems (Laurent et al., 2005b). Also, water from different sources can enter the distribution system at different points. Because of this complexity, it is difficult to know the water residence time at any point and at any time in a drinking water distribution system. However, particularly the residence time of the water, the concentration of organic compounds, decreasing chlorine concentration, the temperature and the physico-chemical characteristics of the pipe materials can influence growth of

bacteria, and thus change the quality of the water during distribution (Niquette et al., 2000). Different kinds of mathematical hydraulic models were developed for calculating the flow rate, water pressure and water residence time in distribution systems (Laurent et al., 2005b). Despite these tools, the exact water residence time in drinking water distribution system is difficult to estimate, since many variables such as pipe diameters or the exact position of the pipes, open or closed valves are required, but mostly information is not available (Laurent et al., 2005b). Nevertheless, models provide a starting point for estimating the water residence time in distribution systems (Laurent et al., 2005b).



**Fig. 3.** Scheme of the components of a drinking water distribution system from the treatment plant/reservoir to the tap (derived from Laurent et al., 2005b).

#### **4.2. Biological stability during drinking water distribution**

Two main approaches are in use to deliver hygienically safe drinking water to the consumer. In many countries a disinfectant residual is maintained during drinking water distribution to prevent microbial growth (WHO, 2006). However, chlorination can lead to taste and odour problems and the reaction of chlorine with organic material in the water can lead to toxic disinfection by-products (Peter and von Gunten, 2009; Sedlak and von Gunten, 2011; von Gunten, 2003b). Some European countries, namely the Netherlands, Germany, Austria and Switzerland aim towards distributing drinking water without a disinfectant residual. When using this approach, bacterial growth must be limited by low nutrient concentrations in the water (Hammes et al., 2010a; Hambsch 1999; van der Kooij et al., 1999). In most European countries biodegradable organic carbon is the growth-limiting nutrient, while in some northern European countries phosphorus is the growth-limiting substrate

(Escobar et al., 2001; LeChevallier et al., 1991; Lehtola et al., 2001; Polanska et al., 2005). Water that does not support significant microbial growth is called “biologically stable” (van der Kooij et al., 1992; Hammes et al., 2010a). Experience in distributing water without a disinfectant residual has shown that this approach works successfully. However, the methodological question still remains as to which parameters should be applied to describe and monitor biological stability of drinking water. Potential parameters that might be used include measures of the nutrient availability in the water or parameters that address microbial biomass. For the determination of the organic carbon content several methods were developed (Laurent et al., 2005c; van der Kooij, 2000) and they are described below. For quantifying biomass, the mostly used parameter is the conventional HPC, even though this method has severe limitations (see above). Only few studies addressed biological stability in situ with advanced methods such as total cell counts or ATP concentration measurements in non-chlorinated drinking water systems. All these studies suggested only little variation in biomass in non-chlorinated distribution systems (van der Wielen and van der Kooij, 2010; Hammes et al., 2010a). Nevertheless, even if the total biomass is stable also changes in the microbial community composition caused by microbial growth or detachment and attachment of microbial cells from and to the biofilms on pipe surfaces could lead to instability in a system. Hence, the analysis of multiple parameters, including total cell concentrations, changes in organic carbon concentrations and changes in microbial community composition would give a more detailed insight into the microbial processes taking place in such systems.

#### ***4.3. Household installation systems***

In contrast to the drinking water distribution system, pipe diameters are small in household installation systems, a large variety of materials are in use, temperatures are elevated and particularly long water stagnation times are frequent (Flemming, 2010). These factors might contribute to a change in chemical and microbial quality; particularly extended water stagnation times might increase the risk for microbial growth (Niquette et al., 2000). To date the impact of these factors on microbial growth and microbial community composition on household level have rarely been studied, although it is of importance to understand the factors influencing the microbial quality so that changes can be prevented or minimized. Also, according to the Council Directive of the European Union, the microbial quality of the water must be met at the point of consumption (Council Directive 98/83/EC). Hence, currently a large study in Germany addressed the impact of the material on the potential for biofilm formation in household installations, and particularly the potential for pathogenic growth (Flemming, 2010). Also, in our group an increase in microbial cell concentration in drinking water of two large office buildings after overnight stagnation was

observed recently (Siebel et al., 2008). However, the basic information on microbial growth occurring on household level is to our knowledge missing. For example basic questions including, whether an increase in microbial cell concentration occurs in each building, whether it is accompanied by a change in the microbial community composition, whether it changes to a higher extend with increasing stagnation time, or how much water volume is affected by such a change, are still not answered.

## 5. Methods used in this study

The methods applied in this thesis to analyze changes in microbial cell concentrations, activity, microbial community composition and organic carbon concentrations/composition during drinking water treatment and distribution are listed in Table 3.

**Table 3.** Methods used to detect changes in microbial community composition and organic carbon composition during drinking water treatment and distribution.

| Parameter                              | Methods used to analyze biomass on the filter material | Methods used to analyze water samples |
|--|--|---------------------------------------|
| Total cell concentration               | not appropriate  | Flow cytometry                        |
| Heterotrophic plate count (HPC)        | not appropriate  | Plating on R <sub>2</sub> A           |
| Activity/Biomass on filter material    | ATP, extracellular enzyme activities, microscopy       | ATP                                   |
| Microbial community composition        | DGGE, 454 Pyrosequencing                               | DGGE, 454 Pyrosequencing              |
| Organic carbon concentrations,-quality | not appropriate  | AOC, TOC, DOC, LC-OCD                 |

### 5.1. Flow cytometry

We have used flow cytometry to detect changes in microbial cell concentrations during drinking water treatment and distribution. Fluorescent staining of microbial cells enables rapid detection and enumeration with flow cytometry, which makes it possible to count all microbial cells in a sample irrespective of their cultivability (Hammes and Egli, 2005; Hoefel et al., 2005a, b). A large range of viability stains to distinguish active from non-active cells exist, however most of these stains still have to be evaluated for individual applications (reviewed by Hammes and Egli, 2010). For drinking water, viability-staining with Propidium Iodide combined with SybrGreenI can be used to distinguish membrane-damaged from membrane-intact cells (Berney et al., 2008; Ramseier et al., 2011). This staining method has proven to work successfully when investigating the kinetics of membrane damage on bacterial cells after treatment with varying disinfectants not only with pure

cultures but also for mixed microbial communities (Ramseier et al., 2011; Berney et al., 2008). Flow cytometry and fluorescent staining has shown that two populations of microbial cells are usually found in water samples from natural environments. Large cells with high nucleic acid (HNA) content and small cells with low nucleic acid (LNA) content can be distinguished by fluorescent intensity and sideward scatter signal in the flow cytometric cluster pattern (Lebaron et al., 2001; Wang et al., 2009). Cultivation of bacteria from both groups has shown the presence of “active” bacteria in each population (Wang et al., 2009). Nevertheless, flow cytometry was until today only applied for cell enumeration in very few drinking water systems (Hammes et al., 2010a; Siebel et al., 2008; Hoefel et al., 2005a, b). Yet, it has much potential and could be particularly applied to analyse changes in cell concentration during drinking water distribution.

## **5.2. *ATP measurements***

ATP analysis was used to assess changes in microbial activity during drinking water treatment and distribution. ATP is as an energy carrying molecule a universal energy currency of “alive” cells, including bacteria. ATP determination is based on the reaction of ATP with the enzyme Luciferin-Luciferase. During this reaction light is produced that can be detected with a luminometer. A sensitivity of the assay as low as  $5 \times 10^{-8}$  nmol ATP can be reached, which is equal to ca. 500 cells in a water sample (Hammes et al., 2010b). Studies suggest that the ATP content of a cell correlates strongly with its biovolume and thus small cells contain less ATP than big cells (Hammes et al., 2010b; Eydal and Pedersen., 2007; Karl, 1980). Also, the ATP content of a cell can vary depending on its activity and the type of cell (Karl, 1980). However, it is to our knowledge not known, whether this is only due to varying cell sizes or to real changes in microbial activities. The measurement of ATP in a water sample has the advantage that only the metabolically active cells are assessed. In some studies ATP was used to analyse changes in activity during drinking water distribution (Hammes et al., 2010a; van der Wielen and van der Kooij., 2010). Also, methods based on ATP were developed to measure the biological activity in biofilters (Velten et al., 2007; Magic-Knezev and van der Kooij 2004). Measuring ATP has the advantage that only the viable biomass in a biofilter is assessed and that due to the easy measurement many samples can be processed in parallel (Velten et al., 2007; Magic-Knezev and van der Kooij 2004; van der Kooij et al., 2003).

## **5.3. *Enzymatic assays***

We tested here whether or not enzymatic assays are useful to analyse and compare the degradation potential of microbial communities in drinking water treatment plants. The determination of

enzymatic activities that are involved in the degradation of organic carbon are one way to address the functioning of microbial communities. The enzymes that are present in a microbial community are assumed to indicate which type of carbon can be potentially degraded. Most bacteria have intracellular enzymes to degrade easy organic carbons such as glucose or acetate; however, for the degradation of complex organic matter such as cellulose or chitin, additional extracellular hydrolytic enzymes are needed that cleave the polymers outside of the cells to monomers that can be taken up (Nybroe et al., 1992). A number of sensitive assays using fluorescently labelled model substrates were developed, which allow detecting low enzyme activities. These assays were applied in numerous studies to assess extracellular hydrolytic enzyme activities in a large range of ecosystems such as soil and lake water, or in engineered systems such as wastewater treatment plants (Sinsabaugh et al., 2003; Nybroe et al., 1992; Li and Chrost, 2006). In drinking water treatment plants enzyme assays were so far applied only to study activities in the water phase (Emtiazi et al., 2004; Hendel et al., 2001; Miettinen et al., 1996), but not for investigating activity on biofilters where most carbon degradation takes place.

#### ***5.4. Organic carbon measurements***

A combination of methods (TOC, DOC, LC-OCD, and AOC) was used to characterize the organic carbon degradation during drinking water treatment and changes in organic carbon concentrations during distribution.

##### ***5.4.1. DOC and LC-OCD***

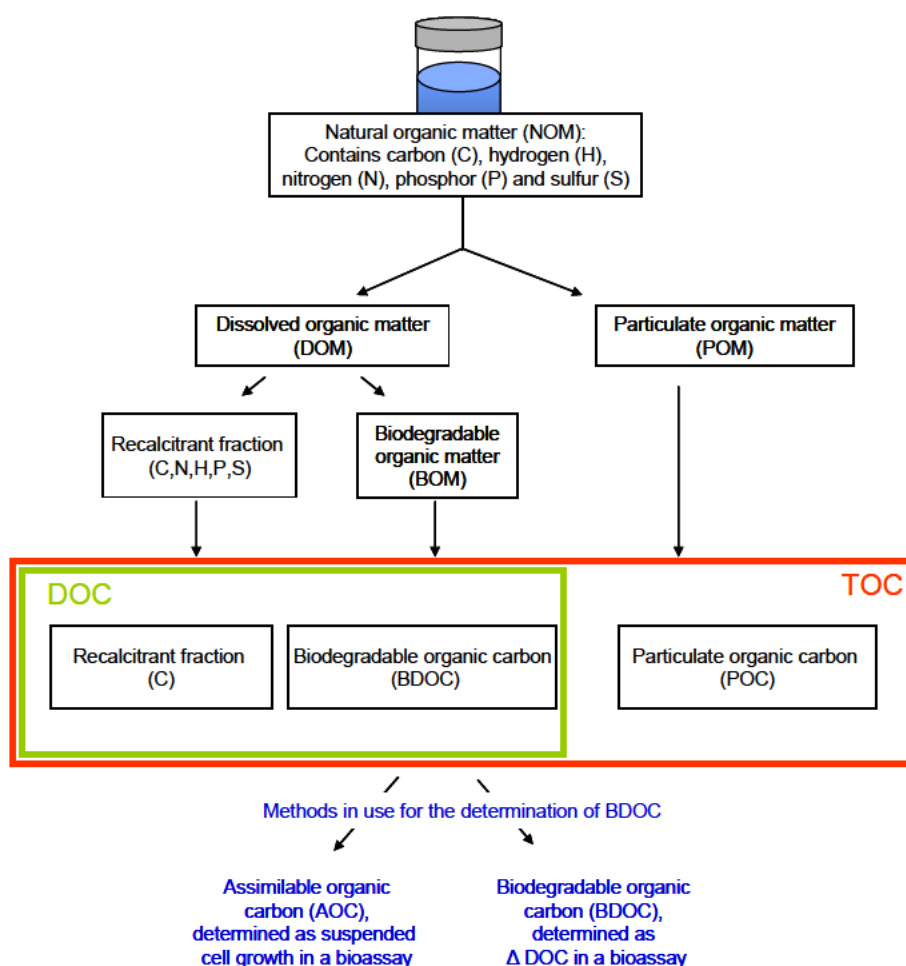
Natural organic matter (NOM) contains carbon, hydrogen, nitrogen, phosphorus and sulfur and can be separated into dissolved organic matter (DOM) and particulate organic matter (POM) (Fig. 4). The part of the DOM that can be consumed by microorganisms is called biodegradable organic matter (BOM). A similar nomenclature is in use for the fractions of total organic carbon (TOC), which is the dominant component of the NOM. In drinking water applications, organic carbon is commonly used as a surrogate for the total amount of NOM (Koudjonou et al., 2005). The total organic carbon (TOC) present in a water sample contains both, particulate organic carbon and DOC (Fig. 4). The particulate fraction can be considered negligible, at least in most treated waters. The biodegradable organic carbon is the fraction of carbon that can be utilized by microorganisms. Dissolved organic carbon includes both, biodegradable organic carbon and non-biodegradable organic carbon and can be analytically measured in a water sample. Before analysis the water sample is filtered through a 0.45 µm filter to separate “dissolved” from “particulate” organic constituents (Kaplan et al., 2005).

Liquid chromatography organic carbon detection (LC-OCD) analysis can be used to analyse the qualitative fractions of DOC in a sample. LC-OCD is based on size exclusion chromatography that allows the separation of DOC into major fractions of different sizes prior to quantification (Huber et al., 2011; Volk et al., 1997). However, this method has the disadvantage that also the classification of carbon fraction is broad, and both biodegradable and recalcitrant DOC can be present in all the LC-OCD subclasses (Volk et al., 1997). Currently there is no analytical method available to measure directly the fractions of organic carbon that are degraded by bacteria. Thus, different biological assays, namely assimilable organic carbon (AOC) assays (van der Kooij et al., 1992; Hammes and Egli, 2005) and biodegradable dissolved organic carbon (BDOC) assays (Laurent et al., 2005c) were developed to assess the amount of biodegradable organic carbon in a water sample.

#### **5.4.2. AOC and BDOC**

AOC represents the organic carbon that is taken up by suspended bacteria during batch growth and converted into new cellular material. In this method a water sample is first 0.2 µm filtered, inoculated with suspended bacteria and incubated for several days. The bacterial cell concentration is determined before and after growth and converted to organic carbon by a theoretical conversion factor ( $1 \times 10^7$  cells/l = 1 µg C/l) (van der Kooij et al., 1992; Hammes and Egli, 2005). In contrast to the AOC assay, in BDOC assays the amount of biodegradable organic carbon is not calculated by a conversion factor from the amount of grown cells, but via the determination of the DOC consumed before and after growth of microbes. The detection limit of this method is considerably higher (0.03-0.1 mg C/l) compared to AOC assays (10 µg/l). Different BDOC assays have been developed that either use batch cultivation with a suspended inoculum, sand colonized by bacteria, or the water is directly run over a “bioreactor” in the form of a column filled with sand or borosilicate beads (reviewed by Kaplan et al., 2005). While both, AOC and BDOC determination assess the fraction of biodegradable organic carbon, AOC was found to constitute only a minor part of the BDOC in many studies (reviewed by Kaplan et al., 2005; Lebanowski and Feuillade, 2009; Frias et al., 1995). These differences might be due to the different inocula used; either mixed microbial communities compared to pure strains, or biofilm bacteria attached to a surface in contrast to suspended microbes. The proportion of carbon sorbing to the surface, disappearing in the extrapolymeric matrix is in the BDOC included (biologically “disappearing” organic carbon). In drinking water distribution networks, both AOC and BDOC have been related to the amount of bacterial growth (Laurent et al., 2005c; van der Kooij, 2000).

We have used DOC and LC-OCD measurements to characterize the organic carbon degradation during drinking water treatment. The BDOC was not determined as described above, but the BDOC was calculated directly from the difference of the DOC concentration in the water before and after each biofiltration step. In water samples from the drinking water network and household installations both, the TOC and AOC were determined. TOC was measured, because the particulate organic carbon in the samples was negligible and because organic carbon might be introduced by contamination during the DOC filtration step into the sample. BDOC was not measured in the network, due to the higher detection limit and hence worse sensitivity of the assay. For the determination of AOC the method of Hammes and Egli (2005) was slightly modified. Samples were not filtered and inoculated, but growth of the natural microbial community present in the water was monitored, in order to prevent AOC contamination in a filtration step.



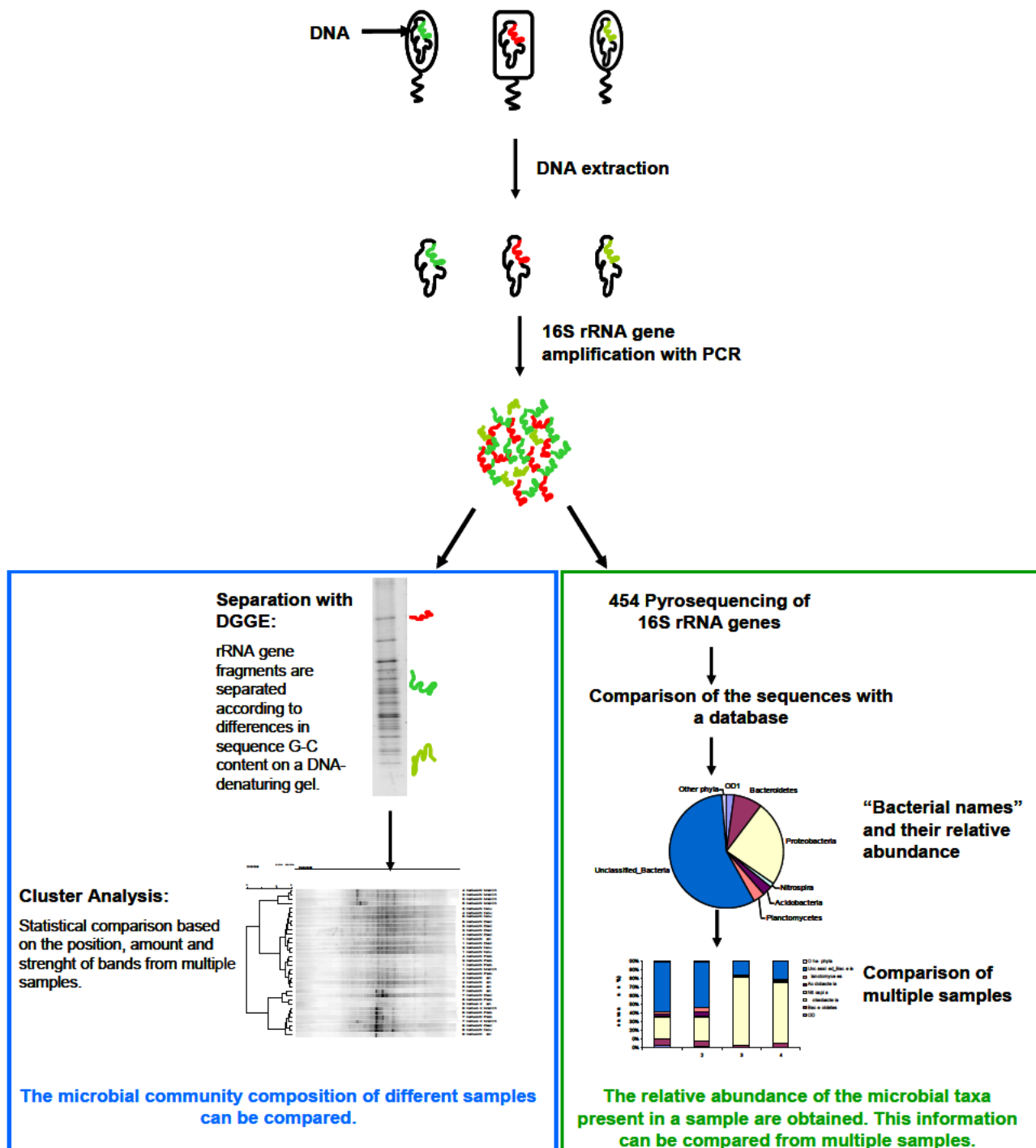
**Fig. 4.** Scheme of the terms in use for organic carbon classification according to Koudjonou et al., 2005.

## **5.5. Microbial community analysis**

### **5.5.1. Choice of methods**

The main objective of the microbial community analysis was to detect changes in microbial community composition between different samples during drinking water treatment and distribution, rather than to obtain detailed information about the comprehensive/exact list of microbial grown species present. We have chosen denaturing gradient gel electrophoresis (DGGE) for the comparison of the microbial community composition of numerous samples, and selected based on these results samples for 454 pyrosequencing to get more detailed information about the microbial community composition in the samples.

For the analysis of the microbial community composition different methods such as FISH and the more sensitive CARD-FISH, molecular fingerprinting methods (e.g. DGGE, T-RFLP, SSCP), and most recently also new generation sequencing methods are in use. FISH and CARD-FISH are based on the detection of certain bacterial groups with specific probes, and the relative percentage of species belonging to this group can be given (reviewed by Bouvier and Giorgio, 2003). Nevertheless, FISH has the disadvantage of the relative wide variation of the effectiveness of the detection of target cells (reviewed by Bouvier and Giorgio, 2003). We did not use CARD-FISH, since in drinking water samples we only detected 30 % of the total amount of bacteria with the general bacterial probe EUB in pre-experiments. Molecular fingerprinting methods are based on the extraction of the bacterial 16S rRNA genes and their amplification (Fig. 5). The 16S rRNA gene contains highly conserved primer-binding sites on the fringes of variable sequences that facilitate genus and species identification of bacteria (reviewed by Weiss and Cozzarelli, 2008; Spiegelman et al., 2005). After amplification of the 16S rRNA genes, these gene fragments can be separated with SSCP, T-RFLP or DGGE. A fingerprint pattern is obtained from each sample and the microbial community composition of numerous samples can be compared (Fig.5). During the last few years new sequencing methods such as 454 pyrosequencing developed rapidly. 454 pyrosequencing has the advantage, that all 16S rRNA gene sequences contained in a sample can be sequenced simultaneously, and thereby not only the microbial community composition of many samples can be compared, but sequences can be also compared to a database and bacteria can be identified (reviewed by Petrosino et al., 2009).



**Fig. 5.** Principles of the molecular methods ((DGGE) and 454 Pyrosequencing) used for microbial community analysis in this thesis.

### 5.5.2. DGGE

DGGE is based on DNA extraction and amplification of 16S rRNA genes by PCR. The difficulty in applying molecular methods for drinking water analysis is the relatively low cell concentrations in the water. It has been shown that a drinking water sample can be already analyzed after filtering 50 ml of water (Burtscher et al., 2009). However, in most studies several litres of water are filtered for

molecular analysis (Hoefel et al., 2005a, b; Eichler et al., 2006; Revetta et al., 2010), which has the advantage that more DNA is obtained, which reduces the chance of contamination and biases and is easier to obtain a good PCR amplification. Further limitations are the incomplete lyses of microbial cells that might occur during DNA extraction and then leads to further biases in the subsequent PCR steps. PCR reactions are very sensitive to reaction conditions and do not always give quantitatively identical results; the number of 16S rRNA genes present in a single organism can differ, and primers must have uniform hybridization efficiency to guarantee the amplification of all targets DNA. Thus an accurate quantification of the consortium is not possible (reviewed by Weiss and Cozzarelli, 2008; Spiegelman et al., 2005). For the separation of the 16S rRNA gene fragments obtained after amplification with PCR DGGE can be applied. By this method PCR-amplified rRNA gene fragments are separated according to differences in sequence G-C content, based on differential mobility through a DNA-denaturing gel. The main limitation of DGGE is the limited size of 500 bp of the DNA fragments that can be separated. Biases associated with PCR could cause relative under- or over-representation of a given taxon in the DGGE profile, and the resolution is limited to DNA from organisms comprising 1 % or more of the community (Muyzer et al., 1993). Because of the existence of multiple copies of rRNA genes in a single organism, multiple bands for a single species are possible. Also, it can be difficult to apply DGGE to extremely complex communities that produce hundreds of bands, which become difficult to visualize (reviewed by Weiss and Cozzarelli, 2008; Spiegelman et al., 2005). Nevertheless, DGGE has proven to be useful for the analysis of microbial communities of a wide range of ecosystems. It was already successfully applied for the analysis of drinking water samples in some studies (Burtscher et al., 2009; Hoefel et al., 2005a, b; Roeder et al., 2010).

### ***5.5.3. Next generation sequencing***

Traditional Sanger DNA sequencing allowed sequence-based identification with a reasonable amount of confidence from relatively long reads (reviewed by Petrosino et al., 2009). However, for microbial community composition analysis of the 16S rRNA gene, clone libraries have to be constructed in order to obtain sequence data, which is labor-intensive when the sample contains a high biodiversity. A fundamentally different approach to traditional Sanger DNA sequencing, namely “DNA pyrosequencing” was developed in the mid 1990s (reviewed by Petrosino et al., 2009). Pyrosequencing occurs by a DNA polymerase-driven generation of inorganic pyrophosphate, with the formation of ATP. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is then detected. The height of each peak (light

signal) is proportional to the number of nucleotides incorporated. Further information can be obtained on [www.pyrosequencing.com](http://www.pyrosequencing.com). The approach offers the possibility to directly sequence all 16S rRNA genes obtained from a sample, which allows the generation of thousands of sequences at once and the sequencing of numerous samples in parallel. Sequences can be analyzed with different taxonomic identification software for bacterial identification. Multiple online databases are already available on the basis of different taxonomic schemes, such as the Ribosomal Database Project II (RDP II), which is based on either the Bergey's taxonomy (oldest and most traditional classification system), or on the Greengenes database that includes multiple taxonomic schemes (reviewed by Petrosino et al., 2009). On the one hand the names of the microbes present in a sample are obtained in case the sequences are known, and, on the other hand, also the microbial community composition of different samples can be compared with statistical software. The great potential for microbial community analysis is illustrated in the numerous studies that applied pyrosequencing for microbial community analysis on samples from a wide range of ecosystems such as soil, marine environments and human guts (Roesch et al., 2007; Qian et al., 2010; Lewis et al., 2010). For the analysis of drinking water samples, pyrosequencing was to our knowledge only applied in one study until today, which revealed a higher diversity in drinking water than previously expected (Hong et al., 2010).

## ***6. Objectives of the thesis***

The role of microorganisms in biofilters used for drinking water treatment and the factors influencing the composition of these microbial communities are largely unknown. Nevertheless, these microbial communities are crucial for the removal of biodegradable organic carbon during the drinking water treatment process and the “stabilization” of the water. Furthermore, the microbial communities in drinking water treatment biofilters can directly affect the composition of the microbial communities in the drinking water that is distributed (Hammes et al., 2010a). This is of particular relevance in case the water is distributed without disinfection residuals. Yet, it is unknown whether or not these microbes finally reach the consumer's tap, or whether the microbial community composition and cell numbers change during distribution in the network and household installations. In order to get more insight into the dynamics and the composition of microbial communities during drinking water treatment and distribution, the following research questions were addressed:

(1) What determines the microbial community composition during drinking water treatment?

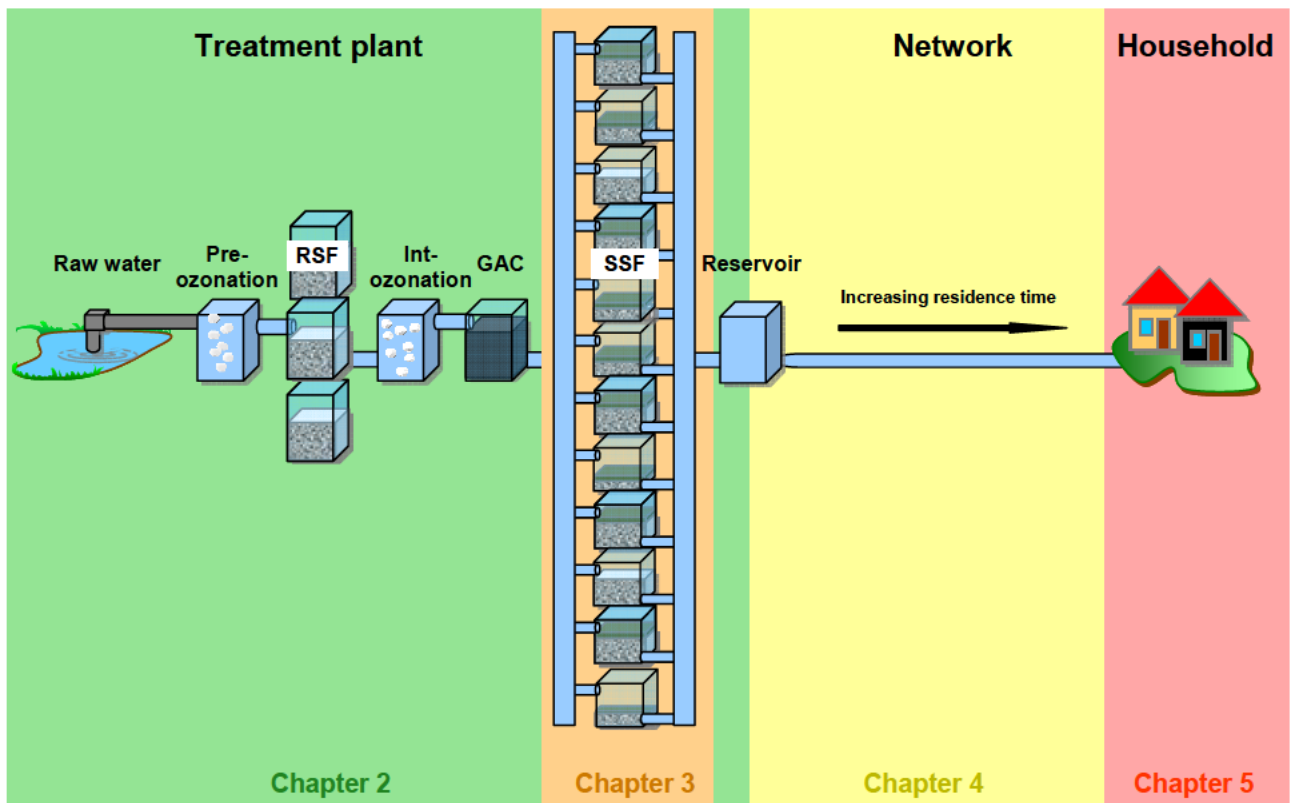
- What is the impact of biofilter age and operation on biomass distribution and microbial community composition of parallel filters?

- Does the microbial community composition change in the course of a multi-step drinking water treatment process?
- Is the performance of the biofilters linked to the microbial community composition?

(2) Is the water biologically stable during distribution/delivery from the drinking water treatment plant to the tap? What does the consumer drink?

- Does the microbial community composition, cell concentration or activity change during drinking water distribution?
- Does the microbial community composition, cell concentration or activity change during stagnation in household installation systems?

The work was done in collaboration with the Zürich Water Supply (WVZ) using the Zürich treatment and distribution network as a model for a non-chlorinated drinking water system (Fig. 6). First samples from filters of all treatment steps and the filter effluents were analyzed to address the dynamics in microbial community composition during treatment and their possible link to the performance of the biofilters (Chapter 2). Further the impact of filter age and operation on the biomass distribution and microbial community composition was studied by analyzing samples from twelve parallel SSF (Chapter 3). For the analysis of biological stability during drinking water distribution, the drinking water network of the city of Zürich was sampled over an extended time period (Chapter 4). To assess the impact of stagnation on microbial growth in household installations, samples from different households were taken after overnight stagnation (Chapter 5).



**Fig. 6.** Outline of this thesis including a simplified scheme of the drinking water treatment plant analyzed.



## 2. Abundance, composition and function of indigenous bacterial communities in a full-scale drinking water treatment plant

### Abstract

Indigenous bacterial communities are integral to biofilters in drinking water treatment systems. In this study, we elucidated the complex microbial community composition of three different biofilter types (rapid sand filters, granular activated carbon and slow sand filters) and their respective effluents in a full-scale multi-step treatment plant. Detailed analyses of organic carbon degradation measured with liquid chromatography organic carbon detection underpinned biodegradation as an essential function of the biofilter biomass. This biomass was present in concentrations in the range of  $1.9 - 5 \times 10^{15}$  cells/m<sup>3</sup> and biomass was phylogenetically, enzymatically and metabolically diverse, suggesting a high potential for the degradation of different and complex organic carbon compounds in all filters. The analysis of the microbial community composition with 454 pyrosequencing showed that similar microbial taxa (predominantly *Proteobacteria*, *Planctomycetes*, *Acidobacteria*, *Bacterioidetes*, *Nitrospira* and a group of unclassified bacteria) were present in all filters and their effluents. This highlights the direct influence of the filter biomass on the microbial community composition of the final drinking water, which is in this case distributed without final disinfection and without the addition of a residual disinfectant. The results of this study shed new light on the complexity of indigenous bacteria colonizing drinking water systems. An improved ecological understanding of biofiltration processes will contribute positively to future optimization and design of drinking water treatment plants.

## 1. Introduction

During the last century, water treatment developed from simple slow sand filters to various integrated, multi-step processes. Today, many multi-step treatment plants combine ozonation steps with several biofiltration steps such as rapid sand filters (RSF), granular activated carbon (GAC) filters, or slow sand filters (SSF) (Hammes et al., 2010a; Baghoth et al., 2010). Ozonation is applied for disinfection and oxidation to control taste and odor, discolor, or eliminate micropollutants from water (von Gunten, 2003a). Easily biodegradable low molecular weight (LMW) byproducts are formed by the oxidative breakdown of natural organic matter (NOM), which changes the carbon composition/quality of the water (von Gunten, 2003a; Volk and LeChevallier, 2002; Hoigné, 1998). Biodegradable carbon is removed during subsequent biofiltration, which reduces the growth potential of microbial populations during drinking water distribution (Prévost et al., 1998; Servais et al., 1995; Mathieu et al., 1992).

Microorganisms that colonize the surfaces of filter materials such as GAC or sand remove the organic matter as part of their metabolic activity (Collins et al., 1992; Rittman and Huck, 1989). Easily degradable organic carbon compounds (e.g., glucose) are readily available for microorganisms, whereas complex organic matter (e.g., cellulose) is first hydrolyzed by extracellular enzymes, before it can be taken up by the cells and metabolized (Sinsabaugh et al., 2003; Nybroe et al., 1992). Hence, easily degradable carbon compounds should be degraded faster than complex polymeric organic carbon compounds during drinking water treatment, which would potentially alter the carbon composition/quality of the water. Such a change in water carbon quality might lead to a change in microbial community composition, since different microbes have different catabolic abilities for certain substrates (Newton et al., 2011). Hence, carbon quality of the water potentially influences the microbial community composition and conversely the microbial community composition should affect the degradation performance and overall functioning of biofilters.

The impact of the water nutrient quality on the microbial community composition on biofilters was shown by previous studies of drinking water pilot plants. For example, different communities were found on biofilters treated with ozonated water compared to non-ozonated water or after the addition of phosphorus to the water (Fonesca et al., 2001; Moll et al., 1998; Li et al., 2010). Accordingly, in a full-scale drinking water treatment plant where ozonation and sequential biofiltration steps alter the water quality, microbial communities may change in the course of the

treatment. In addition, the microbial communities present in a drinking water treatment train can affect the community composition of the final drinking water (Hammes et al., 2010a). However, to our knowledge the detailed composition of microbial communities and their functioning in drinking water treatment plants have not been addressed so far.

Here, the emergences of high-throughput sequencing methods have the potential to expose an entirely new world, which could influence the way treatment is perceived and performed. Particularly in cases where the water is distributed without a final disinfection step, it is of interest to know the factors influencing the microbial community composition. In the present study, we have analyzed a full-scale drinking water treatment plant that produces roughly half of the drinking water of Zürich (CH) by treating surface water through sequential ozonation and filtration steps before the water is distributed without final disinfection (Hammes et al., 2010a). 454 pyrosequencing data of microbial communities were complemented with detailed analysis of biomass, extracellular enzyme activities and organic carbon removal.

## 2. Materials and Methods

### 2.1. Layout of the full-scale drinking water treatment plant

The full-scale plant monitored in this study produces roughly 50 % of the drinking water of Zürich (CH) by treating surface water from Lake Zürich (from 40 m depths) through sequential ozonation and filtration steps. The water is pumped through the treatment system with flow rates ranging between 2420 and 6950 m<sup>3</sup>/h. The treatment train consists of the following steps; (1) pre-ozonation (ozone dose 1.1 mg/l  $\pm$  25%, hydraulic contact time 50 min, ozone residual after contact time ca. 0.15 mg/l); (2) rapid sand filtration (RSF) (double layer filter with 50 cm of pumice stone and 80 cm of quartz sand); (3) intermediate ozonation (ozone dose 0.5 mg/l, hydraulic contact time 26 min, ozone residual after contact time ca. 0.28 mg/l); (4) granular active carbon (GAC) filtration (double layer filter with 130 cm of GAC (Norit ROW 0.8 supra) and 40 cm of quartz sand); (5) slow sand filtration (SSF) (quartz sand ca. 65 cm); and (6) a reservoir in the plant. Following this treatment the water is distributed without additional disinfection. Each filtration step consists of many (12-38) filters operated in parallel (Table 1). Operational parameters are listed in Table 1, chemical water quality parameters are shown in Table 2.

**Table 1.** Operational parameters of the three different filter types in the full-scale treatment plant of Zürich (CH).

|   | <b>Rapid sand filters<br/>(RSF)</b> | <b>Granular activated<br/>carbon<br/>(GAC)</b> | <b>Slow sand filters<br/>(SSF)</b> |
|---|-------------------------------------|--|------------------------------------|
| <b>Number of filters in use</b>                         | 38                                  | 12   | 12                                 |
| <b>Filter area (m<sup>2</sup>)</b>                      | 45                                  | 44   | 1120                               |
| <b>Filter depth (m)</b>                                 | 1.3                                 | 1.7  | 0.65                               |
| <b>Reactor volume per filter (m<sup>3</sup>/filter)</b> | 58.5                                | 74.8   | 728                                |
| <b>Total reactor volume (m<sup>3</sup>)</b>             | 2223                                | 898  | 8736                               |
| <b>Pump rate per filter (m<sup>3</sup>/h)</b>           | 64-183                              | 202-579  | 202-579                            |
| <b>Filter flow rate (m/h)</b>                           | 1.4-4.1                             | 4.6-13.2                                       | 0.2-0.5                            |

**Table 2.** Chemical water quality parameters measured in the influent and effluent before and after each filtration step.

|                        | Ammonium<br>( $\mu\text{g/l}$ ) | Nitrite<br>( $\mu\text{g/l}$ ) | Nitrate<br>( $\text{mg/l}$ ) | Conductivity<br>( $\mu\text{S/cm}$ ) | pH  | Alkalinity<br>( $\text{mmol/l}$ ) | o-P<br>( $\mu\text{g/l}$ ) |
|------------------------|---------------------------------|--------------------------------|------------------------------|--------------------------------------|-----|-----------------------------------|----------------------------|
| Raw water              | 4.38                            | 1                              | 0.8                          | 271                                  | 7.2 | 2.6                               | 9.4                        |
| Pre-Ozonation effluent |                                 |                                | 0.8                          | 272                                  | 7.2 | 2.6                               | <5.0                       |
| RSF effluent           | 2.92                            | 0.24                           | 0.8                          | 279                                  | 7.5 | 2.7                               | 5.9                        |
| Int-Ozonation effluent |                                 |                                | 0.8                          | 278                                  | 7.5 | 2.7                               | <5.0                       |
| GAC effluent           |                                 |                                | 0.9                          | 278                                  | 7.4 | 2.7                               | <5.0                       |
| SSF effluent           | 1.93                            | 0.17                           | 0.9                          | 276                                  | 7.4 | 2.6                               | <5.0                       |

## 2.2. Sampling

Water samples were taken before and after each treatment step as well as from the three different filter types (RSF, GAC, SSF). Water samples (4 l) were collected from the raw water, before and after each filtration step and from the reservoir of the drinking water treatment plant in sterile and carbon-free Schott bottles. Three parallel RSF and SSF were sampled to assess the variation between parallel filters. It was not possible to sample parallel GAC filters due to restricted access to these reactors. From each filter, three samples with a sample volume of 250 ml each were collected from the filter surface at different positions. RSF were sampled during backwashing and GAC filters directly after backwashing to obtain representative samples from the whole filter depths. RSF samples contained pumice stone from the upper layer of the filter and quartz sand from the bottom layer. The two materials were separated by a sieve. Samples from the SSF contained sand and *Schmutzdecke*, which were separated (see below). Each sample was washed three times with 500 ml of 0.22  $\mu\text{m}$  filtered non-chlorinated tap water and the filter material containing attached bacteria was used for further analysis. No visual *Schmutzdecke* was present on the top layer of the SSF. Rather, microscopic *Schmutz*-aggregates clearly distinguishable from the sand were visible in between the sand particles. Samples contained sand, *Schmutz*-aggregates and water. In order to separate the *Schmutz*-aggregates from the sand particles, samples were filled into Schott bottles (1 l), 300 ml of tap water was added and the bottle was gently shaken. The liquid phase containing the *Schmutz*-aggregates was then transferred to a new bottle. This step was repeated several times until the *Schmutz*-aggregates were completely removed from the sand and the volume of the water phase (containing the *Schmutz*-aggregates) was recorded in order to normalise the biomass of the *Schmutz*-aggregates to the sand dry weight (DW) of each sample from where it originated from. For 454 pyrosequencing, enzyme tests and Biolog analyses, three samples from different locations of the same filter were mixed to get a representative sample from the whole filter for analysis. Samples

for community analysis were stored at  $-80\text{ }^{\circ}\text{C}$  until further processing, while all other measurements were done immediately.

### **2.3. Adenosine tri-phosphate (ATP) analysis**

ATP from the filter effluents was measured as described in Hammes et al., 2010b. ATP measurements to estimate active biomass were performed as described in Velten et al., 2007. Filter material (10 g) was rinsed gently three times with 100 ml filtered ( $0.22\text{ }\mu\text{m}$ ) non-chlorinated tap water. The rinsed material (200 mg wt weight) was placed in a sterile Eppendorf tube and submerged in 100  $\mu\text{l}$  of sterile non-chlorinated tap water. Commercial ATP reagent (300  $\mu\text{l}$ ) (BacTiter-Glo™ Microbial Cell Viability Assay, Promega, Madison, USA) was added and the sample was incubated at  $30\text{ }^{\circ}\text{C}$  for 2.5 minutes. The luminescence (relative light units) was measured on a luminometer (GloMax® 20/20, Turner BioSystems, Sunnyvale, CA) and the results were converted to ATP concentrations by means of a calibration curve prepared with pure ATP and heat-sterilized filter material. After analysis, the sand was dried ( $90\text{ }^{\circ}\text{C}$ , 24 h), the dry weight was measured, and the ATP data were normalized to the dry weight. All samples were analyzed in triplicate. The ATP per cubic meter was calculated by multiplying the ATP per milligram dry weight measured from each sample with the measured density (mg dry weight/l) of each filter material (RSF:  $4.5 \times 10^5\text{ mg/l}$ ; GAC:  $4.3 \times 10^5\text{ mg/l}$ ; SSF:  $1.4 \times 10^6\text{ mg/l}$ ).

### **2.4. Flow cytometry**

Flow cytometric analysis of total and intact cell concentrations was done as described in Berney et al. (2008). Briefly, bacterial cells were stained with 10  $\mu\text{l/ml}$  propidium iodide (PI) in combination with SYBR® Green I (SG/PI) to measure membrane-intact cells. Working solutions of the dyes were prepared as follows: SYBR® Green I was diluted 100-fold in anhydrous dimethylsulfoxide (DMSO) and mixed with PI (0.6 mM final concentration). Samples were then incubated in the dark for at least 15 minutes at room temperature before measurement. Samples were diluted 10-fold just before measurement in filtered ( $0.1\text{ }\mu\text{m}$ ; Millex®-GP, Millipore), cell-free bottled mineral water (EVIAN, France), so that the concentration measured with the flow cytometer was always less than  $2 \times 10^5\text{ cells/ml}$ . Flow cytometry was performed by using a portable CyFlow SL flow cytometer (Partec, Hamburg, Germany) equipped with a 20 mW solid state laser emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected in the FL1 channel ( $520 \pm 20\text{ nm}$ ), and red fluorescence was collected in the FL3 channel ( $> 615\text{ nm}$ ). All data were processed with the Flowmax software (Partec) and electronic gating with the software

was used to separate the defined/selected clusters. The specific instrumental gain settings for these measurements were as follows: FL1 = 500, FL3 = 700, speed = 3 (implying an event rate never exceeding 1000 events/sec.). All samples were collected as logarithmic (3 decades) signals and were triggered on the green fluorescence channel (FL1). The collection of data as FL1/FL3 dot plots allowed for optimal distinction between the stained intact microbial cells, permeabilised cells, “high and low nucleic acid bacteria” and instrument noise or sample background (Hammes et al., 2008).

## ***2.5. Dissolved organic carbon (DOC) and fractioning of the NOM by liquid chromatography organic carbon detection (LC-OCD) analysis***

Dissolved organic carbon was measured by an infrared detector after complete oxidation of the natural organic matter (NOM) to CO<sub>2</sub> by Graentzel Thin-Film Reactor (Huber and Frimmel, 1992). The detection limit was 10 µg/l (Meylan et al., 2007). The separation of the NOM into different fractions depending on size was obtained using a size exclusion column (Toyopearl TSK HW 50S, Posoh Bioscience, Tokyo, Japan). All samples were pre-filtered prior to analysis using a washed 0.22 µm filter.

## ***2.6. Biolog***

Filter material (30 g wet weight) was filled into a 500 ml Schott bottle and 300 ml of 0.22 µm filtered and heat-sterilized mineral water (Evian) was added. The samples were sonicated for five minutes at 400 W using the TUC-699 sonication bath (Scherrer AG, Zuzwil, Switzerland) to detach the biomass. The cell suspension (200 µl per well) was then filled into Biolog Eco and Biolog GN2 plates (Biolog, Hayward CA, USA). Biolog Eco plates contain 96 wells with 31 different substrates and an empty control in triplicate, whereas GN2 plates contain 95 different substrates and an empty control. Plates were incubated at 30°C for 4 days; absorbance for color development (OD<sub>460</sub>) and optical density (OD<sub>546</sub>) was measured with a microtiterplate reader (Tecan Infinite<sup>®</sup> 200, Männedorf, Switzerland). All analyses were performed in triplicates.

## ***2.7. Enzyme assays***

### ***2.7.1. Enzyme assays with MUB substrate analogues***

The potential enzyme activities of seven enzymes contributing to the degradation of organic matter were tested with substrates analogues linked to the fluorescent molecule *4-methylumbelliferone*

(*MUB*) (Sigma-Aldrich, Buchs, Switzerland). *4-MUB-phosphate* was used to measure the potential activity of alkaline phospho-monoesterase and *4-MUB-acetate* was used to assess the potential esterase activity. Five potential polysaccharide-degrading activities were determined using *4-MUB- $\beta$ -D-glucopyranosid* and *4-MUB- $\alpha$ -D-glucopyranosid* for  $\alpha$ - and  $\beta$ -glucosidase activities, *4-MUB- $\beta$ -cellobioside* for cellobiohydrolase activity, *4-MUB- $\beta$ -D-xylopyranoide* for xylosidase activity and *4-MUB-N-acetyl- $\beta$ -D-glucosaminid-dihydrate* to assess the potential activity of chitinase. The enzyme assays were performed under optimized reaction conditions: 30 g of sample (from SSF, RSF and GAC) or 30 ml of *Schmutz*-aggregates were filled into a 500 ml Schott bottle and 300 ml Tris buffer (50 mM, pH 8) was added. The samples were sonicated for five minutes at 400 W using the TUC-699 sonication bath (Scherrer AG, Zuzwil, Switzerland) to detach the biomass. The ATP of the sample suspension was determined as described above. Stock solutions of 200  $\mu$ M for all substrates were prepared prior to the test according to Sinsabaugh et al. (2003). The respective substrate analogues (50  $\mu$ l per well) were filled into a 96-well plate and the sample suspension (200  $\mu$ l per well) was added to start the test. Substrate controls (50  $\mu$ l of the respective substrate + 200  $\mu$ l tris buffer), a sample control (200  $\mu$ l cell suspension + 50  $\mu$ l tris buffer) and a quenched *MUB* standard in the range of 0-100  $\mu$ M (50  $\mu$ l of *MUB* + 200  $\mu$ l sample suspension) was added to each 96-well plate. Each test was performed with four replicates. In pre-tests the linear increase with time of all measured enzyme activities was observed and the time required for each enzyme assay was determined. The fluorescence was measured with a microtitreplate reader (Tecan Infinite<sup>®</sup> 200, Männedorf, Switzerland) at 445 nm after 23 hours incubation at 4°C. Only the esterase activity was already determined after 1.5 h due to its high activity and the instability of the substrate at longer incubation times. From each test the OD<sub>445</sub> value measured at the beginning was subtracted from the OD<sub>445</sub> value measured at the end of the test after subtraction of the controls. Fluorescence intensity was converted to *MUB* by the standard curve and activity was expressed in nmol substrate/(h x g ATP).

### **2.7.2. Enzyme assays with *L-DOPA***

Potential polyphenol oxidase (PPO) and peroxidase (PO) activities were tested using *L-3,4-dihydroxyphenylalanine (L-DOPA)* as a substrate analogue and *L-DOPA* plus hydrogen peroxide for the measurement of the peroxidase activity. Those enzyme assays are based on the higher absorbance of *L-DOPA* after being oxidized (Sinsabaugh et al., 2003). The test was performed similar as for the *MUB* substrate analogues, only the deviations are described below. A 25 mM stock solution for *L-DOPA* was prepared prior to the test (Sinsabaugh et al., 2003). For the polyphenoloxidase test 50  $\mu$ l *L-DOPA* were used as a substrate and for the polyphenolperoxidase

test 50  $\mu$ l *L-DOPA* plus 10  $\mu$ l of 0.3 % hydrogen peroxide were used. Absorbance was measured at 460 nm at the beginning of the test and after three hours of incubation at 4 °C by a microtitreplate reader. The increase in absorbance was converted to *L-DOPA* by the micromolar extinction coefficient of 7.9/ $\mu$ mol under the conditions of the assay.

## **2.8. Microbial community analysis**

### **2.8.1. Denaturing gradient gel electrophoresis (DGGE)**

DNA from filter material was extracted as described in Boon et al., 2000. DNA was amplified using the general bacterial primers 338F-GC and 518R (Muyzer et al., 1993). PCR amplification and DGGE was performed as described elsewhere (Lautenschlager et al., 2010; Muyzer et al., 1993). Similarities were calculated by the Pearson correlation, taking into account band intensity and band position. The clustering algorithm of unpaired pair group method (UPGMA) using arithmetic averages was used to calculate dendrograms.

### **2.8.2. 454 Pyrosequencing**

Each water sample (4 l) was filtered through a 0.2  $\mu$ m polycarbonate filter and filters were stored at -80 °C until further processing. DNA was extracted using FastDNA Spin Kit (MPBio, Solon, OH) following manufacturer's instructions. The 16S rRNA gene was PCR-amplified in 50  $\mu$ l reaction volumes in S1000 Thermal Cycler (BioRad, Hercules, CA, USA) using Bullseye standard Taq DNA polymerase 2.0 x master mix (MIDSCI, St. Louis, MO, USA) according to the manufacturer's protocol with the following universal primers that have Roche standard barcodes 1) FA-MIDs-515F and FB-909R and 2) FB-MIDs-515F and FA-909R (Wang and Qian, 2009). PCR was carried out with the following parameters: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 40 s, 56 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were detected by 1.5 % agarose gel electrophoresis and the correctly sized DNA bands were excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, St. Louis, MO, USA) according to manufacturer's instructions. Equal amounts of purified PCR products were pooled for subsequent 454 pyrosequencing on the Titanium platform (Roche/454 Life Sciences, Switzerland) at the W.M. Keck Center, part of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

### ***Data Analysis***

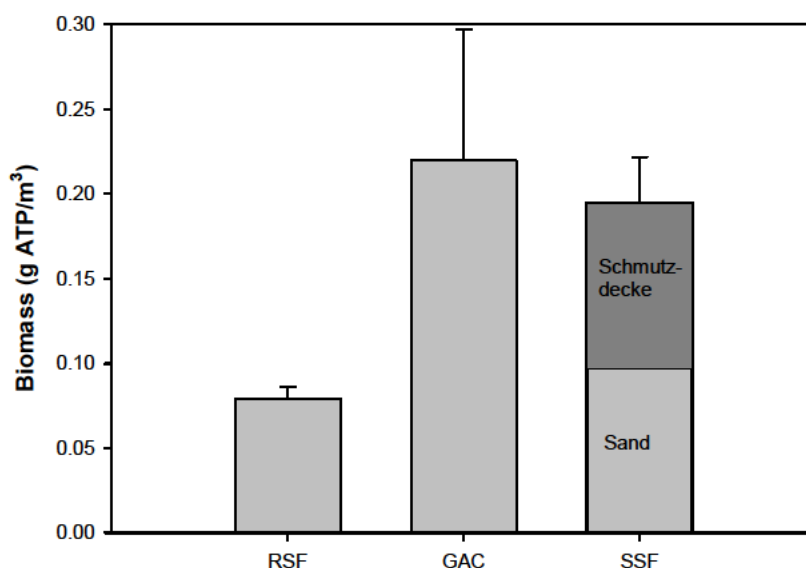
The pyrotags were sorted by barcodes (MIDs) and the sequences were analyzed following the Ribosomal Database Project pyrosequencing pipeline (<http://pyro.cme.msu.edu/>; Cole et al., 2009). First the sequences were trimmed using the initial data processing tool with default parameters (max number of N's = 0 and minimum average quality score = 20) using forward and reverse primer sequences. The trimmed sequences were aligned via RDP aligner and the sequences were subjected to RDP complete linkage clustering. The clustered sequence data was then used to estimate microbial community diversity based on the number of OTUs, Shannon-Weaver ( $H'$ ) index, and Chao 1 estimator with RDP analysis tools at a cut-off value of 97 % sequence similarity. RDP classifier was used for taxonomic assignments of the sequences at 95 % confidence level (Cole et al., 2009).

### 3. Results and Discussion

#### 3.1. Biomass concentrations in biological filters

Comparable densities of microbes were found in all three different biofilters of the treatment plant. The biomass concentration of each filter at three different positions was analyzed. Additionally, samples were also taken from three parallel filters of the same treatment step (for RSF and SSF). The RSF contained approximately three times less biomass ( $0.079 \pm 0.0074$  g ATP/m<sup>3</sup>; n = 9) than GAC ( $0.22 \pm 0.077$  g ATP/m<sup>3</sup>; n = 3) and SSF ( $0.2 \pm 0.027$  g ATP/m<sup>3</sup>; n = 9) (Fig. 1). Based on the ATP content per cubic meter (ATP/m<sup>3</sup>) and the average cellular ATP content (ATP/cell) of the water effluents from the biofilters, the amount of cells per cubic meter (cells/m<sup>3</sup>) on each filter material was calculated. Cell concentrations were in the range of  $1.9 - 5 \times 10^{15}$  cells/m<sup>3</sup>, which was in accordance with previously detected cell concentrations in drinking water biofilters ( $10^{15}$ - $10^{16}$  cells/m<sup>3</sup>) (Hammes et al., 2011; Velten et al., 2007; Magic-Knezev et al., 2004; Mauclaire et al., 2004). The pumice stone fraction of the RSF was separated from the quartz sand, but no difference in biomass per gram was measured between these two materials, which suggests only little variation in biomass with filter depth (data not shown). Other studies found no decrease in biomass concentration, or a decrease of 50 % biomass with filter depth on different filter media, with varying backwashing regimes (Wang et al., 1995; Moll and Summers., 1999). Thus the biomass decrease with filter depth might depend on whether and how often filters are backwashed. In terms of total filter biomass, most microbial activity should be located in the SSF when considering an approximately 20-fold larger surface area and 10-fold higher filter volume of the SSF compared to the GAC and RSF (Table 1). However, microbial biomass in the SSF might be overestimated with the ATP measurements due to a higher biomass concentration at the top of the filter (Eighmy et al., 1992). Such a gradient of the biomass should not establish in RSF and GAC filters, because these filters are regularly backwashed. In previous studies, various factors such as the amount of biodegradable organic carbon (BDOC) in the water phase, but also filter material (sand and anthracite) were found to influence the biomass concentration by a factor of 2-8, and can thus explain the slight variations between the three different filter types (Servais et al., 2005; Wang et al., 1995). Also, the empty bed contact time (EBCT), defined as the contact time in the media portion of an empty filter bed (volume of particles in the bed/flow rate), has been found to directly influence the BDOC removal and should hence indirectly influence the amount of biomass on a filter (Servais et al., 2005). Our findings clearly show microbial activity in each filter of the multi-step treatment plant, which is indicative for biodegradable carbon compound degradation. This was not necessarily obvious, since especially RSF are usually not seen as a biological filtration step, but are designed as

straining filters for turbidity removal, where biological processes are regarded as limited (Gimbel et al., 2006).



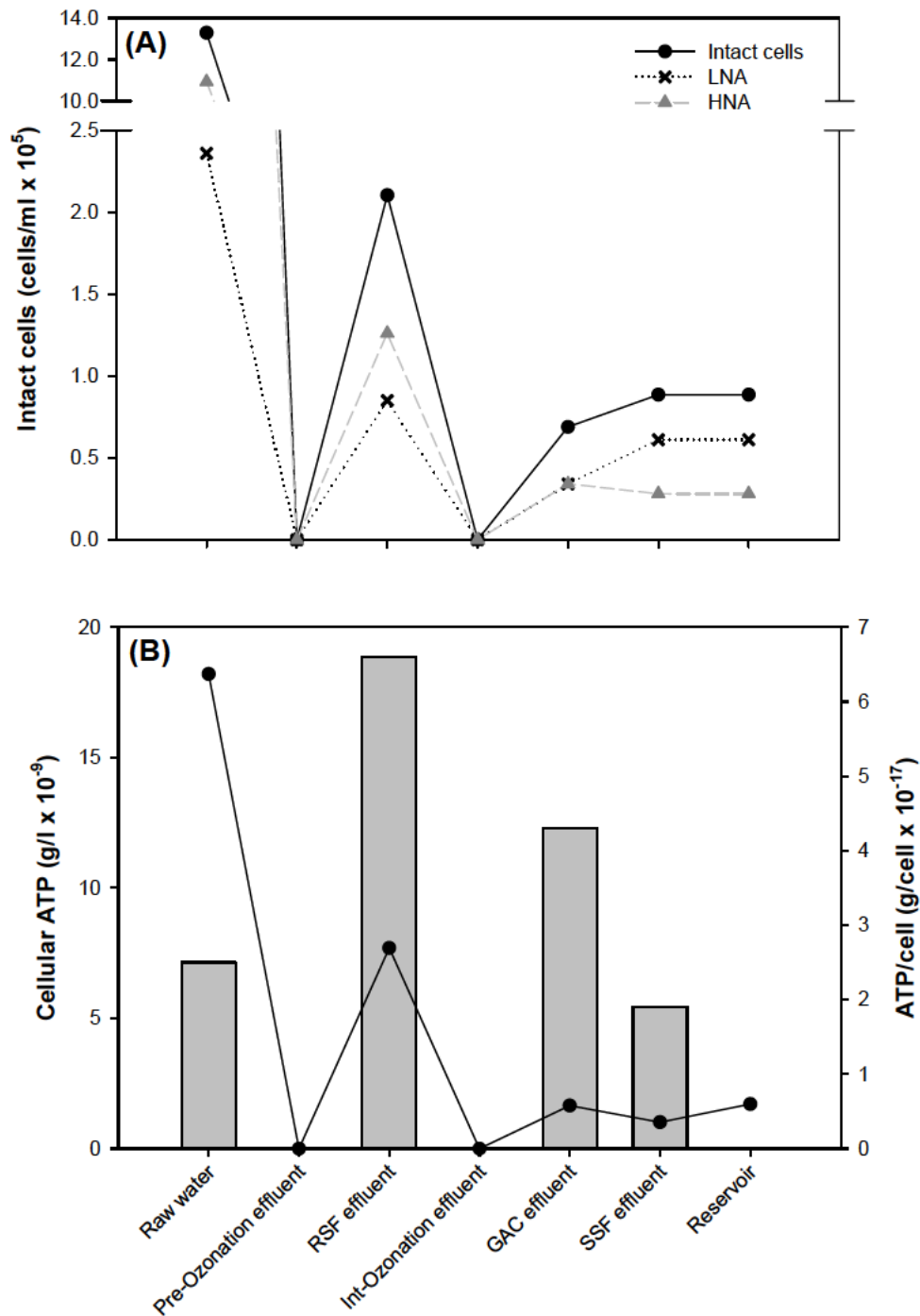
**Figure 1.** Biomass measurements from rapid sand filters (RSF), granular activated carbon filters (GAC) and slow sand filters (SSF). Error bars represent the standard deviations for a minimum of three samples.

### 3.2. Changes in planktonic biomass concentrations during drinking water treatment

FCM and ATP analysis showed expected fluctuations in planktonic biomass concentrations during treatment, with dramatic reduction/removal after both ozonation steps and clear increases after biofiltration (Fig. 2A, B). The HPC method was shown in a previous study to give only few counts (below 10 CFU/ml) in this drinking water treatment plant and is thus not suitable to sufficiently describe changes that might occur during treatment (Hammes et al., 2010a). These results were in accordance with previous measurements from the same plant (Hammes et al., 2010a). No bacterial cells were detectable in the influent of the RSF and GAC filters (ozonated water), whereas in the filter effluents  $2.1 \times 10^5$  and  $6.9 \times 10^4$  intact cells/ml were present (Fig. 2A). This implies that all these cells must have originated from the biofilters. The GAC effluent (inclusive bacteria) flows directly into the SSF, where cell concentrations further increased to  $9.3 \times 10^4$  cells/ml (Fig. 2A). The bacteria in the SSF effluent therefore either originated from the GAC filters and passed through the SSF filter, or originated from the SSF (Hammes et al., 2010a). It is not possible to say whether complete turnover occurs in the SSF, or whether a significant fraction of GAC bacteria pass through this filter. In the reservoir, cell concentrations remained stable.

In flow cytometric clustering patterns, two distinct populations of the so-called “high nucleic acid” (HNA) and “low nucleic acid” (LNA) content bacteria are commonly observed in fresh water environments with FCM after staining with fluorescent dyes (Lautenschlager et al., 2010; Wang et al., 2009; Lebaron et al., 2001). These two clusters were also clearly visible in all samples analyzed from the drinking water treatment train. Interestingly, a clear shift from more HNA bacteria in the RSF (HNA/LNA=1.5), to equal abundance in the GAC (HNA/LNA=1), to more LNA bacteria in the SSF (HNA/LNA=0.45) was observed in the course of the treatment process (Fig. 2A). It was described previously that HNA cells are considerably larger than LNA cells (Wang et al., 2009). The decreasing filter pore size from RSF to SSF might allow smaller LNA bacteria to pass the filters than bigger HNA bacteria. Alternatively, it might be possible that LNA bacteria grow preferably in GAC filters and SSF, or that selective grazing of HNA bacteria leads to the change in microbial community composition during the course of the treatment process (Boenigk et al., 2004).

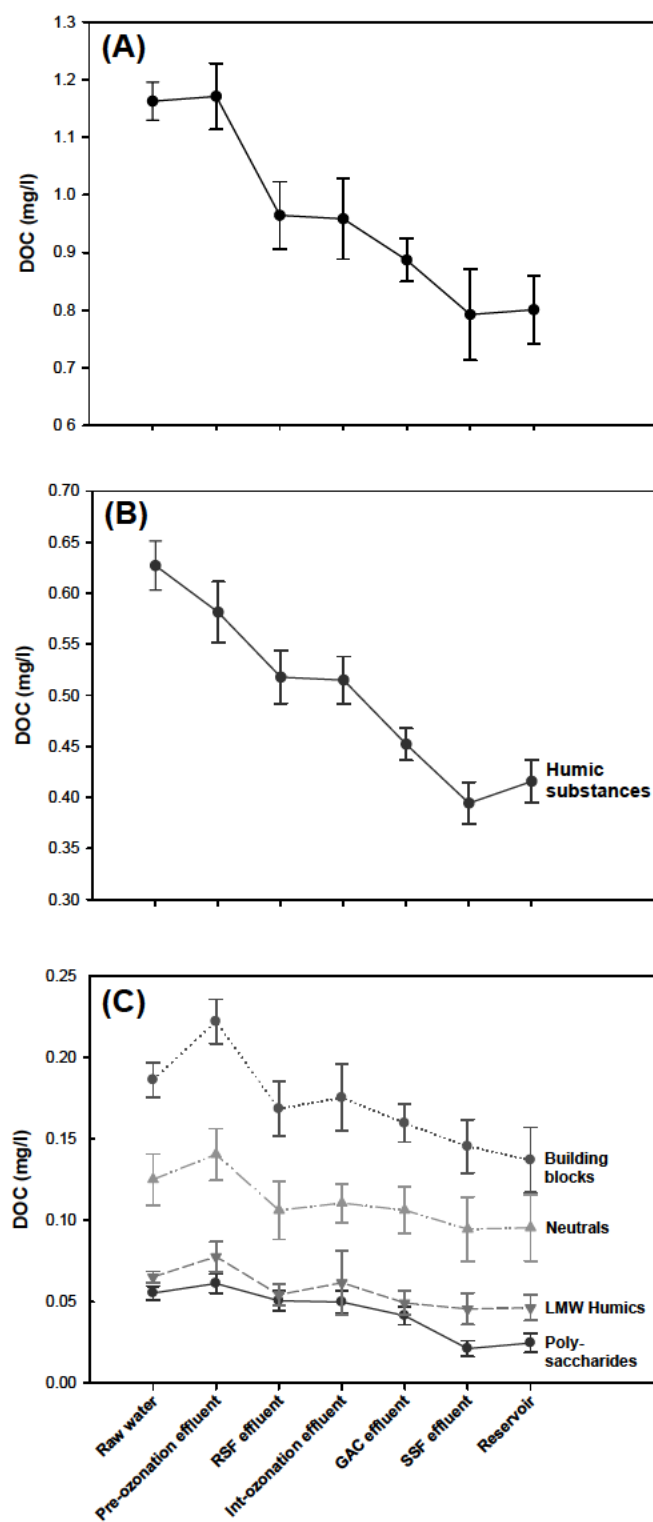
The same trend as observed in intact cell concentrations was also observed in ATP concentrations (Fig. 2B). However, the cellular ATP content (ATP/cell) was considerably lower in the raw water than in the RSF effluent. This might have been due to a shift in microbial community composition (see below), since the ATP content of a cell can vary according to its activity, type and size (Karl, 1980). In addition, the cellular ATP content decreased from the RSF effluent ( $6.60 \times 10^{-17}$  g ATP/cell) to the GAC ( $4.3 \times 10^{-17}$  g ATP/cell) and SSF effluent ( $1.9 \times 10^{-17}$  g ATP/cell) (Fig. 2B). ATP has been found previously to correlate with the biovolume of the cells, thus the shift to smaller LNA bacteria during filtration (discussed above) most likely lead to the decrease in the cellular ATP content in the course of the treatment (Hammes et al., 2010b; Lautenschlager et al., 2010; Wang et al., 2009; Eydal and Pederson 2007; Karl, 1980). To conclude, intact cell concentrations and ATP measurements showed that the cells in the water phase must have originated from the biofilters since the vast majority of cells of the raw water were eliminated by the ozonation processes. In addition, the change in the cellular ATP content and also the shift towards more LNA bacteria in the course of the treatment indicated a possible shift in microbial community composition. Importantly, this demonstrates clearly that ATP and FCM should be viewed as complimentary rather than competing methods for drinking water analysis.



**Figure 2.** Changes in total intact cell concentrations (●), and intact cell concentrations of low nucleid acid (LNA) (x) and high nucleid acid (HNA) (▲) bacteria (A). Changes in adenosine tris-phosphate (ATP) (●) concentrations and ATP/cell (grey bars) concentrations during the drinking water treatment process (B).

### 3.3. Removal of dissolved organic carbon (DOC)

Liquid chromatography organic carbon detection (LC-OCD) analysis is based on size exclusion chromatography that allows the separation of natural organic matter (NOM) into major fractions of different sizes prior to quantification (Huber et al., 2011; Volk et al., 1997). LC-OCD showed that the increase in cell concentrations during each filtration step was accompanied by a general decrease in DOC and a decrease in specific DOC fractions. The DOC decreased by 0.36 mg/l (31 %) from 1.16 mg/l to 0.8 mg/l during the entire drinking water treatment process (Fig. 3A). Most DOC was removed during RSF (210 µg/l), whereas only little was removed in SSF (95 µg/l) and GAC (72 µg/l) filters. The DOC was mainly composed of humic substances ( $54 \pm 4$  %), but also fractions of building blocks ( $16 \pm 6$  %), low molecular weight (LMW) humics ( $11 \pm 12$  %), LMW organics ( $6 \pm 5$  %), neutrals ( $9 \pm 4$  %) and polysaccharides ( $5 \pm 8$  %) were found. This is still a rather broad classification, and both biodegradable and recalcitrant DOC can be present in all the LC-OCD subclasses. The majority of the total DOC (84 %) removed was humic substances (Fig. 3B), which were also found in other studies to be the most important part of biodegradable organic carbon (Volk et al., 1997; Gremm and Kaplan, 1998). Approximately the same amount of humic substances was degraded in each filter (57 - 63 µg/l). However, the fractions of building blocks (53 µg/l), neutrals (23 µg/l) and LMW humics (34 µg/l) removed were mainly degraded during RSF, while polysaccharides were mainly removed during SSF (20 µg/l) (Fig. 3C). This indicated that different fractions of organic carbon were removed during each filtration step, with the more complex substances at the end of the treatment train. For the degradation of polysaccharides, extracellular enzymes are needed for the conversion into easily degradable organic carbon (Sinsabaugh et al., 2003; Nybroe et al., 1992). The longer residence time of the water in the SSF (Table 1), might have facilitated the degradation of complex organic matter, which could not be removed in previous filtration steps. Several studies have shown that biodegradable organic carbon leads to growth during drinking water distribution (Prévost et al., 1998; Servais et al., 1995; Mathieu et al., 1992). For example, only 10 µg/l assimilable organic carbon (AOC), which is the carbon fraction that can be used by planktonic bacteria during batch growth, leads to growth of  $1 \times 10^5$  cells/ml (Hammes and Egli 2005). However, the AOC constituted only a minor part (18 %) of the removed DOC in a previous study of the same plant (Hammes et al., 2010a; Hammes and Egli 2005). This stresses the importance of removing as many carbon fractions as possible from the water during drinking water treatment, particularly through biodegradation steps, and to assess the removal with multiple methods.



**Figure 3.** Dissolved organic carbon degradation during drinking water treatment measured by size exclusion chromatography (A). The degradation of the total organic carbon and divided into fractions (B) and (C). Error bars represent the standard deviations of eight samples taken during the course of one year.

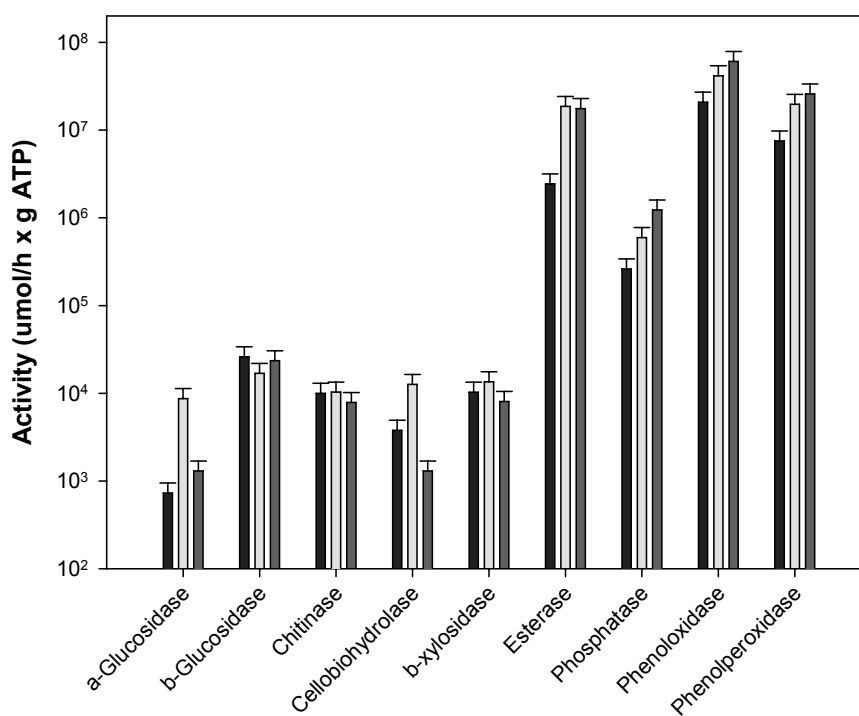
### 3.4. Efficiency in DOC removal of the different filter types

The DOC removal rate (mg/h) was calculated for each process unit by multiplying the flow rate ( $\text{m}^3/\text{h}$ ) with the removed DOC ( $\text{mg}/\text{m}^3$ ). Approximately two times more DOC was removed during RSF (979 g/h) than during GAC filtration (396 g/h) and SSF (451 g/h). In order to calculate the efficiency in DOC removal per cubic meter, the DOC removal per hour was divided by each respective filter volume (Table 1). The efficiency in DOC removal per cubic meter was 8.6-fold higher in RSF ( $0.44 \text{ g} / (\text{h} \times \text{m}^3)$ ) and GAC filters ( $0.44 \text{ g} / (\text{h} \times \text{m}^3)$ ) compared to SSF ( $0.051 \text{ g} / (\text{h} \times \text{m}^3)$ ). The filter flow rate in the SSF is considerably lower compared to the GAC and RSF (Table 1), which leads to a relatively longer residence time of the water in the SSF and a higher EBCT, which should lead to more BDOC removal (Servais et al., 2005). The low efficiency of the SSF suggested that at the time the water has reached the SSF, most of the BDOC was already degraded. However, the low DOC removal per cubic meter in the SSF in contrast to the high biomass concentration (see above) does not suggest, that the differences in biomass concentration of the different filter types were caused by the amount of BDOC removal. Regular backwashing of RSF and GAC leads to continuous removal of bacteria from these filter materials and causes a comparably lower biomass concentration on the filters compared to the non-backwashed SSF. SSF may not be very effective, but it is a critical final polishing step.

### 3.5. Enzyme activities in the biofilters

Enzyme activities that are involved in the degradation of complex organic carbon such as cellulose or chitin ( $\beta$ -1.4-glucosidase,  $\alpha$ -1.4-glucosidase, chitinase, cellobiohydrolase,  $\beta$ -xylosidase), phenolic compounds such as lignin (phenoloxidase and phenolperoxidase), the hydrolysis of organic phosphate compounds (alkaline phospho-monoesterase), or that indicate general heterotrophic activity (esterase) were measured (Sinsabaugh et al., 2003; Hendel et al., 2001; Schnürer and Rosswall, 1982). Since SSF are not backwashed and have a fine pore size, a *Schmutzdecke* usually develops, in which extracellular substances were found to accumulate (Huisman and Wood, 1974). Also the LC-OCD measurements (see 3.3) indicated that polysaccharides are better degraded in the SSF than GAC filters and RSF. Thus higher activities of extracellular enzymes were expected in samples from the SSF compared to the other filters. The measured enzyme activities were normalized to ATP, to relate activity to biomass, since we have observed a high correlation of ATP and enzyme activities (data not shown). Irrespective of the filter type, higher specific enzyme activities were measured for esterase, phosphatase, phenoloxidase and phenolperoxidase ( $10^5 - 10^8 \mu\text{mol}/(\text{h} \times \text{g ATP})$ ) than for the glucosidases, chitinase, cellobiohydrolase and  $\beta$ -xylosidase ( $10^2 -$

$10^3 \mu\text{mol}/(\text{h} \times \text{g ATP})$ ) (Fig. 4). Such differences in enzyme activities are typical for natural environments; higher phosphatase and esterase activities than  $\beta$ -glucosidase activities were also found in groundwater, lake water or river water in numerous studies (Emtiazi et al., 2004; Hendel et al., 2001; Miettinen et al., 1996). Unexpectedly, specific enzyme activities ( $\mu\text{mol}/(\text{h} \times \text{g ATP})$ ) did not vary significantly between the different filter materials (Fig. 4). Similarly, natural biofilms formed during drinking water production from surface water embankment filtration did not differ in specific enzyme activities (Emtiazi et al., 2004; Nybroe et al., 1992). Also, in a wastewater treatment plant enzyme activities were found to highly correlate with bacterial abundance, but not with specific process parameters, confirming our results (Nybroe et al., 1992). In our study, the same microbial taxa were found on the three different filter types (see below), which might be the reason for the similar potential enzyme activities. To conclude, these enzyme tests suggest that the microbial communities of each biofilter population have a similar potential for the breakdown of polymers. The higher degradation of polysaccharides in the SSF (see above) might be rather influenced by the longer residence time of the water in this filter and the dense porosity of the sand in which organic compounds can get captured.



**Figure 4.** Average enzyme activities measured at 4°C in RSF (black bars), GAC (light grey bars) and SSF (dark grey bar) samples normalized to ATP (A). The average of the enzymatic activities from sand and *Schmutzdecke* are shown. Error bars represent the 30 % standard deviation.

### ***3.6. Substrate degradation potential of the biomass***

Different amounts and types of substrates from Biolog GN2 and ECO plates could be used by RSF, GAC, and SSF microbial communities (Table 3). Differences in the substrate utilization pattern on Biolog plates of filters treating non-ozonated NOM compared to filters treating ozonated water were observed in another study, where the water quality influenced the microbial community composition. This suggested that the microbial community composition influences the substrate utilization pattern (Moll et al., 1998). We hypothesized that the SSF microbial community can degrade more complex substrates, since the population could not favor the easy degradable substrates that are available in the RSF and GAC step due to the ozonation process (see above). However, such a pattern was not observed with Biolog assay. Surprisingly, the least substrates could be utilized by the communities from GAC filters. The reason for this is not obvious. The one third lower microbial richness found in the GAC filter (see below) might be the reason for the lower substrate utilization potential from GAC samples, since one can assume that the diversity of a community in a sample should be related to metabolic versatility. Possibly also the different operational parameters, e.g. the higher filter flow rate compared to RSF and SSF (Table 1), caused the difference. In contrast to the enzyme activity assays (see above), the Biolog result might reflect species that contribute only a minor fraction to the community in the sample, but are able to grow on the substrates. In contrast, we assume that the short incubation time and low temperature of the enzyme activity assays should not lead to microbial growth and thus represent rather the potential for organic carbon degradation/conversion of the dominant microbial groups in the sample. Also, the substrate concentration in Biolog plates is with 5-15 mM considerably higher than in oligotrophic environments. It still needs to be tested whether substrate utilization patterns obtained at high concentrations also apply to low substrate concentrations. Hence a Biolog assay is in our opinion not useful to describe the actual functioning of the microbial community in the system, although it might show the potential a microbial community has to adapt to different kinds of substrates.

**Table 3.** Percentage of substrates that can potentially be utilized of bacteria derived from GAC, RSF and SSF samples measured with the Biolog ECO and GN 2 plates after 4 days incubation at 30°C. The detailed description for substrates that could be utilized is given for Biolog ECO plates. Substrates that could be utilized are indicated with +.

|                                     | RSF | GAC | SSF | Schmutzdecke |
|-------------------------------------|-----|-----|-----|--------------|
| Percentage of substrates utilized:  |     |     |     |              |
| GN 2 plates (%)                     | 54  | 20  | 59  | 56           |
| Eco plates (%)                      | 53  | 22  | 50  | 63           |
| D-Mannitol                          | +   |     | +   | +            |
| L-Asparagine                        | +   |     | +   | +            |
| D-Glucosaminic Acid                 | +   | +   | +   | +            |
| 4-Hydroxybenzoic acid               | +   |     | +   | +            |
| L-Arginine                          | +   |     | +   | +            |
| Tween 80                            | +   | +   | +   | +            |
| N-acetyl-D-Glucosamine              | +   | +   | +   | +            |
| Tween 40                            | +   |     | +   | +            |
| D-Glacturonic acid                  | +   |     | +   | +            |
| L-Serine                            | +   |     | +   | +            |
| Pyruvic acid methyl ester           | +   | +   | +   | +            |
| D-Malic acid                        |     |     | +   | +            |
| Putrescine                          | +   |     | +   | +            |
| D-Galactonic acid $\gamma$ -lactone | +   |     | +   | +            |
| L-Threonine                         |     |     |     | +            |
| Glycyl-L-Glutamic acid              | +   |     | +   | +            |
| Itaconic acid                       |     |     | +   | +            |
| D-Xylose                            | +   |     | +   | +            |
| L-Phenylalanine                     | +   |     | +   | +            |
| $\alpha$ -Ketobutyric acid          |     |     |     | +            |
| $\beta$ -Methyl-D-Glucoside         |     |     |     | +            |
| $\gamma$ -Gydrxybutyric acid        |     |     |     | +            |
| i-Erythriol                         |     |     |     | +            |
| D-Cellobiose                        | +   | +   |     | +            |
| D,L,- $\alpha$ -Glycerol Phosphate  |     |     | +   |              |
| $\alpha$ -D-Lactose                 |     | +   |     |              |
| Phenylethylamine                    |     |     |     |              |
| Glycogen                            |     |     |     |              |
| $\alpha$ -Cyclodextrin              | +   | +   |     |              |

### 3.7. Changes in microbial community composition during the drinking water treatment

#### 3.7.1. Comparison of the microbial community composition of parallel filters

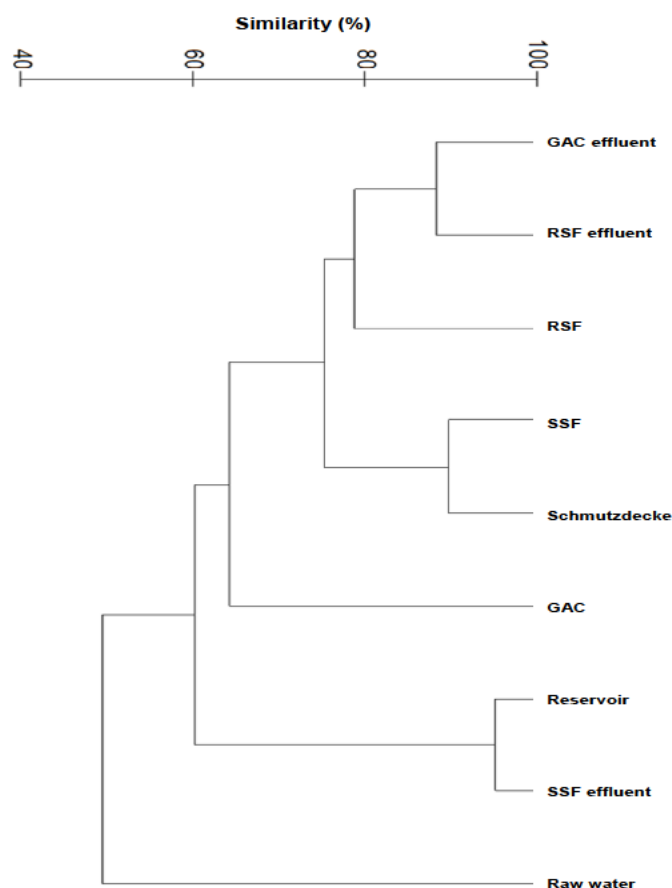
Little is known about the functional bacterial communities that colonize drinking water biofilters and contribute to the high quality water treatment. Clustering analysis of the DGGE pattern suggested a high similarity (> 95 %) of the major/dominant microbial communities obtained from parallel filters of the same treatment steps and also from samples obtained from different locations within the same filter (Supplementary information Fig. S1). However, microbial communities from

the three different biofiltration steps clustered separately. Community changes between the different filters were in the range of 25 - 30 % (Supplementary information Fig. S1). For a more in depth analysis of these changes, we have chosen one sample of each filtration step for a detailed microbial community analysis with 454 pyrosequencing.

With DGGE the least diversity was detected on the GAC (24 bands), whereas a higher diversity was detected on RSF (38 bands), sand (38 bands) and *Schmutzdecke* (43 bands) samples. The pyrosequencing data analyzed with the abundance-based nonparametric species richness estimator, Chao1 (Chao, 1984), also indicated a higher richness on RSF (900 Chao1) and SSF (900 Chao1) than on the GAC filter (600 Chao 1). The discrepancy in diversity to the DGGE result is probably due to only few (ca. 50) sequences that were present more than 1 % (Fig. 7) and with DGGE only species that are abundant more than 1 % can be detected (Muyzer et al., 1993). This is coherent to a study, where rare species contributed a significant portion (75 %) of the diversity in samples from a membrane tank when analyzed with pyrosequencing (Kwon et al., 2011). The general high diversity suggests a high metabolic flexibility of the microbial communities, which might allow the microbial community to adapt to possible changes in the water quality (Wittebolle et al., 2009).

The microbial communities on treatment plant filters most probably developed irrespective of the microbial communities that were present in the raw water, since most of these bacteria were destroyed during ozonation (see above). The microbes in the biofilters might originate from the filter material (which is not sterile), from a first flushing of the treatment plant with tap water before taken into operation, or from cells surviving the ozonation process. Interestingly, *Actinobacteria* that were highly present (40 %) in the raw water did not grow again after ozonation (Fig. 6A). *Actinobacteria* are often the numerically dominant phylum in lakes (Newton et al., 2011). One explanation for their absence in the biofilters might be that *Actinobacteria* do not attach preferentially to surfaces, due to their free-living style and also the different environment in the biofilters might have introduced a selective pressure. Alternatively the carbon source they were growing on might have been altered during ozonation. Similar to the DGGE data, cluster analysis of the pyrosequencing data showed that the microbial communities of the three filter types differed (Fig. 5). However, after both ozonation steps a community composed of the same major microbial taxa developed in all filter types, namely *Proteobacteria*, but also *Planctomycetes*, *Acidobacteria*, *Bacteroidetes*, *Nitrospira*, and a group of unclassified bacteria (Fig. 6A, Fig. 7). These bacterial groups are usually found in freshwater environments and are capable of utilizing a large variety of substrates (Newton et al., 2011). The differences in the clustering could be ascribed to variation in the relative abundance of the taxa between the three filter types and the varied species richness (see

above). We propose that the different substrate availability in each filter, such as more easily degradable substances in the RSF than in GAC and SSF (Fig. 3), likely affected the relative abundance of each bacterial group. A higher proportion of *Proteobacteria* was found on the GAC filter (67 %) in comparison to the SSF (34 %) and RSF (50 %), which was due to an increase of both,  $\alpha$ -*Proteobacteria* (+ 12-16 % higher) and  $\beta$ -*Proteobacteria* (+ 13-14 %) (Fig. 6A, B). Also, noticeably more *Acidobacteria* were found on the SSF (19 %) in comparison to RSF (6 %) and GAC (5 %) filters (Fig. 6A). *Acidobacteria* as well as  $\alpha$ -*Proteobacteria* and  $\beta$ -*Proteobacteria* are commonly found in freshwater and freshwater sediments.  $\alpha$ -*Proteobacteria* were reported to be competitive under low nutrient concentrations and are capable of degrading complex organic compounds; whereas  $\beta$ -*Proteobacteria* are fast growing and nutrient loving (Newton et al., 2011). Yet, generally very little is known about the role these members play in the environment, thus it is impossible at this point to relate the bacterial groups to certain functions (Newton et al., 2011). Nevertheless, within the  $\alpha$ -*Proteobacteria*, relatively high abundance of the genus *Nitrospira* on the RSF (9 %), GAC (4 %) and SSF (3 %) indicate a potential for nitrification (Fig. 6A). Nitrification has been observed to occur during GAC filtration in treatment plants with ammonia concentrations ranging from 20-260  $\mu\text{g NH}_4^+/\text{l}$  in the raw water (Andersson et al., 2001; Kasuga et al., 2010). These concentrations were considerable higher than the ammonia concentrations of the raw water in our study (4  $\mu\text{g NH}_4^+/\text{l}$ ), where nitrification is not an essential treatment process. The relative abundance of non-classified bacteria was slightly higher on SSF (24 %) than in the RSF (18 %) and GAC filter (8 %) (Fig. 6A). The big fraction of non-classified bacteria on the drinking water treatment plant filters suggests that these bacteria are probably important for the treatment process. Future metagenomic analysis could give more insight into the function of these microbial communities. The similar microbial taxa on all treatment plant filters suggest that the water quality (chemical/carbon composition) mainly determines the composition of the microbial communities. The changes in the relative abundance are probably due to the changes in the water quality in the course of the treatment (Fig. 3). This was also proposed by other studies, where phospholipid fatty acid and molecular analysis revealed differences in microbial community composition between biofilters treated with non-ozonated and ozonated water (Fonesca et al., 2001; Moll et al., 1998).

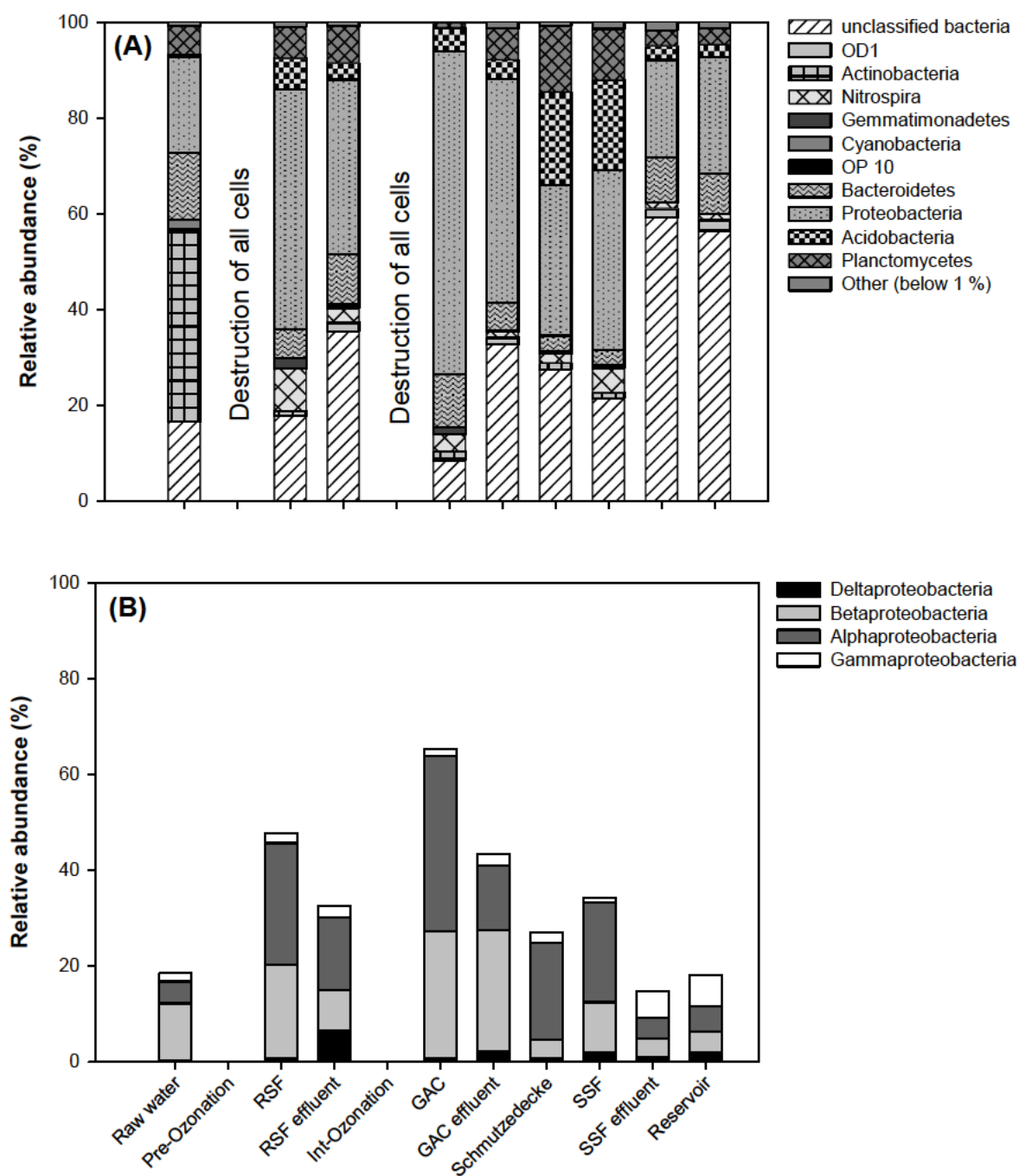


**Fig. 5.** Cluster analysis from 454 pyrosequences data from RSF, GAC filter, SSF and their effluents based on the phylum level.

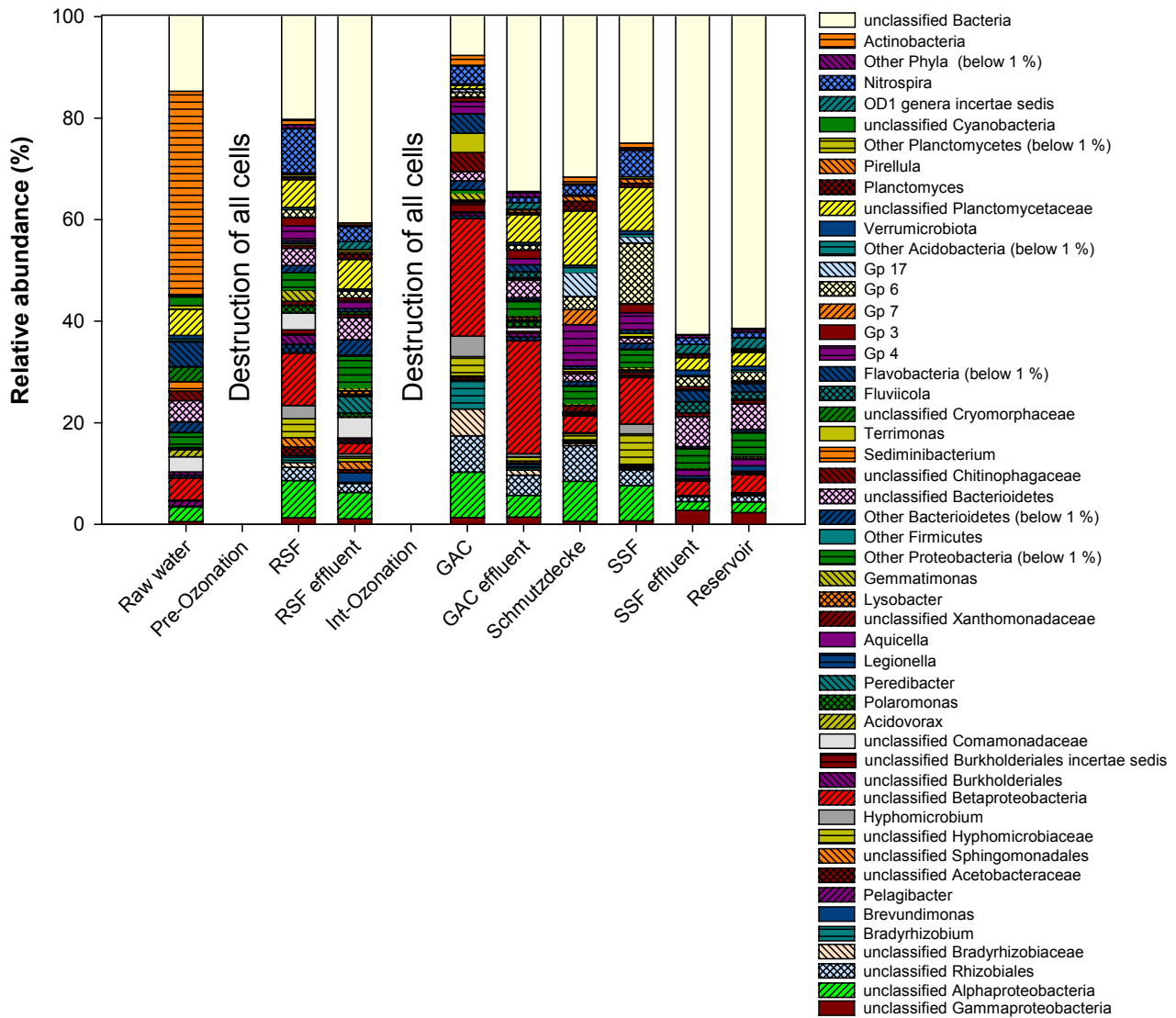
### 3.7.2. Comparison of the filter community to the effluent community

We compared the microbial community composition on the filters with the microbial community composition in the water phase. Particularly the impact of the filters on the microbial community composition of the SSF effluent is of interest, because these bacteria remain in the water that is distributed and delivered to the consumer without disinfectant (Hammes et al., 2010a; network paper). The cluster analysis showed that filter communities were different to the filter effluent communities (Fig. 5). However, the effluent populations reflected the filter populations, e.g., the higher abundance of *Proteobacteria* observed on the GAC filter (67 %) resulted in a higher abundance of *Proteobacteria* (47 %) in the GAC effluent compared to RSF effluent (20 %) and SSF effluent (36 %) (Fig. 6A). Yet, bacteria that could not be classified were more abundant in the filter effluents (33-59 %) in comparison to the communities on the filters (8-27 %), with most unclassified bacteria in the SSF effluent (59 %) (Fig. 6A). Also, the higher richness in the filter effluents (1000 Chao1 in RSF and GAC effluents, >2000 Chao1 in the SSF effluent) compared to the filters (600-900 Chao1) showed that the microbial community composition changed between

filters and their effluents. It would be conceivable that the group of unclassified bacteria was highly diverse, since its increase correlated with the increase of diversity. From GAC effluent to SSF effluent the part of the non-classified bacteria increased (+ 27 %), while the fraction of  $\beta$ -*Proteobacteria* decreased (Fig. 6A). This shows that the SSF considerably influenced the microbial community composition of the final drinking water, which is supported by the increase in LNA bacteria abundance from the GAC effluent to the SSF effluent (see 3.2). The different relative abundance of bacterial taxa on the filters in comparison to the filter effluents might be due to some bacteria rather living attached than others. It would be also conceivable that the microbial community composition changes with filter depths as observed previously (Moll et al., 1998). However, since RSF and GAC filters are regularly backwashed, which should lead to a mixing of the microbial communities, this explanation is less likely. Although it was possible to analyze the differences between the biofilm and the liquid phase microbial communities, the further reasons causing these changes still need to be elucidated as well as the role of the non-classified bacteria during drinking water treatment.



**Figure 6.** 454 pyrosequencing analysis from samples from filters and filter effluents of the drinking water treatment train. The relative abundance of phyla (A) and classes of *Proteobacteria* (B) is shown.



**Figure 7.** 454 pyrosequencing analysis from filter and filter effluent samples of the drinking water treatment train. The relative abundance of all genera/families that were present with at least 1 % in one of the samples is shown.

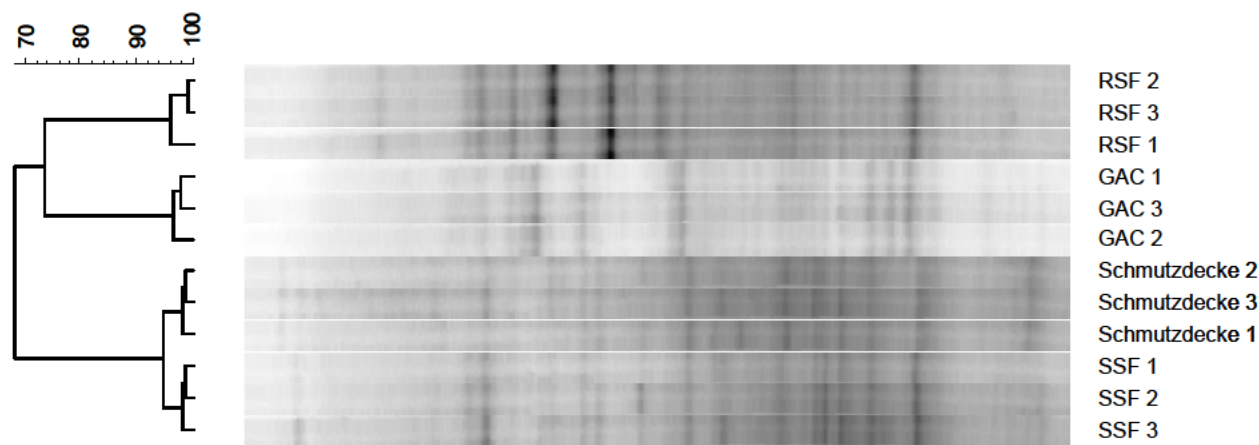
#### **4. Conclusions**

- This study provides a first detailed view on the complex microbial community that inhabit drinking water biofilters and which contribute to the function/performance of these systems.
- GAC, RSF and SSF are densely colonized with bacteria. The same dominant microbial groups were found on each filter type, suggesting that the general water quality determined the broader microbial community composition.
- A high phylogenetic, enzymatic and metabolic diversity in the filters suggests that the filters have a high potential to degrade different kinds of substrates.
- However, a large fraction of these bacteria is still unclassified, which necessitates further research on the identity, ecology and function of these organisms in biofiltration systems.

#### **Acknowledgements**

We would like to thank Sebastian Meylan and Jacqueline Traber for the liquid chromatography organic carbon detection (LC-OCD) measurements and analysis. We are grateful to the financial support of the EU project TECHNEAU (018320) research grant and the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia.

Supplementary information



**Figure S1.** Cluster analysis of denaturing gradient gel electrophoresis (DGGE) patterns from samples of three different RSF and SSF as well as three samples from a GAC filter (Pearson-UPGMA) (A).

### **3. Influence of filter age and operation on the biomass distribution, activity and microbial community composition of covered slow sand filters**

#### **Abstract**

Slow sand filters (SSFs) are often used in full-scale drinking water treatment plants as the final treatment step and indigenous microbial communities colonizing the filters are essential for their optimal performance. Usually multiple SSFs are operated in parallel, but these filters can differ considerably with respect to their operational parameters. We compared 12 parallel SSFs, varying mainly in filter age and the time since the last scraping, with respect to the impact of these factors on the biomass concentration, activity and microbial community composition. Adenosine triphosphate (ATP) measurements showed that the biomass increased with filter age during the first five years of filter operation. In filters aged between 10 and 24 years the biomass remained constant and was mostly influenced by scraping. A direct link between the amount of biomass and the potential for organic carbon degradation was demonstrated by a strong correlation of extracellular enzyme activities with ATP concentrations. The microbial community composition was very similar in the 12 SSFs and hardly any influence of filter age and scraping was found. This suggested that the water inflow quality as the most constant factor determined the microbial community composition in these filters. The results elucidate the impact of operational practices on the biomass distribution and microbial ecology of SSFs, which might influence the microbial community composition of the final drinking water, in case the water is distributed without disinfection.

## 1. Introduction

The use of engineered biofiltration systems in Europe dates back to the early 1800s when John Gibb built the first slow sand filter (SSF) in order to supply his bleachery and the town of Paisley (UK) with water (Baker and Taras, 1981). This new technology was shortly afterwards applied in the cities of London (UK) and Hamburg (DE) for the treatment of river water before supplying it to the public. Today SSFs are in use worldwide to treat drinking water in various cities in industrialized countries, for example Amsterdam (NL) and Zürich (CH). In these cities, multistep disinfection-biofiltration processes were gradually introduced in the treatment trains and slow sand filtration is typically applied as a final treatment step before distributing the water without the addition of a disinfectant residual (Hammes et al., 2010a; Rittman and Huck, 1989).

SSFs are broadly defined as filtration systems consisting of sand as porous media (0.15 - 1 mm particle size), colonized by indigenous microbial communities and operated with a slow filtration rate (0.1 - 0.3 m/h). Their initially-intended and most obvious function is the straining of particles and turbidity, which is due to the fine size of the sand and the slow filtration rate (Huisman and Wood, 1974). An important function, namely pathogen removal, was discovered serendipitously under unfortunate circumstances in Hamburg in 1892, when people receiving untreated river water suffered from a cholera epidemic, whereas people receiving filtered drinking water did not suffer from the epidemic (Huisman and Wood, 1974). While the exact mechanisms of pathogen removal remain unresolved to date, several studies attributed this function to a combination of bacteriophage by zooplankton, straining and/or adsorption (Hijnen et al., 2007; Weber-Shirk et al., 1999; Huisman and Wood, 1974). A third function of SSFs is the removal of dissolved organic matter by microorganisms that was observed in several studies (Hammes et al., 2010a; Baghoth et al., 2009; Collins et al., 1992; Fox et al., 1984). In drinking water treatment plants where SSF is used as a final treatment step, this final “polishing” of organic carbon can contribute considerably to the microbial quality and biological stability of the water during distribution.

Although microbial processes are critical to optimal SSF operation, a detailed description and understanding of the distribution and composition of the microbial biomass driving the above-mentioned processes is still developing. Moreover, SSFs are typically not designed, monitored or operated from a microbiological perspective. Biomass develops in SSFs in two main phases: while a biofilm forms directly on the sand fraction throughout the filter, an additional layer of biomass together with other material accumulates on top of the filter and in between the sand particles of the upper layer as a result of physical straining in the absence of backwashing. This so-called

“*Schmutzdecke*” or “dirt layer” is a rather vague concept. It was previously defined as the “matrix in which microorganisms, macroorganisms and particulate organic matter get trapped” or simply as the upper layer (5-10 cm) of a SSF (Joubert and Pillay, 2008; Mauclaire et al., 2006; Eighmy et al., 1992; Huisman and Wood, 1974). The microbial and mineral composition of the material that comprises this layer was until now not characterized in detail. It is generally believed that most biomass is located in the *Schmutzdecke* and that this fraction contributes the most to the functioning of a SSF (Huisman and Wood, 1974). However, quantitative and qualitative data of the biomass distribution and activity in sand and *Schmutzdecke* supporting this statement remain limited. This is firstly due to time restraints when studying SSFs on laboratory scale, since in practice they are often in continuous operation (thus continuous biomass development) for a period of decades. Secondly, studies on full-scale are usually hampered by restrictions on access to the filters during operation. Thirdly, a lack of simple routine methods for microbial biomass, activity and community composition might be a reason for the limited amount of field-scale investigations in the past. However, a clear understanding of the biomass distribution and activity in SSFs would improve the understanding of their functioning and provide information for optimizing operational practices.

In practice, multiple SSFs are often operated in parallel as a single process unit, and the operation of the individual filters can vary considerably with respect to the actual filter age, filter height, flow rate and the time passed since the last scraping-removal of the *Schmutzdecke*. This leads to the fundamental question whether operational differences affect the development and composition of the biomass in biofilters. This is also of interest, because the microbial community composition in the filters might influence the microbial community composition of the final drinking water, in case the water is distributed after SSF without final disinfection. (Hammes et al., 2010a). In the present study, we assessed the impact of the actual filter age and the age relative to the last scraping (scraping age) on the biomass distribution and composition of 12 parallel SSFs from a full-scale treatment plant. In this plant the water is treated through sequential ozonation and biofiltration steps and SSF as the final treatment step (Hammes et al., 2010a). The biomass on the sand, in the *Schmutz*-aggregates between the sand, and in the water phase was characterized with microscopy and quantified separately with cultivation-independent measurements. Enzyme activities for the breakdown of organic matter and the removal of the total organic carbon of each filter were measured, while the microbial community composition was analysed by denaturing gradient gel electrophoresis (DGGE).

## 2. Materials and Methods

### 2.1. Sampling and separation of *Schmutz*-aggregates and sand

Twelve full-scale SSFs from the treatment plant of Lengg (Zurich, CH) were sampled at the filter surface. Unfortunately, it was not possible to collect depth profiles of the filters due to the disruption that this would cause to the integrity of the full-scale operational filters. No visual *Schmutzdecke* was present on the top layer of the filters. Rather, microscopic *Schmutz*-aggregates clearly distinguishable from the sand were visible in between the sand particles (Fig. 1A). From each filter, three samples with a sample volume of 250 ml each were scraped from the filter surface, containing sand, *Schmutz*-aggregates and water. In order to separate the *Schmutz*-aggregates from the sand particles, samples were then filled into Schott bottles (1 l), 300 ml of tap water was added and the bottle was gently shaken. The liquid phase containing the *Schmutz*-aggregates was then transferred to a new bottle. This step was repeated several times until the *Schmutz*-aggregates were completely removed from the sand and the volume of the water phase (containing the *Schmutz*-aggregates) was recorded in order to normalise the biomass of the *Schmutz*-aggregates to the sand dry weight (DW) of each sample from where it originated from. Samples for community analysis were stored at  $-20^{\circ}\text{C}$  until further processing, while all other measurements were processed immediately.

### 2.2. Microscopy

Samples were stained with SYBR Gold nucleic acid stain (1/1000 diluted stock solution; Invitrogen, CH) alone and in combination with the fluorescently labelled lectin Concanavalin A, conjugated with tetramethyl rhodamine isothiocyanate (TRITC) (Invitrogen, CH). Samples were first stained overnight with SYBR Gold and washed three times with filtered tap water prior to staining for 2 h with concanavalin A (10  $\mu\text{g/ml}$  final concentration). Confocal laser scanning microscopy (CLSM) images were captured with a Leica SP5 microscope equipped with a 25 x 0.95 objective. The excitation wavelengths were 488 nm for SYBR Gold and 515 nm for Concanavalin A and the emission was either detected at 510 – 530 nm for SYBR Gold or at 630 - 680 nm for Concanavalin A. Phase contrast and epifluorescent microscopy images were taken with a 10 x objective (Leica PH1). For scanning electron microscopy (SEM), samples were fixed for 1 h with 2.5 % formaldehyde, washed with filtered tap water and serially dehydrated with ethanol up to 100 %, then dried at critical point and thereafter sputter-coated with 10 nm gold before analysis.

### **2.3. Determination of dry weight, ash weight and mineral analysis**

The dry weight from the *Schmutz*-aggregates and the sand was determined after 2 days drying at 90°C. To determine the percentage of minerals in the *Schmutz*-aggregates, the samples were heated to 450°C for 4 h hours prior to determination of the ash weight. The ratio of ash weight to dry weight was taken to calculate the percentage of inorganics in the *Schmutz*-aggregates. Mineral analysis of the *Schmutz*-aggregates was performed on the high-resolution sector field inductively coupled plasma-mass spectrometer (Element 2 ICP-MS) (Thermo Scientific).

### **2.4. Adenosine tri-phosphate (ATP) analysis**

Total ATP on the sand was determined as described in Velten et al. (2007). In short, 10 g of the sand was rinsed gently three-times with 100 ml non-chlorinated tap water. Thereafter, 200 mg of the rinsed sand was placed in a sterile Eppendorf tube, and submerged with 100 µL of non-chlorinated tap water. 300 µL of a commercial ATP reagent (BacTiter-Glo™ Microbial Cell Viability Assay, Promega, Madison, USA) was then added and the sample was incubated at 30°C. After 2.5 minutes of incubation, the luminescence (relative light units) was measured on a luminometer (GloMax® 20/20, Turner BioSystems, Sunnyvale, CA) and the results were converted to ATP concentrations by means of a calibration curve prepared with pure ATP and heat-sterilized sand. After analysis, the sand was dried (90 °C, 24 h); the dry weight was measured, and used for normalization of the ATP data. The *Schmutz*-aggregates were sonicated for 5 minutes at 400 W using the TUC-699 sonication bath (Scherrer AG, Zuzwil, Switzerland) and subsequently diluted 10-fold in sterile nanopure water. ATP was then analysed as described in Hammes et al. (2010b). In short, the diluted sample (500 µl) and the ATP reagent (50 µl) were warmed to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were then combined and the luminescence was measured after 20 sec reaction time at 30°C. The data were collected as relative light units (RLU) and converted to ATP concentrations by means of a calibration curve. ATP was measured in triplicate for all samples. The ATP from the *Schmutz*-aggregates was normalized to the dry weight of the sand for each sample.

## 2.5. Enzyme assays

The potential enzyme activities of seven enzymes contributing to the degradation of organic matter were tested with substrates analogues linked to the fluorescent molecule *4-methylumbelliferone* (*MUB*) (Sigma-Aldrich, Buchs, Switzerland). *4-MUB-phosphate* was used to measure the potential activity of alkaline phospho-monoesterase and *4-MUB-acetate* was used to assess the potential esterase activity. Five potential polysaccharide-degrading activities were determined using *4-MUB- $\beta$ -D-glucopyranosid* and *4-MUB- $\alpha$ -D-glucopyranosid* for  $\alpha$ - and  $\beta$ -glucosidase activities, *4-MUB- $\beta$ -cellobioside* for cellobiohydrolase activity, *4-MUB- $\beta$ -D-xylopyranoide* for xylosidase activity and *4-MUB-N-acetyl- $\beta$ -D-glucosaminid-dihydrate* to assess the potential activity of chitinase. Potential polyphenol oxidase (PPO) and peroxidase (PO) activities were tested using *L-3,4-dihydroxyphenylalanine* (*L-DOPA*) as a substrate analogue and *L-DOPA* plus hydrogen peroxide for the measurement of the peroxidase activity. The enzyme assays were performed as described in chapter 2.

## 2.6. Flow cytometry

Total cell concentrations were determined by flow cytometry as described in Hammes et al. (2010a). Briefly, bacterial cells (1 ml) were stained with 10  $\mu$ l/ml SYBR<sup>®</sup> Green I (1/100 dilution in dimethylsulfoxide (DMSO); Invitrogen) and incubated in the dark for at least 15 minutes before measurement. Flow cytometry was performed using a PASIII flow cytometer (Partec, Hamburg, Germany) equipped with a 25 mW solid state laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected in the FL1 channel ( $520 \pm 20$  nm), and red fluorescence collected in the FL3 channel ( $> 615$  nm). All data were processed with the Flowmax software (Partec), and electronic gating with the software was used to separate the desired clusters. The specific instrumental gain settings for these measurements were as follows: FL1 = 500, FL3 = 700, speed = 3 (implying an event rate never exceeding 1000 events/sec). All samples were collected as logarithmic (3 decades) signals and were triggered on the green fluorescence channel (FL1). The collection of data as FL1/FL3 dot plots allowed for optimal distinction between the stained microbial cells, “high and low nucleic acid bacteria” and instrument noise or sample background.

## **2.7. Community analysis with DGGE**

DNA was extracted with the Soil DNA Isolation Kit (UltraClean® Soil DNA Isolation Kit, MoBio, Carlsbad, CA, USA) following the provided instructions. 4 µl of the extracted DNA was amplified using the general forward primer 338F-GC and the reverse primer 518r as described previously (Lautenschlager et al., 2010; Muyzer et al., 1993). DGGE was performed using the Biorad DCode system (Biorad, Reinach, Switzerland). PCR fragments (20 µl) were mixed with loading dye (10 µl) and loaded onto 8 % polyacrylamide gels (w/v) with a gradient from 45 - 60 %. The electrophoresis was run for 16 h at 60°C and 38 V. Gels were stained in SYBR® Green I nucleic acid gel stain (1:10000 dilution) for 20 min with gentle agitation and photographed. DGGE patterns were clustered using the Gel Compare II 6.0 software (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation and results in a distance matrix. Samples from sand and *Schmutz*-aggregates were run on two separate gels. To ensure a correct clustering, samples were run together with a ladder and one sand sample was loaded as a reference on both gels. The clustering algorithm of unpaired pair group method (UPGMA) using arithmetic averages was used to calculate dendrograms.

### 3. Results and Discussion

#### 3.1. Filter description

In the full-scale drinking water treatment system of Zürich, two ozonation steps are combined with three biofiltration steps, namely rapid sand filtration (RSF), granular activated carbon (GAC) and slow sand filtration (SSF) before the water is distributed without the addition of a residual disinfectant (Hammes et al., 2010a). However, a treatment step in a full-scale plant usually does not comprise a single process unit. In this case, 12 SSFs with a filter surface of 1120 m<sup>2</sup> each and a total surface area of 13440 m<sup>2</sup>, which equals the surface area of two soccer fields, are operated in parallel. Even more impressive is the surface area available for bacterial colonization on the sand grains. In total, the 12 SSFs contain about  $3.5 \times 10^{13}$  sand particles with an average surface area of approximately 0.67 mm<sup>2</sup> each, which equals a total surface area of  $2.4 \times 10^7$  m<sup>2</sup>. All filters are operated at the same temperature (6 °C) and treat the same water, namely the mixed effluent of the GAC reactors. However, at the time of the present study, the filter age, defined as the time since each filter was taken into operation, ranged from 2 months to 24 years (Table 1). Additional operational practices have caused further variations between the filters; some old filters have been scraped several times, whereas younger filters were never scraped, which led to varying filter heights in the range of 50 to 90 cm. Also, the time period since the previous scraping, defined as the scraping age, varied considerably and different flow rates (average flow rate: 0.35 m/h) developed due to filter headloss (Table 1). The present study focused specifically on the influence of these operational differences on the microbial composition and function of the SSF.

**Table 1.** Operational parameters of the 12 slow sand filters (SSFs) from the drinking water treatment plant of the city of Zürich.

| Filter | Filter age<br>(years) | Last<br>scraping<br>(years) | Number of<br>scrapings | Filter<br>height<br>(cm) | Filter<br>headloss<br>(cm) | Total organic<br>carbon (TOC)<br>removal (mg/l) <sup>a</sup> | Total cell<br>concentration<br>(TCC)<br>increase <sup>b</sup> |
|--------|-----------------------|-----------------------------|------------------------|--------------------------|----------------------------|--|---|
| A      | 0.1                   | 0.1                         | 0                      | 90                       | 17                         | 0.17   | $3.2 \times 10^4$   |
| B      | 0.8                   | 0.8                         | 0                      | 90                       | 24                         | 0.28   | $1.8 \times 10^4$   |
| C      | 12.6                  | 0.1                         | 1                      | 74                       | 20                         | 0.19   | $4.7 \times 10^4$   |
| D      | 4.5                   | 4.5                         | 0                      | 85                       | 38                         | 0.30   | $5.1 \times 10^4$   |
| E      | 5.7                   | 5.7                         | 0                      | 85                       | 44                         | 0.35   | $3.2 \times 10^4$   |
| F      | 17.0                  | 4.0                         | 2                      | 79                       | 73                         | 0.36   | $3.8 \times 10^4$   |
| G      | 17.1                  | 2.7                         | 2                      | 72                       | 67                         | 0.36   | $1.6 \times 10^4$   |
| H      | 21.1                  | 1.3                         | 3                      | 58                       | 61                         | 0.34   | $4.4 \times 10^4$   |
| I      | 14.4                  | 4.8                         | 1                      | 77                       | 105                        | 0.41   | $4.1 \times 10^4$   |
| J      | 19.1                  | 0.7                         | 4                      | 69                       | 39                         | 0.38   | $2.3 \times 10^4$   |
| K      | 13.1                  | 3.4                         | 1                      | 80                       | 60                         | 0.25   | $2.5 \times 10^4$   |
| L      | 24.1                  | 2.9                         | 4                      | 50                       | 63                         | 0.27   | $3.5 \times 10^4$   |

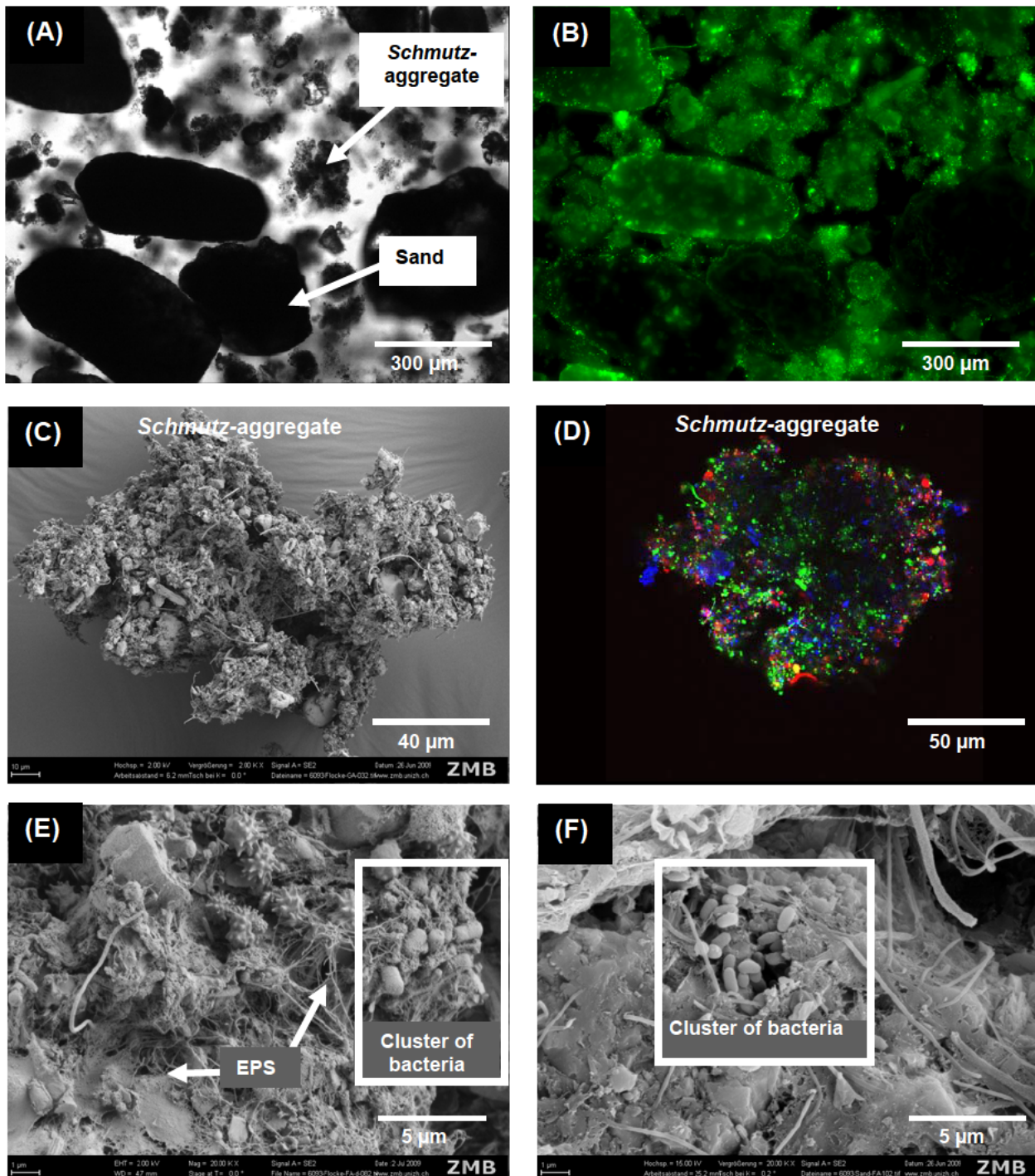
<sup>a</sup> The TOC in the mixed influent of all filters was 1.10 mg/l.

<sup>b</sup> The TCC in the mixed influent of all filters was  $7.90 \times 10^4$  cells/ml.

### 3.2. Qualitative characterization of sand and Schmutz-aggregates with microscopy

Microscopic images were taken to gain a visual impression of the microbial colonization on sand particles and *Schmutz*-aggregates. The overview image taken in phase-contrast shows the small (50-150 µm diameter) but clearly discernable *Schmutz*-aggregates in relation to the larger sand grains (300-500 µm diameter) (Fig. 1A). The *Schmutz*-aggregates fill the pores in between the sand particles, which suggest that the filter performance should enhance with *Schmutz*-aggregate development. The same image was taken with fluorescent microscopy, and revealed after staining with a fluorescent dye that both sand and *Schmutz*-aggregates were densely colonized with microbes (Fig. 1B). A close-up assessment of a *Schmutz*-aggregate with scanning electron microscopy (SEM) showed that these aggregates consist of different kinds of particles with varying sizes accumulating together (Fig. 1C). Light reflection in the confocal laser scanning microscope (CLSM) indicated that many of these particles are inorganic compounds (probably CaCO<sub>3</sub>) (Fig. 1D). The accumulation of exopolymeric substances (EPS) were previously observed in SSFs (Joubert and Pillay, 2008). We stained a *Schmutz*-aggregate with concanavalin A that binds to polysaccharides, which are part of the EPS, in combination with a fluorescent dye binding to DNA. With CLSM clear spots of polysaccharides and bacteria became visible within the aggregate (Fig.

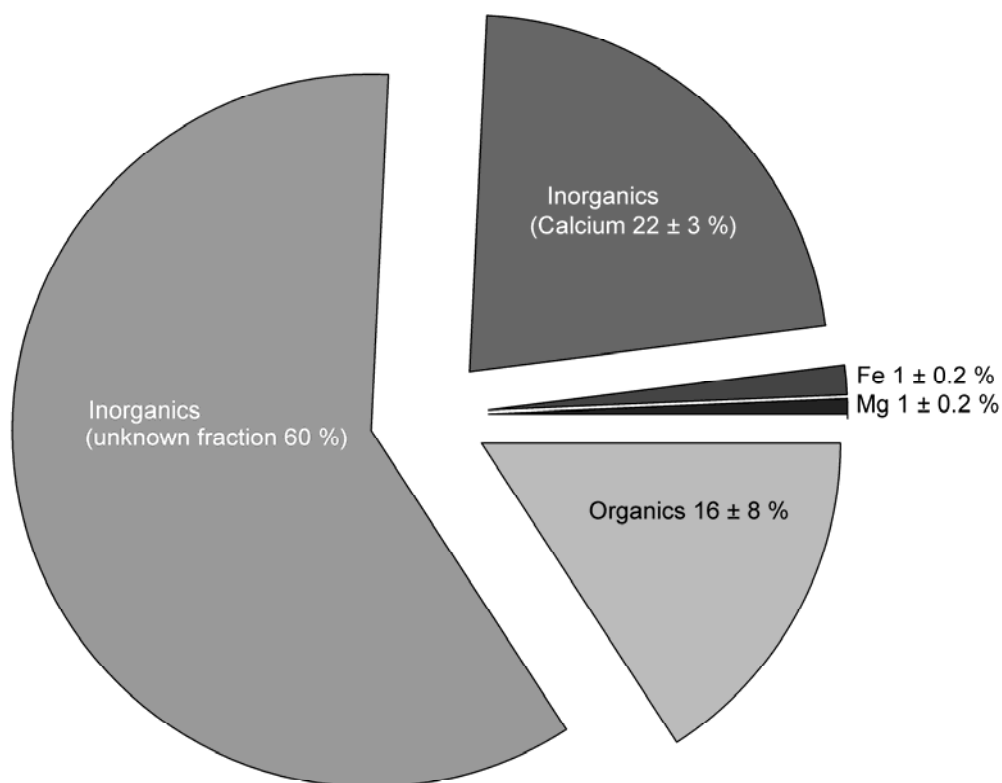
1D), with EPS predominantly on the outer perimeter of the aggregate. The EPS form a network structure that could be observed by detailed SEM images on *Schmutz*-aggregates, thus capturing and binding together all kinds of particles and bacteria accumulating in the pores in between the sand particles (Fig. 1E, F). On the SEM pictures also many similar bacteria of the same size and shape were observed on a relatively small surface area of only a few micrometers forming bacterial clusters (Fig. 1E, F). This accumulation of the same kind of bacteria can be attributed to bacterial growth and random attachment of bacteria on the sand and the *Schmutz*-aggregates. The presence of bacteria on both sand and *Schmutz*-aggregates further indicates that each fraction contributes to the functioning of a SSF.



**Figure 1.** Microscopy images from sand and *Schmutz*-aggregates taken in phase contrast (A) and colonization with bacteria (green) after staining with a fluorescent dye (B). Close-up of a *Schmutz*-aggregate taken with scanning electronic microscopy (SEM) (C) and fluorescent confocal microscopy (D). Bacteria are coloured in green, exopolymeric substances (EPS) in red and the reflected light in blue (D). Detailed images of the microbial colonization of *Schmutz*-aggregates (E) and sand (F) taken by SEM.

### 3.3. Mineral analysis of the Schmutz-aggregates

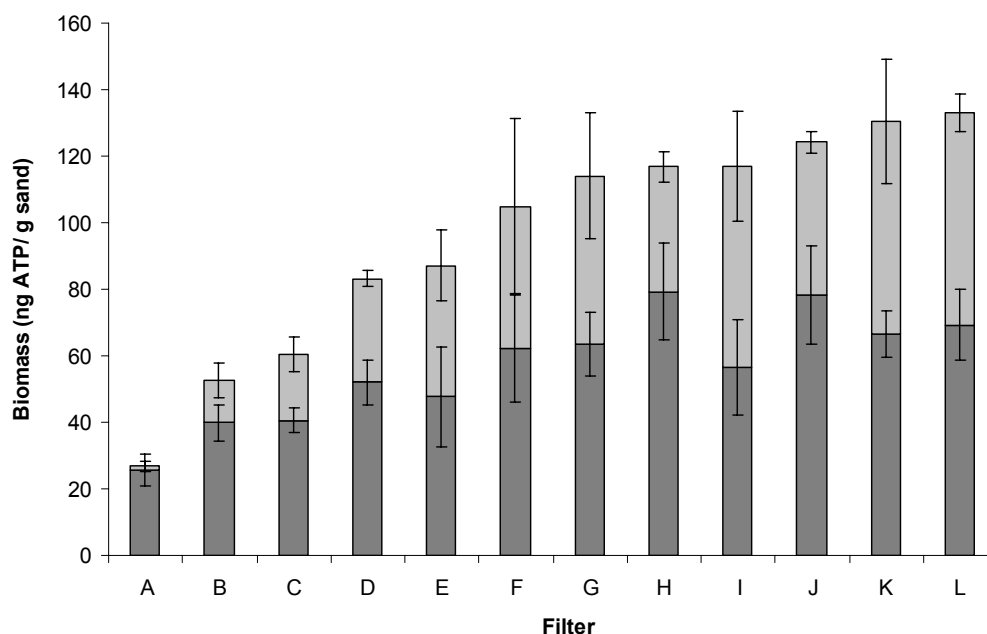
Visually the separated *Schmutz*-aggregates appeared as an accumulation of organic substances, similar to activated sludge flocs. However, analysis of the dry weight, which includes inorganic and organic substances, and the ash weight, which only comprises inorganic substances, revealed that the *Schmutz*-aggregates comprised on average  $84 \pm 8\%$  inorganic substances ranging from 74 % in the older filters, up to 99 % in the newest filter (Fig. 2). The high percentage of inorganic compounds supports the microscopy observations (Fig. 1D) and particularly the high percentage of inorganic material in the newest filter suggests that biomass had not yet developed in the *Schmutz*-aggregates of the filter. The largest fraction of the analysed minerals was calcium ( $22 \pm 3\%$ ), but also small amounts of magnesium ( $0.7 \pm 0.2\%$ ), manganese ( $0.2 \pm 0.07\%$ ), and iron ( $1.3 \pm 0.22\%$ ) were found. Further small concentrations (below 0.05 %) of chromium, copper, zinc and nickel were present. The major part of the unidentified inorganic compounds (60%) was most probably carbonates and phosphates present in mineral form. These data demonstrate the important physical straining function of the *Schmutz*-aggregates, which enhances if after a certain run time of the filter a considerable amount of *Schmutz*-aggregates developed, and which would be affected by its removal through scraping.



**Figure 2.** The *Schmutz*-aggregates were fractioned into organic and inorganic compounds. Average values and standard deviations from 12 *Schmutz*-aggregate samples are given.

### 3.4. Biomass distribution in the sand and *Schmutz-aggregates*

We have selected ATP measurements to assess the microbial biomass in the biofilters, since it allows an accurate and reproducible assessment of the microbial activity in biofilters, has proven to be a very good indicator of viable biomass in heterogeneous aquatic systems, and makes it possible to process many samples in parallel (Velten et al., 2007; Magic-Knezev et al., 2004; van der Kooij et al., 2003). The total biomass concentration on the sand particles and in the *Schmutz-aggregates* was highly variable in the parallel-operated SSFs (Fig. 3). In the samples analysed, the *Schmutz-aggregates* were located in between the sand grains, and we therefore normalized the amount of *Schmutz-aggregate* biomass to the amount of sand dry weight of each respective sample. This made it possible to directly compare the amount of sand biomass to the amount of *Schmutz-aggregate* biomass. A five-fold difference in total biomass between the filter with the most biomass (135 ng ATP/g sand) and the least biomass (25 ng ATP/g sand) was observed (Fig. 3). The separation of the biomass into sand and *Schmutz-aggregates* showed considerable variation in the extent of biomass development in particularly the *Schmutz-aggregates* (4.7 to 52 %) (Fig. 3). Nevertheless, in most filters the sand biomass comprised between 50-75 % of the total biomass. This suggests that the sand biomass in the upper layer of a SSF contributes at least to the same extent to the biomass as the *Schmutz-aggregates*, which is to some extent controversial to the common view that the most activity in SSFs is found in the *Schmutzdecke* (Page et al., 2006; Huisman and Wood 1974). However, this statement probably developed due to the rather vague definition of the *Schmutzdecke* concept.



**Figure 3.** Total biomass in the top 5 cm of the 12 slow sand filters (SSFs) (Table 1) separated into biomass from sand (dark grey bars) and biomass from *Schmutz*-aggregates (light grey bars). Error bars represent the standard deviation of triplicate samples. All data were normalised to the sand dry weight of each sample.

### 3.5. Influence of filter age on the biomass concentration on the sand and *Schmutz*-aggregates

The most obvious operational differences that can cause variations between the 12 SSFs in the present study were the filter age and scraping age. Hence, we analyzed the biomass data with respect to these parameters. First, the impact of filter age on the amount of biomass on the sand is described for filters that were never scraped. A new SSF is normally constructed with washed sand from an old filter and contained already 25 ng ATP/g sand (0.1 year), whereas nearly no *Schmutz*-aggregates were present (Fig. 4A, B). Considering the average biomass concentration on the sand of 80 ng ATP/g sand in an old filter (Fig. 4A), only approximately 70 % of the sand biomass was removed by washing, but the *Schmutz*-aggregates were removed completely. Until a filter age of five years, a slow but linear increase in biomass of approximately 4.5 ng ATP/g sand on the sand and 7 ng ATP/g sand in the *Schmutz*-aggregates was observed. The faster development of the *Schmutz*-aggregate biomass might be due to accumulation (straining) of organic matter and microorganisms from the water phase in the developing exopolymeric matrix. A six-fold linear increase of biomass during the first three months after filter setup was observed in a previous study (Campos et al., 2002). Based on our data, hardly any increase in biomass would be measureable in such a short time period. The faster development of the biomass in the previous study might be

ascribed to the higher dissolved organic carbon (DOC) concentrations (1.5 and 3 mg/l) in the SSF influent compared to the low DOC concentrations (0.7-1 mg/l) and very low AOC concentration (approx. 30 µg/l) in our system (Campos et al., 2002; Hammes et al., 2010a). A 4.5 year old filter contained approximately three times more total biomass (83 ng ATP/g sand) compared to a new filter (Fig. 3, Filter D). This biomass concentration was already close to the average biomass concentration of  $113 \pm 23$  ng ATP/g sand in SSFs older than ten years (Fig. 3; Fig. 4A, B). In older filters the biomass on the sand and the *Schmutz*-aggregates did not increase further with filter age, which might be due to the scraping of these filters to maintain the desirable flow rate (Fig. 4A, B). Likewise, the low nutrient concentrations in the SSF influent (above) may be a limiting factor for biomass formation and development. The high standard deviations on the sand biomass in older filters (10-30 %) suggest some heterogeneity in the biomass distribution on the filter surface, which might have developed due to uneven scraping. Particularly the biomass concentration in the *Schmutz*-aggregates was highly variable in the range of 20 to 64 ng ATP/g sand in filters older than 10 years. The accumulation of organic matter, microorganisms and all kinds of particles from the water phase in the exopolymeric matrix should lead to the faster biomass development in the *Schmutz*-aggregates compared to the sand and would favour the development of the *Schmutz*-aggregates on the filter top. Hence a high gradient in *Schmutz*-aggregate biomass with filter depths should logically establish. Scraping might consequently affect the *Schmutz*-aggregate biomass more than the sand biomass and hence cause comparably higher variations and a considerably decrease in biomass with filter depths (discussed below).

### ***3.6. Influence of scraping age on the biomass concentration on the sand and Schmutz-aggregates***

The impact of scraping on the biomass could be observed firstly by comparing filters with the same filter age (13 years), but different scraping ages (0.1 years and 3.4 years) (Fig. 4A, B). The sand biomass in the freshly scraped filter was 40 % lower (41 ng ATP/g sand) than in the ripened filter (67 ng/ATP g sand) and the *Schmutz*-aggregate biomass was as much as 70 % lower (44 ng ATP/g sand difference) (Fig. 4A, B). These decreases show indirectly the decrease in biomass concentration with filter depths, since the top layer (5-10 cm) of the SSF is normally removed during scraping. When estimating the time for the development of the *Schmutz*-aggregates an increase of 13 ng ATP/g sand per year would be necessary in order to reach an increase of 44 ng ATP/g sand after 3.4 years, assuming a linear increase. However, since unfortunately only two data points were available it might be that the maximum biomass was already reached faster. This was an inevitable limitation of studying a full-scale system, and the data should therefore be treated with

care. However, this suggests that in older filters, the *Schmutz*-aggregates develop faster than when a new filter is taken into operation (ca. 7 ng ATP/g sand per year, see 3.5). In addition, it is obvious that the *Schmutz*-aggregates in the old, freshly scraped filter (Fig. 4B, Filter C, 13 years) still contains 20 ng ATP/g sand, whereas the new filter (Fig. 3, Filter A, 0.1 year) does not contain any *Schmutz*-aggregate biomass (Fig. 4B). This clearly shows that the *Schmutz*-aggregates penetrate with filter age also into the depths of the filter and will never be completely removed by scraping (Fig. 4B). This is supported by the fact that in younger filters the biomass concentration correlates with the pressure loss of the filter, whereas filters older than ten years have to be scraped on a regular basis due to filter clogging (Supplementary information, Fig. S1). Although this necessitates more frequent scraping of old filters, it also guarantees faster recovery to “normal” operational conditions. Complementary to our data, a slight decrease in biomass with filter depths (below 50 %) were observed in the upper layer of covered SSF beds, receiving pre-treated water similar to the SSF of the present study (Campos et al., 2002; Mauclair et al., 2004). In contrast, Eighmy et al. (1992) studied the biomass distribution with filter depths in various covered SSFs, receiving untreated water and observed a drastic initial decrease of approx. 90 % in biomass in the upper five centimetres before the biomass distribution was stable across the whole filter bed (until 30-60 cm filter depths). The more drastic decrease observed there might be ascribed to higher DOC concentrations in the SSF influent and particularly to the fact that the water was not pre-treated (Eighmy et al., 1992). The amount of biomass in the filter should be related to the removal of biodegradable organic matter leading to microbial growth (Hammes et al., 2010a; Collins et al., 1992). Based on the literature data and the data of the present study, scraping would only remove a small fraction of the total filter biomass considering the dimension of a SSF.

### ***3.7. Predicting the total biomass in a SSF based on filter age and scraping age***

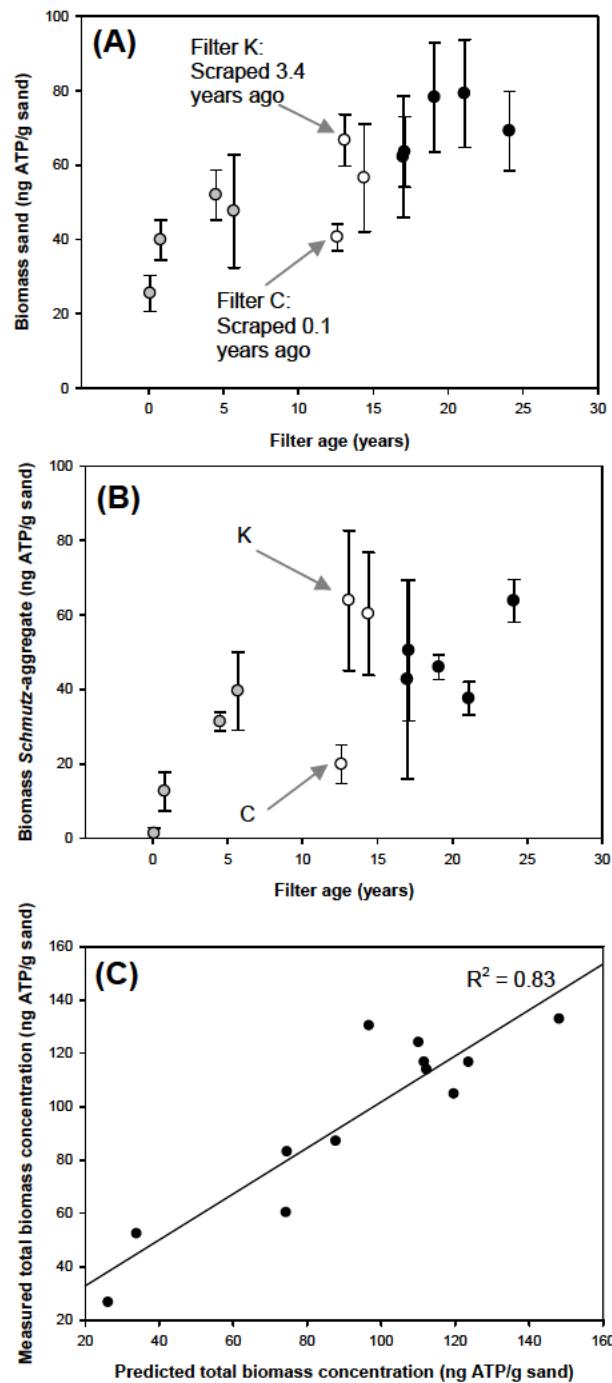
Based on the obtained data we developed a straightforward equation that estimates the total biomass concentration in the top filter layer (Fig. 3) of this particular system, based on filter age and scraping age. The measured biomass concentration of 25 ng ATP/g sand was used as the biomass concentration at time point zero (new filter). We assumed a linear increase in total biomass concentration of 11.5 ng ATP/g sand per year ((4.5 (sand) + 7 (*Schmutz*) ng ATP/g sand) and a decrease in total biomass of 55 % ((40 % (sand) + 70 % (*Schmutz*)) / 2) by scraping. For simplicity it was assumed, that the biomass develops after scraping in the same rate as with filter age and no differentiation between sand and *Schmutz*-aggregate development was made. We estimated the total biomass by the biomass growth before scraping (Eq. 1) minus the loss of biomass due to scraping (Eq. 2) plus the biomass growth in the years following the scraping (Eq. 3).

$$\frac{25 \text{ ng ATP}}{\text{g sand}} + \left( \frac{11.5 \text{ ng ATP}}{\text{g sand} \times \text{year}} \times \frac{\text{filter age}(\text{years}) - \text{scraping age}(\text{years})}{1} \right) \quad \text{Eq. 1}$$

$$0.55 \times (\text{Eq 1}) \quad \text{Eq. 2}$$

$$\frac{11.5 \text{ ng ATP}}{\text{g sand} \times \text{year}} \times \frac{\text{scraping age}(\text{years})}{1} \quad \text{Eq. 3}$$

By applying this equation on the actual filter and scraping ages of the 12 SSFs, we found a good correlation ( $R^2 = 0.83$ ) with the actual measured biomass concentrations (Fig. 4C). For filters that were never scraped, only Equation 1 was applied, and for filters that were already scraped several times, only the last scraping age was used. The clear correlation shows that the SSF biomass concentration is clearly influenced by both, scraping age and filter age.



**Figure 4.** Influence of filter age on the biomass development of the sand (A) and the *Schmutz*-aggregates (B) on filters that were never scraped (●), scraped only once (○) or scraped more often (●). The impact of scraping is shown by the arrows indicating two 13 year old filters that were scraped 0.1 (Filter C) and 3.4 years (Filter K) ago, respectively. The filters are labelled according to Table 1. The correlation between predicted biomass concentration and measured biomass concentration (C). Error bars represent the standard deviation of triplicate samples. All data were normalised to the sand dry weight of each sample.

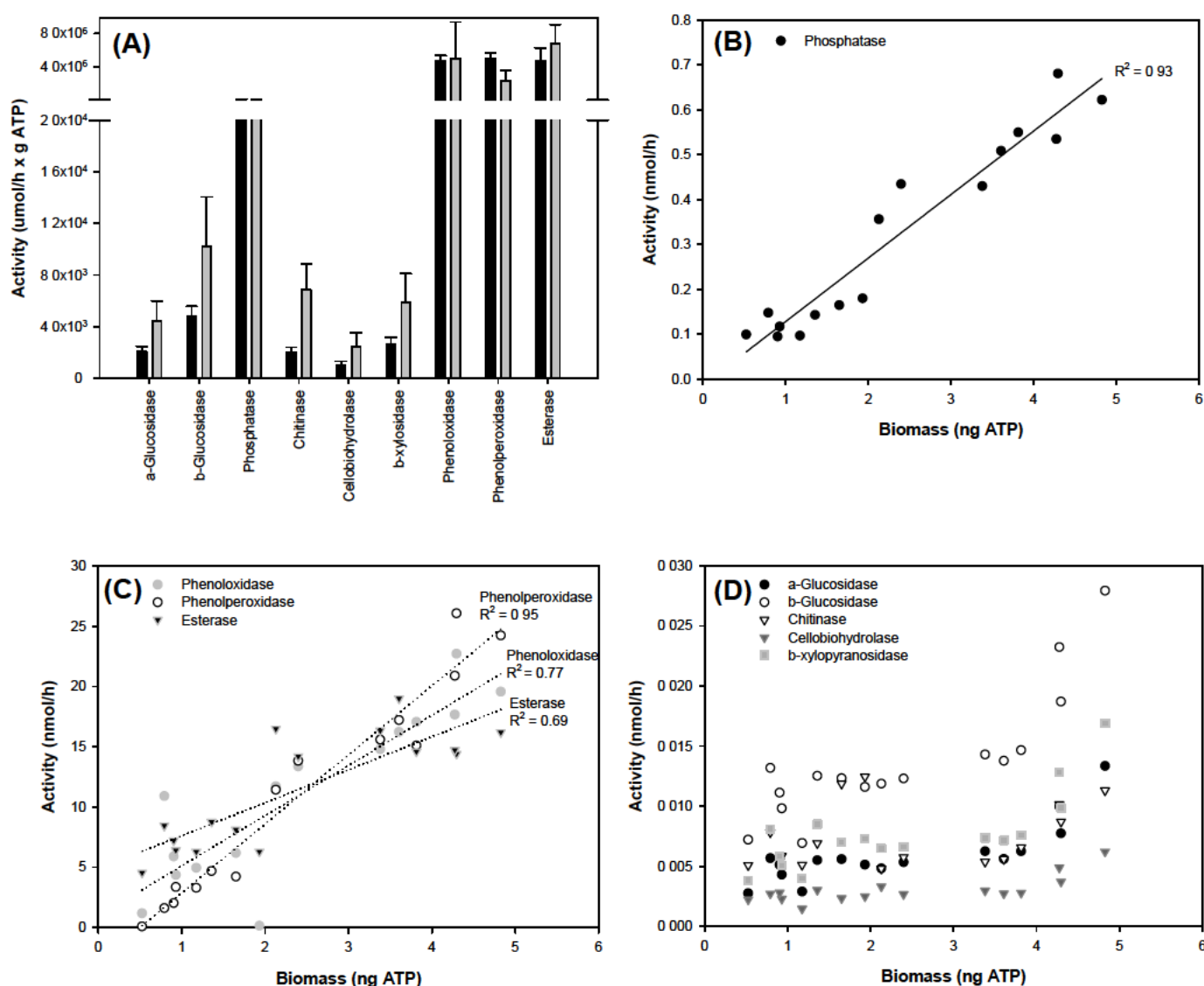
### 3.8. Enzyme activities

Enzyme activities from eight different SSF samples were measured by using fluorogenic methylumbelliferyl (*MUB*) substrates, which provide specific bonds for the naturally occurring enzymes to degrade. The fluorescent *MUB* is released during the degradation process and can be easily quantified. Hydrolytic extracellular enzymes that contribute to the degradation of polysaccharides such as cellulose or chitin were selected, including  $\beta$ -1.4-glucosidase,  $\alpha$ -1.4-glucosidase, chitinase, cellobiohydrolase and  $\beta$ -xylosidase (Sinsabaugh et al., 2003). Other extracellular- and intracellular occurring enzymes that were assayed were esterase, phosphatase, phenoloxidase and phenolperoxidase. Esterase activity is rather an indicator for general heterotrophic activity and does not yield information on the degradation of specific substances (Schnürer and Rosswall, 1982).

We normalized the measured enzyme activities to ATP to relate activity to biomass and analysed differences in activities between *Schmutz*-aggregates and sand samples. Generally, higher activities in the range of  $1.4 - 57 \times 10^5 \mu\text{mol}/(\text{h} \times \text{g ATP})$  were found for the esterase, phosphatase, phenoloxidase and phenolperoxidase compared to the relatively low activities in the range of  $1.7 - 4.5 \times 10^3 \mu\text{mol}/(\text{h} \times \text{g ATP})$  of the extracellular enzymes  $\alpha$ -glucosidase,  $\beta$ -glucosidase, chitinase, cellobiohydrolase and xylosidase (Fig. 5A). Activities of extracellular enzymes were slightly higher in the *Schmutz*-aggregates compared to the sand. This might be due to the accumulation of such enzymes in the exopolymeric substances of the *Schmutz*-aggregates. Compared to soil environments the measured enzyme activities are relatively low. In soil environments from a large range of ecosystems, specific enzyme activities in the range of 2 - 3000 nmol/(h x g soil) were found, when measured at temperatures between 15 and 20°C (Sinsabaugh et al., 2008). The activities in the SSFs of the present study were with 0.2 - 48 nmol/(h x g sand) relatively low. This is not surprising considering the low concentrations of organic carbon in the drinking water and also the low operational temperature of the SSFs at which the assay was performed.

A good correlation was found between ATP concentrations and the activities of enzymes that were highly active, namely phenoloxidase ( $R^2 = 0.77$ ), phenolperoxidase ( $R^2 = 0.95$ ), esterase ( $R^2 = 0.69$ ) and phosphatase ( $R^2 = 0.93$ ) (Fig. 5 B and C). Also activities for  $\alpha$ - and  $\beta$ -glucosidases, chitinase, cellobiohydrolase and xylosidase correlated positively with biomass concentrations (Fig. 5D). However, especially at low ATP concentrations, hardly any increase in activity was observed with increased biomass, which might be due to the low enzyme activities that were close to the detection

limit of the assay. In general, the correlations show that ATP is a good indicator for microbial activity and especially a good indicator for the biodegradation potential. Specific phosphatase and  $\beta$ -glucosidase activities were previously found to decrease in the water phase during passage of a coarse gravel filter before remaining stable throughout the whole SSF bed. The authors conclude that the decrease can be attributed to the depletion of easily degradable phosphorus and carbohydrates, which indicates that organic carbon is actively removed by bacteria during SSF (Hendel et al., 2001). The presence of the measured enzymes suggests clearly that the potential exist for the degradation of a broad range of organic carbon substrates in SSFs.



**Figure 5.** Average enzyme activities measured at 4°C in sand (dark) and *Schmutz*-aggregates (light) normalized to ATP (A). Error bars represent the standard deviations of eight samples. Correlation of ATP and enzyme activities measured separately for the sand and the *Schmutz*-aggregates (B, C, D). The standard deviations for all measurements were below 10 % ( $n = 4$ ).

### 3.9. Organic carbon removal

We further analysed whether the amount of biomass in the SSFs was related to the TOC removal in the filters (Table 1). The total TOC decreased on average from 1.07 mg/l to 0.79 mg/l during slow sand filtration. A trend of higher TOC removal at higher biomass concentrations was observed. In the filter with the smallest amount of biomass (27 ng ATP/g sand) the least TOC removal (0.17 mg/l) was observed, whereas in the filter with the most biomass (116 ng ATP/g sand) 0.4 mg/l TOC was removed. For a 4.5-year-old filter containing in total 80 ng ATP/g sand the TOC removal (0.3 mg/l) is already close to the maximum, which suggests that the maximum removal capacity is reached earlier than filter ripening. The removal of biodegradable organic carbon and dissolved organic carbon in SSFs was previously related to temperature (Welte and Montiel, 1996). At high temperatures (9 - 23 °C) a higher removal was observed than at temperatures below 8 °C. The authors stated that this is due to higher biological activity at high temperatures and that below 8 °C the removal was due to adsorption (Welte and Montiel, 1996). In the present study, TOC, biomass and enzyme activity measurements at 4 °C suggest that also at low temperatures (6 °C) organic carbon removal is due to biological activity and not solely due to adsorption.

### 3.10. Planktonic biomass concentrations

SSFs are commonly seen as a disinfection step that prevents microbial passage, before the water is distributed (Huisman and Wood, 1974). Several studies on pathogen removal during SSF suggest log scale reductions of pathogens including viruses, bacteria and protozoan oocysts during slow sand filtration (Hijnen et al., 2007; Weber-Shirk et al., 1998). However, this does not mean that the SSF effluent is sterile or even lower in bacterial numbers than the influent. We measured an increase in the total cell concentration from  $8.2 \times 10^4$  cells/ml in the SSF influent to  $1.13 \times 10^5$  cells/ml in the filter effluent (Table 1) resulting in an average increase of  $3.1 \times 10^4$  cells/ml. This increase can be due to influent bacteria passing through the SSF completely, together with continuous microbial growth and detachment from the filters. Alternatively it can be due to a complete turnover of cells, i.e. straining and die-off of influent bacteria, followed by regrowth of indigenous bacteria during filter passage. In the present study microbial growth in the filter was indicated by the microbial clusters in the microscopy images and the increase in filter biomass with time. A passage of bacteria through the SSF seems very likely considering that a significant fraction of natural freshwater bacterial communities, namely low nucleic acid bacteria (LNA) can pass through 0.45, 0.22 and 0.1 µm filters (Wang et al., 2007) and the nominal pore size of SSFs is considered to be considerable larger. Especially the passage of small LNA bacteria through the SSF

pores was suggested by the flow cytometric data, where a higher ratio of small LNA bacteria to large high nucleic acid (HNA) bacteria in the SSF effluent (LNA/HNA: 2.2) compared to the SSF influent (LNA/HNA: 0.8) was observed. This change was due to an increase of  $2.3 \times 10^4$  cells/ml in LNA bacteria and a decrease of  $1.4 \times 10^4$  cells/ml in HNA bacteria in the water during the passage through the filter. Furthermore, important predators for bacteria that can be present in the filters were found to favour large bacteria and to spare ultramicrobacteria (Boenigk et al., 2004). Hence, especially small bacteria should have a good chance to “survive” the treatment process.

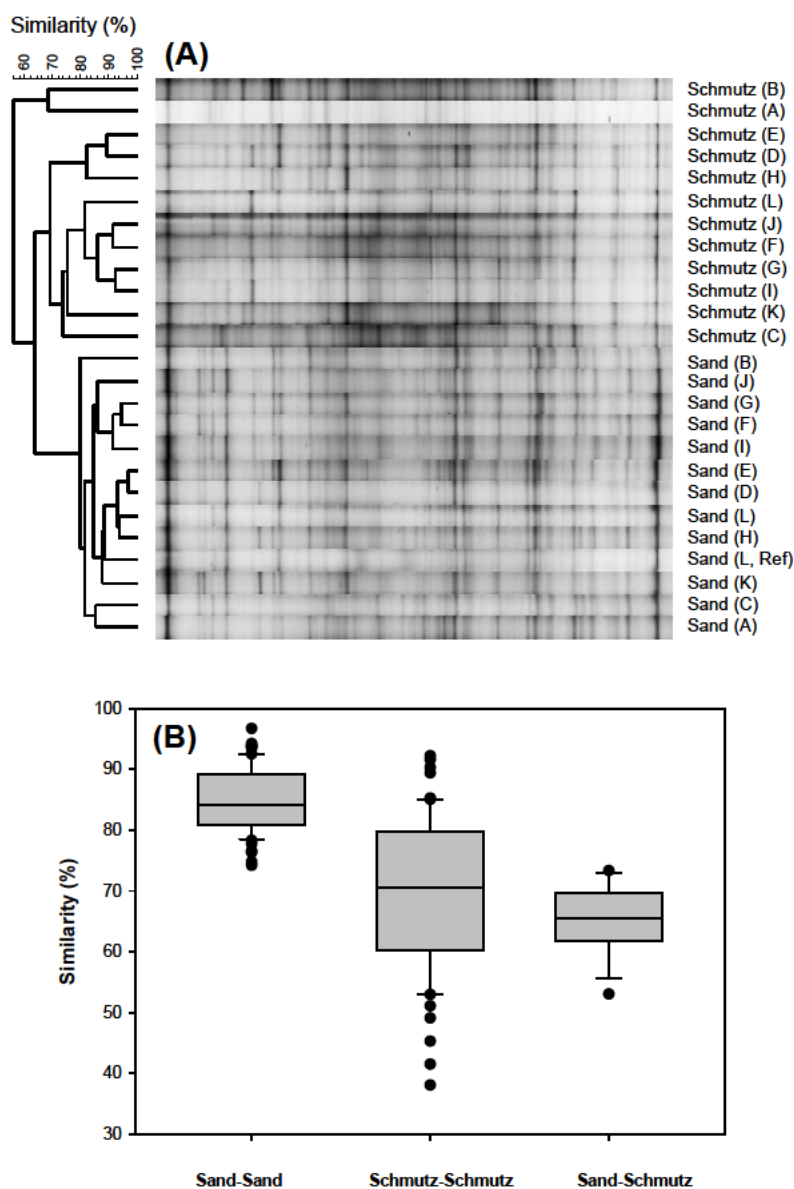
### ***3.11. Influence of filter age on the microbial community composition of sand and Schmutz-aggregates***

The microbial communities developing on SSF are of particular interest, firstly because they affect organic carbon removal and secondly because they are released into the final drinking water. Moreover, the filters are operated in excess of 20 years, thus allowing an interesting view on long-term ecological development. We analyzed the microbial communities from the sand and *Schmutz*-aggregates of each filter with molecular tools to explore the variation in community composition between the 12 SSFs operated in parallel. All parallel filters received as a consistent “inoculum” the same influent, whereas differences in filter age, scraping age, flow rates, filter history or other unknown factors could influence the microbial community composition. In the latter cases one would expect considerable variations in the microbial community composition, whereas one would predict only little or no differences if the water quality was the main factor determining microbial community composition.

The microbial community composition was analyzed by DGGE, which shows bacteria present more than 1 % in a sample (Muyzer et al., 1993). Clustering by Pearson and UPGMA was used to compare the DGGE patterns from all samples. Note that all samples that show more than 90 % similarity can be regarded as equal. The microbial community on the *Schmutz*-aggregates was slightly different compared to the microbial community on the sand samples. This can be clearly seen in the two separate clusters of sand and *Schmutz*-aggregates (Fig. 6A). However, the similarity between the clusters is relatively high (almost 60 %) and by comparing the band patterns it is evident that most bands present in the sand were also present in the *Schmutz*-aggregates. The changes were mainly due to more species present in the *Schmutz*-aggregates compared to the sand. This was also reflected in the higher richness (number of bands) in *Schmutz*-aggregate samples ( $44 \pm 4$ ) than in sand samples ( $30 \pm 5$ ). The difference might be due to the fact that a new SSF contains already 30 % of biomass on the sand (Fig. 3), whereas the biomass in the *Schmutz*-

aggregates only developed with increasing filter age. Hence the microbial community composition of the *Schmutz*-aggregate biomass might be more influenced by seasonal and time- dependant changes than that of the sand microbial community.

The “sand” microbial community from the 12 filters was very similar, with 75 % of the samples in the range of 80 - 90 % similarity (Fig. 6B). For comparison, the similarity of a replicate sample was 85 %. This suggests that due to the same water quality running over the filters, the same microbial community developed and operational parameters hardly influenced the microbial community composition. Quantitatively, the biomass concentration on the sand also changed little in time, and was less affected by scraping and filter renewal than the biomass in the *Schmutz*-aggregates (Fig. 4A, B). No correlation between changes of the microbial community composition and filter scraping occurred, which further suggests, that the microbial community composition of the sand does not change with filter depths. More variability was found when comparing the *Schmutz*-aggregate communities towards each other, from which 75 % of the samples were in the range of 60 - 80 % similarity (Fig. 4B). It might be possible, that such variations are due to slight variations over time in the microbial community composition of the influent and microbes getting trapped in the exopolymeric substances of the *Schmutz*-aggregates. In this case the scraping age should influence the microbial community composition in the *Schmutz*-aggregates. This might also explain the differences observed between sand and *Schmutz*-aggregates samples from the same filter. Lower similarities (53 - 73 %) were observed when comparing each sand sample to its respective *Schmutz*-aggregate sample (Fig. 6B). However, the observed variations were too small to be linked to the amount of biomass, scraping age or age of the filters. To conclude, the variations in the composition of the sand and the *Schmutz*-aggregate biomass were only small and hence suggest no major differences in the functioning with respect to organic carbon removal of sand and *Schmutz*-aggregates and between the parallel operated filters.



**Figure 6.** Cluster analysis of denaturing gradient gel electrophoresis (DGGE) patterns from 12 SSFs (A-L, Table 1) from the total community of sand and *Schmutz*-aggregates (= *Schmutz*) samples. Sand sample L was run on both of the two separate gels and used as a reference for clustering (A). Similarities of the bacterial community composition (in %) between all sand samples (sand-sand;  $n = 66$ ); all *Schmutz*-aggregate samples (*Schmutz* – *Schmutz*;  $n = 66$ ) and the similarities from each sand sample to its respective *Schmutz*-aggregate sample (Sand-*Schmutz*;  $n = 12$ ) are shown (B). Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values ( $n = 10$ ).

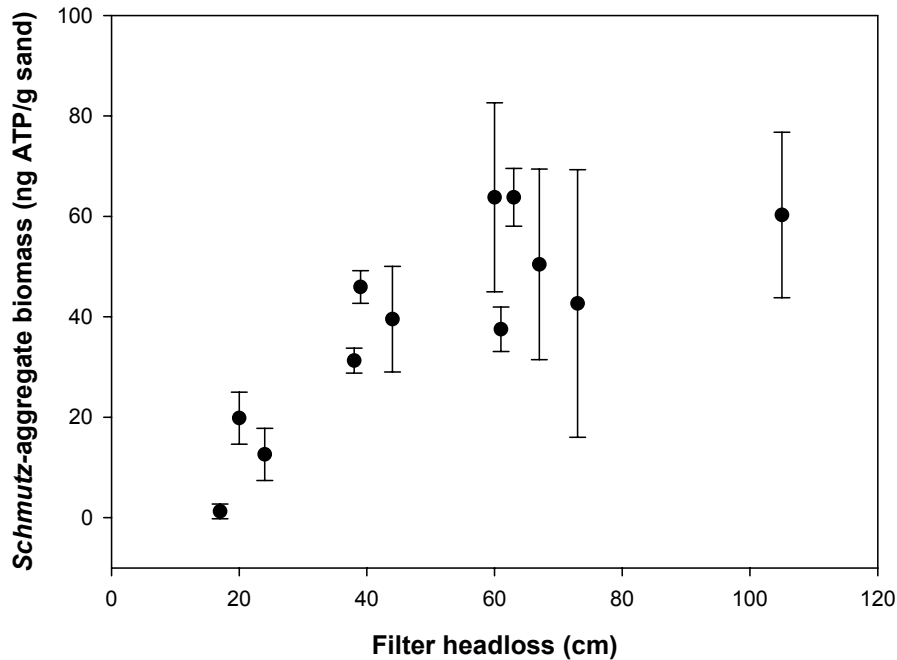
#### **4. Conclusions**

- The results elucidate the impact of operational practices on the biomass distribution and microbial ecology of SSFs, which might influence the microbial community composition of the final drinking water, in case the water is distributed without disinfection.
- Enzyme activities for organic carbon removal correlate strongly with ATP. The higher the ATP content the higher the potential for organic carbon removal in a filter should be.
- Hardly any differences in the microbial community composition between the different filters were observed, suggesting that the microbial community composition was hardly influenced by filter age or scraping. This implies that the water quality of the water inflow as the most constant factor dictated the microbial community composition in the filters.

#### **Acknowledgements**

The authors would like to thank Jakob Helbing for help during sampling, Hans-Ulrich Weilenmann and David Kistler for technical support and Aline Frossard for support with the enzymatic assays. We are grateful to the financial support of the EU project TECHNEAU (018320) research grant.

## Supplementary Information

1. Influence of *Schmutz*-flock biomass on filter headloss

**Figure S1.** Filter headloss in relation to the *Schmutz*-aggregate biomass. Exopolymeric substances (EPS) in the *Schmutzdecke* were previously found to cause filter clogging (Mauclaire et al., 2004). Hence it is questionable whether the maximal biomass in the *Schmutz*-aggregates is required for optimal filter performance. In our study the biomass concentration in the *Schmutz*-aggregates correlated with filter headloss until a biomass concentration of 40 ng ATP/ g sand was reached. A *Schmutz*-aggregate biomass of 30 ng ATP/g sand reached after 4.5 years only led to a pressure loss of 40 cm and was still acceptable for filter performance, whereas at higher biomass concentrations pressure losses increased up to 100 cm before the filters got finally scraped. However, not only the biomass concentration can account for the pressure loss, since no correlation between pressure loss and biomass in the *Schmutz*-aggregates was found in filters containing between 40 and 60 ng ATP/g sand in the *Schmutz*-aggregates. In those filters, the pressure loss might then rather depend on the scraping age, since more *Schmutz*-aggregate and EPS content should also increase the accumulation of particles and consequently the increase in pressure loss. In addition, the accumulation of particles in the depths of the SSF bed can contribute to the filter headloss.

#### 4. A multi-parameter approach towards assessing biological stability in drinking water distribution networks

##### Abstract

Biological stability refers to the inability of drinking water to support microbial growth, which implies that the concentration and composition of microbial cells do not change during water distribution. In the present study, we monitored several parameters related to biological stability in water samples with varied retention times (0-52 h) of a non-chlorinated drinking water distribution network for a period of four months, with repetition two years later. Remarkably stable total cell concentrations (TCC) of  $(1.0 \pm 0.15) \times 10^5$  cells/ml in water samples from the reservoir and throughout most of the distribution network were measured with flow cytometry. This stability was also observed in the concentration of adenosine tri-phosphate (ATP), heterotrophic plate counts (HPC), dissolved organic carbon (DOC) and assimilable organic carbon (AOC). The microbial communities analysed with denaturing gradient gel electrophoresis (DGGE) and 454 pyrosequencing showed more than 80 % similarity at these sampling locations and during the time period of two years. At two sampling locations having the longest water retention times, a slight but significant increase in the TCC to  $1.3 \times 10^5$  cells/ml was observed. This increase was reflected by a clear shift in the microbial community profiles and specifically by an increase from 2 to 60 % in the relative abundance of members belonging to the family of *Comamonadaceae*. These findings suggest that planktonic cell enumeration can be complemented with microbial community analysis when assessing biological stability in drinking water distribution systems.

## 1. Introduction

*“Water entering the distribution system must be microbiologically safe and ideally should also be biologically stable”* (WHO, 2006). To ensure these requirements are achieved, source water protection and multiple barriers for pathogens are established in the water treatment train for maintaining a good water quality in the distribution system. In many countries disinfectants are added to the water in order to maintain a residual disinfectant as a protection against low-level contamination and growth (WHO, 2006). Alternatively, in some European countries such as Switzerland, The Netherlands, Austria, and Germany, the aim is to distribute a high quality “biologically stable” water, in which microbial growth is restricted by the availability of growth supporting nutrients (Hammes et al., 2010a; Hambsch 1999; van der Kooij et al., 1999).

Irrespective of the distribution philosophy, appropriate methods are needed to monitor the biological stability in drinking water systems. Measuring the microbial growth potential of a water sample is one way to measure biological stability. Different methods such as assimilable organic carbon (AOC) measurements, biofilm formation rate (BFR) assays, biodegradable dissolved organic carbon (BDOC) and pathogen growth potential (PGP) assays were developed for this purpose (Vital et al., 2010; Laurent et al., 2005a; Escobar and Randall 2001; van der Kooij 2000; Hammes and Egli, 2005). These parameters in some cases have been related to the degree of growth observed in drinking water distribution systems and recommendations for “biologically stable” water were suggested for AOC ( $< 10 \mu\text{g/l}$ ) and BDOC ( $< 100 \mu\text{g/l}$ ) concentrations (Laurent et al., 2005c; van der Kooij, 2000). Nevertheless, during drinking water distribution, multiple factors such as piping material, flow velocity, biofilms, or in the worst case the intrusion of contaminated water from subsurface material, might influence the microbial composition of the water (Laurent et al., 2005b, 2005c; WHO, 2006). Neither of the above mentioned growth potential methods is likely to detect or predict such events. Also, the sensitivity of organic carbon assays is generally low, since a considerable fraction of organic carbon (ca. 90 %) is not easily degradable, thus masking small changes caused by consumption due to bacterial growth.

Biological stability implies that neither cell concentration, nor activity and nor microbial community composition changes in the water during distribution. The direct approach to assess these changes is to sample a network at spatially separated locations and to compare the quality of the water samples directly with each other. Water utilities routinely use only the heterotrophic plate count (HPC) method for this purpose, although it is well known that the HPC usually represents less than 1 % of the total microbial population (van der Kooij et al., 2003; Siebel et al., 2008). Only few

studies addressed biological stability *in situ* with advanced methods such as measuring total cell counts or ATP concentrations in non-chlorinated drinking water systems (van der Wielen and van der Kooij, 2010; Hammes et al., 2010a). In some studies, also DNA fingerprint methods such as denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) were used to assess the composition of the microbial community in drinking water distribution systems (Burtscher et al., 2009; Eichler et al., 2006). However, these studies focused on the analysis of the composition and did not address the question of biological stability. Moreover, for in depths analysis of the microbial community composition with traditional Sanger-sequencing of the 16S rRNA gene, clone libraries needed to be constructed in order to obtain sequence data. This process can be labor-intensive when the sample contains a high biodiversity in which a large number of sequences needs to be analyzed. For this reason, sequence identification was often restricted to a limited number of samples and, to our knowledge, only in few studies a large amount of 16S rRNA gene sequences were analyzed from water samples of chlorinated networks (Revetta et al., 2010; Eichler et al., 2006). Today, new generation sequencing methods, e.g. pyrosequencing, offer the possibility to directly sequence all 16S rRNA genes obtained from a sample, which allows the generation of thousands of sequences at once and the sequencing of numerous samples in parallel. Using the pyrosequencing method, Hong and co-workers (2010) showed that diversity in the drinking water distribution systems is higher than previously expected. The assessment of multiple parameters, such as total cell counts combined with community composition analysis, would give a more comprehensive overview of possible changes that might occur during drinking water distribution.

In this study, the reservoir directly after treatment and six spatially separated locations in the distribution network of Zürich (CH) containing non-chlorinated water with varied hydraulic retention times (6 - 52 hours) were sampled during four months and again two years later to assess the long-term stability of the distribution system. The total cell number (and viability) of planktonic cells were monitored with flow cytometry (FCM), the HPC and the concentration of ATP, while the composition of the microbial communities were analyzed with DGGE and 454 pyrosequencing. DOC and AOC concentrations were also measured to detect changes in organic carbon concentrations. This multi-parametric approach to monitor biological stability in a drinking water distribution network should reveal not only quantitative changes in the microbial cell concentration, but also possible changes in microbial community composition that might occur during distribution.

## 2. Materials and Methods

### 2.1. Sampling

Water was sampled from the full-scale distribution network of Zürich (CH) receiving non-chlorinated drinking water produced from a multi-step drinking water treatment facility (Hammes et al., 2010a). The reservoir and six points of the connected distribution network, containing water with increasing hydraulic retention times (6 - 52 hours) were sampled six times in the time period from November 2008 until February 2009 and once in September 2010. Water samples from the network were obtained from public fountains where water flowed continuously. Water samples were collected in sterile and carbon-free Schott bottles (2 l). An additional sample of 500 ml was taken for the analysis of water chemistry (Table 1). The temperature was measured directly at each sampling location. All samples were processed within one day, while samples for DGGE and pyrosequencing were filtered (2 l) and stored at -20 °C until further analysis.

**Table 1.** Chemical water quality parameters measured in the drinking water network. Average values and standard deviations from six measurements are indicated for each sampling point.

| Number | Retention-time (h) | Temperature (°C) | Nitrate (mg N/l) | Conductivity (µS/cm) | pH        | Alkalinity (mmol/l) | o-P (µg P/l) |
|--------|--------------------|------------------|------------------|----------------------|-----------|---------------------|--------------|
| (1)    | 0                  | 7.1 ± 0.8        | 0.9 ± 0.4        | 271 ± 6.3            | 7.0 ± 0.2 | 2.7 ± 0.0           | 8.3 ± 4.4    |
| (2)    | 6                  | 7.6 ± 1.0        | 0.9 ± 0.3        | 273 ± 5.6            | 7.0 ± 0.2 | 2.7 ± 0.0           | 10.7 ± 6.4   |
| (3)    | 27                 | 7.2 ± 1.4        | 0.9 ± 0.6        | 273 ± 6.4            | 7.0 ± 0.2 | 2.7 ± 0.1           | 8.5 ± 4.8    |
| (4)    | 29                 | 7.3 ± 1.5        | 0.9 ± 0.4        | 271 ± 4.6            | 7.0 ± 0.2 | 2.7 ± 0.0           | 8.8 ± 4.4    |
| (5)    | 44                 | 7.7 ± 1.1        | 1.0 ± 0.7        | 272 ± 6.1            | 7.0 ± 0.2 | 2.7 ± 0.1           | 8.7 ± 4.1    |
| (6)    | 49                 | 7.3 ± 1.4        | 0.9 ± 0.5        | 272 ± 4.2            | 7.2 ± 0.3 | 2.7 ± 0.0           | 8.9 ± 4.7    |
| (7)    | 52                 | 7.6 ± 1.1        | 0.9 ± 0.4        | 273 ± 5.0            | 7.0 ± 0.2 | 2.7 ± 0.0           | 8.9 ± 4.3    |

### 2.2. Adenosine tri-phosphate (ATP) analysis

Total ATP was determined using the BacTiter-Glo™ reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA) as described elsewhere (Hammes et al., 2010b). Simultaneously a water sample (500 µl) and the ATP reagent (50 µl) were warmed (1 min) to 38 °C in separate sterile Eppendorf tubes. The sample and the reagent were then combined and the luminescence was measured after 20 sec. reaction time at 38 °C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). Cellular ATP and free ATP were distinguished by

filtering each sample through a 0.1 µm sterile syringe filter (Millex®-GP, Millipore), and then repeating the analysis described above. ATP was measured in triplicate for all samples, and the standard deviation of the measurement was below 4 %.

### ***2.3. Intact and total cell counts***

Flow cytometric analysis of the total and intact cell concentrations was done as described in Berney et al. (2008). Briefly, bacterial cells were stained with 10 µl/ml propidium iodide (PI) in combination with SYBR® Green I (SG/PI) to measure membrane-intact cells. Working solutions of the dyes were prepared as follows: SYBR® Green I was diluted 100-fold in anhydrous dimethylsulfoxide (DMSO) and mixed with PI (0.6 mM final concentration). Samples were then incubated in the dark for at least 15 minutes before measurement. Samples were diluted 10-fold just before measurement in filtered (0.1 µm; Millex®-GP, Millipore), cell-free bottled mineral water (EVIAN, France), so that the concentration measured with the flow cytometer was always less than  $2 \times 10^5$  cells/ml. Flow cytometry was performed by using a portable CyFlow SL flow cytometer (Partec, Hamburg, Germany) equipped with a 20 mW solid state laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware.

### ***2.4. Heterotrophic plate counts (HPC)***

The HPC method was performed with R2 agar (R2A) since it was observed previously that R2A gave more colony counts than conventional plate count agar (PCA), which is recommended in the Swiss guidelines for drinking water (Uhl and Schaule, 2004; SLMB 2000, 56, E.1). In short, 1 ml of the water sample was transferred to a sterile Petri dish and mixed with about 15 ml warm R2A, (Oxoid, Cambridge, UK). The agar was kept at 42 °C before use. The plates were incubated at 30 °C for 7 days and then colony forming units (CFU) were counted manually. All measurements were done in triplicate.

### ***2.5. Microbial community analysis***

#### ***2.5.1. Denaturing gradient gel electrophoresis (DGGE)***

DNA was extracted with the DNA water Isolation Kit (UltraClean™ Water DNA Isolation Kit, MoBio, Carlsbad, CA, USA) following the provided instructions. Each water sample (2 l) was filtered on 0.2 µm filters (MoBio) and stored at -20 °C until further processing. DNA was first

amplified using the general bacterial primers 63F and 1378R followed by amplification with the primers 338F-GC and 518R (Heuer et al., 1997; Muyzer et al., 1993). PCR amplification and DGGE was performed as described elsewhere (Lautenschlager et al., 2010). DGGE gel images were analyzed using the Bionumerics 5.1 software (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation, taking into account band intensity and band position. The clustering algorithm of unpaired pair group method (UPGMA) using arithmetic averages was used to calculate dendrograms.

### ***2.5.2. 454 Pyrosequencing***

DNA from biomass obtained on 0.2 µm filters was extracted using Schmidt's protocol (Schmidt et al., 1991). The 16S rRNA gene was PCR-amplified in 50 µl reaction volumes in S1000 Thermal Cycler (BioRad, Hercules, CA, USA) using Bullseye standard Taq DNA polymerase 2.0 x master mix (MIDSCI, St. Louis, MO, USA) according to the manufacturer's protocol with the following universal primers that have Roche standard barcodes 1) FA-MIDs-515F and FB-909R and 2) FB-MIDs-515F and FA-909R (Wang and Qian, 2009). PCR was carried out with the following parameters: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 40 s, 56 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were detected on 1.5 % agarose gel electrophoresis and the correctly sized DNA bands were excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, St. Louis, MO, USA) according to manufacturer's instructions. Equal amounts of purified PCR products were pooled for subsequent 454 pyrosequencing on the Titanium platform (Roche/454 Life Sciences, Switzerland) at the W.M. Keck Center, part of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

### ***Data Analysis***

The pyrotags were sorted by barcodes (MIDs) and the sequences were analyzed following the Ribosomal Database Project pyrosequencing pipeline (<http://pyro.cme.msu.edu/>; Cole et al., 2009). First the sequences were trimmed using the initial data processing tool with default parameters (max number of N's = 0 and minimum average quality score = 20) using forward and reverse primer sequences. The trimmed sequences were aligned via RDP aligner and the sequences were subjected to RDP complete linkage clustering. The clustered sequence data was then used to estimate microbial community diversity based on the number of OTUs, Shannon-Weaver (H') index, and Chao 1 estimator with RDP analysis tools at a cut-off value of 97 % sequence similarity. RDP

classifier was used for taxonomic assignments of the sequences at 95 % confidence level (Cole et al., 2009). The total sequences were combined and representative sequences were sorted via RDP dereplication tool (maximum distance = 5). The resulting sequences were again subjected to RDP classifier. The sequences in the unclassified bacterial group were extracted and aligned via Greengenes alignment tool ([http://greengenes.lbl.gov/cgi-bin/nph-NAST\\_align.cgi](http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi)). The resulting alignment file and Greengenes' 16S rRNA gene database were input into ARB software package for phylogenetic tree construction.

### 3. Results and Discussion

#### 3.1. Sampling location and retention times

We have sampled the reservoir of the drinking water treatment plant and six locations within the distribution system with estimated water retention times ranging from 0-52 h that received water from this plant. The sampling locations were selected based on advice from the local water utility, taking into account the flow of water and pipe diameters in the system. The water pipes in this network were made of steel, grey irons, polyethylene, cement, ductile cast iron pipe coated, and ductile cast iron pipe cement mortar. However, the different materials were randomly distributed in the network and thus could not easily be linked to any of the measured parameter. Samples were taken between November 2008 and February 2009, and the sampling was repeated in September 2010. The origin of the water was confirmed by conductivity and water chemistry parameters (e.g., nitrate, pH, alkalinity and phosphate) that would vary between different water sources (Table 1). All water chemistry parameters were similar at all sampling locations and during the whole sampling period (Table 1).

#### 3.2. Planktonic cell concentrations and activity

##### 3.2.1. Flow cytometry (FCM) and heterotrophic plate counts (HPC)

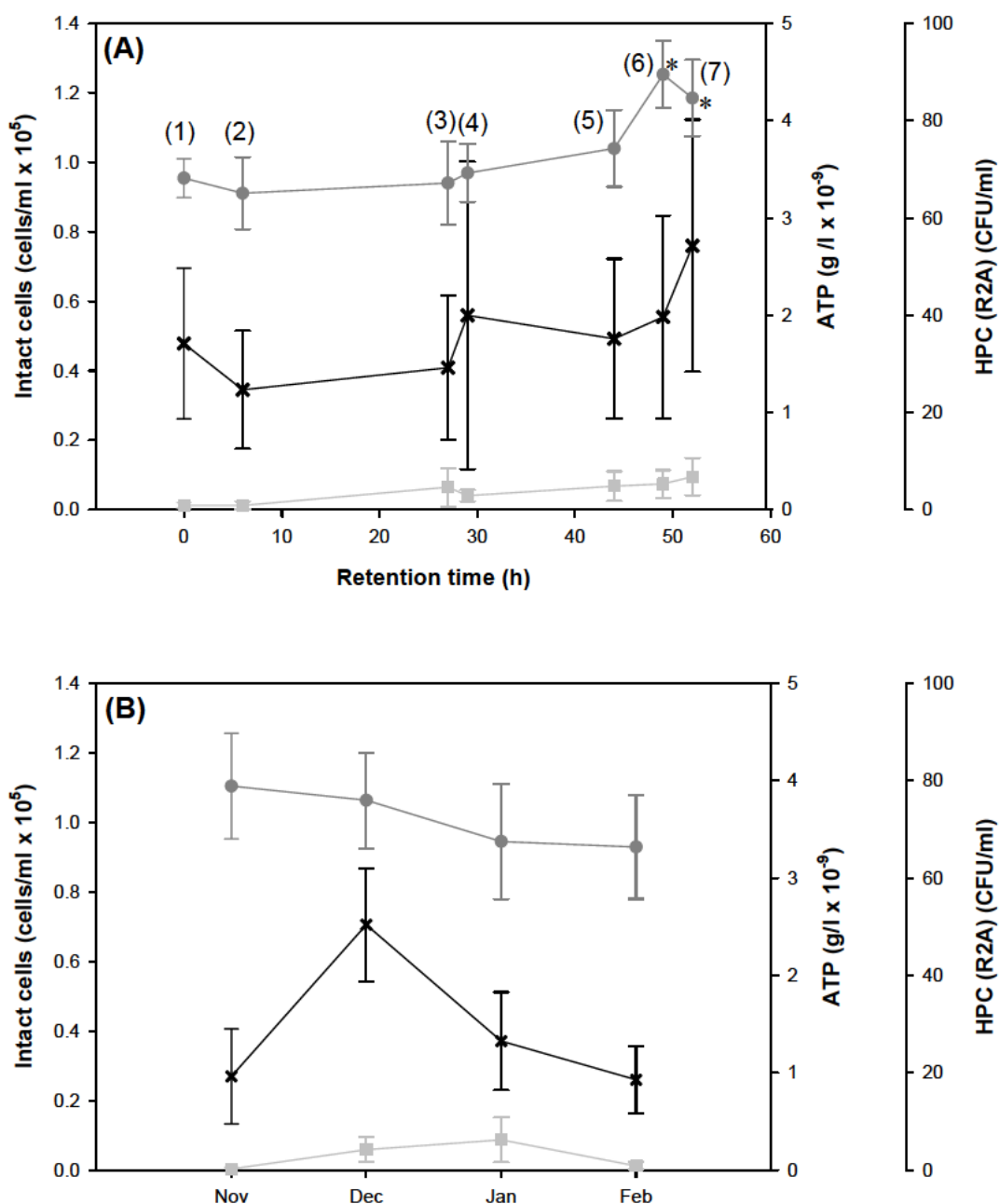
Planktonic cell concentrations in the range of  $10^3$ - $10^5$  cells/ml are usually found in drinking water (Hammes et al., 2010a; Burtscher et al., 2009; Hoefel et al., 2005a, b). FCM analysis of intact cells was used to analyze the biological stability with respect to microbial cell concentrations. Directly after treatment, the drinking water in the reservoir contained  $(0.95 \pm 0.06) \times 10^5$  intact cells/ml ( $n = 6$ ). With increasing water residence time this cell concentration initially remained constant during distribution. However, at the two highest water retention times (49 and 52 h), intact cell

concentrations were slightly but significantly ( $p < 0.05$ ,  $n = 6$ ) increased to  $(1.27 \pm 0.09) \times 10^5$  and  $(1.21 \pm 0.11) \times 10^5$  cells/ml, respectively (Fig. 1A). This low level of biological instability could only be linked to the water retention time in the network; no obvious relation to pipe material, pipe diameter or any other parameter was found. Also, this increase was a relatively small change (ca. 20-30 %) compared to a 2-5 fold increase in total cell concentration that was previously observed in household installations during overnight stagnation and with increasing residence times in unstable drinking water distribution systems in Canada and France (Lautenschlager et al., 2010; Laurent et al., 2005c). During the four months, and even two years later, no substantial changes in intact cell concentrations were observed at the individual locations, suggesting a very high biostability of the treated water (Fig. 1B). HPC (R2A) were below 10 colony forming units (CFU)/ml at all sampling locations and times, and did only increase slightly with water retention time (Fig. 1A, B). R2A has been previously found to give a considerably higher HPC result than conventional plate count agar (PCA) (Uhl and Schaule, 2004; Reasoner and Geldreich, 1985). The authors state that a method yielding higher counts is better suited to detect upcoming growth than a method yielding lower counts (Uhl and Schaule, 2004; Reasoner and Geldreich, 1985). However, only bacteria that can utilize the respective culturing media and grow at the given incubation temperature can be analyzed with HPC (Sartory et al., 2004). The chance of measuring a change in cell concentrations during distribution should increase with the amount of species that can be detected. Total cell concentration measurements with FCM thus have clear benefits, since all bacteria are detected irrespective of their cultivability (Hammes et al., 2010a). Of course, small changes can be masked by the general high abundance of bacteria, but our results showed that a 20 % change in cell concentration can be clearly detected.

### **3.2.2. Adenosine tri-phosphate (ATP) analysis**

Bacterial cell-bound ATP concentrations were determined to assess changes in viable biomass and/or microbial activity. Bacterial ATP concentrations were stable at  $(1.8 \pm 1.0) \times 10^{-9}$  g/l ATP at all sampling points, except for a slight increase at 52 h water retention time (Fig. 1A). The high standard deviation was caused by a 2-fold higher ATP concentration measured in December (Fig. 1B). The percentage of extracellular ATP was on average  $42 \pm 8$  % of the total ATP, which emphasizes the importance of including free ATP into the analysis when measuring drinking water samples. The change in intact cell concentrations detected after 49 h water retention time was not reflected in ATP concentrations (Fig. 1). This might be due to the lower sensitivity of ATP measurements compared to FCM when assessing small changes. An increase of  $2 \times 10^4$  cells/ml against a background of  $1 \times 10^5$  cells/ml is close to the detection limit of the ATP method.

Particularly small cells with low nucleic acid (LNA) content (so-called “LNA bacteria”) were previously found to contain very low amounts of ATP per cell and growth of such bacteria would hardly lead to a detectable increase in ATP (Hammes et al., 2010b; Wang et al., 2009). In addition, differences in the activity, size, and type of individual cells influence the total ATP content of a water sample thus making it difficult to relate ATP concentrations to cell concentrations (Hammes et al., 2010b; Karl, 1980). In a recent study in the Netherlands, the effect of distance to the treatment plant on ATP concentrations was analyzed (van der Wielen and van der Kooij, 2010). The authors found varying ATP concentrations (both increases and decreases) in water sampled at a short-, middle-, and long- distance from the treatment plant with some seasonal variation. On household level a clear increase in ATP concentration (2-8 fold) after overnight stagnation was measured previously (Lautenschlager et al., 2010; Siebel et al., 2008). These results suggested that the determination of ATP is suitable to analyze relatively large changes in cell concentration/activity occurring during drinking water distribution, whereas small changes would hardly be detected. Overall a high biostability with respect to intact cell concentrations, ATP and HPC was observed. Only at two sampling locations, a slight but significant change was observed in intact cell concentrations.



**Figure 1.** Changes in intact cell (●), adenosine tri-phosphate (ATP) concentrations (x) and heterotrophic plate counts (HPC) (■) during drinking water distribution sorted by increasing water retention times from November 2008 until February 2009. Samples are numbered from (1) - (7) according to Table 1. Error bars indicate standard deviations from six measurements. A paired student t-test (\* $P < 0.05$ ,  $n = 6$ ) was used to calculate changes for each network point to the reservoir **(A)**. Average values of samples (1) - (7) from each month with error bars indicating standard deviations from seven sampling locations **(B)**.

### **3.2.3. Total organic carbon and assimilable organic carbon (AOC) analysis**

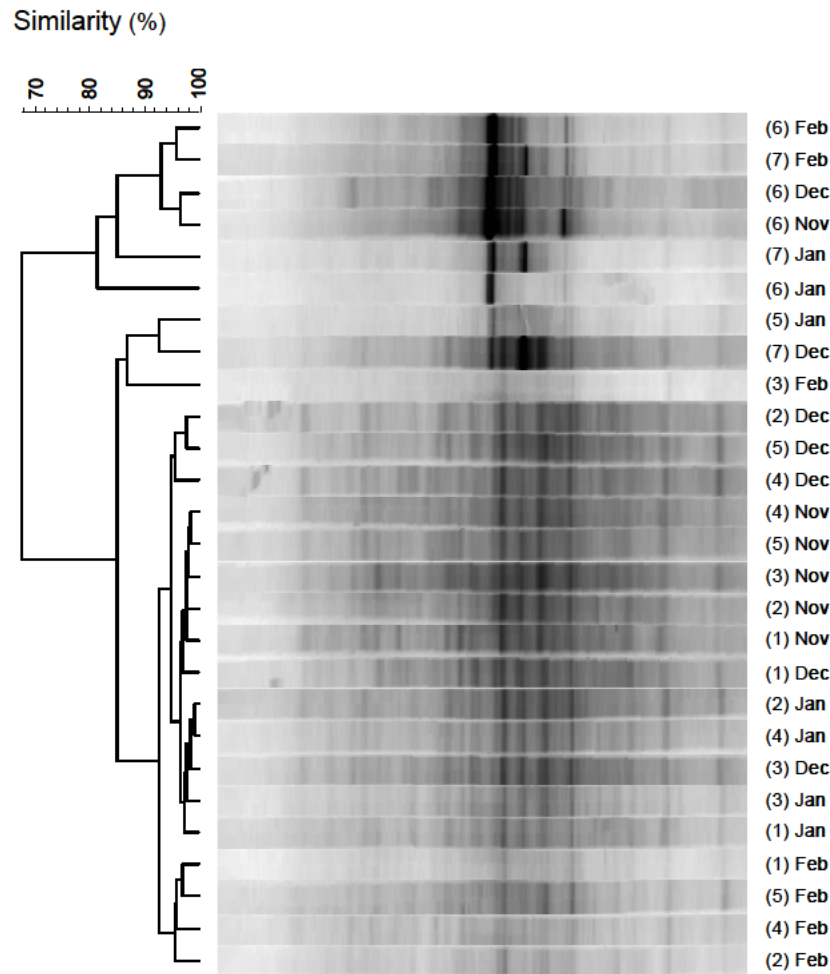
An increase in cell concentration should be reflected by a decrease in carbon in case of heterotrophic growth. For example, an increase in cell number in the water phase was previously attributed to the biodegradation of organic matter in the biofilm that leads to microbial growth and subsequent release of bacteria (Block, 1992), and in a more recent study also to planktonic growth in the water phase (Boe-Hansen et al., 2002). Assimilable organic carbon (AOC) concentrations in all the water samples were below the detection limit (10 µg/l) and consequently the increase in intact cell concentration could not be linked to a decrease in AOC. The total organic carbon concentration (TOC) was on average  $0.88 \pm 0.032$  mg/l, and no measurable change in TOC was detected in samples with varied water retention times and sampling date. The observed increase of  $2 \times 10^4$  cells/ml (Fig. 1) would correspond to a decrease of only 2-20 µg/l organic carbon (Hammes et al., 2010a), which is too small to be detected by either the AOC or TOC method. These results suggest that organic carbon measurements are not useful to detect changes in a drinking water distribution system, where only small changes in nutrient concentrations and microbial growth are expected. However, it was previously shown that organic carbon degradation (in the form of BDOC) can also be used as a parameter for biological stability in unstable drinking water (BDOC = 200-700 µg/l), but this parameter can not be applied when BDOC concentrations are low (Laurent et al., 2005c).

### **3.3. Microbial community composition analysed with DGGE and pyrosequencing**

The microbial community composition of the water from all sampling locations was compared in order to assess possible changes during distribution. Despite that cell concentrations remained rather stable in the bulk water, one could argue that changes in community composition might be caused by microbial growth or turnover as a result of continuous attachment and detachment of bacteria to and from the pipes. Piping materials were reported to have an impact on the development of microbial communities in biofilms and subsequently on the planktonic microbial cells in the bulk water (Lehtola et al., 2004). The water pipes in the distribution network of the present study were composed of various materials ranging from steel and cement mortar to polyethylene.

### ***3.3.1. Denaturing Gradient Gel Electrophoresis (DGGE)***

DGGE analysis of all samples revealed that the microbial community composition directly present after drinking water treatment remained very stable in the distribution network with respect to water retention time and over the entire sampling period of four months. Almost all samples from locations 1-5 (Fig. 2) showed more than 90 % Pearson similarity towards each other when the clustering method of unpaired pair group method (UPGMA) was applied. By using the same clustering method, Burtscher and coworkers (2009) found similarities between 85 to 95 % when analyzing replicate water samples and water from the same sampling location with varying water volumes. Thus, it can be assumed that microbial communities with a similarity of 85 % or higher can be regarded as having the same composition. However, the samples derived from the two distant sampling locations having 49 and 52 h retention time clustered separately (Fig. 2). The change was due to differences in the presence and abundance of 1-2 dominant bands, which suggests preferential growth of a few particular species. Again, no obvious relation between these changes in community composition and the pipe material were found. Interestingly, the change in microbial community composition correlated with the increase in intact cell concentration (Fig. 1).

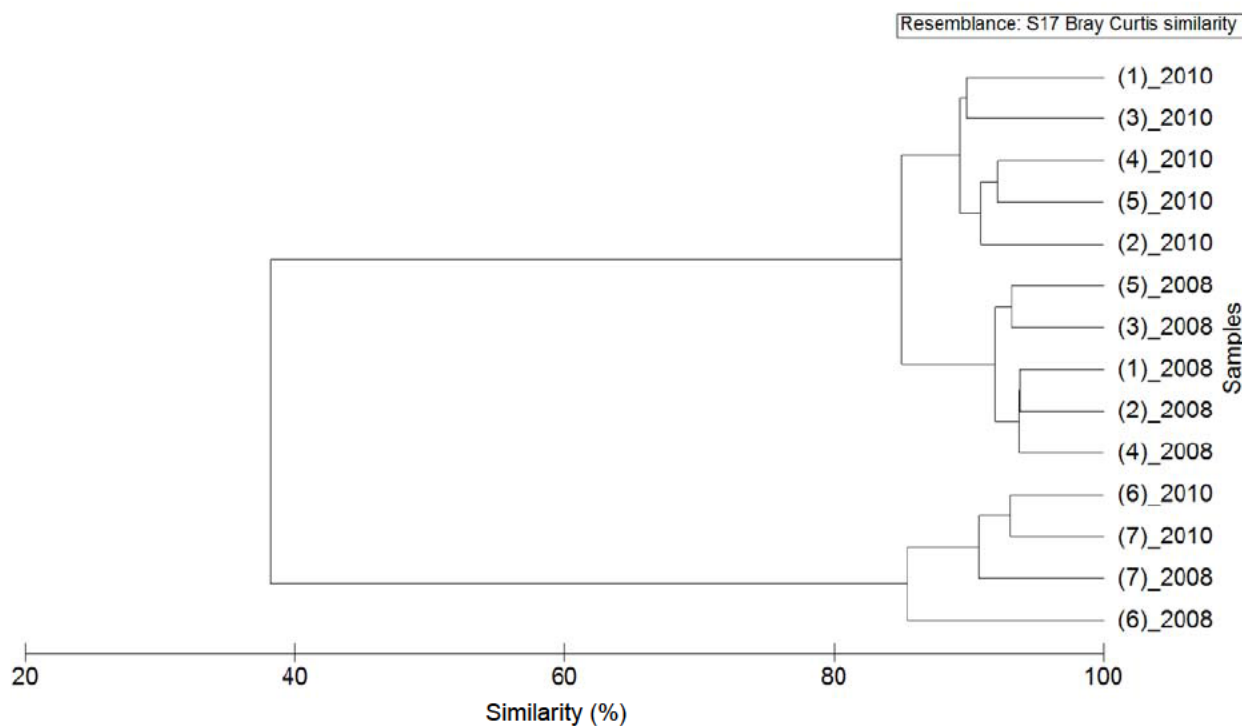


**Figure 2.** Cluster analysis of denaturing gradient gel electrophoresis (DGGE) patterns (Pearson, UPGMA) from the drinking water network. Samples are numbered from (1) - (7) according to Table 1. The mid-term stability from November 2008 until February 2009 is shown.

### 3.3.2. 454 Pyrosequencing

454 pyrosequencing of 16S rRNA genes offers the possibility to gain a detailed insight into the microbial species composition of a water sample (Hong et al., 2010). Based on the high stability over time suggested by the DGGE result, one sample from each sampling location between November 2008 and February 2009 was selected for pyrosequencing. To further analyze the long-term stability of the drinking water distribution network, water samples were taken from the network at the same locations again in September 2010. The clustering analysis of all samples analyzed with pyrosequencing showed more than 80 % similarity. Again, only water samples from the two locations having the longest water retention times clustered separately from the other samples (Fig. 3), further supporting the DGGE results (Fig. 2). Though the samples collected in 2010 clustered separately from the samples from 2008, the similarity in microbial community

composition was more than 80 % (Fig. 3). This shows that during drinking water treatment in this plant constantly biological stable water is produced, that hardly varies in the microbial community composition over time.

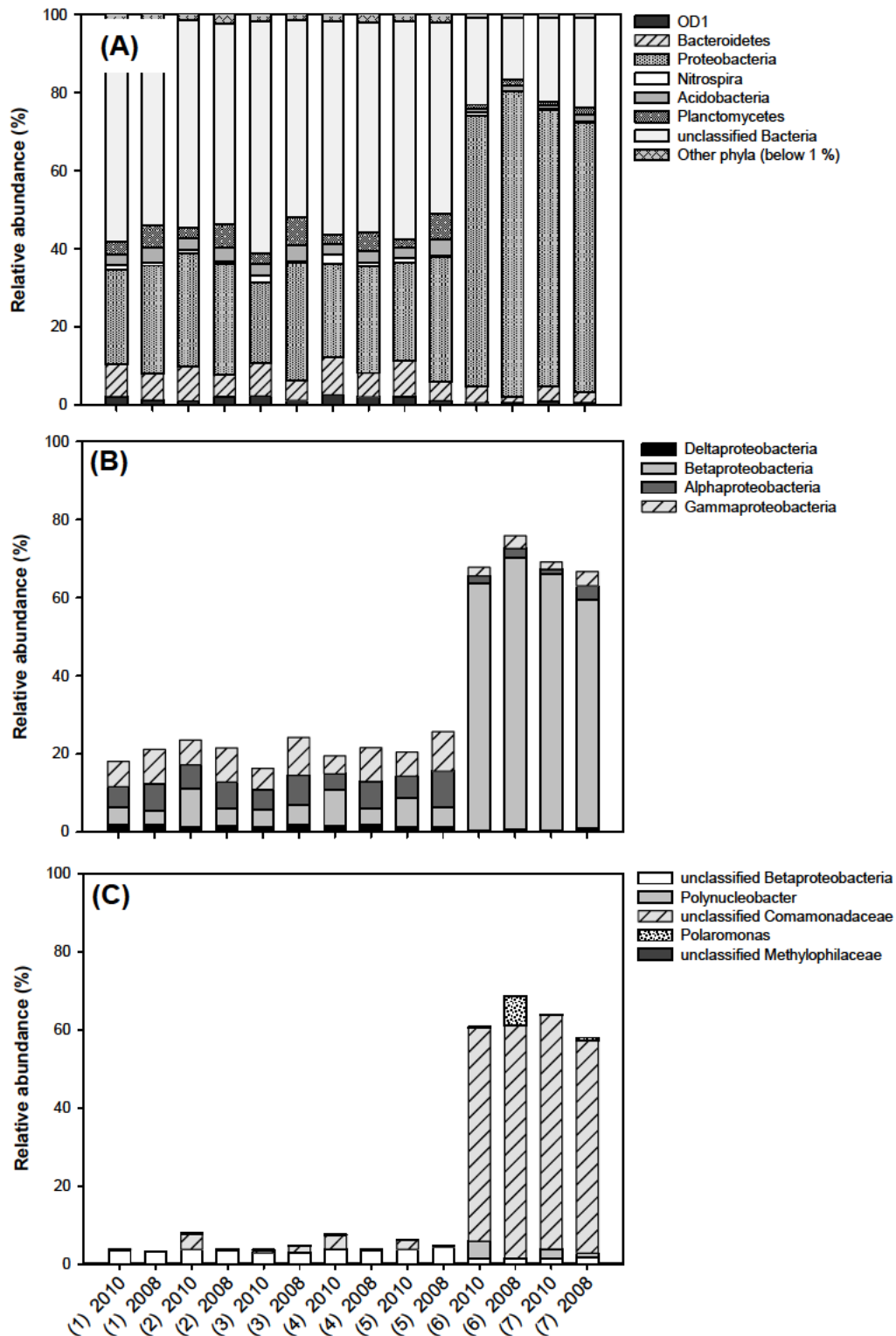


**Figure 3.** Cluster analysis from 454 pyrosequencing data from water samples of each sampling point from December 2008 and September 2010. Samples are numbered from (1) - (7) according to Table 1.

The microbial community in the drinking water reservoir was dominated by *Proteobacteria* (20 %) and bacteria that could not be classified (> 50 %) with the RDP database. However, further in-depth phylogenetic analysis using the ARB program revealed that these sequences belong to 21 novel candidate phyla and a few divergent phylotypes of unknown affiliation, which highlights the extremely rich and largely unexplored bacterial diversity in drinking water (Supplemental material, Fig. S1). In addition, members belonging to the phyla *Bacteroidetes*, *Nitrospira*, *Acidobacteria*, *OD1* and *Planctomycetes* were detected (Fig. 4A). A more detailed overview of all bacterial taxa that were present at more than 1 % is given in Figure 5. The detected groups are typical in freshwater environments and were previously found in drinking water distribution networks (Hong et al., 2010; Eichler et al., 2006). Similar to our findings, previous studies showed that the majority of phylotypes in chlorinated drinking water were related to uncultured bacterial taxa and difficult-to-classify bacterial sequences (Revetta et al., 2010; Eichler et al., 2006). This shows that until now cultivation-dependent identification methods have failed to elucidate the diversity in drinking water.

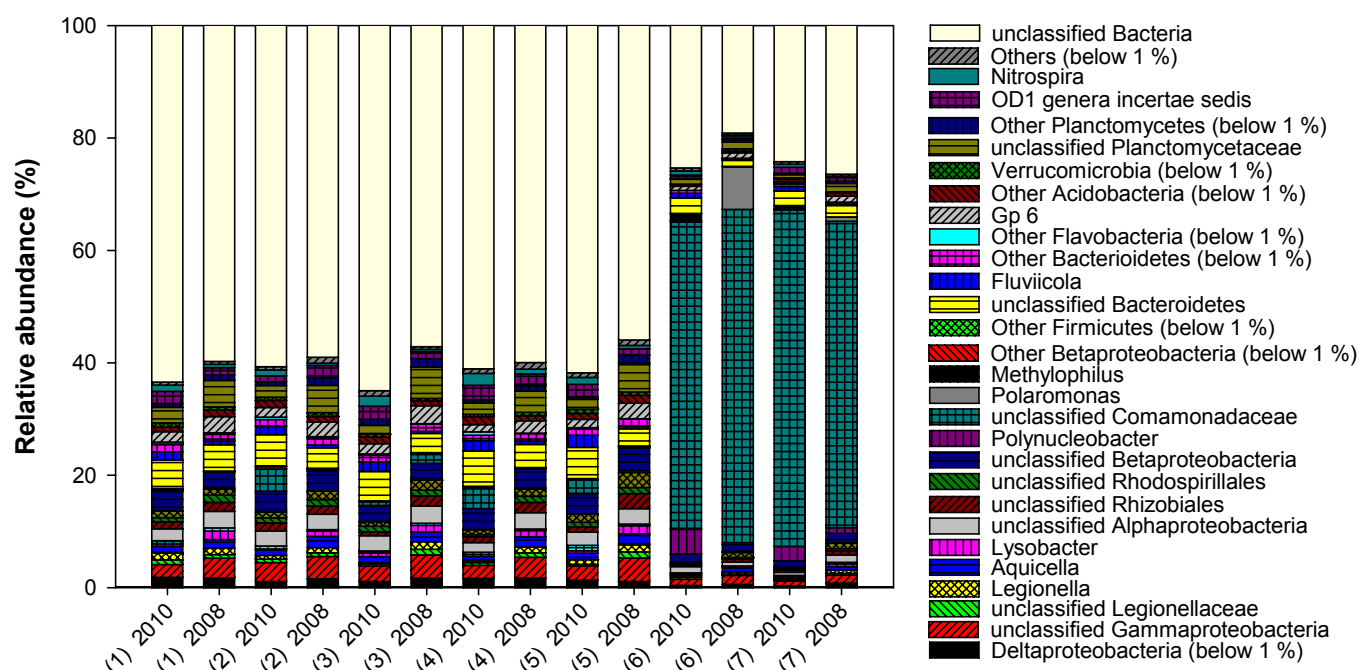
However, approaches that use different growth media with a composition more related to their natural environments, natural water, or adjusted nutrient levels, have shown to be successful for the cultivation of new bacterial species (Wang et al., 2009; Stevenson et al., 2004; Button et al., 1993). For a better understanding of the potential role of bacteria in drinking water, further attempts to cultivate and characterize these bacteria are necessary.

We analyzed the sequence data for major changes in microbial community composition during drinking water distribution. At the two locations having the longest retention times a significant increase in the relative abundance of *Proteobacteria* (from 20 to 70 %) occurred (Fig. 4A). The change in community composition with longer water retention time was linked to an increase in  $\beta$ -*Proteobacteria*, specifically to unclassified *Comamonadaceae* formerly included in the *Pseudomonas* group (Fig. 4B and 4C). Members of the *Comamonadaceae* group were found in many different environments including drinking water, lake water, activated sludge or soil and they are able to utilize a large variety of substrates (Burtscher et al., 2009; Pernthaler et al., 1998; Dias et al., 1964). The substrates that the *Comamonadaceae*-like bacteria grow on in drinking water still need to be elucidated. The majority of the sequences ( $n = 59$ ; total number of clones = 79) obtained via 16S rRNA clone library had  $> 97$  % sequence identity to uncultured *Comamonadaceae* clone sequences from marine environments, such as glacier, karstic spring water, and lake water (Supplemental material, Fig. S2). Taking into account the parallel increase in cell concentrations at these sampling locations (Fig. 1), the increase in *Comamonadaceae* would be equivalent to  $2 \times 10^4$  cells/ml, but may need to be proven using qPCR or FISH that directly target the 16S rRNA gene sequence of this population. However, the observation suggests that a small increase in cell concentration ( $2\text{--}3 \times 10^4$  cells/ml) can already cause a clear and detectable shift in microbial community composition.



**Figure 4.** 454 pyrosequencing analysis from water samples of each sampling point from December 2008 and September 2010. The relative abundance of phyla (A), *Proteobacteria* (B) and  $\beta$ -*Proteobacteria* (C) is shown. Samples are numbered from (1) - (7) according to Table 1.

Based on the abundance-based non-parametric species richness estimator, Chao1 (Chao, 1984), a very high diversity (more than 1500 Chao1) was observed in all water samples, and the diversity is considerably higher than the species richness between 100 to 350 Chao1 previously observed in a chlorinated drinking water system (Hong et al., 2010). Compared to the species richness of 25 bands detected with DGGE in this study (Fig. 2) and previous reports (Burtscher et al., 2009; Eichler et al., 2006), immensely more diversity was revealed with pyrosequencing. These differences in richness can be explained by the high resolution of DNA sequences obtained via pyrosequencing. Only 30 of the sequences obtained with pyrosequencing were present at a relative abundance of more than 1 % (Fig. 5). This suggests that many bacterial populations in drinking water are present in very low percentages, which cannot be detected with the conventional DNA fingerprinting methods (Muyzer et al., 1993). Pyrosequencing offers the opportunity to obtain sequence information that goes below the detection limit of conventional fingerprinting methods. Thus, in addition to only comparing the bacterial community structure with DGGE, the bacterial community composition can be analyzed with in depths pyrosequencing, which allows a comparison of the sequences and identification at the same time.



**Figure 5.** 454 pyrosequencing analysis from water samples of each sampling point from December 2008 and September 2010. The relative abundance of all sequences that were present more than 1 % in at least one of the samples is shown. Sequences that were present in a lower amount were summarized to the respective bacterial taxon (e.g. other *Gammaproteobacteria*). Samples are numbered from (1) - (7) according to Table 1.

### ***3.4. Implications of the results***

All the parameters investigated in this study including concentrations of cells, ATP and organic carbon, DGGE and pyrosequencing, suggest a remarkable level of biological stability over a large distance within the distribution network (short temporal and spatial), and span over several months (mid temporal) to two years (long term). Such a high stability in microbial community composition over a distance of a few kilometers was also detected previously in drinking water systems in Austria and Germany during the time span studied (Burtscher et al., 2009; Eichler et al., 2006). These observations suggest that the microbial community in the drinking water that finally reaches the consumer is still the same as in the reservoir located at the treatment plant. Significant change may then occur on household level (Lautenschlager et al., 2010; Siebel et al., 2008). In the present study, the bacteria in the reservoir likely originate from the biofilters in the plant (Hammes et al., 2010a). The high biological stability during distribution suggests that if engineers succeed in producing biologically stable water in the treatment process, the microbial community composition and cell concentration in the whole distribution system can be controlled. However, also the maintenance of intact pipes with materials that do not support microbial growth in the distribution network seems to be essential. For example, a correlation was reported between the abundance of fixed and suspended bacteria in drinking water distribution systems and it was assumed that the bacteria in the bulk water originated primarily from the detachment of biofilm-associated cells (Laurent et al., 2005c). Since the abundance of biofilm-associated bacteria in the distribution systems studied was in the range of  $0.3 - 2.6 \times 10^7$  cells/cm<sup>2</sup>, this appears to be also a logical explanation (Laurent et al., 2005b). However, the high stability in microbial community composition over a large distance observed here questions a major impact of pipe biofilms on the bulk water. Either a similar microbial community exists in pipe biofilms and bulk water throughout the drinking water distribution network, or pipe biofilms have only a minor impact on the microbial community composition of the bulk water, which were not detected with the methods applied. Future studies that directly compare pipe biofilm communities with the microbial communities in the bulk water in drinking water distribution systems could give more insight into the interaction of the pipe biofilms and the water phase. Furthermore, the similar microbial community composition and cell concentration in the drinking water treatment reservoir over the whole time span of two years shows that only little variation in the community composition of the treated water occurs. This suggests a high temporal stability of the microbial communities in the biofilters the cells are originating from.

#### **4. Conclusions**

The present study showed the value of a multi-parametric approach to assess biological stability in drinking water distribution systems. The microbial quality in a non-chlorinated drinking water distribution network was stable over extended time periods with respect to intact cell concentrations, ATP measurements and microbial communities. A small change was detected at longer retention times from the total cell counts with both flow cytometry and microbial community analysis, whereas this change was not detected with ATP, HPC or organic carbon measurements. Total cell concentrations show the quantitative extend of the change, while pyrosequencing offers the opportunity to analyze the change qualitatively. The use of multiple tools in combination provide the opportunity to describe changes and stability during drinking water distribution in much more detailed than what was previously possible.

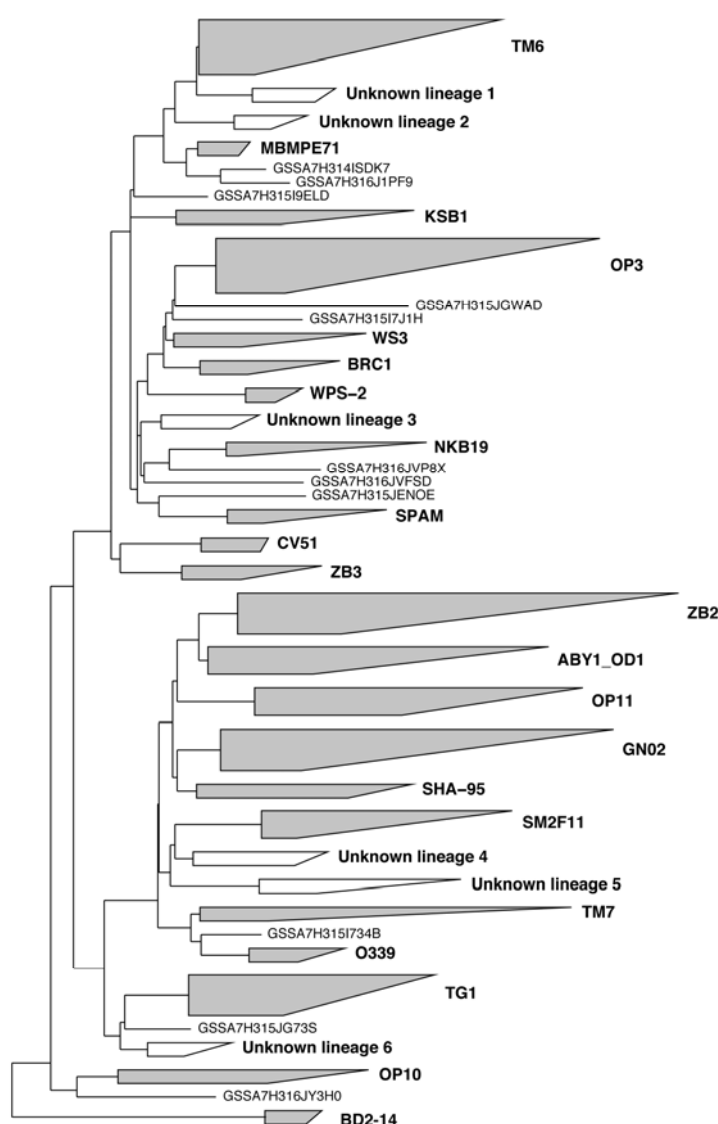
#### **Acknowledgements**

We would like to thank Dr. Hideyuki Tamaki (at UIUC) for his help with ARB phylogenetic construction. We are grateful to the financial support of the EU project TECHNEAU (018320) and the King Abdullah University of Science and Technology (KAUST) in Saudi Arabia.

## Supplementary information

### 1. In-depth phylogenetic analysis using the ARB software

The sequences in the unclassified bacterial group with the RDP database were extracted and aligned via Greengenes alignment tool ([http://greengenes.lbl.gov/cgi-bin/nph-NAST\\_align.cgi](http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi)). The resulting alignment file and Greengenes' 16S rRNA gene database were input into ARB software package for phylogenetic tree construction.



**Figure S1.** Phylogenetic tree of the unclassified bacterial 16S rRNA gene sequences (with the RDP database) obtained via pyrosequencing. Wedges in grey represent classified sequences and white wedges indicate sequences that still could not be classified (unknown lineage). The thickness of the bars indicates the relative abundance of sequences.

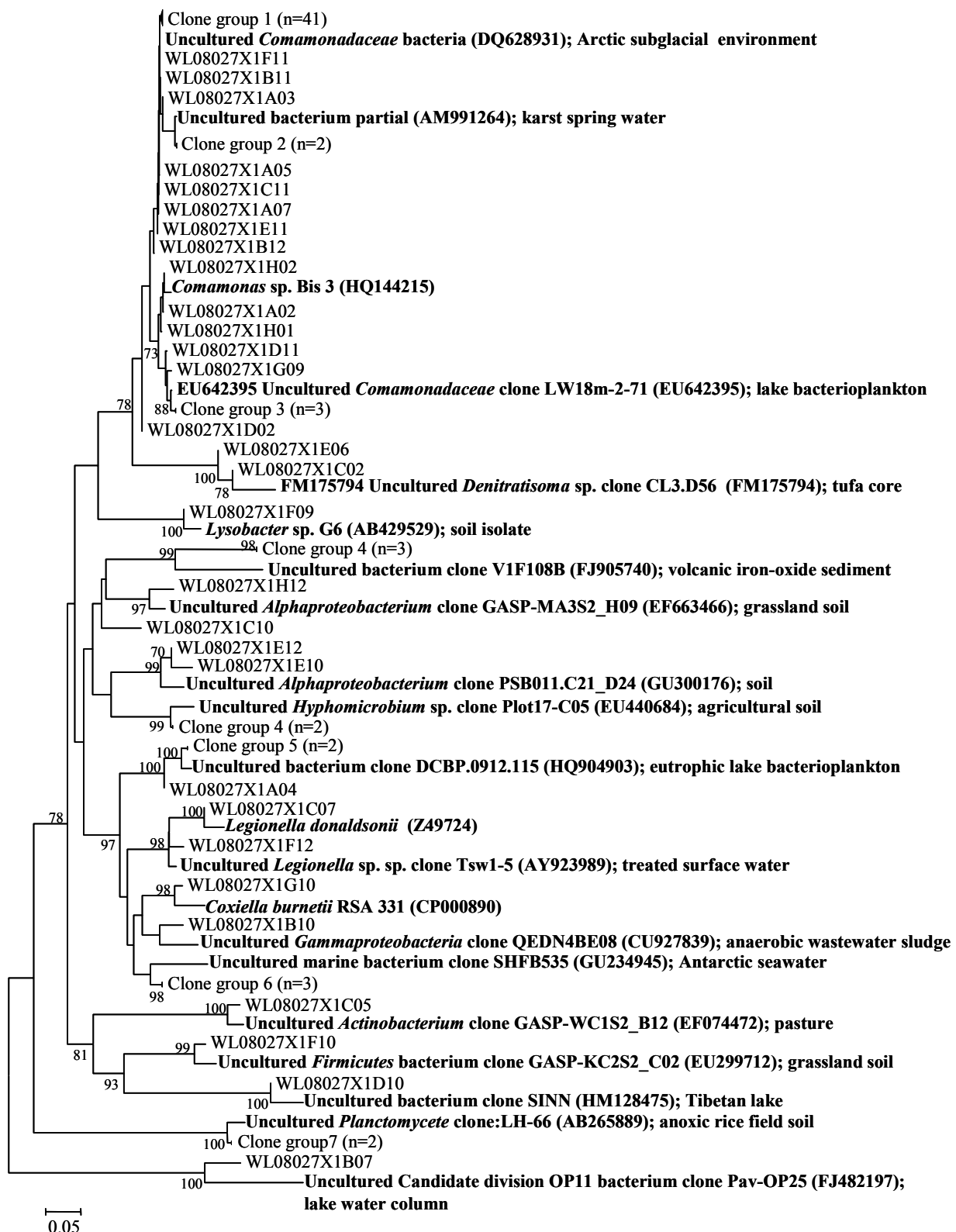
## 2. 16S rRNA gene clone library

### 2.1. 16S rRNA gene clone library set up and sequence determination

A 16S rRNA gene clone library was set up to further analyze the predominant bacterial group detected from DGGE and pyrosequencing analysis in sample “(6)\_2008”. The 16S rRNA genes were amplified with the universal bacterial primer pair FD1 (5’ AGA GTT TGA TCC TGG CTC AG 3’) and 1540R (5’ AAG GAG GTG ATCCAG CC 3’). The triplicate PCR reactions (25 µl) contained 12.5 Bullseye standard Taq DNA polymerase 2.0 x master mix (MidSci), 1 µl each primer, 9.5 µl sterilized Milli-Q water, 1 µl DNA template (5-10 ng). The PCR parameters were as follows: 80 °C for 1.5 min; 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. The PCR reactions were combined prior to cloning. An aliquot (5 µl) of the PCR products was run in a 0.8 % TAE agarose gel stained with ethidium bromide. The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System. The purified fragments were cloned using the pGEM-T Easy Vector System (Promega) according to manufacturer’s instructions. White colonies were randomly selected (n = 96) and the cloned inserts were amplified with vector-specific primers (M13 forward and M13 reverse) using the PCR parameters described above. The PCR products were purified with a Montage PCR<sub>u96</sub> plate according to manufacturer’s instructions (Millipore, Bedford, MA). DNA sequences were determined with an internal primer (universal reverse primer 907R, 5’CCG TCA ATT CMT TTR AGT T3’) using an ABI 3730XL capillary sequencer at the W.M. Keck Center, part of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

### 16S rRNA gene sequence analysis

The quality of the DNA sequences was analyzed with Greengenes web application “Trim tool” ([http://greengenes.lbl.gov/cgi-bin/nph-trim\\_fasta\\_by\\_qual.cgi](http://greengenes.lbl.gov/cgi-bin/nph-trim_fasta_by_qual.cgi)). Sequence identification was done via comparison to known sequences in NCBI GenBank with the BLASTN server ([http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE\\_TYPE=BlastHome](http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome)). All clone sequences and reference sequences were aligned and a phylogenetic tree was constructed with the Neighbor-joining method and a Maximum Composite Likelihood model (bootstrap: 500 replicates) with MEGA v. 4.1.



**Figure S2.** Phylogenetic relationship of cloned 16S rRNA genes and reference sequences (in bold) from NCBI (accession numbers in parenthesis) from sample “(6) 2008”. The source of the clone provided by NCBI is indicated.

## **5. Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition**

### **Abstract**

Drinking water quality is routinely monitored in the distribution network but not inside households at the point of consumption. Fluctuating temperatures, residence times (stagnation), pipe materials and decreasing pipe diameters can promote bacterial growth in buildings. To test the influence of stagnation in households on the bacterial cell concentrations and composition, water was sampled from ten separate households after overnight stagnation and after flushing the taps. Cell concentrations measured by flow cytometry increased (2-3 fold) in all water samples after stagnation. This increase was also observed in adenosine tri-phosphate (ATP) concentrations (2-18 fold) and heterotrophic plate counts (4-580 fold). An observed increase in cell biovolume and ATP-per-cell concentrations furthermore suggests that the increase in cell concentrations was due to microbial growth. After five minutes flushing of the taps, cell concentrations and water temperature decreased to the level generally found in the drinking water network. Denaturing gradient gel electrophoresis also showed a change in the microbial composition after stagnation. This study showed that water stagnation in household pipes results in considerable microbial changes. While hygienic risk was not directly assessed, it emphasizes the need for the development of good material validation methods, recommendations and spot tests for in-house water installations. However, a simple mitigation strategy would be a short flushing of taps prior to use.

## 1. Introduction

The Council Directive of the European Union states that water supplied from a distribution network should fulfill quality requirements (including microbiological parameters) “*at the point, within premises or an establishment, at which it emerges from the taps that are normally used for human consumption*” (Council Directive 98/83/EC). Similarly, according to the Swiss Health Directive, drinking water is regarded as “consumable” when it meets hygienic and microbial requirements at the point of consumption, again implying specifically the household tap (Verordnung des Eidgenössischen Departments des Innern, 2005). However, in most European countries, drinking water quality is not monitored routinely at household level but rather directly in the distribution system, as waterworks and authorities have limited access to private homes, as well as limited control over household plumbing and operation. Household pipes can, in fact, have a considerable impact on the water quality, which was already shown in several large-scale studies addressing heavy metal concentrations in tap water after overnight stagnation (Zietz et al., 2007; Zietz et al., 2003; Haider et al., 2002). These studies all reported increased concentrations of lead, cadmium, copper and nickel after stagnation in household tap water in Germany and Austria.

The impact of stagnation on the microbiological quality is not as clear. In the distribution network, bacterial growth is limited by low nutrient concentrations, disinfectant residuals, low temperatures and short residence times (Servais et al., 1992; Kerneys et al., 1995; Niquette et al., 2000). However, it is possible that the microbial quality may change in household pipes, where higher temperatures, longer residence times, depletion of disinfectant residuals and nutrient contamination from pipe material or from the household environment could lead to bacterial growth. This may cause problems, since bacterial growth can lead to adverse aesthetic changes such as the development of taste, odour and colour in drinking water (Mallevalle and Suffet, 1987). Moreover, it bears the potential risk of pathogenic proliferation. The presence of coliform bacteria in distribution systems was previously associated with temperature, disinfectant type and the concentration of assimilable organic carbon (AOC), all factors which might change during stagnation (LeChevallier et al., 1996; WHO, 2006). Similar observations have been reported for *Aeromonas hydrophila*, *Mycobacteria*, *Escherichia coli* O157 and *Vibrio cholerae* (van der Kooij and Hijnen, 1988; Torvinen et al., 2004; Vital et al., 2008; Vital et al., 2007). It is likely that those conditions that limit growth of general heterotrophic bacteria also influence growth of pathogens. Therefore, a clear understanding of regrowth in general is of considerable value.

Bacterial regrowth on household level was shown in a study in Tuscon (USA) where increased heterotrophic plate count (HPC) concentrations above guideline values were found in the consumers' taps after stagnation compared to the distribution network (Pepper et al., 2004). However, the study was limited to culturable bacteria, which represent only a small fraction of the total microbial community (van der Kooij, 2003; Siebel et al., 2008; Hammes et al., 2008). A previous study from our group also found significantly higher cell numbers in tap water in the morning compared to the evening in two office buildings, indicating overnight growth (Siebel et al., 2008). However, this study was restricted to two adjacent office buildings and it was not addressed whether changes in cell concentrations were accompanied by a change in microbial communities.

To assess the impact of stagnation on the microbial water quality in more detail, taps from ten separate households fed with non-chlorinated drinking water from the same distribution system were sampled after overnight stagnation and then after brief flushing. Bacterial cell concentrations and activity were determined with flow cytometry, HPC and adenosine tri-phosphate (ATP) measurements, while microbial diversity was analysed with denaturing gradient gel electrophoresis (DGGE). The aim of the study was to assess the extent of overnight regrowth on drinking water in various households and the influence of regrowth on the stability of the microbial community.

## 2. Materials and Methods

### 2.1. Sampling

Schott bottles (1 l and 2 l) were used for sampling. All glassware used in the study was sterile and carbon-free as described previously (Greenberg *et al.*, 1993). Drinking water samples were collected from ten cold water taps in separate households in Dübendorf (CH), fed with water from the same distribution network with water consisting of 49 % groundwater (untreated), 50 % lake water (ozonation and sequential biofiltration) and 1 % spring water. The water is distributed without the addition of any residual disinfectants. From every house, the first litre of cold tap water in the morning after overnight stagnation (8-20 h) was sampled, as well as two litres of cold tap water after gently flushing the same tap for five minutes (approximately 30 l). The flushing time was selected as the average time it took for the water to reach network temperature. Temperature was measured after the second water sample was taken. The temperature of the first litre sample was not measured, to avoid carbon contamination to the water sample. Two additional samples were taken directly from the drinking water network in Dübendorf. General water quality parameters are listed in Table 1.

**Table 1.** General water quality parameters measured in flushed, stagnated and the two water samples taken directly from the drinking water network.

|                             | <b>Flushed</b> | <b>Network 1</b> | <b>Network 2</b> | <b>Stagnated</b> |
|-----------------------------|----------------|------------------|------------------|------------------|
|                             | <b>Average</b> | <b>Average</b>   | <b>Average</b>   | <b>Average</b>   |
| <b>Temperature (°C)</b>     | 9.1 ± 2.8      | 6                | 10               | *                |
| <b>TOC (mg/l)</b>           | 0.8 ± 0.0      | 0.76             | 0.78             | 0.8 ± 0.0        |
| <b>Nitrate (mg/l)</b>       | 3.4 ± 0.3      | 3.2              | 3.3              | 3.3 ± 0.1        |
| <b>Conductivity (µS/cm)</b> | 461 ± 28       | 450              | 459              | 450 ± 10         |
| <b>pH</b>                   | 7.9 ± 0.2      | 7.9              | 8                | 8.0 ± 0.1        |

\*see figure 4 for typical stagnation temperature

### 2.2. Fluorescence staining and flow cytometry

Flow cytometric analysis was done as described in Berney *et al.* (2008). Briefly, bacterial cells were stained with 10 µl/ml propidium iodide in combination with SYBR<sup>®</sup> Green I (SG/PI) to measure membrane intact cells, and with 10 µl/ml SYBR<sup>®</sup> Green I only to measure the total cell concentration. Working solutions of the dyes were prepared as follows: SYBR<sup>®</sup> Green I was diluted

100 fold in anhydrous dimethylsulfoxide (DMSO) and when used in combination with PI, mixed with PI (30 mM) at a ratio of 1:50. Samples were then incubated in the dark for at least 15 minutes before measurement. Samples were diluted 1/10 just before measurement in filtered, cell free (0.1  $\mu\text{m}$ ; Millex<sup>®</sup>-GP, Millipore) bottled mineral water (EVIAN, France), so that the concentration measured with the flow cytometer was always less than  $2 \times 10^5$  cells/ml. Flow cytometry was performed using a portable CyFlow SL flow cytometer (Partec, Hamburg, Germany) equipped with a 20 mW solid state laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected in the FL1 channel ( $520 \pm 20$  nm), and red fluorescence collected in the FL3 channel ( $> 615$  nm). All data were processed with the Flowmax software (Partec), and electronic gating with the software was used to separate the desired clusters. The specific instrumental gain settings for these measurements were as follows: FL1 = 500, FL3 = 700, speed = 3 (implying an event rate never exceeding 1000 events  $\text{sec}^{-1}$ ). All samples were collected as logarithmic (3 decades) signals and were triggered on the green fluorescence channel (FL1). The collection of data as FL1/FL3 dot plots allowed for optimal distinction between the stained intact microbial cells, permeabilised cells, “high and low nucleic acid bacteria” and instrument noise or sample background (Hammes et al., 2008). Biovolume was calculated as described in Hammes et al. (2010b).

### ***2.3. Adenosine tri-phosphate (ATP) analysis***

Total ATP was determined using the BacTiter-Glo<sup>™</sup> reagent (Promega Corporation, Madison, WI, USA) and a luminometer (Glomax, Turner Biosystems, Sunnyvale, CA) as described elsewhere (Hammes et al., 2010b). A water sample (500  $\mu\text{l}$ ) and the ATP reagent (50  $\mu\text{l}$ ) were warmed (1 min) to 38 °C simultaneously in separate sterile Eppendorf tubes. The sample and the reagent were then combined and then the luminescence was measured after 20 sec reaction time at 38°C. The data were collected as relative light units (RLU) and converted to ATP (nM) by means of a calibration curve made with a known ATP standard (Promega). Cellular ATP and free ATP were distinguished by filtering each sample through a 0.1  $\mu\text{m}$  sterile syringe filter (Millex<sup>®</sup>-GP, Millipore), and then repeating the analysis described above. ATP was measured in triplicate for all samples, and the standard deviation of the measurement was below 4 %.

### ***2.4. Heterotrophic plate counts (HPC)***

The HPC method was performed according to the Swiss guidelines for drinking water (SLMB 2000, 56, E.1). In short: 1 ml of the water sample was transferred to a sterile Petri dish and mixed with

about 15 ml plate count agar (PCA, Oxoid, Cambridge, UK). The agar was kept at 42 °C before use. The plates were incubated at 30 °C for 72 h and then counted with an automatic plate reader (aCOLyte, SYNBIOSIS, Cambridge, UK). All measurements were done in triplicate.

### ***2.5. Localization of bacterial growth in household taps***

Water from three taps in separate households was sampled step-wise after overnight stagnation. Samples were taken one after another (20 - 100 ml) from the first two litres of tap water. To reach water from the network the tap was then gently flushed for five minutes (approx. 30 l). Cell concentrations and temperatures were measured in all samples

### ***2.6. Influence of stagnation time on bacterial growth***

The influence of stagnation time on bacterial cell concentrations in tap water was determined in a single household tap. A tap was flushed gently for five minutes before keeping it closed for varying stagnation intervals (1 - 74 h). A 100 ml sample was taken after flushing and after each stagnation interval. Cell concentrations were determined with flow cytometry as described above. The tap was always opened to the same extent to ensure an equal flow rate. The increase in cell concentrations was calculated by subtracting the cell concentration of the flushed water sample from the stagnated water sample. Temperature was measured in all samples.

### ***2.7. Growth on biodegradable carbon***

A simplified version of the assimilable organic carbon (AOC) assay developed in our group (Vital et al., 2007) was used to assess regrowth potential in the water samples. Triplicate samples of each water were filled into sterile, carbon-free vials and incubated without any further treatment for 3 days at 30 °C. Total cell concentrations of the indigenous microbial community were determined before and after incubation with flow cytometry (as described above). The net-growth was used as indicator of the AOC concentration left in the sample.

### ***2.8. Community analysis with DGGE***

DNA was extracted with the DNA water Isolation Kit (UltraClean™ Water DNA Isolation Kit, MoBio, Carlsbad, CA, USA) following the provided instructions. 1 l from the first litre samples and 2 l from the flushed water samples were filtered on 0.2 µm filters (MoBio) and stored at -20 °C

until further processing. PCR was performed using a Taq polymerase kit (Fermentas GmbH, St-Leon Rot, Germany). To increase the sensitivity of DNA amplification, nested PCR was performed. In the first PCR step DNA was amplified using the general bacterial primers 63F and 1378r (Heuer et al., 1997). In the second PCR step the primers 338F-GC and 518r (Muyzer et al., 1993) were used. Final concentrations of the components in the PCR mix were: 0.2  $\mu$ M of each primer, 200  $\mu$ M of each deoxynucleotide triphosphate, 0.125 U Taq Polymerase, 1x Taq-polymerase 10x reaction buffer (MgCl<sub>2</sub>-free) and 1.5 mM MgCl<sub>2</sub>. In the first round 10  $\mu$ l of template were added to 15  $\mu$ l of mastermix. PCR products were then diluted appropriately and 1  $\mu$ l was added to 24  $\mu$ l master mix in the second PCR round. PCR amplification was done with the following program: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 53°C for 1 min, extension at 72 °C for 2 min and a single final extension at 72 °C for 10 min. After each PCR step the size of the product was verified on a 1 % agarose gel. DGGE based on the protocol of Muyzer et al. (1993) was performed using an Ingeny phor U2 system (Ingeny, Leiden, The Netherlands). PCR fragments (7  $\mu$ l) were mixed with loading dye (3  $\mu$ l) and loaded onto 8 % polyacrylamide gels (w/v) (100 % denaturant contains 7 M urea and 40 % deionized formamide ) in 1 x TAE (20 mM Tris, 10 mM Acetate, 0.5 mM EDTA). To separate the DNA fragments gels with a gradient from 45 - 60 % were used. The electrophoresis was run for 16 h at 60°C and 120 V. Gels were stained in SYBR<sup>®</sup> Green I nucleic acid gel stain (1:10000 dilution) for 20 min with gentle agitation and photographed. DGGE patterns were clustered using the Bionumerics 5.1 software (Applied Maths, Kortrijk, Belgium). Similarities were calculated by the Pearson correlation, taking into account band intensity and band position. The similarity among replicates of the DGGE method was 90%. The clustering algorithm of Ward (Ward 1963) was used to calculate dendrograms. Dynamics were calculated according to Marzorati et al., 2008. Gini coefficients were calculated as described in Mertens and coworkers (2005).

### 3. Results and Discussion

#### 3.1. Choice of samples and methods

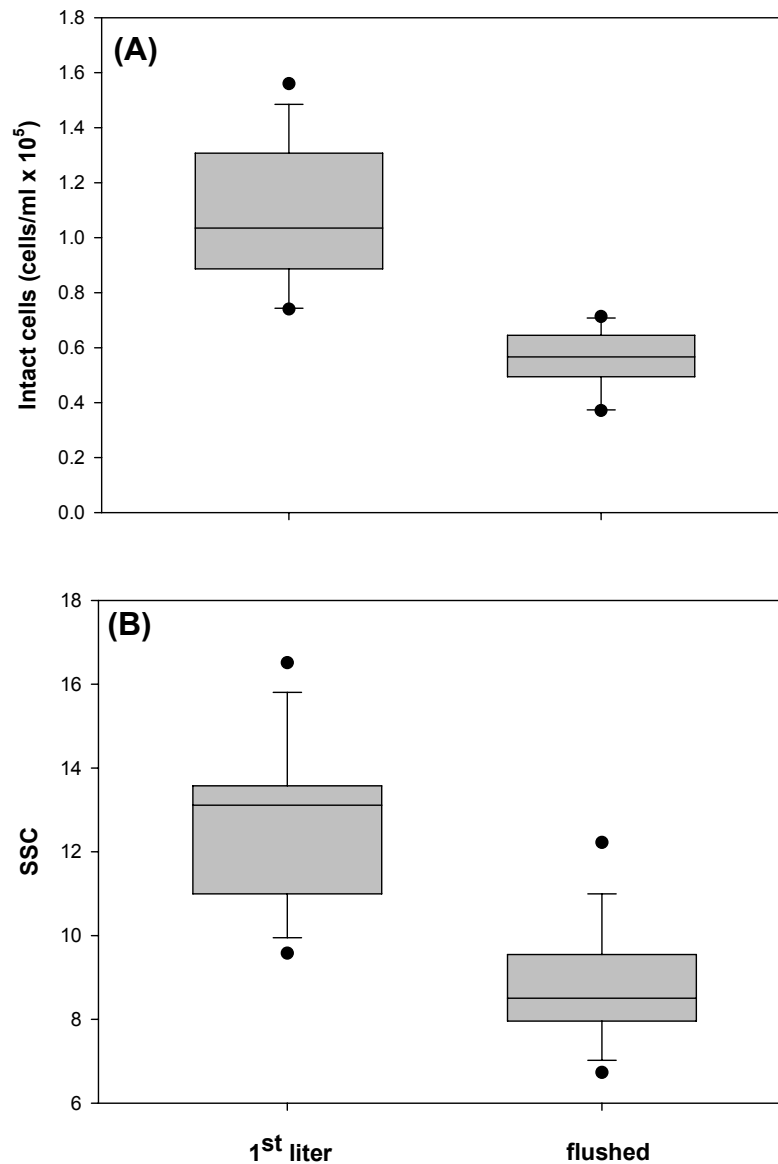
The ten sampled households were chosen randomly and included both single-family houses and multiple-family apartment buildings. Since every house was different in terms of age, pipe material, size, pipe volume, and inside temperature, variations between separate houses were expected in stagnated water samples. Measurements of temperature and cell concentrations suggested that five minutes of gentle flushing (equal to approximately 30 l) was sufficient to obtain ‘network quality’ water (Fig. 1., Table 1). Therefore, less variability between flushed water samples was expected. Bacterial cell concentrations were assessed from all samples with both cultivation-based and cultivation-independent methods.

#### 3.2. Bacterial growth during stagnation measured with FCM

The entire indigenous microbial community in a water sample can be visualised with fluorescent dyes coupled with FCM enumeration. This allows assessment of all microorganisms, including so-called unculturable heterotrophic bacteria, autotrophic bacteria and so-called “viable-but-not-culturable” (VBNC) cells (Rinta-Kanto et al., 2004; van der Kooij, 2003). We used SYBR<sup>®</sup> Green I/Propidium Iodide to exclude cells with severely damaged membranes from the analysis (Hammes et al., 2010b; Berney et al., 2008; Hoefel et al., 2005a, b). In each house a clear increase in intact cell concentrations was observed after overnight stagnation (Fig. 1A). The increase of the cell concentrations varied between 1.6 - 3.2 times in the different houses, with an average cell concentration of  $(1.1 \pm 0.25) \times 10^5$  cells/ml after stagnation and a decrease to  $(5.6 \pm 1.0) \times 10^4$  cells ml<sup>-1</sup> after flushing (Fig. 1A). The latter average was similar to average values of samples taken directly from the network ( $5.9 \times 10^4$  cells/ml). On average, 85 % of cells in all water samples were membrane intact, irrespective of whether it were stagnated samples or flushed samples (for details see supplementary information, Fig. S1). This was expected, since the water was not subject to final disinfection during treatment or residual disinfectants during distribution (Berney et al., 2008). In this case, the difference between intact cell concentration and total cell concentration did not alter the data interpretation. In contrast, we also analysed another network where a low dose of chlorine dioxide (0.05 mg/l) was used for network protection (for details see supplementary information, Fig. S2). In that particular case, the percentage of intact cells increased concomitantly with the increase in cell concentrations from 34 to 57 % following overnight stagnation (Fig. S2). This highlights the

importance of including a viability parameter in such analysis, and also demonstrates that bacterial growth in buildings is not only a problem in non-chlorinated drinking water systems, but a general problem. Currently no drinking water guidelines exist for total or intact bacterial cell counts. However, the data presented in Fig. 1A suggest that this is a straightforward and robust parameter to assess changes in the total microbial community during stagnation.

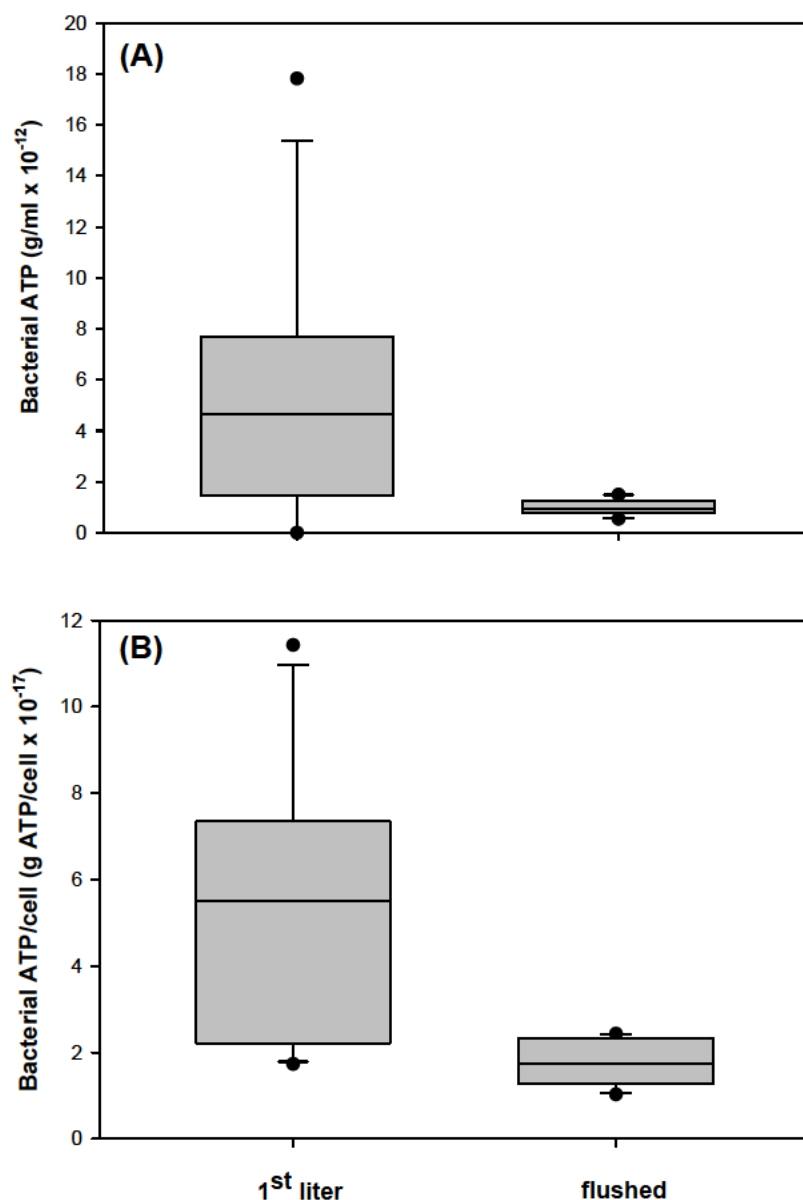
The bacterial sideward scatter signals (SSC) from FCM measurements was found to correlate well with the biovolume of cells (Servais et al., 2003; Felip et al., 2007; Hammes et al., 2010b). SSC values of cells from the flushed and stagnated water samples were compared. Bacteria in stagnated water samples had an average SSC signal of  $12.6 \pm 1.87$  arbitrary units (a.u.), which was significantly higher than that of bacteria in flushed water samples ( $8.32 \pm 0.84$  a.u.) (Fig. 1B). The estimated biovolume from these values was  $0.15 \pm 0.06 \mu\text{m}^3/\text{cell}$  (stagnated) and  $0.06 \pm 0.03 \mu\text{m}^3/\text{cell}$  (flushed), respectively. Since the biovolume of cells usually increases during growth (Stephenson, 1949), it is probable that growing cells were responsible for the observed increase of cell concentrations. The change in the average cell size was also reflected in a change in the clustering pattern (for details see supplementary information, Fig. S3). Two distinct populations of so-called “high nucleic acid” (HNA) and “low nucleic acid” (LNA) content bacteria are commonly observed in natural aquatic environments with FCM after staining with fluorescent dyes (Wang et al., 2009; Lebaron et al., 2001), and these two clusters were clearly visible in all samples analysed (Fig. S3). A significant ( $p < 0.05$ ) increase in the proportion of HNA bacteria to LNA bacteria from  $48 \pm 16$  % in flushed water samples to  $66 \pm 16$  % in stagnated water samples suggested that HNA bacteria were growing preferably during stagnation. Wang and coworkers (2009) showed that HNA cells are considerably larger than LNA cells; therefore, this shift probably also contributes to the increase of the average SSC values. However, we also observed an increase in intact cell concentrations and SSC values of LNA populations after stagnation, which demonstrated that also LNA bacteria were growing (for details see supplementary information, Fig. S4). This is in accordance with the emerging view, that LNA as well as HNA bacteria are actively growing cells (Bouvier et al., 2007; Wang et al., 2009). These data show that measurements of cell size and specific FCM “fingerprints” provide complimentary information to the straightforward cell concentration values.



**Figure 1.** Intact cell concentrations (A) and sideward scatter values indicating average cell size (B) from the 1<sup>st</sup> litres of tap water after overnight stagnation compared to samples taken from the same tap after flushing for five minutes. Data are from 10 different households belonging to the same distribution network. Intact cell concentrations were measured with SYBR<sup>®</sup> Green I/propidium iodide staining and flow cytometry. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values (n = 10).

### 3.3. Bacterial growth during stagnation measured with ATP

Bacterial (cell-bound) ATP concentrations and free ATP concentrations were determined. Bacterial ATP concentrations increased (on average 6-fold) in stagnated water samples ( $(6.32 \pm 4.92) \times 10^{-12}$  g/ml) compared to concentrations after flushing ( $(1.01 \pm 0.32) \times 10^{-12}$  g/ml) (Fig. 2A). The percentage of free ATP in stagnated water samples was considerably lower ( $26 \pm 18$  %) than in flushed water samples ( $51 \pm 13$  %). This difference was due to a constant amount of free ATP in flushed and stagnated water samples and an increase in bacterial ATP during stagnation. The increase can be ascribed to the increase of total cell concentrations (Fig. 1A), and possibly to differences in the activity, size and type of individual cells (Karl, 1980). In case the increase was solely due to the increase in total cell concentrations, the ATP-per-cell concentration of stagnated and flushed water samples should have been the same (Fig. 2B). We have calculated average values of  $5.42 \pm 3.17 \times 10^{-17}$  g ATP/cell in stagnated and  $(1.81 \pm 0.54) \times 10^{-17}$  g ATP/cell in flushed water samples (Fig. 2B), which are in the same range as values previously recorded for low nutrient environments (Karl, 1980; Eydal and Pedersen, 2007; Hammes et al., 2008; Wang et al., 2009). The fact that three-fold higher ATP-per-cell concentrations in stagnated than in flushed water samples were recorded, shows that also a change in cellular ATP amounts of individual cells occurred (Fig. 2B). A significant correlation between cellular ATP concentrations and cellular biovolume was previously reported (Karl, 1980; Eydal and Pederson 2007; Hammes et al. 2010b). We have measured a significant increase in average biovolume during stagnation (Fig.1B). When the biovolume is taken into consideration, the estimated ATP-per-biovolume values for first litres ( $(3.5 \pm 1.5) \times 10^{-16}$  g/ $\mu\text{m}^3$ ) and flushed water samples ( $(3.6 \pm 1.6) \times 10^{-16}$  g/ $\mu\text{m}^3$ ) showed no statistical difference. The data shows the complimentary nature of cultivation-independent FCM and ATP data. While ATP measurements are fast and easy to handle, considerable care should be taken with the potential impact of cell size (biovolume) and free-ATP in the interpretation of ATP data.

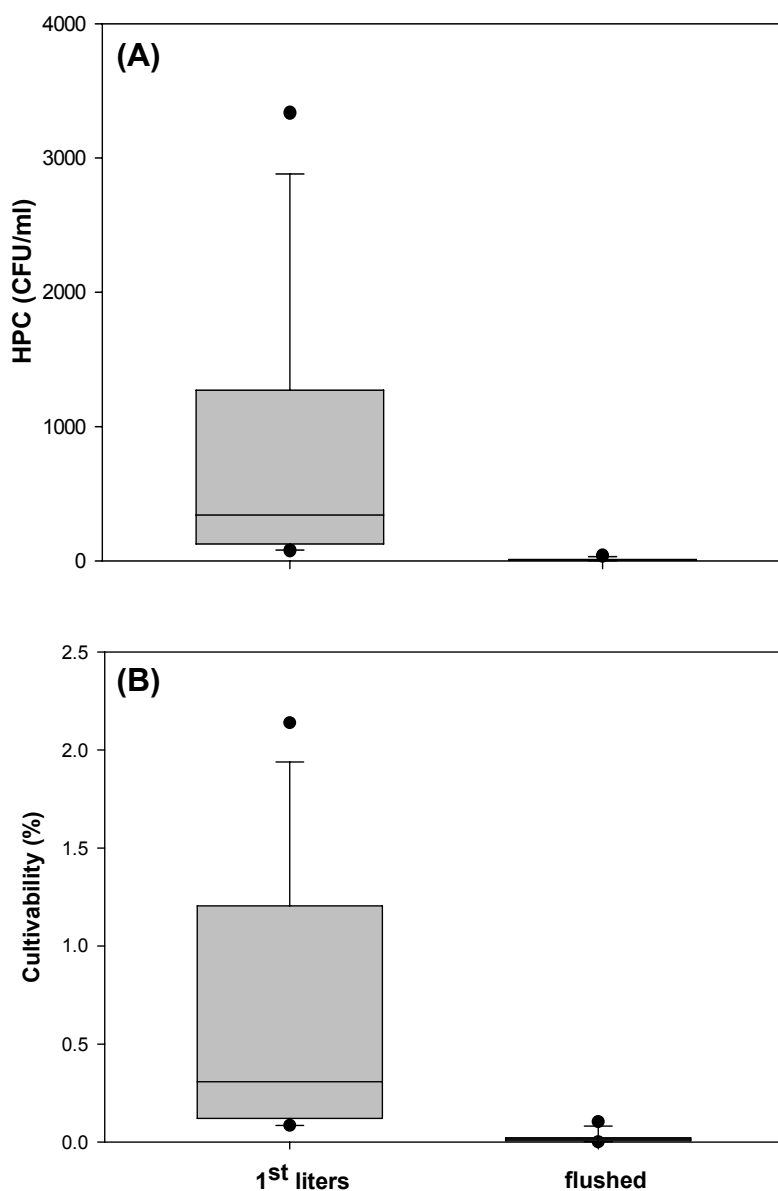


**Figure 2.** Adenosine tris-phosphate concentrations (ATP) **(A)** and ATP/cell concentrations **(B)** from the 1<sup>st</sup> litres of tap water after overnight stagnation compared to samples taken from the same tap after flushing for five minutes. Data are from 10 different households belonging to the same distribution network. ATP concentrations were determined by using the Bac Titer-Glo Microbial Cell Viability Assay. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values ( $n = 10$ ).

### 3.4. Bacterial growth during stagnation measured with HPC

The general microbial quality of drinking water is usually monitored with HPC analysis (Allen et al., 2004; Pepper et al., 2004). In Switzerland, the official guideline states that 300 colony forming units (CFU)/ml in the network should not be exceeded (SLMB, 2000). However, in stagnated water samples, elevated HPC concentrations ( $(0.87 \pm 1.0 \times 10^3)$  CFU/ml) were measured (Fig. 3A); in six

out of ten houses those concentrations were above the recommended limit. After flushing, the HPC concentration decreased in all houses below the limit to  $9.1 \pm 9.8$  CFU/ml. The high standard deviation in stagnated water samples indicates the very high variability of HPC values between the different houses. HPC were 4 - 580 fold higher in stagnated than in flushed water samples. Such a high variability and drastic increase of HPC in the consumer's tap was also observed previously (Pepper et al., 2004). In contrast, the increase in intact cell concentrations and ATP concentrations in stagnated water samples was only 1.6 - 3.2 fold (Fig. 1A) and 1.7 - 18 (Fig. 2A) fold respectively. When using HPC one should consider that heterotrophic plate counts usually comprise a fraction well below 1 % of total cell counts in drinking water (van der Kooij, 2003; Hammes et al., 2008). In the present study we observed culturability of  $0.02 \pm 0.03$  % in flushed water samples and an increase up to  $0.62 \pm 0.68$  % in stagnated water samples, which was a 31-fold increase in culturability (Fig. 3B). This increase was most probably due to culturable species growing preferably during overnight stagnation. Wang et al. (2009) has shown LNA to be unculturable on the conventional growth media used in the present study. Therefore, the observed shift to more HNA bacteria during stagnation (for details see supplementary information, Fig. S3) could have contributed to the observed increase in culturability. Generally, despite the low culturability the growth event was clearly reflected in the HPC.

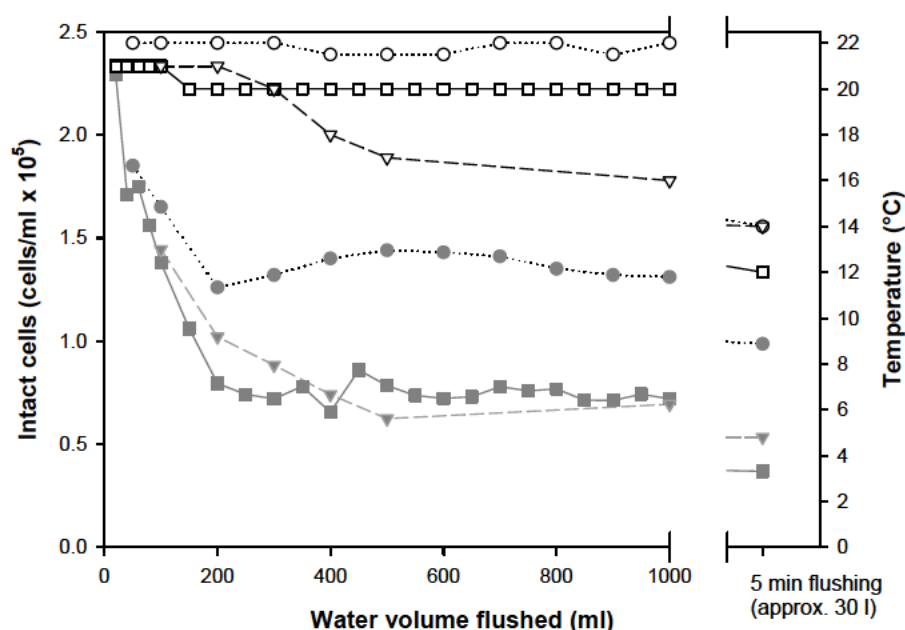


**Figure 3.** Heterotrophic plate counts (HPC) (A) and percentage of culturable cells from total cell concentrations (B) from the 1<sup>st</sup> litre of tap water after overnight stagnation compared to samples taken from the same tap after flushing for five minutes. Data are from 10 different households belonging to the same distribution network. Colony forming units (CFU) were determined after 3 days incubation on PCA at 30°C. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values (n = 10).

### 3.5. Localization of bacterial growth in household taps

Water from three taps in separate households was sampled step-wise to localize bacterial growth after overnight stagnation (Fig. 4). In one example, the bacterial cell concentrations decreased rapidly from  $2.3 \times 10^5$  cells/ml in the first 20 ml to  $7.9 \times 10^4$  cells/ml after only 200 ml and then gradually down to  $4.9 \times 10^4$  cells/ml after 2 l flushing. A final drop in cell concentration ( $3.7 \times 10^4$

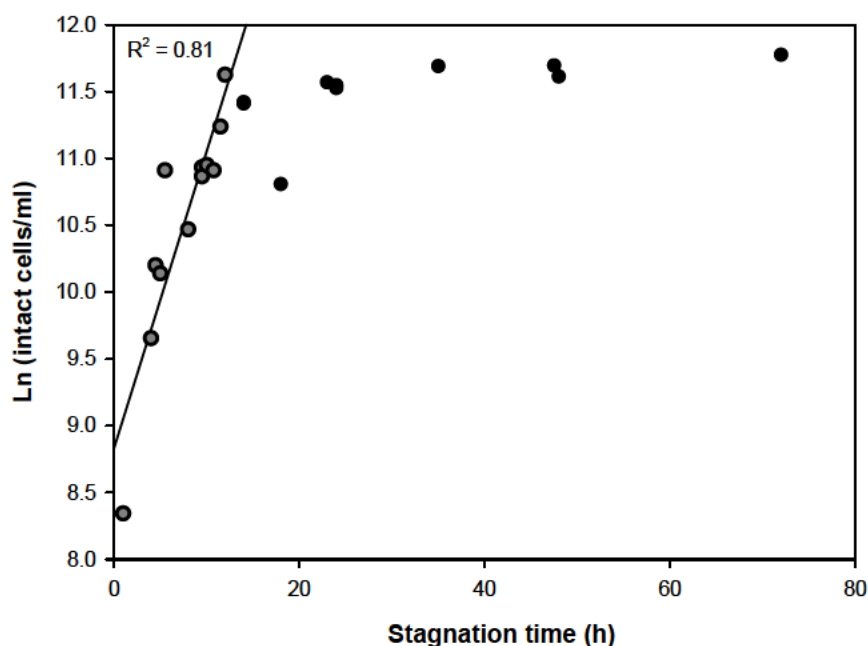
cells/ml) and temperature (from 19.5 °C to 12 °C) indicated that ‘network quality’ water was only reached after flushing of five minutes (approximately 30 l). The same trend was observed in all three taps sampled (Fig. 4). An integration of the decline in cell concentrations during the first 200 ml of tap water after stagnation shows clearly that these first millilitres contributed a considerably amount of cells (13 - 30 %) to the cell concentration measured in the first litre of tap water following overnight stagnation. There was no direct correlation between the total cell concentrations and the water temperatures. The data described above were obtained from three household taps, and it is evident that the amount of water affected by bacterial growth can vary considerably between different buildings. For example, we have observed an increase in cell concentration up to  $4.5 \times 10^5$  cells/ml after overnight stagnation in a new building with cell concentrations only starting to decrease after one litre of flushing (data not shown). The amount of water affected and the extent of growth depend on the piping material, pipe diameters, the size and age of the building, the temperature, volatile organic carbon contamination in the house and the distance to the next network node (Servais et al., 1992; Kerneys et al., 1995; Niquette et al., 2000). However, a very simple mitigation strategy is a short flushing of the tap before use.



**Figure 4.** Decrease of intact bacterial cell concentrations (filled symbols) and temperatures (open symbols) in the first two litres of household tap water in the morning following overnight stagnation. The samples were taken stepwise from three taps in separate households and measured with flow cytometry. The temperatures were measured immediately after taking the samples.

### 3.6. Influence of stagnation time on bacterial growth

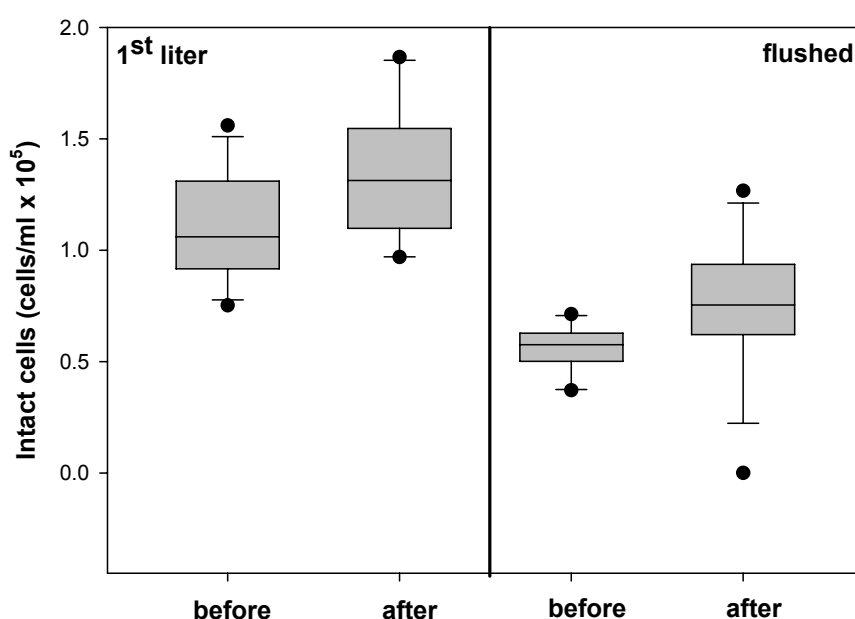
The influence of stagnation time on bacterial cell concentrations in a single tap was tested. Cell concentrations increased at a rate of 0.22/h until 12 h of stagnation (Fig. 5). Longer stagnation times did not lead to a further increase in cell concentrations, suggesting that bacterial growth was substrate limited. Temperature of the water samples was constant between 20 and 22 °C during the time period the tap was sampled. It is common sense that the underlying reason for the observed cell increases is bacterial growth; if only detachment of cells, without additional growth occurred, the regular use of a tap would eventually result in a complete removing of bacteria from the pipes. Similarly, steady increases of cell concentrations during stagnation in a model distribution system were reported previously (Boe-Hansen et al., 2002; Lehtola et al., 2007). In one of the studies growth only occurred until 12 hours stagnation time, the authors argued that further growth was limited by assimilable organic carbon (AOC) (Boe-Hansen et al., 2002).



**Figure 5.** Increase of intact bacterial cell concentration after varying stagnation times in the same household tap measured with flow cytometry. Intact cell concentrations increased until 12 h stagnation time and those data points were used for regression (●). The tap was flushed and then kept closed for the specific stagnation time. The increase in intact cell concentration is the cell concentration from the stagnated water sample minus the cell concentration from the flushed water sample.

### 3.7. Regrowth on biodegradable carbon

Assimilable organic carbon (AOC) is regarded as one of the main factors governing heterotrophic growth in drinking water (van der Kooij and Hijnen, 1988). We have measured whether bacterial growth was promoted by AOC in the water, by direct incubation of the water from flushed and first litre samples from the ten households for four days. Cell concentrations in flushed water samples only increased slightly ( $(2.4 \pm 2.1) \times 10^4$  cells/ml) during incubation (Fig. 6). This increase would be equal to an AOC concentration of about 2  $\mu\text{g/l}$  (Vital et al., 2007). Comparing these numbers with the increase in cell concentrations of the first litres ( $5.4 \pm 2.1 \times 10^4$  cells/ml; Fig. 6) after stagnation in household taps, it is clear that AOC can only account partially (45 %) for the increase in cell concentration. In stagnated water samples the increase during incubation was even less ( $1.8 \pm 3.3$  cells/ml) (Fig. 6), suggesting that bacteria used up AOC during stagnation. We also measured total organic carbon before and after flushing, but no difference between flushed and stagnated water samples was detected (Table 1). However, the amount of growth during stagnation was probably not enough to allow detection of DOC changes.

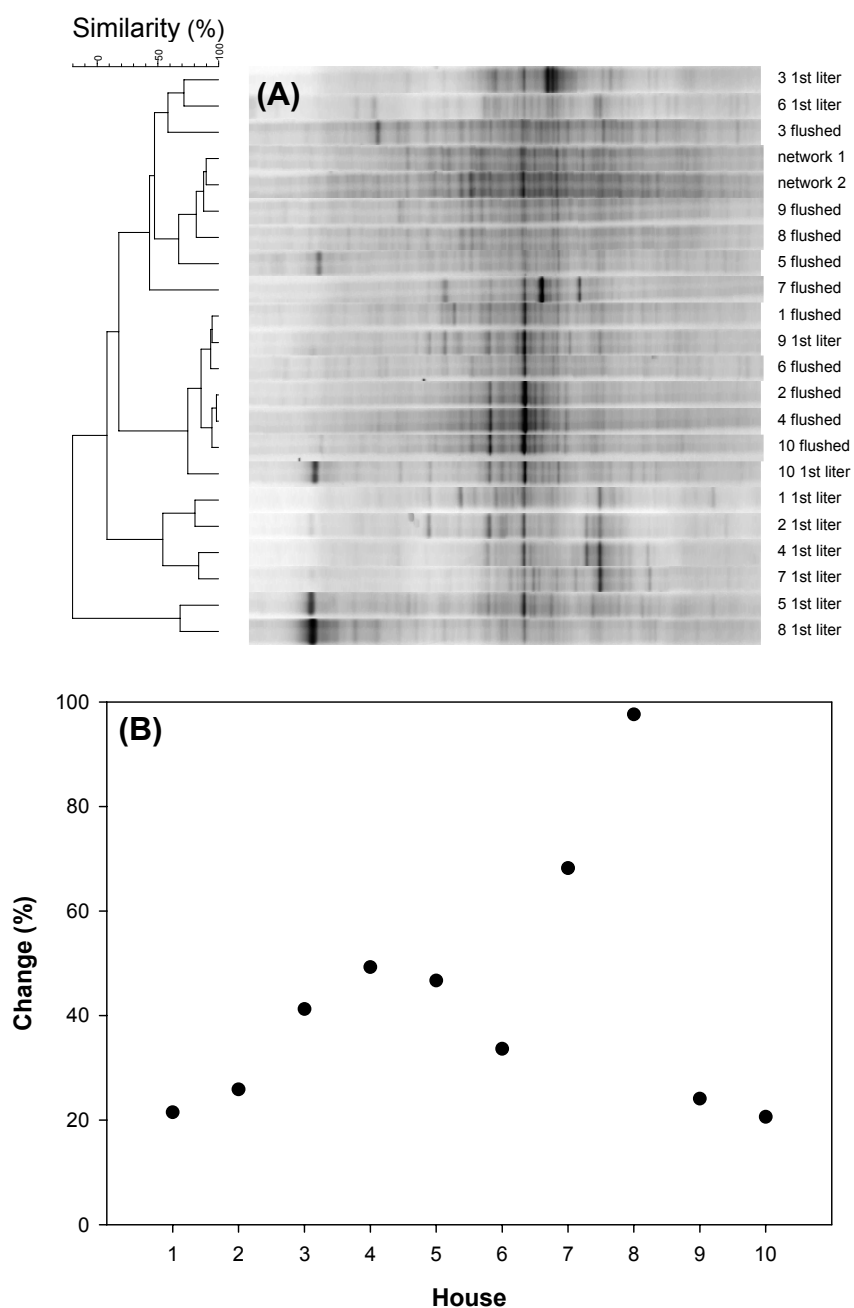


**Figure 6.** Ranges of intact bacterial cell concentration from tap water from 10 separate households measured immediately after sampling (“before”) compared to the cell concentrations after incubation at 30°C for 4 days (“after”). Samples were taken from the first litres of tap water in the morning and from the same taps after flushing for 5 min. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values (n = 10).

Growth in new buildings might be also promoted by nutrients leaking from the pipes. Commonly used piping materials in house drinking water installations are copper, galvanized steel and polymer materials. Several of such piping materials (PEX, HCPE and PVC) have been reported to release volatile compounds as migration products in test drinking water samples (Skjevrak et al. 2003). Several of such materials were shown to promote bacterial growth when being in contact with drinking water (Enkiri et al., 2006; van der Kooij et al., 2003). In addition, differences in the extent of biofilm formation between materials as PVCu, PE40, gray iron, galvanized steel, stainless steel and PEX were observed in model drinking water systems (Niquette et al., 2000; Lehtola et al., 2007). While this is an important issue in new buildings, the cessation of growth (Fig. 5) suggests a short-term nutrient limitation, which would not be consistent with continuous leaking from pipe material.

### ***3.8. Influence of stagnation on microbial community composition***

To test whether bacterial growth in household taps caused a change in the microbial composition we analysed the total microbial community with DGGE. Bacteria in drinking water systems were found to be as diverse as in other typical freshwater systems. Previously communities dominated by *Proteobacteria* (*Alpha*-, *Beta*-, *Gammaproteobacteria*), *Cyanobacteria* and *Bacteroidetes* were observed in drinking water systems (Kahlisch et al., 2010). Species as *Pseudomonas*, *Flavobacterium*, *Aeromonas*, *Acinetobacter*, *Corynebacterium*, sulphatobacteria and ferrobacteria were frequently detected (Laurent et al., 2005b; Hoefel et al., 2005a, b). The purpose of the present study was not to identify specific species in the tap water, but to determine whether stagnation causes a change in the global microbial community composition. We compared the band patterns of flushed and stagnated water samples according to their similarities based on clustering (Fig. 7A). Each stagnated water sample was found in a separate cluster as the flushed water sample from the same tap, clearly indicating that the community changed considerably during stagnation. Expressed in percentages, those differences varied between small community changes of 20 % in three houses up to large changes between 50 and 100 % in most of the houses (Fig. 7B). Community changes on household level were also observed previously when a shift to a higher percentage of Gram-negative to Gram-positive bacteria was monitored on heterotrophic plate counts from the distribution network to the tap (Pepper et al., 2004).



**Figure 7.** Cluster analysis of denaturing gradient gel electrophoresis (DGGE) patterns from household tap water from the total community of stagnated (1<sup>st</sup> litre), flushed and two water samples from the distribution network. The first number is the household tap from which the water was sampled (A). Changes of bacterial community composition (in %) between stagnated and flushed water samples (B). Data is from 10 different households belonging to the same distribution network.

Only slight changes in richness (indicated by the number of bands) and evenness of the populations during stagnation were observed. On average  $37 \pm 18$  DGGE bands were present in flushed water samples and decreased slightly to  $28 \pm 9$  during stagnation (not significant,  $p > 0.05$ ). The Gini coefficient describes the degree of evenness of a community; the higher the Gini coefficient, the more uneven a community is (Wittebolle et al., 2009). In case all species are present in the same number (same band intensities) the community is even with a Gini coefficient of 0. Flushed populations were relatively uneven with a Gini coefficient of  $0.69 \pm 0.19$ . The increase of the Gini coefficient to  $0.77 \pm 0.09$  during stagnation showed that the community became slightly more uneven (not significant,  $p > 0.05$ ). This decrease of evenness was due to some species getting more dominant in the samples probably due to growth, since a doubling of the cell concentrations was measured by flow cytometry.

The changes in microbial populations during stagnation could have been either due to planktonically growing bacteria or due to microbial growth and subsequent detachment in the biofilms of the pipes. Growth and detachment of cells from biofilms are implied by bands present in stagnated water samples, which were not present in flushed water samples (e.g. houses four, seven and eight). Meanwhile, factors like piping material can also have an impact on the development of the microbial communities in the biofilm. This was shown in a study in which the development of different microbial communities on PE and copper pipes in the biofilm as well as the outlet water was observed in a model distribution system (Lehtola et al., 2004). We did not investigate this further, since biofilms in Swiss household taps cannot be accessed easily.

A change in community composition following stagnation was seen in most households. However, we observed that the DGGE pattern from some of the water samples, taken after flushing, varied considerably from each other and from the pattern of the network samples. This is in stark contrast to the cell concentration measurements (Fig. 1 - 3), and suggests localized influences on microbial composition in the flushed samples representing “network quality” water. Due to the nature of the sampling, we could not ascertain whether the different community structure was a result of household influences (network quality not reached with 5 minutes flushing) or a result of localised network influences (variations in the material, and stagnation time in a full scale distribution network).

The visible community change during stagnation stresses the importance not to neglect changes on household level when assessing the consumer’s risk. Stagnation clearly can have a considerable impact, not only on cell concentrations but also on community composition. Further development of

good validation methods and guidelines regarding the microbiological quality of in-house water installations are essential, while additional information about the influence of specific piping materials on microbial growth is required.

#### **4. Conclusions**

- The results show clearly that a change in microbial cell concentration and composition occurred during stagnation. Cell concentration and microbial composition varied between different houses.
- A short flushing of the tap leads to a decrease in cell concentration and is, therefore, a good mitigation strategy.
- Water quality is usually monitored in the network, but since changes on household level occur, it is important to rethink sampling strategies for risk assessment, specifically when addressing “worst-case scenarios”.
- Further development of material validation methods, recommendations and spot tests for in-house water installations are important.

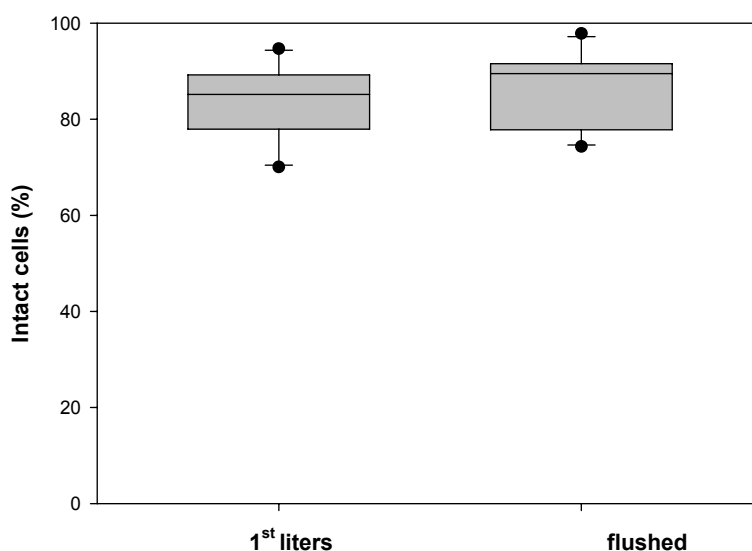
#### **Acknowledgements**

The authors are grateful to the financial support of the EU project TECHNEAU (018320) research grant as well as the financial support from the Flemish Fund for Scientific Research (FWO-Vlaanderen, GP.005.09N).

## Supplementary information

### 1. Percentage of intact cell concentration to total cell concentration in non-chlorinated tap water

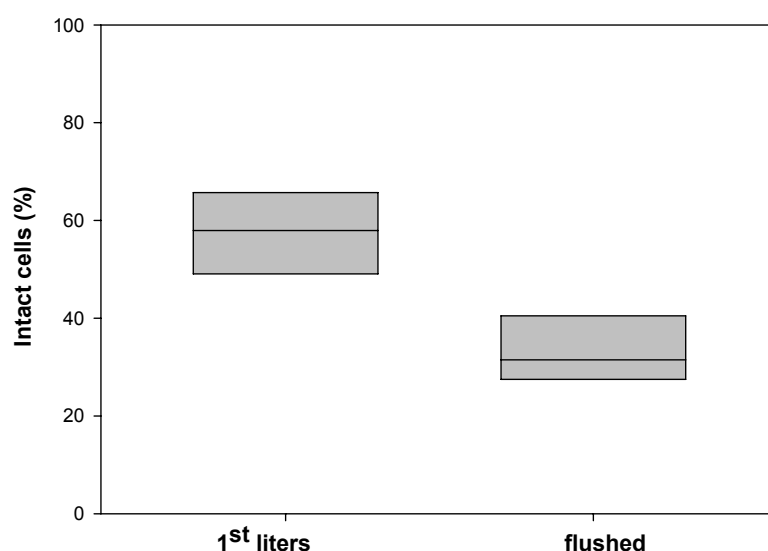
The non-chlorinated water (analyzed in the present study), approximately 85 % of the total bacteria showed intact membranes. No significant difference was observed between flushed and stagnated water samples (Fig. S1).



**Figure S1.** Percentage of intact cell concentrations from the total cell concentration from the 1<sup>st</sup> litres of tap water after overnight stagnation, compared to samples taken from the same tap after flushing for five minutes. Data are from 10 different households belonging to the same distribution network. Intact cells were stained with SYBR<sup>®</sup> Green I/propidium iodide staining, whereas total cell concentrations were only stained with SYBR<sup>®</sup> Green I and measured with flow cytometry. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values (n = 10).

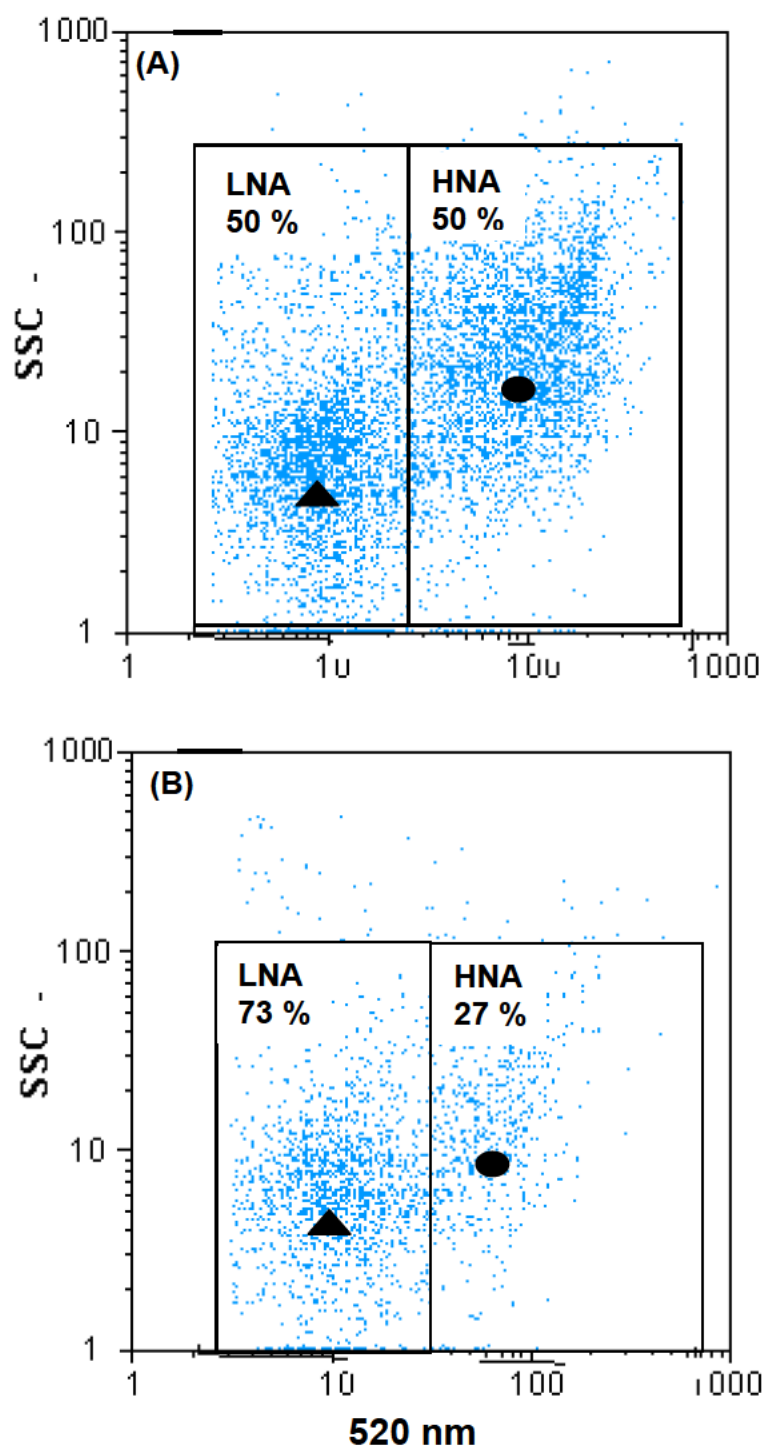
## 2. Percentage of intact cell concentration to total cell concentration in chlorinated tap water

Household water samples from an additional network distributing chlorinated drinking water were analyzed. Disinfection with  $\text{ClO}_2$  causes cell membrane damage. This was clearly seen in the low percentage of intact cells in the flushed water samples (34 %). After stagnation and growth a clear and significant increase in the percentage of intact cells was observed in all households (57 %). This example shows that staining with SYBR<sup>®</sup> Green I/propidium iodide is a useful method to exclude cell membrane damaged cells when assessing bacterial cell concentrations in chlorinated drinking waters, and that overnight bacterial regrowth is a general phenomenon.



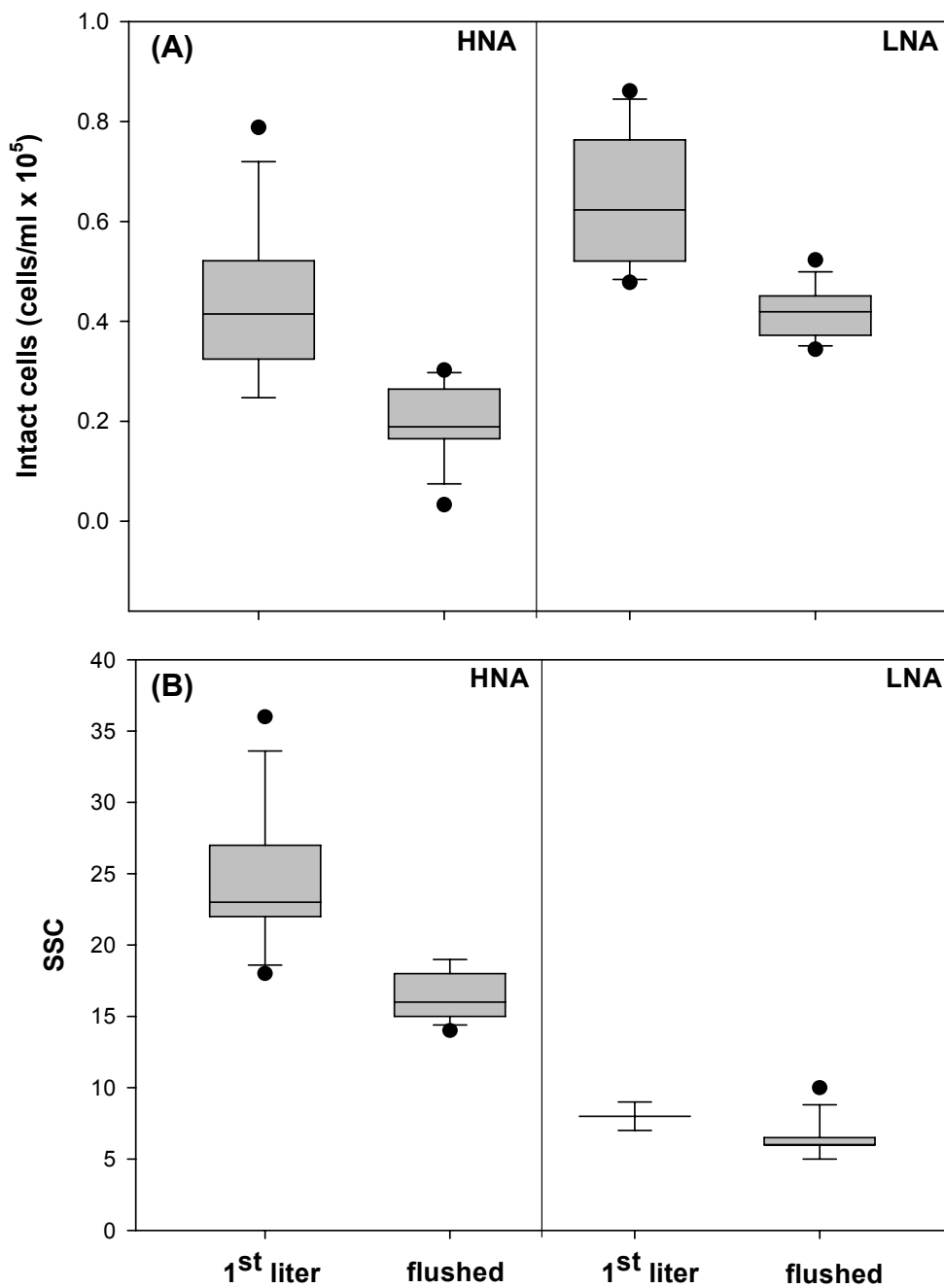
**Figure S2.** Percentage of intact cell concentrations from the total cell concentration from the 1<sup>st</sup> litres of tap water after overnight stagnation compared to samples taken from the same tap after flushing for five minutes. Data are from 6 different households belonging to the same distribution network distributing water with residual disinfectant (0.05 mg/l  $\text{ClO}_2$ ). Intact cells were stained with SYBR<sup>®</sup> Green I/propidium iodide staining, whereas total cell concentrations were only stained with SYBR<sup>®</sup> Green I and measured with flow cytometry.

### 3. Changes in flow cytometric pattern following overnight stagnation



**Figure S3.** FCM plot of a stagnated (A) and flushed (B) water sample. A shift from low nucleic acid bacteria (LNA) to a higher proportion of high nucleic acid bacteria (HNA) was observed by the sideward scatter signal (SSC). The Gmean of the sideward scatter signal of HNA (●) and LNA (▲) is indicated.

#### 4. Changes in HNA/LNA intact cell concentrations and flow cytometric side scatter values.



**Figure S4.** Increase in intact cell concentrations (A) and the average Gmean of the sideward scatter signals (SSC) (B) of high nucleic acid (HNA) and low nucleic acid (LNA) bacteria in 1<sup>st</sup> litre samples compared to samples after flushing. Data are from 10 different households belonging to the same distribution network. Grey bars represent the 75 percentiles, whisker lines represent the 90 percentiles and black dots represent the outlier values (n = 10).



## 6. General conclusions and outlook

In this thesis the microbial community composition of non-chlorinated drinking water was analyzed from the raw water via the drinking water treatment plant to the consumer's tap. The objectives of the thesis were to (1) determine the factors influencing the microbial community composition of the biofilters in the drinking water treatment plant and of the final drinking water; (2) analyze whether this microbial community composition influences the function of the biofilters; (3) study whether the microbial community composition changes during drinking water distribution in the network or during stagnation in house installations. These questions were studied using the drinking water treatment plant "Lengg" of the city of Zürich, the adherent distribution network as well as separate household taps. In this drinking water treatment plant lake water from 40 m depths is pre-ozonated (also for disinfection) before being treated by rapid sand filtration (RSF), intermediated ozonation, granular active carbon (GAC) filtration and slow sand filtration (SSF). Afterwards the water is distributed with  $10^5$  cells/ml without the addition of a residual disinfectant.

### *1. Drinking water treatment*

#### *1.1. Factors determining the microbial community composition during drinking water treatment*

**Microbial community analysis indicated that the water quality determines the microbial community composition during drinking water treatment.**

This was to our knowledge the first study addressing the microbial community composition in biofilters and their effluents in such detail in a full-scale drinking water treatment plant. Similar microbial taxa were present in RSF, GAC and SSF and their filter effluents. DOC and LC-OCD measurements showed that a main function of these indigenous bacteria is the degradation of DOC, which serves as the nutrient source for the bacteria. This suggests that the carbon composition of the water influences the nutrient competition of these bacteria and as a result the microbial community composition in the biofilters. This was also supported by the comparison of the microbial community composition of twelve parallel SSFs. DGGE analysis revealed a similar major microbial community composition on these filters, although the filters were operated differently (e.g. different flow rates) and varied in filter age (0-24 years). Hence, one must conclude that the water quality (carbon composition) of the water influent, which was the same for all filters, determined the microbial community composition. The influence of the water quality on the microbial community

composition was also reported in previous studies, where in pilot plant biofilters a different microbial community composition was found after treatment with ozonated water in comparison to non-ozonated water (Fonesca et al., 2001; Moll et al., 1998). Also in our study RSF and GAC filters received ozonated water, while the SSF received non-ozonated water and nutrients were constantly removed in the course of the treatment. This change in water quality, which was also observed by LC-OCD measurements, might have caused the variation in relative abundance of the phyla/taxa on the three filter types. However, as observed by Fonesca, Moll and coworkers, the DGGE patterns of the three different filter types differed. Only the 454 pyrosequencing data allowed a deeper analysis of the changes in microbial community composition and demonstrated that differences were a result of the variation in relative abundance of the phyla/taxa and not of a completely different microbial community. The factors determining the microbial community composition in the drinking water treatment plant are of interest, since they also influence the microbial community composition of the final drinking water. Also, the microbial community composition on the biofilters might have an implication for their functioning. Knowing the factors influencing the microbial community composition, might allow us to relate possible changes in community composition in the water to the cause. Future studies that compare many different treatment systems might give more insight into the factors influencing the microbial community composition. However, almost all drinking water treatment plants differ with respect to the water source and the amount and kind of treatment steps in use. Therefore, studies on pilot-scale under defined conditions could give further insight into factors, such as filter material or filter flow rate that might influence the microbial community composition during drinking water treatment.

### *1.2. Functioning of microbial communities during drinking water treatment*

**DOC was removed during each filtration step (RSF, GAC and SSF). The biomass from each filtration step has the potential to degrade complex organic carbon compounds.**

DOC and LC-OCD analysis/data showed that one major function of each biofiltration step is the degradation of organic carbon. Furthermore, results from LC-OCD measurements demonstrated that different fractions of organic carbon were degraded during each biofiltration step. For example, polysaccharides were mainly degraded during SSF, whereas building blocks, neutrals and LMW humics were mainly removed during RSF and GAC filtration. While the efficiency for DOC removal of the SSF was comparably low, LC-OCD analysis showed that a fraction of carbon was removed, which would otherwise be present in the final drinking water and might there lead to microbial growth. Hence, it is quite certain that this last SSF increases the biostability of the final

drinking water. The presence of active biomass on each filter, and the activity of hydrolytic extracellular enzymes clearly showed that each filtration step acts as a biofiltration step. Because of the variations in biomass between different filters, the limited amount of samples that we were allowed to collect from RSF and GAC filters, the lack of depth profiles and the varying operation regimes of the filters (e.g., RSF and GAC are backwashed, SSF not), it was not possible to obtain a complete mass balance of the total amount of biomass in each filtration step from the DOC removal.

The function of the microbial communities in biofilters, their activity, and their potential for organic carbon removal has not been addressed so far. We wanted to know whether or not the differences in organic carbon degradation in the different filtration steps can be attributed to the different microbial community composition (the varying relative abundance of the similar microbial taxa) of the filters. In general, extracellular enzyme assays showed that complex organic carbon compounds can be potentially degraded in each biofiltration step and not only in the SSF. The SSF study showed that enzymatic activities increased proportionally to the ATP content of the samples, which allowed us to normalize enzymatic activities obtained from the three different filtration steps to ATP. However, enzyme activities exhibited by the biomass from each filtration step were very similar, indicating no significant differences in their function. This suggests that the observed differences in organic carbon removal were not caused by a different functioning of the biomass. Other factors such as the filter flow rate or the characteristics of the SSF might have lead to the degradation of more complex carbons in these filters. This, for example, implies that a SSF can not be replaced by, e.g., a RSF. Of course, it is possible that differences in function were not reflected in the enzyme assays chosen. Biolog Plates indicated differences for the degradation capacity for carbon sources. Since this assay also relies on growth of the inoculated community it does not necessarily reflect, which of the substrates in the filters might be actually degraded. To conclude, both methods did not allow determining whether or not the different microbial community composition on the filters was responsible for the differences in organic carbon removal.

Proteomic studies might possibly give a deeper insight into functional differences; however, also here might then be hidden by a large background of proteins for the degradation of easily degradable organic carbon in every cell. Alternatively, metagenomic studies (analyzing the whole microbial genomes for differences in the presence of certain genes) might give additional insights into microbial community function. However, it is questionable whether differences are detected with such an approach, if it is not known for which genes/functions the data should be analyzed. The direct and most practical way to test the influence of different microbial communities on the potential for organic carbon degradation during drinking water treatment would be to run two

biofilters in parallel that differ in their microbial community composition and to analyze the amount and fractions of organic carbon that are removed. Different microbial communities can for example be obtained by running ozonated water over one filter and non-ozonated water over another filter. After establishment of a microbial community, which might take several months, the same water could be run over both filters. Such a filter set-up would not only show whether the microbial communities differ in their carbon degradation potential, but it would be also possible to observe whether or not the microbial community adapts to the changing conditions and - if yes- in which time span. Such information is of relevance for a water supplier when a new filter is taken into operation.

### ***1.3. Origin of microbial communities in the final drinking water***

|   |
|---|
| <b>Bacteria in the final drinking water originated from GAC and/or SSF.</b> |
|---|

Following the microbial community composition from the raw water to the reservoir in the drinking water treatment plant demonstrated that the raw water community did not determine the composition on the filters. The microbial community composition on the biofilters was different from the lake water microbial community. *Actinobacteria* that were mainly abundant in the lake water were only very rarely present in the RSF after ozonation (Chapter 3). Probably the alterations in carbon composition after ozonation and the different environment in the biofilters lead to a different selection pressure. The origin of the microbial strains that established themselves on biofilters is not clear. They might originate from the filter material (which is not sterile at the beginning), from a first flushing of the treatment plant with tap water before it is taken into operation, or from cells surviving the ozonation process. The measurement of intact cell concentrations indicated that the microbes present in the final drinking water originated from the GAC and SSFs of the drinking water treatment plant. However, it was not possible to determine whether these microbes originated from the GAC or the SSF, since the microbial community composition (with respect to the taxa that were present) was very similar. The increase in unclassifiable cells and the change in the flow cytometric pattern during SSF only suggest that the SSF also influences the final microbial community composition in the drinking water, a finding that is not really surprising. However, the study clearly showed that SSF do not remove all microbial cells, as it is sometimes assumed based on the reported removal of pathogens in SSFs (Huisman and Wood, 1974). It might be possible to analyze in a pilot-scale study whether or not bacteria from a

GAC can pass the SSF by staining the GAC community and to then analyze whether these stained cells pass the SSF.

## 2. Stability

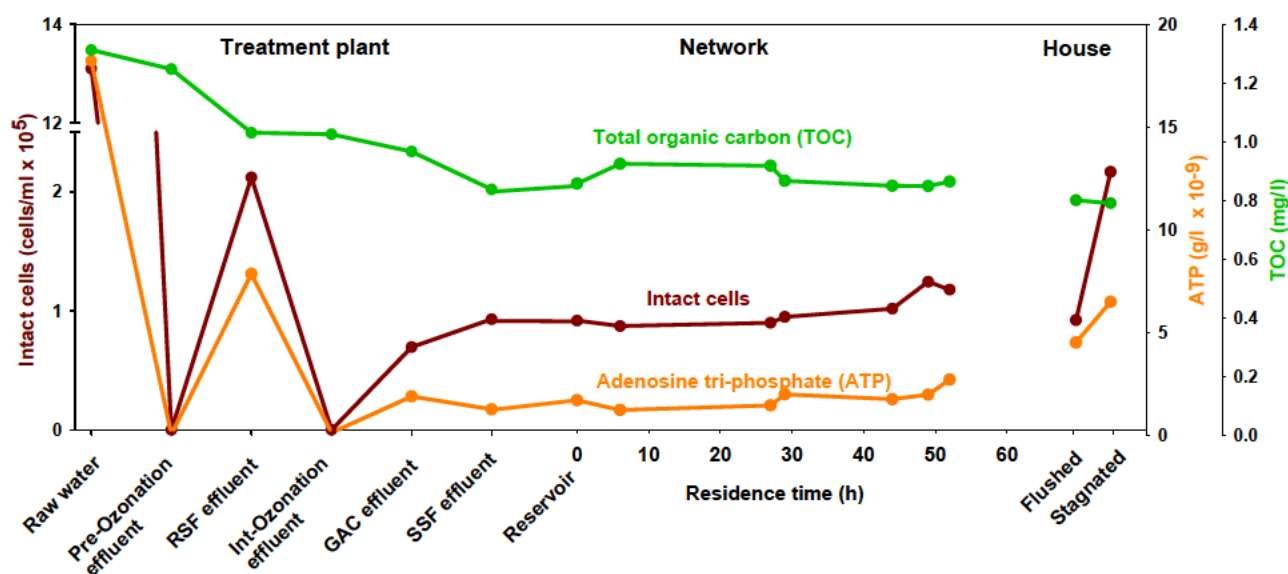
### 2.1. Drinking water distribution network

**Drinking water was stable with increasing residence time during distribution, only at the two sampling points containing water with the longest water retention time a slight change due to an increase in species belonging to the family of *Comamonadaceae* occurred.**

The flow cytometry dot-plots showed that the cells in the drinking water reservoir must have been released and/or flushed off from the GAC and SSFs. This was also reflected in the presence of the same microbial taxa in the final drinking water as well as the GAC and SSFs. At six locations in the drinking water distribution system with increasing residence time, the microbial community composition remained stable with respect to intact cell concentration, ATP concentration, total organic carbon concentration and microbial community composition (Fig. 1). This implies that in this particular system the water that reaches the consumer's house, is still of the same microbial quality as that released from the treatment plant. This is normally also where the responsibility of the water supplier ends. With respect to biological stability, the pyrosequencing showed the same result as the DGGE patterns, although both analyses were done independently and by different people. This enormously increases the credibility of these results. The very stable microbial community composition in the distributed water suggests that during distribution the biofilm bacteria on pipe surfaces do not influence bulk water microbial community composition, or, that biofilms on the pipe surfaces have a similar composition as the microbial communities in the bulk water. The latter explanation is less unlikely than the former, nevertheless further studies are required that address this issue.

At the two locations containing water with the longest retention times, a slight increase in cell concentration and a change in microbial community composition were observed. Using pyrosequencing analysis, this increase in cell concentration of  $2 \times 10^4$  cells/ml could be attributed to an increase from 2 to 60 % of members belonging to the family of *Comamonadaceae*. The advantage of pyrosequencing here is that the change can be attributed to the growth of a hygienically irrelevant species, which directly implies that no health risk was connected to the

change. The observed increase in cell concentration corresponds to a decrease of in between 2-20  $\mu\text{g/l}$  organic carbon only (Hammes et al., 2010a). This change can, and was not detected, neither by the AOC nor the TOC method. This demonstrates that compared to organic carbon determination methods, the direct assessment of changes in cell concentration or community composition increases the sensitivity for detecting changes. The observed change might have been due to the longer residence time in the system leading to microbial growth in the water or in the biofilms on the pipes, and/or the release of cells from the biofilms; yet, also other explanations, such as a change in microbial community composition in a drinking water reservoir, might be possible. Future studies should, for example, directly compare communities of biofilms with those in the bulk water phase to address the impact of biofilms on the microbial community composition in the water. Alternatively, samples could be taken over a certain time period before a cleaning of the pipes during which biofilms are removed by flushing. Comparing the microbial community composition in the water before and after the flushing, might thus show whether the microbial community composition on the pipes is different of the water phase.



**Fig. 1.** This figure from combined chapters 2, 4, 5 showing the changes in intact cell concentrations measured with flow cytometry, adenosine tri-phosphate (ATP), and TOC concentrations, from the raw water to the distribution network in Zürich. Household samples are from an open network (Dübendorf); hence samples cannot directly be compared to the Zürich network, but to water samples after flushing the taps.

**A high biological stability was observed over a time period of four months and the same microbial community composition and cell concentration was also measured two years later.**

This local situation, namely a stable bulk at most parts in the distribution system and an increase and change at the two sampling points containing water with the longest retention times was observed during the time period of four months and again two years later. This suggests that the filters in the drinking water treatment plant buffer against possible changes in water quality that might occur in the raw water, which then leads to the production of stable drinking water. This is supported by the report of Hammes et al., 2010a, who showed that the cell concentration after RSF varies greatly over a time period of 18 months, whereas cell concentrations after GAC and SSF changed only slightly. However since also filter backwashing can influence the final cell concentration in the drinking water, it must be assumed that particularly the SSF, which is the only filter not being backwashed, leads to the high stability in cell concentrations in the drinking water over time.

**The microbial community composition at the consumer's tap depends on various factors such as treatment or disinfectant regimes.**

So far changes in microbial community composition were not investigated in non-chlorinated drinking water systems for the analysis of biological stability. However, a few distribution networks that receive only slightly chlorinated water have been studied. In Austria, Burtscher and coworkers (2009) obtained similar DGGE patterns at 10 points in a system distributing water with chlorine concentrations below 0.1 mg/l, such low chlorine concentrations have little effect and the high similarity of DGGE patterns in another system support our findings. Nevertheless, the study of Burtscher and coworkers (2009) was restricted to microbial community analyses, with the aim to compare the HPC communities present with the total communities. Cell concentrations fluctuated greatly in the range of  $1 \times 10^5$ -  $4 \times 10^5$  cells/ml. This might result from the method used as cell concentrations were determined by microscopic counting (Table 1). More studies addressing the microbial community composition in drinking water were conducted in chlorinated systems. In contrast to non-chlorinated systems, chlorinated systems can be presumed to contain a large part of dead cells; however, these cells might still contain intact DNA and are consequently included when molecular methods are applied. Hence, the aims of these studies varied tremendously from, e.g.,

developing methods to distinguish between alive and dead cells in chlorinated water, developing new molecular detection methods for pathogens, or to gain new insight into microbial ecology of drinking water systems (Table 1). For example, it was found that the source water microbial community composition significantly influenced the final microbial community composition in the water, when water was treated by flocculation and sand filtration only (Eichler et al., 2006). This study was based on extraction of 16S rRNA genes but also of 16S rRNA, to demonstrate that also active cells were present in the water. In contrast, in our study no direct influence of the microbial community composition of the raw water on that in the final drinking water was observed. This is most probably due to the different treatment process and shows that depending on the treatment process and disinfection method applied, different factors can contribute to the final microbial community composition in drinking water. Also, chlorination and chloramination has shown to cause a shift in the microbial community composition and a drastic decrease in diversity (Hoefel et al., 2005 a, b) (Table 1). Hoefel and colleagues also detected microbial growth after a decrease of the residual disinfectant in drinking water distribution systems. This was also observed in our group, when an increase in cell concentration from  $1 \times 10^5$  -  $5 \times 10^5$  cells/ml was measured in a chlorinated system containing high DOC concentrations (Hammes, personal communication). Generally, these studies demonstrate that the microbial community composition present in the water when it reaches the consumer depends on various factors: The treatment process, whether the water is distributed with or without a residual disinfectant, and finally the biological stability during drinking water distribution.

|   |
|---|
| <b>Microbial communities were highly diverse.</b> |
|---|

The microbial community composition in the water was very diverse: 30 - 50 of the sequences obtained with pyrosequencing were present at a relative abundance of more than 1 %. Generally, less diversity was observed in chlorinated systems than in the non-chlorinated system of the present study (Table 1) (Hong et al., 2010; Eichler et al., 2006; Hoefel et al., 2005 a, b). Since the bacteria in the final drinking water originated directly from the drinking water treatment plant, it is most likely that these microbes grew on the substrates (mainly carbon compounds) that were present in the water. Natural water is composed of a mixture of many different individual organic carbon compounds that are readily available for growth (Münster, 1993). A high number of different substrates and the capability of many bacteria to use different substrates simultaneously (Ihssen and Egli, 2005; Kovarova-Kovar and Egli 1998), might contribute to the high diversity in drinking water. Such a high diversity might protect the water from growth of pathogens during distribution.

For example, it was observed that pathogens, such as *E. coli* O157 and *Vibrio cholerae* can not grow in natural waters, if natural bacteria are present, whereas growth was observed in sterile natural waters (Vital, 2010). The higher the diversity, the higher the probability should be that in case of nutrient contamination of the system, a natural bacterium is present that can grow faster than a pathogenic strain. Hence, the chance for pathogens in a system to proliferate should be reduced by the presence of a highly diverse natural microbial community. Also in slightly chlorinated systems or after depletion of the chlorine during distribution “live” bacteria were found in several studies; however, diversity was reduced in all cases (Williams et al., 2004; Hoefel et al., 2005 a, b). This raises the question, whether a low chlorine concentration that either does not lead to a complete killing of all bacterial cells, or which is not maintained during distribution, is useful or whether it even leads to a higher risk for pathogenic growth. A good way to address this issue would be to monitor a pilot-plant system and to spike pathogens into the water when it is non-chlorinated and repeat the test with the same water but slightly chlorinated. In full-scale distribution systems it might be interesting to compare chlorinated and non-chlorinated systems and particularly the occurrence/frequency of uncontrolled microbial growth. However, usually many parameters, such as the water quality and the water treatment, differ between systems and this makes it difficult to link differences to one particular factor.

**Microbial communities during drinking water treatment and distribution were dominated by *Proteobacteria* and a large fraction of sequences could not be classified.**

In addition to the analysis of the biological stability, pyrosequencing gave a detailed insight into the species composition of the drinking water produced at the drinking water treatment plant “Lengg” in Zürich. In the SSF effluent and also the distribution network, most bacteria belonged to the group of the *Proteobacteria* but also *Bacteroidetes*, *Nitrospira*, *Acidobacteria*, *ODI* and *Planctomycetes* were present. Noticeable was the high percentage (> 50 %) of bacterial sequences that could not be classified. Similar to our findings, previous studies showed that the majority of phylotypes in chlorinated drinking water were related to uncultured bacterial taxa and difficult-to-classify bacterial sequences (Hong et al., 2010; Revetta et al., 2010; Eichler et al., 2006). When comparing the microbial community composition of the drinking water with other published studies, it is obvious, that the dominant bacterial groups in the total community differ between studies (Table 1) (Hong et al., 2010; Revetta et al., 2010; Roeder et al., 2010; Burtscher et al., 2009; Eichler et al., 2006; Hoefel et al., 2005a, b). Only *Proteobacteria*, which are present in most freshwater

environments, seem to dominate in all systems (Hong et al., 2010; Revetta et al., 2010; Roeder et al., 2010; Burtscher et al., 2009; Eichler et al., 2006; Hoefel et al., 2005a, b) (Table 1). Since particularly the raw water quality or the microbial community composition of the raw water (discussed above) can influence the composition, the differences in the microbial community composition of different drinking water distribution systems are probably as diverse as the differences between different freshwater habitats. The microbial composition can thus differ, but is not necessarily of any health relevance. For a better understanding of the potential role of bacteria in drinking water, further attempts to cultivate and characterize these bacteria are necessary. In general, detailed information concerning the microbial community composition was obtained with pyrosequencing. Although the many “names” do not tell us much at this stage, they allow a comparison of different samples and to detect changes. In comparison to the DGGE patterns, a better insight was obtained into the extent of the changes that occurred. Pathogens can potentially be detected by pyrosequencing in the future, as soon as it will be possible to sequence longer fragments of the 16S rRNA genes. In our study, *Legionella* were present in the drinking water at a very low percentage (1 %) and were then identified with a clone library to belong to uncultured *Legionella*, but also to the potentially pathogenic *Legionella donaldonii*. Nevertheless, up to date information is missing on how many pathogenic strains are present in the environment that were not detected previously but can be detected with the more sensitive methods today. Possibly such strains are present everywhere and are not of much risk. Also, cells detected by 16S rRNA gene sequencing methods might be dead. For the Zürich drinking water, we know from experience that these strains do not pose a detectable health risk, since thousands of people drink the water everyday without health-related problems reported.

**Table 1.** Overview of studies addressing the microbial community composition in drinking water.

|  | Goals of the study   | Methods applied  | Sample origin/Treatment of the water   | Main Outcome/Dominant taxa detected in the total microbial community   |
|--|--|--|--|--|
| <b>Burtscher et al., 2009</b>  | Detailed investigation of the HPC vs. the <i>in situ</i> bacterial community structure.                                    | <ul style="list-style-type: none"> <li>- 16S rRNA gene amplification, separation with DGGE</li> <li>- 16S rRNA gene amplification of HPC colonies and separation with DGGE</li> <li>- Analysis of total and HPC communities</li> <li>- HPC, direct count with microscopy</li> </ul>  | <ul style="list-style-type: none"> <li>- 10 sampling locations alongside a complex water distribution pipe system at two time points</li> </ul> <b>Treatment:</b> ClO <sub>2</sub> (< 0 - 0.1 mg/l)  | <ul style="list-style-type: none"> <li>- Only slight changes in microbial community composition at different sampling dates and sampling locations</li> <li>- Total community was completely different to HPC community on the DGGE</li> </ul> <b>Dominant taxa detected:</b> <i>β-Proteobacteria</i> , <i>α-Proteobacteria</i> , <i>Comamonadaceae</i> , <i>Caulobacter</i> , <i>Bdellovibrio</i> spp.<br><b>Diversity:</b> 8-12 OTU  |
| <b>Eichler et al., 2006</b><br><br><b>Henne et al., 2008</b><br><br><b>Kahlisch et al., 2010</b> | <b>Eichler:</b><br>Gain new insights into the microbial ecology of drinking water systems                                  | <b>Eichler:</b><br><ul style="list-style-type: none"> <li>- 16S rRNA and 16S rRNA gene amplification, separation with Single Strand Conformational Polymorphism (SSCP)</li> <li>- Analysis of the total community</li> </ul>   | <b>Eichler:</b><br><ul style="list-style-type: none"> <li>- Two surface water reservoirs</li> <li>- Tap water samples</li> </ul>   | <b>Eichler:</b><br><ul style="list-style-type: none"> <li>- Significant influence of two source waters on the composition of the microflora reaching the consumer</li> <li>- Chlorination of the processed raw water strongly affected bacterial community structure</li> <li>- Bacterial community remained rather constant from the storage containers to the tap</li> <li>- Different pattern obtained from rRNA and rRNA gene SSCP</li> </ul>  |
| <b>All three studies were conducted with water from the same drinking water taps.</b>            | <b>Henne/Kahlisch:</b><br>The Development of new molecular detection technologies of microbial pathogens in drinking water | <b>Henne:</b><br><ul style="list-style-type: none"> <li>- 16S rRNA gene amplification, separation with SSCP</li> <li>- Analysis of the total community</li> </ul> <b>Kahlisch:</b><br><ul style="list-style-type: none"> <li>- Live/Dead staining and sorting with FACS</li> <li>- 16S rRNA amplification before and after sorting, separation with SSCP</li> </ul>                  | <b>Henne:</b><br><ul style="list-style-type: none"> <li>- Tap water from autumn 2006 to spring 2008.</li> </ul> <b>Kahlisch:</b><br><ul style="list-style-type: none"> <li>- Tap water at 3 different days</li> </ul> <b>Treatment (in all 3 studies):</b><br>Chlorinated (0.2 - 0.7 mg/l) | <b>Henne:</b><br><ul style="list-style-type: none"> <li>- Seasonal pattern in microbial community composition detected</li> </ul> <b>Kahlisch:</b><br><ul style="list-style-type: none"> <li>- 32 % of phylotypes occurred only in the “alive” sorted fraction; 21 % only in dead, 46 % in both fractions</li> </ul> <b>Dominant taxa detected:</b> <i>α-Proteobacteria</i> , <i>β-Proteobacteria</i> , <i>Bacteroidetes</i> , the majority of phylotypes were related to uncultured bacterial taxa<br><b>Diversity:</b> 71 phylotypes |
| <b>Feazel et al., 2009</b>   | Determining the composition of shower head biofilms and waters   | <ul style="list-style-type: none"> <li>- rRNA gene amplification</li> <li>- Clone libraries and sequencing</li> <li>- Quantitative PCR: to detect <i>Legionella pneumophila</i> and <i>Mycobacterium avium</i></li> <li>- ITS gene sequencing of samples containing <i>M. avium</i> for <i>Mycobacterium</i> spp.-specific amplification of the 16S-23S rRNA ITS sequence</li> </ul> | <ul style="list-style-type: none"> <li>- 45 showerhead sites (total of 52 samples) throughout the United States between May 2006 and January 2008</li> <li>- Biofilm samples and water samples were taken</li> </ul>   | <ul style="list-style-type: none"> <li>- Variable and complex microbial assemblages were found inside showerheads, most genus- or species-level relatedness groups are commonly found in water and soil</li> <li>- <i>Mycobacterium avium</i> (potential pathogen) was identified in 20 % of showerhead biofilms, with an abundance of 32 % of the library when observed (&gt; 100-fold above background water contents)</li> </ul> <b>Dominant taxa detected:</b> <i>Actinobacteria</i> , <i>Proteobacteria</i> , <i>Firmicutes</i>   |

|                                 | Aims of the studies  | Methods applied  | Sample origin/Treatment of the water  | Main Outcome/ Dominant taxa detected in the total microbial community  |
|---------------------------------|--|--|---|--|
| <b>Hoefel et al., 2005 a, b</b> | b) Profiling of bacterial populations at different stages of a water treatment process<br>b) Flow cytometric cell sorting coupled with PCR-DGGE was used to study a water treatment plant and distribution system employing chloramination during an instability event | - CFDA and Live/Dead staining and sorting with FACs<br>- 16S rRNA amplification before and after sorting, separation with DGGE<br>- Analysis of total and HPC communities<br>- FISH<br>- HPC<br>- AOB specific nested PCR<br>- NOB specific 16S rRNA gene-directed PCR | - Samples from the raw water to the distribution system from two different systems (a and b)<br><b>Treatment:</b><br>a) Chlorination: 1.06 mg/l chlorine in the finished water, 0.4 mg/l in the distribution system<br>b) Chloramination: 3.14 mg/l chlorine in the finished water, < 0.1 mg/l in the distribution system | - Level of active bacteria increased to 10 <sup>5</sup> cells/ml after chloramine residual had diminished<br>- <i>Nitrosomonas</i> species were present but not in the associated water treatment plant<br>- Total community was completely different to HPC community on the DGGE.<br><b>Dominant taxa detected:</b> $\beta$ - <i>Proteobacteria</i> , also $\alpha$ - <i>Proteobacteria</i> and <i>Nitrobacter</i><br><b>Diversity:</b> 2-20 bands |
| <b>Hong et al., 2010</b>        | Explore the possibilities of 454 pyrosequencing for drinking water sample analysis.  | - 16 rRNA gene amplification<br>- 454 pyrosequencing of the total community  | - Water meters from two private households at two sampling dates<br><b>Treatment:</b><br>Chlorinated (concentration not given)  | - Differences in bacterial diversity and composition were observed between two water meters of a drinking water distribution system<br><b>Dominant taxa detected:</b> <i>Firmicutes</i> , <i>Deinococcus-Thermus</i> , <i>Bacteroidetes</i> , <i>Actinobacteria</i> , <i>Proteobacteria</i><br><b>Diversity:</b> 208 and 133 Chao1   |
| <b>Revetta et al., 2010</b>     | Examine the structure and composition of active bacterial communities in bulk drinking water samples   | - 16S rRNA amplification<br>- Clone libraries and sequencing<br>- Analysis of total and HPC communities (21 different colonies were subcultured from which seven different colony morphotypes were analyzed with 16S rRNA gene analysis)                               | - 1 single point of use tap over 3 months<br><b>Treatment:</b><br>Chlorinated (0.67-1.01 mg/l)  | - Suggested which bacterial groups are present in drinking water.<br>- all seven isolates belonged to the $\alpha$ - <i>Proteobacteria</i><br><b>Dominant taxa detected:</b> 57 % of the sequences difficult to classify, <i>Proteobacteria</i> , <i>Cyanobacteria</i> , <i>Actinobacteria</i> , <i>Bacteroidetes</i> , <i>Planctomycetes</i><br><b>Diversity:</b> 60 OTU  |
| <b>Roeder et al., 2010</b>      | Effect of installation materials on biofilm populations in drinking water systems.   | - 16S rRNA gene amplification, separation with DGGE<br>- Clone libraries and sequencing  | - Biofilms from growth supporting materials used for drinking water installations. Samples from flow through systems.<br><b>Treatment:</b> Water from different sources/locations   | - Diversity depended on the material<br>- Composition of the subdivisions was influenced by the tested material<br><b>Dominant taxa detected:</b> <i>Proteobacteria</i>  |
| <b>Williams et al., 2004</b>    | Comparison of bacterial community composition from chlorinated and chloraminated water.  | - 16S rRNA gene amplification<br>- Cloning, sequencing (between 70 and 100 sequences were analyzed).   | - Drinking water from a test facility (US)<br><b>Treatment:</b><br>Chlorinated (1 mg/l), chloraminated (0.06-0.22 mg/l)   | <b>Dominant taxa detected:</b> $\alpha$ - <i>Proteobacteria</i> in distribution system water under two different disinfectant residuals  |

## 2.2. Household

**Overnight stagnation in household taps caused microbial growth and changes in microbial community composition.**

In general, our study suggests that the microbial community reaching the consumer's house originated from the final biofilters of the drinking water treatment plant in this particular system. However, we demonstrated that changes in microbial quality can occur in household installation systems. Considerable changes in microbial cell numbers, activity and microbial community composition occurred at household level after overnight stagnation (Fig. 1). Such changes were observed in houses receiving non-chlorinated drinking water, but also in houses from different locations that received water from different sources (different cities), e.g., groundwater, and also slightly chlorinated water. This showed that microbial growth during stagnation occurs in every household (tested so far). However, the study also demonstrated that after a short flushing of the tap, cell concentrations are comparable to those present in the drinking water network. Also, the detailed analyses of water samples of different taps revealed that mainly in the first few hundred millilitres increased cell concentrations were present. The increase might be caused by either planktonic growth occurring in the water phase, or growth occurring in the pipe biofilm and the constant release of cells during stagnation. The ratio of cells in biofilms and in the bulk water should largely depend on the diameter of the pipes; hence, the biofilm should have more influence in a pipe with small diameter compared to a large diameter pipe. Also, the elevated temperatures in-house might promote microbial growth that is less likely to occur in the distribution network. However, so far the main cause for the growth is still unknown. One reason for this lack of information is certainly that only little carbon is necessary (10-20 µg/l) to cause the observed changes; it is difficult to detect such small carbon concentrations with the available methods to date. Carbon compounds leaking from pipe materials or from armature sealings might be a good reason. Hence, certain armatures might be suited better than others. The presence of higher cell concentrations in the first few hundred millilitres implies that growth occurs also in armatures. This was, for example, shown in shower head biofilms, where also opportunistic pathogens were found to be enriched (Faezel et al., 2009; Table 1). In Germany, a large study was conducted during the last five years with the aim to better characterize the importance of biofilms in drinking water installations as sources of contamination for hygienically relevant bacteria better and to determine possibilities for prevention and their elimination (Flemming, 2010). It was reported that the microbial composition of biofilms in a drinking water installation can depend on a range of different factors including material in use,

drinking water quality, water temperature or disinfectant; also pathogens, such as *Legionella pneumophila* and *Pseudomonas aeruginosa*, were found to be able to become part of pipe biofilms (was tested on pilot-scale) and to be released into stagnating water (Flemming, 2010). An epidemiological study showed that problems are often due to aged materials in drinking water systems that are not secure anymore from contamination from outside. Also inappropriate operation of the drinking water systems in public buildings was reported to lead to hygienic problems (Flemming, 2010). In our group it was found that some materials promote microbial growth or biofilm formation more than others (Koetsch et al., 2010). Hence, further research that leads to recommendations for certain materials might be useful for the improvement of household installations.

### **3. Potential of the applied methods for practice**

This was the first study, where biological stability in a non-chlorinated drinking water network was addressed with multiple parameters including cell concentration and activity but also changes in microbial community composition. This study was restricted to one drinking water distribution network and it demonstrates how “biologically stable” water can be characterized with the applied methods in an “ideal system”. However, it is only possible to judge the water quality or biological stability in drinking water systems with the “new methods” developed recently when some experience has been collected with them; so far no comparison to “normal” values is possible. Flow cytometry as well as ATP determinations are easy to handle, the result is obtained fast, and thus these methods are suitable for routine methods. Molecular methods, such as pyrosequencing and DGGE are too labor-intensive and susceptible for routine analysis and will most probably remain predominantly at the level of research tools. However, these tools can be useful to further analyse in detail changes detected by flow cytometry or ATP. Pyrosequencing data might then show whether the change was due to “pathogenic” growth or growth of “natural” bacteria, as it was the case in our network study. This might be also realistic in the future, since companies already offer 454 pyrosequencing and this could include also data analysis. Flow cytometry is useful as a routine parameter to detect changes in microbial cell concentrations during drinking water distribution or to monitor process efficiencies such as ozonation. The pure enumeration of cells in a water sample cannot be interpreted on its own but has to be compared to other samples from the same system. For the usage of FCM and ATP as routine parameters and the judgement of the extent of changes (e.g. whether a change is small or big), further analysis of different systems could contribute to an easier evaluation of data.

---

## References

- Allen, M. J., S. C. Edberg, and D. J. Reasoner.** 2004. Heterotrophic plate count bacteria-what is their significance in drinking water? *International Journal of Food Microbiology* **92**: 265-274.
- Andersson, A., P. Laurent, A. Kihn, M. Prévost, and P. Servais.** 2001. Impact of temperature on nitrification in biological activated carbon (BAC) filters used for drinking water treatment. *Water Research* **35**: 2923-2934.
- Baghoth, S. A., M. Dignum, A. Grefte, J. Kroesbergen, and G. L. Amy.** 2009. Characterization of NOM in a drinking water treatment process train with no disinfectant residual. *Water Science and Technology: Water Supply* **9**: 379-386.
- Baghoth, S. A., S. K. Sharma, and G. L. Amy.** 2010. Tracking natural organic matter (NOM) in a drinking water treatment plant using fluorescence excitation emission matrices and PARAFAC. *Water Research* **45**: 797-809.
- Baker, M. N., and M. J. Taras.** 1981. *Quest for Pure Water: the history of water purification from the earliest records to the twentieth century*. Published by the American Water Works Association.
- Berney, M., M. Vital, I. Hulshoff, H. U. Weilenmann, T. Egli, and F. Hammes.** 2008. Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research* **42**: 4010-4018.
- Block, J.C.** 1992. Biofilms in drinking water distribution systems, in Melo L.F., T.R. Bott, M. Fletcher and B. Capdeville: *Biofilms-Science and Technology*, 1992. NATO ASI Series.
- Boe-Hansen, R., H. J. Albrechtsen, E. Arvin, and C. Jorgensen.** 2002. Bulk water phase and biofilm growth in drinking water at low nutrient conditions. *Water Research* **36**: 4477-4486.
- Boenigk, J., P. Stadler, A. Wiedlroither, and M. W. Hahn.** 2004. Strain-specific differences in the grazing sensitivities of closely related ultramicrobacteria affiliated with the *Polynucleobacter* cluster. *Applied and Environmental Microbiology* **70**: 5787-5793.

- Boon, N., J. Goris, P. De Vos, W. Verstraete, and E. M. Top.** 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. *Applied and Environmental Microbiology* **66**: 2906-2913.
- Bouvier, T., and P. A. Del Giorgio.** 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): a quantitative review of published reports. *FEMS Microbiology Ecology* **44**: 3-15.
- Bouvier, T., P. A. Del Giorgio, and J. M. Gasol.** 2007. A comparative study of the cytometric characteristics of high and low nucleic-acid bacterioplankton cells from different aquatic ecosystems. *Environmental Microbiology* **9**: 2050-2066.
- Burtscher, M. M., F. Zibuschka, R. L. Mach, G. Lindner, and A. H. Farnleitner.** 2009. Heterotrophic plate count vs. in situ bacterial 16S rRNA gene amplicon profiles from drinking water reveal completely different communities with distinct spatial and temporal allocations in a distribution net. *Water SA (Online)* **35**: 495-504.
- Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson.** 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Applied and Environmental Microbiology* **59**: 881-891.
- Campos, L. C., M. F. J. Su, N. J. D. Graham, and S. R. Smith.** 2002. Biomass development in slow sand filters. *Water Research* **36**:4543-4551.
- Chao, A.,** 1984. Nonparametric-estimation of the number of classes in a population. *Scandinavian Journal of Statistics* **11**: 265-270.
- Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, and G. M. Garrity.** 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* **37**: D141.
- Collins, M. R., T. T. Eighmy, J. M. J. Fenstermacher, and S. K. Spanos.** 1992. Removing natural organic matter by conventional slow sand filtration. *Journal of the American Water Works Association* **84**: 80-90.

**Council directive 98/83/EC of November 1998:** on the quality of: drinking water directive: Article 6, paragraph 1a.

**Dias, F. F., and J. V. Bhat.** 1964. Microbial ecology of activated sludge: I. Dominant bacteria. *Applied and Environmental Microbiology* **12**: 412-417.

**EDI,** 2009. Hygieneverordnung des EDI (HyV) 817.024.1,23. November 2005, Stand 25. Mai 2009.

**Eichler, S., R. Christen, C. Holtje, P. Westphal, J. Botel, I. Brettar, A. Mehling, and M. G. Höfle.** 2006. Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting. *Applied and Environmental Microbiology* **72**: 1858-1872.

**Eighmy, T. T., M. Robin Collins, S. K. Spanos, and J. Fenstermacher.** 1992. Microbial populations, activities and carbon metabolism in slow sand filters. *Water Research* **26**: 1319-1328.

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

**Enkiri, F., Legrand, J-Y., Squinazi, F., Ponelle, Leroy, P.** 2006. Assessment of microbial support potential of six materials used in drinking water distribution systems. *European Journal of Water Quality* **37**: 175-188.

**Escobar, I. C., A. A. Randall, and J. S. Taylor.** 2001. Bacterial growth in distribution systems: effect of assimilable organic carbon and biodegradable dissolved organic carbon. *Environmental Science and Technology* **35**: 3442-3447.

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

**Eydal, H. S., and K. Pedersen.** 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3-1000 m. *Journal of Microbiological Methods* **70**: 363-373.

- Feazel, L. M., L. K. Baumgartner, K. L. Peterson, D. N. Frank, J. K. Harris, and N. R. Pace.** 2009. Opportunistic pathogens enriched in showerhead biofilms. *Proceedings of the National Academy of Sciences* **106**: 16393-16399.
- Felip, M., S. Andreatta, R. Sommaruga, V. Straskrabova, and J. Catalan.** 2007. Suitability of flow cytometry for estimating bacterial biovolume in natural plankton samples: comparison with microscopy data. *Applied Environmental Microbiology* **73**: 4508-4514.
- Flemming, H. C.** 2010. Vermeidung und Sanierung von Trinkwasser-Kontaminationen durch Hygienisch relevante Mikroorganismen aus Biofilmen in Hausinstallationen. IWW Rheinisch-Westfälisches Institut für Wasserforschung Gemeinnützige GMBH. Eigenverlag.
- Fonseca, A. C., R. Scott Summers, and M. T. Hernandez.** 2001. Comparative measurements of microbial activity in drinking water biofilters. *Water Research* **35**: 3817-3824.
- Fox, K. R., R. J. Miltner, G. S. Logsdon, D. L. Dicks, and L. F. Drolet.** 1984. Pilot-plant studies of slow-rate filtration. *Journal of the American Water Works Association* **76**: 62-68.
- Frías, J., F. Ribas, and F. Lucena.** 1995. Comparison of methods for the measurement of biodegradable organic carbon and assimilable organic carbon in water. *Water Research* **29**: 2785-2788.
- Gimbel, R., N. Graham, and M. R. Collins.** 2006. Recent progress in slow sand and alternative biofiltration processes. IWA Publishing, London, UK. ISBN 9781843391203.
- Greenberg, A. E., L. S. Clesceri, and A. D. Eaton.** 1993. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C. 18th ed.
- Gremm, T. J., and L. A. Kaplan.** 1998. Dissolved carbohydrate concentration, composition, and bioavailability to microbial heterotrophs in stream water. *Acta Hydrochimica et Hydrobiologica* **26**: 167-171.
- Haider, T., M. Haider, W. Wruss, R. Sommer, and M. Kundi.** 2002. Lead in drinking water of Vienna in comparison to other European countries and accordance with recent guidelines. *International Journal of Hygiene and Environmental Health* **205**: 399-403.

- Hambsch, B.** 1999. Distributing groundwater without a disinfectant residual. *Journal of the American Water Works Association* **91**: 81-85.
- Hammes, F. A., and T. Egli.** 2005. New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum. *Environmental Science and Technology* **39**: 3289-3294.
- Hammes, F., and T. Egli.** 2010. Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. *Analytical and Bioanalytical Chemistry* **397**:1083-1095.
- <sup>a</sup>**Hammes, F., C. Berger, O. Köster, and T. Egli.** 2010. Assessing biological stability of drinking water without disinfectant residuals in a full-scale water supply system. *Journal of Water Supply: Research and Technology. AQUA* **59**: 31-40.
- <sup>b</sup>**Hammes, F., F. Goldschmidt, M. Vital, Y. Wang, and T. Egli** 2010. Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments. *Water Research* **44**: 3915 - 3923.
- Hammes, F., M. Berney, Y. Wang, M. Vital, O. Köster, and T. Egli.** 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Research* **42**: 269-277.
- Hammes, F., N. Boon, M. Vital, P. Ross, A. Magic-Knezev, and M. Dignum.** 2011. Bacterial colonization of pellet softening reactors used during drinking water treatment. *Applied and Environmental Microbiology* **77**: 1041-1048.
- Hendel, B., J. Marxsen, D. Fiebig, and G. Preuß.** 2001. Extracellular enzyme activities during slow sand filtration in a water recharge plant. *Water Research* **35**: 2484-2488.
- Henne, K., L. Kahlisch, J. Draheim, I. Brettar, and M. Hofle.** 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. *Water Science and Technology: Water Supply* **8 (5)**: 527–532.
- Heuer, H., M. Krsek, P. Baker, K. Smalla, and E. M. Wellington.** 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-

electrophoretic separation in denaturing gradients. *Applied Environmental Microbiology* **63**: 3233-3241.

**Hijnen, W. A. M., Y. J. Dullemont, J. F. Schijven, A. J. Hanzens-Brouwer, M. Rosielle, and G. Medema.** 2007. Removal and fate of *Cryptosporidium parvum*, *Clostridium perfringens* and small-sized centric diatoms (*Stephanodiscus hantzschii*) in slow sand filters. *Water Research* **41**: 2151-2162.

**Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* **33**: 1225-1228.

<sup>a</sup>**Hoefel, D., P. T. Monis, W. L. Grooby, S. Andrews, and C. P. Saint.** 2005. Profiling bacterial survival through a water treatment process and subsequent distribution system. *Journal of Applied Microbiology* **99**: 175-186.

<sup>b</sup>**Hoefel, D., P. T. Monis, W. L. Grooby, S. Andrews, and C. P. Saint.** 2005. Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system. *Applied and Environmental Microbiology* **71**:6479-6488.

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

**Hoigne, J.** 1998. Chemistry of aqueous ozone and transformation of pollutants by ozonation and advanced oxidation processes. *The Handbook of Environmental Chemistry* **5**: 83-142.

**Hong, P. Y., C. Hwang, F. Ling, G. L. Andersen, M. W. LeChevallier, and W. T. Liu.** 2010. Analysis of bacterial biofilm communities in water meters of a drinking water distribution system via pyrosequencing. *Applied and Environmental Microbiology* **76**: 5631-5635.

**Huber, S. A., A. Balz, M. Abert, and W. Pronk.** 2011. Characterisation of aquatic humic and non-humic matter with size-exclusion chromatography-organic carbon detection-organic nitrogen detection (LC-OCD-OND). *Water Research* **45**: 879-885.

- Huber, S. A., and F. H. Frimmel.** 1992. A new method for the characterization of organic carbon in aquatic systems. *International Journal of Environmental Analytical Chemistry* **49**: 49-57.
- Huisman, L., W. E. Wood, and O. World Health.** 1974. Slow sand filtration. World Health Organization Geneva.
- Ihssen, J., and T. Egli.** 2005. Global physiological analysis of carbon and energy limited growing *Escherichia coli* confirms a high degree of catabolic flexibility and preparedness for mixed substrate utilization. *Environmental Microbiology* **7**: 1568-1581.
- Joubert, E. D., and B. Pillay.** 2008. Visualisation of the microbial colonisation of a slow sand filter using an Environmental Scanning Electron Microscope. *Electronic Journal of Biotechnology* **11**: 119-125.
- Kahlisch, L., K. Henne, L. Groebe, J. Draheim, M. G. Hofle, and I. Brettar** 2010. Molecular analysis of the bacterial drinking water community with respect to live/dead status. *Water Science and Technology* **61**: 9-14.
- Kaplan, L., M. Ribas, F., Reasoner, D.** 2005. Techniques for measuring biodegradable organic matter, in Prévost, M., P. Laurent., P. Servais., and J.C. Joret: Biodegradable organic matter in drinking water treatment and distribution., American Water Works Association Chapter **2**, pp. 147-190.
- Karl, D. M.** 1980. Cellular nucleotide measurements and applications in microbial ecology. *Microbiological Reviews* **44**: 739-796.
- Kasuga, I., H. Nakagaki, F. Kurisu, and H. Furumai** 2010. Predominance of ammonia-oxidizing archaea on granular activated carbon used in a full-scale advanced drinking water treatment plant. *Water Research* **44**: 5039-5049.
- Kerneys, A., F. Nakache, A. Deguin, and M. Feinberg.** 1995. The effects of water residence time on the biological quality in a distribution network. *Water Research* **29**: 1719-1727.

**Koch R.** 1883. Über die neuen Untersuchungsmethoden zum Nachweis der Mikrokosmen in Boden, Luft und Wasser. In: Gesammelte Werke von Robert Koch (G. Faffky, E.Pfuhl, J. Schalbe, Herausg.). Erster Band, pp. 274-284. Verlag Georg Thieme, Leipzig, 1912.

**Koetsch S., T. Egli, and M. Bucheli-Witschel** 2010. Beurteilung von Kunststoffen in Kontakt mit Trinkwasser. *Gas und Abwasser* **9**: 797-810.

**Koudjonou B., M. Prévost., N. Merlet** 2005d. Characterization of organic matter in water resources and supplies, in Prévost M., P. Laurent., P. Servais., and J.C. Joret: Biodegradable organic matter in drinking water treatment and distribution., American Water Works Association Chapter **1**, pp. 1-36.

**Kovarova-Kovar, K., and T. Egli.** 1998. Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiology and Molecular Biology Reviews* **62**: 646-666.

**Kwon, S., E. Moon, T. S. Kim, S. Hong, and H. D. Park.** 2011. Pyrosequencing demonstrated complex microbial communities in a membrane filtration system for a drinking water treatment plant. *Microbes and Environments/JSME*. **26**: 149-155.

<sup>a</sup>**Laurent, P., B. Barbeau, and P. Servais.** 2005. Evaluating the impacts of treatment modifications on regrowth potential in distribution systems: a new screening procedure using water quality modeling. *Urban Water Journal* **2**: 81-92.

<sup>b</sup>**Laurent, P., M.C Besner, P. Servais, V. Gauthier, M. Prévost and A. Camper** 2005. Water quality in drinking water distribution systems, in Prévost M., P. Laurent., P. Servais., and J.C. Joret: Biodegradable organic matter in drinking water treatment and distribution. American Water Works Association, Chapter **5**, pp.205-268.

<sup>c</sup>**Laurent, P., P. Servais, V. Gauthier, M. Prévost, J.C. Joret, and J.C. Block.** 2005. Biodegradable organic matter and bacteria in drinking water distribution systems, in Prévost M., P. Laurent., P. Servais., and J.C. Joret: Biodegradable organic matter in drinking water treatment and distribution., American Water Works Association Chapter **4**, pp. 147-190.

- Lautenschlager, K., N. Boon, Y. Wang, T. Egli, and F. Hammes.** 2010. Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. *Water Research* **44**: 4868-4877.
- Lebanowski, J., Feuillade, G.** 2009. Combination of Biodegradable Organic Matter Quantification and XAD-Fractionation as Effective Working Parameter for the Study of Biodegradability in Environmental and Anthropic Samples. *Chemosphere* **74**: 605-611.
- Lebaron, P., P. Servais, H. Agogue, C. Courties, and F. Joux.** 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied and Environmental Microbiology* **67**: 1775-1782.
- LeChevallier, M. W., N. J. Welch, and D. B. Smith.** 1996. Full-scale studies of factors related to coliform regrowth in drinking water. *Applied and Environmental Microbiology* **62**: 2201-11.
- LeChevallier, M. W., W. Schulz, and R. G. Lee.** 1991. Bacterial nutrients in drinking water. *Applied and Environmental Microbiology* **57**:857-862.
- Lehtola, M. J., I. T. Miettinen, A. Hirvonen, T. Vartiainen, and P. J. Martikainen.** 2007. Estimates of microbial quality and concentration of copper in distributed drinking water are highly dependent on sampling strategy. *International Journal of Hygiene and Environmental Health* **210**: 725-732.
- Lehtola, M. J., I. T. Miettinen, M. M. Keinanen, T. K. Kekki, O. Laine, A. Hirvonen, T. Vartiainen, and P. J. Martikainen.** 2004. Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. *Water Research* **38**: 3769-3779.
- Lehtola, M. J., I. T. Miettinen, T. Vartiainen, T. Myllykangas, and P. J. Martikainen.** 2001. Microbially available organic carbon, phosphorus, and microbial growth in ozonated drinking water. *Water Research* **35**: 1635-1640.

- Lewis, J. D., G. D. Wu, Y. Y. Chen, C. Hoffmann, K. Bittinger, S. A. Keilbaugh, L. Nessel, R. W. Berkowsky, J. Chen, and H. Li.** 2010. W1832 Diversity in Human Gut Microbiome Composition Assessed by Pyrosequencing: Comparison of Sampling Methods. *Gastroenterology* **138**:749-749.
- Li, X., G. Upadhyaya, W. Yuen, J. Brown, E. Morgenroth, and L. Raskin.** 2010 Changes in microbial community structure and function of drinking water treatment bioreactors upon phosphorus addition. *Applied and Environmental Microbiology*: 01232-10.
- Li, Y., and R. J. Chrost.** 2006. Microbial enzymatic activities in aerobic activated sludge model reactors. *Enzyme and Microbial Technology* **39**: 568-572.
- Magic-Knezev, A., and D. Van Der Kooij.** 2004. Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. *Water Research* **38**: 3971-3979.
- Mallevalle, J., and I. Suffet.** 1987. Identification and treatment of tastes and odors in drinking Water. AWWA Research Foundation.
- Marzorati, M., L. Wittebolle, N. Boon, D. Daffonchio, and W. Verstraete.** 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Microbiology* **10**: 1571-81.
- Mathieu, L., J. L. Paquin, J. C. Block, G. Randon, J. Maillard, and D. Reasoner.** 1992. Parameters governing bacterial growth in water distribution systems. *Revue des Sciences de l'Eau* **5**: 91-91.
- Mauclore, L., A. Schürmann, and F. Mermillod-Blondin.** 2006. Influence of hydraulic conductivity on communities of microorganisms and invertebrates in porous media: a case study in drinking water slow sand filters. *Aquatic Sciences - Research Across Boundaries* **68**: 100-108.
- Mauclore, L., A. Schurmann, M. Thullner, J. Zeyer, and S. Gammeter.** 2004. Sand filtration in a water treatment plant: biological parameters responsible for clogging. *Aqua* **53**: 93-108.

- Mertens, B., N. Boon, and W. Verstraete.** 2005. Stereospecific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. *Environmental Microbiology* **7**: 660-669.
- Meylan, S., F. Hammes, J. Traber, E. Salhi, U. von Gunten, and W. Pronk.** 2007. Permeability of low molecular weight organics through nanofiltration membranes. *Water Research* **41**: 3968-3976.
- Miettinen, I. T., T. Vartiainen, and P. J. Martikainen.** 1996. Bacterial enzyme activities in groundwater during bank filtration of lake water. *Water Research* **30**: 2495-2501.
- Moll, D. M., and R. Scott Summers.** 1999. Assessment of drinking water filter microbial communities using taxonomic and metabolic profiles. *Water Science and Technology* **39**: 83-89.
- Moll, D. M., R. S. Summers, and A. Breen.** 1998. Microbial characterization of biological filters used for drinking water treatment. *Applied and Environmental Microbiology* **64**: 2755-2759.
- Morita, R.Y.** 1993. Bioavailability of energy and the starvation state. In: *Starvation in bacteria*. Kjelleberg S. (ed.) New York and London: Plenum Press. pp 1-23.
- Münster, U.** 1993. Concentrations and fluxes of organic carbon substrates in the aquatic environment. *Antonie van Leeuwenhoek* **63**: 243-274.
- Muyzer, G., E. C. de Waal, A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.
- Newton, R. J., S. E. Jones, A. Eiler, K. D. McMahon, and S. Bertilsson.** 2011. A guide to the natural history of freshwater lake bacteria. *Microbiology and Molecular Biology Reviews* **75**: 14-49.
- Niquette, P., Servais, P., Savoir.** 2000. Impacts of pipe materials on densities of fixed bacterial biomass in a drinking water distribution system. *Water Research* **34**: 1952-1956.
- Nybroe, O., P. E. Jørgensen, and M. Henze.** 1992. Enzyme activities in wastewater and activated sludge. *Water Research* **26**: 579-584.

- OECD** 2003 *Safer Drinking Water: Improving the Assessment of Microbial Safety*. Organization for Economic Co-operation and Development, Paris, France.
- Page, D., S. Waskelin, J. H. Van Leeuwen, and P. Dillon.** 2006. Review of biofiltration processes relevant to water reclamation via aquifers. CSIRO Land and Water Science Report.
- Pepper, I. L., P. Rusin, D. R. Quintanar, C. Haney, K. L. Josephson, and C. P. Gerba.** 2004. Tracking the concentration of heterotrophic plate count bacteria from the source to the consumer's tap. *International Journal of Food Microbiology* **92**: 289-95.
- Pernthaler, J., F. O. Glockner, S. Unterholzner, A. Alfreider, R. Psenner, and R. Amann.** 1998. Seasonal community and population dynamics of pelagic bacteria and archaea in a high mountain lake. *Applied and Environmental Microbiology* **64**: 4299-4306.
- Peter, A., von Gunten, U.** 2009. Taste and Odour Problems Generated in Distribution Systems: a Case Study on the Formation of 2,5,6-trichloroanisole. *Journal of Water Supply: Research and Technology-AQUA* **58**: 386-394.
- Petrosino, J. F., S. Highlander, R. A. Luna, R. A. Gibbs, and J. Versalovic.** 2009. Metagenomic pyrosequencing and microbial identification. *Clinical Chemistry* **55**: 856-866.
- Polanska, M., K. Huysman, and C. van Keer.** 2005. Investigation of assimilable organic carbon (AOC) in Flemish drinking water. *Water research* **39**: 2259-2266.
- Prévost, M., A. Rompré, J. Coallier, P. Servais, P. Laurent, B. Clément, and P. Lafrance.** 1998. Suspended bacterial biomass and activity in full-scale drinking water distribution systems: impact of water treatment. *Water Research* **32**: 1393-1406.
- Qian, P. Y., Y. Wang, O. O. Lee, S. C. K. Lau, J. Yang, F. F. Lafi, A. Al-Suwailem, and T. Y. H. Wong** 2011. Vertical stratification of microbial communities in the Red Sea revealed by 16S rDNA pyrosequencing. *The ISME Journal* **5**: 507-518.

- Ramseier, M. K., U. von Gunten, P. Freihofer, and F. Hammes.** 2011 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 Research* **45**: 1490-1500.
- Reasoner, D. J., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**: 1-7.
- Revetta, R. P., A. Pemberton, R. Lamendella, B. Iker, and J. W. Santo Domingo.** 2010. Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. *Water Research* **44**: 1353-1360.
- Rinta-Kanto, J. M., M. J. Lehtola, T. Vartiainen, and P. J. Martikainen.** 2004. Rapid enumeration of virus-like particles in drinking water samples using SYBR green I-staining. *Water Research* **38**: 2614-2618.
- Rittman, B.E. and P.M. Huck.** 1989. Biological treatment of public water supplies. *CRC Critical Review in Environmental Control CCECAU* **19**: 119-184.
- Roeder, R. S., K. Heeg, P. Tarne, J. K. Benolken, G. Schaule, B. Bendinger, H. C. Flemming, and U. Szewzyk** 2010. Influence of Materials, Water Qualities and Disinfection Methods on the Drinking Water Biofilm Community. *Water Practice and Technology* **5**.
- Roesch, L. F. W., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie, and E. W. Triplett.** 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME journal* **1**: 283-290.
- Sartory, D. P.** 2004. Heterotrophic plate count monitoring of treated drinking water in the UK: a useful operational tool\* 1. *International Journal of Food Microbiology* **92**: 297-306.
- Schardinger, F.** 1892: Über das Vorkommen Gärung erregender Spaltpilze im Trinkwasser und ihre Bedeutung für die hygienische Beurteilung desselben. *Wiener Klein Wochenschr.* **5**: 403-405.
- Schmidt, T.M., E.F. DeLong, N.R. Pace.** 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *Journal of Bacteriology* **173**: 4371-4378.

- Schnürer, J., and T. Rosswall.** 1982. Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology* **43**: 1256-1261.
- Sedlak, D. L., and U. von Gunten** 2011. The Chlorine Dilemma. *Science* **331**: 42-43.
- Servais, P., E. O. Casamayor, C. Courties, P. Catala, N. Parthuisot, and P. Lebaron.** 2003. Activity and diversity of bacterial cells with high and low nucleic acid content. *Aquatic Microbial Ecology* **33**: 41-52.
- Servais, P., G. Billen, P. Laurent, Y. Levi, and G. Randon.** 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**: 69-89.
- Servais, P., M. Prévost, P. Laurent, J.C. Joret, S. Summers, B. Hambsch and C. Ventresque.** 2005. Biodegradable organic matter in drinking water treatment, in Prévost M., P. Laurent., P. Servais., and J.C. Joret: Biodegradable organic matter in drinking water treatment and distribution. American Water Works Association, Chapter **3**, pp. 61-130.
- Servais, P., P. Laurent, and G. Randon.** 1995. Comparison of the bacterial dynamics in various French distribution systems. *Aqua - Journal of Water Supply: Research and Technology* **44**: 10-17.
- Siebel, E., Y. Wang, T. Egli, and F. Hammes.** 2008. Correlations between total cell concentration, total adenosine tri-phosphate concentration and heterotrophic plate counts during microbial monitoring of drinking water. *Drinking Water Engineering and Science* **1**: 1-6.
- Sinsabaugh, R. L., C. L. Lauber, M. N. Weintraub, B. Ahmed, S. D. Allison, C. Crenshaw, A. R. Contosta, D. Cusack, S. Frey, and M. E. Gallo.** 2008. Stoichiometry of soil enzyme activity at global scale. *Ecology Letters* **11**: 1252-1264.
- Sinsabaugh, R. L., K. Saiya-Cork, T. Long, M. P. Osgood, D. A. Neher, D. R. Zak, and R. J. Norby.** 2003. Soil microbial activity in a Liquidambar plantation unresponsive to CO<sub>2</sub>-driven increases in primary production. *Applied Soil Ecology* **24**: 263-271.

- Skjevrak, I., A. Due, K. O. Gjerstad, and H. Herikstad.** 2003. Volatile organic components migrating from plastic pipes (HDPE, PEX and PVC) into drinking water. *Water Research* **37**: 1912-20.
- SLMB.** 2000. Mikrobiologie von Lebensmitteln und Futtermitteln - Horizontales Verfahren für die Zählung von Mikroorganismen-Koloniezählverfahren bei 30 Grad Celcius. Schweizer Lebensmittelhandbuch.
- Spiegelman, D., G. Whissell, C.W. Greer** 2005. A survey of the methods for the characterization of microbial consortia and communities. *Canadian Journal of Microbiology* **51**(5): 355-386.
- Staley, J. T., and A. Konopka.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Reviews in Microbiology* **39**: 321-346.
- Stephenson, M.** 1949. Growth and nutrition. *Bact Metabol* 3rd ed., Longmans, Green and Co., London, pp 159 - 178.
- Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt, and J. A. Breznak.** 2004. New strategies for cultivation and detection of previously uncultured microbes. *Applied and Environmental Microbiology* **70**: 4748-4755.
- Torvinen, E., S. Suomalainen, M. J. Lehtola, I. T. Miettinen, O. Zacheus, L. Paulin, M. L. Katila, and P. J. Martikainen.** 2004. *Mycobacteria* in water and loose deposits of drinking water distribution systems in Finland. *Applied Environmental Microbiology* **70**: 1973-1981.
- Uhl, W., and G. Schaule.** 2004. Establishment of HPC (R2A) for regrowth control in non-chlorinated distribution systems. *International Journal of Food Microbiology* **92**: 317-325.
- Van der Kooij, D.** 2000. Biological stability: a multidimensional quality aspect of treated water. *Water, Air, & Soil Pollution* **123**: 25-34.
- Van der Kooij, D.,** 1992. Assimilable Organic-Carbon as an Indicator of Bacterial Regrowth. *Journal American Water Works Association* **84**: 57-65.

- Van der Kooij, D., and W. A. Hijnen.** 1988. Nutritional versatility and growth kinetics of an *Aeromonas hydrophila* strain isolated from drinking water. *Applied Environmental Microbiology* **54**: 2842-2851.
- Van der Kooij, D., J. S. Vrouwenvelder, and H. R. Veenendaal.** 2003. Elucidation and control of biofilm formation processes in water treatment and distribution using the Unified Biofilm Approach. *Water Science and Technology* **47**: 83-90.
- Van der Kooij, L., van Lieverloo, J.H.M., Schellart, J.A. and P. Hiemstra.** 1999. Distributing drinking water without disinfectant: highest achievement or height of folly? *Aqua* **48**: 31-37.
- Van der Wielen, P. W. J. J., and D. van der Kooij.** 2010. Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. *Water Research* **77**: 634-641.
- Velten, S., F. Hammes, M. Boller, and T. Egli.** 2007. Rapid and direct estimation of active biomass on granular activated carbon through adenosine tri-phosphate (ATP) determination. *Water Research* **41**: 1973-1983.
- Verordnung des Eidgenössischen Departement des Innern (EDI)** 817.022.102 über Trink-, Q.-u. M. 23. November 2005; Artikel 3.
- Vital, M.** 2010 Growth of pathogenic bacteria in freshwater and their competition with the autochthonous bacterial flora. PhD thesis.
- Vital, M., D. Stucki, T. Egli, and F. Hammes.** 2010. Evaluating the growth potential of pathogenic bacteria in water. *Applied and Environmental Microbiology* **76**: 6477-6494.
- Vital, M., F. Hammes, and T. Egli.** 2008. *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. *Environmental Microbiology* **10**: 2387-2396.
- Vital, M., H. P. Fuchslin, F. Hammes, and T. Egli.** 2007. Growth of *Vibrio cholerae* O1 Ogawa Eltor in freshwater. *Microbiology* **153**: 1993-2001.

- Volk, C. J., and M. W. LeChevallier.** 2002. Effects of conventional treatment on AOC and BDOC levels. *Journal of the American Water Works Association* **94**: 112-123.
- Volk, C. J., C. B. Volk, and L. A. Kaplan.** 1997. Chemical composition of biodegradable dissolved organic matter in streamwater. *Limnology and Oceanography* **42**: 39-44.
- <sup>a</sup>**Von Gunten, U.** 2003. Ozonation of drinking water: Part I. Oxidation kinetics and product formation. *Water Research* **37**: 1443-1467.
- <sup>b</sup>**Von Gunten, U.** 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. *Water Research* **37**: 1469-1487.
- Wang, J. Z., R. S. Summers, and R. J. Miltner.** 1995. Biofiltration performance: part 1, relationship to biomass. *Journal of the American Water Works Association* **87**: 55-63.
- Wang, Y. and Qian, P.Y.** 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* **4**: e7401.
- Wang, Y., F. Hammes, N. Boon, and T. Egli.** 2007. Quantification of the filterability of freshwater bacteria through 0.45, 0.22, and 0.1 µm pore size filters and shape-dependent enrichment of filterable bacterial communities. *Water Science and Technology* **41**: 7080-7086.
- Wang, Y., F. Hammes, N. Boon, M. Chami, and T. Egli.** 2009. Isolation and characterization of low nucleic acid (LNA)-content bacteria. *The ISME Journal* **3**: 889-902.
- Ward, J. H.** 1963. Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association* **58**: 236-244.
- Weber-Shirk, M. L., and R. I. Dick.** 1999. Bacterivory by a chrysophyte in slow sand filters. *Water Research* **33**: 631-638.
- Weiss J., Cozzarelli I.** 2008. Biodegradation in contaminated Aquifers: Incorporating Microbial/Molecular Methods. *Groundwater* **46** (2): 305-322.

- Welte, B., and A. Montiel** 1996. Removal of BDOC by Slow Sand Filtration: Comparison with Granular Activated Carbon and Effect of Temperature. *Advances in Slow Sand and Alternative Biological Filtration* from Nigel Graham and Robin Collins, Wiley 1996.
- Williams, M. M., J. W. S. Domingo, M. C. Meckes, C. A. Kelty, and H. S. Rochon.** 2004. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. *Journal of Applied Microbiology* **96**: 954-964.
- Wittebolle, L., M. Marzorati, L. Clement, A. Balloi, D. Daffonchio, K. Heylen, P. De Vos, W. Verstraete, and N. Boon.** 2009. Initial community evenness favours functionality under selective stress. *Nature* **458**: 623-626.
- World Health Organization (WHO).** 2008: Guidelines for drinking water quality (3rd edition) incorporating the 1st and 2nd addenda. Volume 1, Recommendations (electronic resource). Geneva, Switzerland.
- World Health Organization (WHO).** 2006. Guidelines for Drinking-water Quality: incorporating first addendum. Vol.1, Recommendations. - 3<sup>rd</sup> ed., Geneva, Switzerland.
- Zietz, B. P., J. D. de Vergara, and H. Dunkelberg.** 2003. Copper concentrations in tap water and possible effects on infant's health-results of a study in Lower Saxony, Germany. *Environmental Research* **92**: 129-138.
- Zietz, B. P., J. Lass, and R. Suchenwirth.** 2007. Assessment and management of tap water lead contamination in Lower Saxony, Germany. *International Journal of Environmental Health Research* **17**: 407-418.

## Curriculum Vitae

Karin Lautenschlager

born November 18, 1980

in Mühlacker, Germany

|           |   |
|-----------|---|
| 1997-2000 | Theodor-Heuss-Gymnasium Mühlacker, Germany  |
| 2000-2001 | Freiwillig Ökologisches Jahr, Nationalparkhaus Rosenhaus, Wangerooge, Germany   |
| 2001-2006 | Studies of Biology at the University of Konstanz, Germany   |
| 2005-2006 | Diploma work at the chair of microbial ecology at the University of Konstanz, Germany   |
| 2006-2007 | Research assistant at the chair of microbial ecology at the University of Konstanz, Germany   |
| 2007-2011 | Teaching assistant at the Swiss Federal Institute of Technology (ETH), Zürich, Switzerland  |
| 2007-2011 | Doctoral theses at the Swiss Federal Institute of Aquatic Science and Technology (Eawag/ETH), Dübendorf, Switzerland<br>Defense on September 15, 2011 |