‘The existing literature in connection with the Bacteriology of Water is so extensive, that we venture to think an urgent necessity has arisen for a work in which this subject is specially discussed.’

Percy Frankland, 1894

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GROWTH AND FLOW CYTOMETRIC MONITORING
OF BACTERIA IN DRINKING WATER

IR. SAM VAN NEVEL

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Environmental Technology
Dutch translation of the title
Groei en flowcytometrische monitoring van bacteriën in drinkwater

Funding
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Cover illustration


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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorbance spectrophotometry</td>
</tr>
<tr>
<td>AOC</td>
<td>Assimilable organic carbon</td>
</tr>
<tr>
<td>API</td>
<td>Analytical profile index</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AWWA</td>
<td>American Water Works Association</td>
</tr>
<tr>
<td>BDOC</td>
<td>Biodegradable dissolved organic carbon</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological oxygen demand</td>
</tr>
<tr>
<td>CARD-FISH</td>
<td>Catalysed reporter deposition fluorescent <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CODt</td>
<td>Total chemical oxygen demand</td>
</tr>
<tr>
<td>cTEP</td>
<td>Colloidal transparent exopolymer particles</td>
</tr>
<tr>
<td>DALY</td>
<td>Disability-adjusted life years</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DW</td>
<td>Drinking water</td>
</tr>
<tr>
<td>DWC</td>
<td>Drinking water community</td>
</tr>
<tr>
<td>DWDS</td>
<td>Drinking water distribution system</td>
</tr>
<tr>
<td>EFM</td>
<td>Epifluorescence microscopy</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substance</td>
</tr>
<tr>
<td>FCM</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FDA</td>
<td>Fisher discriminant analysis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>FL1, FL2...</td>
<td>Fluorescence detectors of a flow cytometer, numbered starting from the lowest wavelength</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter detector</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$f_x$</td>
<td>Calibration factor for expressing alcian blue absorbance as xanthan gum</td>
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<tr>
<td>HPC</td>
<td>Heterotrophic plate count</td>
</tr>
<tr>
<td>IC</td>
<td>Indigenous community</td>
</tr>
<tr>
<td>ICC</td>
<td>Intact cell counts</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>MAP</td>
<td>Microbially available phosphorus</td>
</tr>
<tr>
<td>MBR</td>
<td>Membrane bioreactor</td>
</tr>
<tr>
<td>MF</td>
<td>Membrane filtration</td>
</tr>
<tr>
<td>MPN</td>
<td>Most probable number</td>
</tr>
<tr>
<td>MTF</td>
<td>Multiple tube fermentation</td>
</tr>
<tr>
<td>NF</td>
<td>Nanofiltration</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>PACI</td>
<td>Poly aluminium chloride</td>
</tr>
<tr>
<td>PC</td>
<td>Principal components</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>Propidium monazide</td>
</tr>
<tr>
<td>pTEP</td>
<td>Particular transparent exopolymer particles</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>R2a</td>
<td>Reasoner’s 2a (agar)</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>SCADA</td>
<td>Supervisory control and data acquisition</td>
</tr>
<tr>
<td>SG</td>
<td>SYBR Green I</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter detector</td>
</tr>
<tr>
<td>STP</td>
<td>Sewage treatment plant</td>
</tr>
<tr>
<td>Notation</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>SW</td>
<td>Surface water</td>
</tr>
<tr>
<td>SWC</td>
<td>Surface water community</td>
</tr>
<tr>
<td>TCC</td>
<td>Total cell counts</td>
</tr>
<tr>
<td>TEP</td>
<td>Transparent exopolymer particles</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature gradient gel electrophoresis</td>
</tr>
<tr>
<td>TOC</td>
<td>Total organic carbon</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
</tr>
<tr>
<td>VBNC</td>
<td>Viable but non-culturable</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WPC</td>
<td>Water plate count (agar)</td>
</tr>
</tbody>
</table>
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GENERAL INTRODUCTION
‘There is no question that our health has improved spectacularly in the past century. It did not happen because of medicine or medical science, but much of the credit should go to the plumbers and engineers’

Lewis Thomas, Fragile Species, 1984
CHAPTER 1

GENERAL INTRODUCTION ON BACTERIOLOGY IN DRINKING WATER DISTRIBUTION SYSTEMS (DWDS)

Parts of this chapter are redrafted after:


1. Description of bacteria in DWDS

High-quality drinking water is far from sterile, contrary to popular belief. But this should be of no concern. Microorganisms are ubiquitous and are of major importance in many natural processes, including the carbon and nitrogen cycle, the decay of organic material but also the digestion in our gut. Only a minor fraction can negatively affect human health. These should not be alarming, but we have to pay attention to the presence of microorganisms in drinking water. We have to do effort, to fully understand the processes that affect microbial growth in drinking water and anticipate on the factors allowing pathogen presence. Microorganisms in drinking water and the distribution system can include bacteria, fungi, viruses, protozoa and algae, but next to these, even small invertebrates like crustaceans inhabit DWDS, grazing on the previous groups. However, this work will focus on the presence, growth and detection of the first group, bacteria.

1.1 Bacterial presence in four phases in drinking water

Bacteria are present in the bulk phase of drinking water, but the idea is already widespread that the biofilm phase on the pipe walls is at least of equal importance for bacterial growth (Flemming 2002, LeChevallier et al. 1987). What is less known however, is the extensive bacterial presence in suspended solids and loose deposits in the DWDS. According to Liu et al.
(2013b), bacteria are present in four phases: bulk water, pipe wall biofilm, suspended solids and loose deposits. Suspended solids exist of particulate matter transported throughout the network, while loose deposits are an accumulation of particulate matter, settled on the pipe bottom. Liu et al. (2014) estimated the biomass in each of these phases and attributed 2% of the biomass to the bulk water and the therein suspended solids, while biofilms and loose deposits took 98% of the biomass for their account. Remarkably, biofilms and loose deposits contributed in similar amounts to the total biomass (Liu et al. 2014). Several authors had indeed already indicated the importance of loose deposits as reservoir for bacteria and nutrients (Douterelo et al. 2014b, Gauthier et al. 1999, Lehtola et al. 2004), although it is negotiable whether one cannot just appoint this fraction also as biofilm material, starting from the functional biofilm definition ‘...microorganisms attached to a surface and/or to each other and embedded in a polymeric matrix ...’ (Donlan 2002, Douterelo et al. 2014b).

1.2 Commonly found bacteria in drinking water

An overview of the commonly found and reported bacterial phyla and genera, including the opportunistic pathogens, is given in Table 1-1. The following discussion will discriminate between the different phases, as discussed in §1.1.

Liu et al. (2014) analysed stable communities in each of the four phases in a non-chlorinated system and concluded bulk drinking water to be distinctly different from the other three, rather similar phases. They hypothesized therefore that bulk water bacteria originated mainly from the raw water and biological filtration systems, as stated before (Pinto et al. 2012), and not from the pipe biofilm, as stated by others (Flemming 2002). The similarity between the bacterial communities in suspended solids and pipe wall biofilms should be of no surprise, since suspended solids can originate from sloughed biofilm material (Liu et al. 2014). Each of these surface-associated phases makes the bacteria more resistant against hostile conditions such as disinfection (Costerton et al. 1999, Flemming et al. 2014, Gauthier et al. 1999), while the extracellular polymeric substance (EPS) layer in biofilms provides additional protection against grazing by protozoa (Weitere et al. 2005).
Table 1-1: Dominant bacteria and opportunistic pathogens in DWDS. *: ‘Opportunistic pathogens’ only cause disease in immunocompromised people via the waterborne route, while ‘pathogens’ cause disease in the general population (van der Wielen et al. 2014). Table initially based on Liu et al. (2013b).

<table>
<thead>
<tr>
<th>Commonly found bacteria</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phyla</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Genera</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Opportunistic pathogens</strong></td>
<td>(van der Wielen et al. 2014, WHO 2011)</td>
</tr>
<tr>
<td>Acinetobacter, Aeromonas, Burkholderia, Chlamydia, Klebsiella, Legionella, Mycobacterium, Pseudomonas, Stenotrophomonas, Yersinia, etc.</td>
<td></td>
</tr>
<tr>
<td><strong>Pathogens</strong></td>
<td>(NHMRC and ARMC 1996)</td>
</tr>
<tr>
<td>Campylobacter, enterovirulent E. coli, Salmonella, Vibrio cholera, etc.</td>
<td></td>
</tr>
</tbody>
</table>

Pinto et al. (2014) reported strong seasonal variation in the bacterial community composition in the bulk water phase of a chloraminated network. The water showed a high species richness but was dominated by *Alpha*- and *Betaproteobacteria*, where the *Alphaproteobacteria* had their maximum relative abundance in winter and *Betaproteobacteria* in summer. Chloraminated networks typically harbour a few species related to nitrogen cycling, e.g. *Nitrospira* ssp. and *Nitrosomonas* ssp. (Hoefel et al. 2005, Wang et al. 2014), leading to a decreased chloramine residual (Regan et al. 2002).

Chlorinated networks are reported to be dominated by *Alpha-, Beta- and Deltaproteobacteria, Cyanobacteria, Actinobacteria* and *Firmicutes* (Gomez-Alvarez et al. 2012, Henne et al. 2013).
Liu et al. (2014) sampled a network without disinfection and concluded *Polaromonas* (*Betaproteobacteria*) to be the most abundant species in the bulk water phase. The biofilm phase generally shows a lower species diversity compared to the bulk water phase (Henne et al. 2013). As for the bulk water phase, biofilms in chloraminated networks were found to be dominated by *Alpha*- and *Betaproteobacteria*. Both groups were as well found to be dominant in biofilms in chlorinated networks, as well as *Gammaproteobacteria* (Henne et al. 2013, Williams et al. 2005). Chlorine-resistant mycobacteria have a relative advantage in chlorinated systems and were therefore found in high concentrations in both (Chiao et al. 2014, Emtiazi et al. 2004, Williams et al. 2005). Interestingly, Henne et al. (2013) concluded that bacterial core communities in older biofilms were dictated by the bulk water and not by the pipe material, resulting in a spatially similar core community in one network with different pipe materials. In non-chlorinated networks, biofilms were dominated *Sphingomonas* ssp., metabolically versatile *Alphaproteobacteria* which have high-substrate affinity and are therefore commonly found in oligotrophic environments (Liu et al. 2014).

### 1.3 Consequences

Bacterial presence and regrowth in DWDS can lead to various types of problems, dangers and discomfort. First, there are the possible implications for drinking water safety and human health. Secondly, bacteria can affect network integrity and hydrology, and hereby possibly drinking water safety. Finally, taste and odour formation can lead to discomfort, customer complaints and again, possibly affect drinking water safety. Each of the previous categories will be discussed in more detail below.

#### 1.3.1 Health-based implications

Elevated bacterial counts are generally accepted to have no direct public-health significance. However, sudden changes in bacterial counts are caused by something, which can and will have its implications. It can be, for example (van der Kooij and van der Wielen 2014a):

- An indication of failing filtration, allowing more bacteria and nutrients entering the DWDS;
- A sign of failing disinfection or loss of disinfectant residual in the network;
- External contamination of the system caused by pipe breakage or other events.
Each of these situations is not discriminating between non-pathogenic and pathogenic strains: in case of filter malfunctioning, membrane rupture or failing disinfection, any bacteria will be able to pass. When pathogens are present in the raw water bacterial community, they will make it to the drinking water. Elevated nutrient concentrations in the network result in bacterial growth and whenever the disinfectant residual concentration is insufficient and pathogens are present, they can proliferate. The low disinfectant residual concentrations can even be caused by bacterial growth itself, for example nitrifying bacteria decomposing monochloramine (Skadsen 1993). External contaminations of the network can be caused by sewage, rainwater, groundwater or surface water entering the DWDS, and all of these water types can carry pathogens. The water intrusion happens typically via leaking or burst pipes, during pipe repair or via wrongly-connected (household) plumbing. Aging of drinking water networks, especially in urban areas, is causing increased leakage and opens the possibility for water intrusion. The London water suppliers for example are transporting water through century-old piping through the centre of the city and report more than 25% of water loss due to leakage, a percentage which is not exceptional in European cities (Lallana 2003, Pearce 2012). Pressure losses turn leaks into a potential portal for entry of groundwater, contaminated with faecal indicator bacteria, into the network (LeChevallier et al. 2002). This stresses the importance of maintaining overpressure in the network.

Several opportunistic pathogens, typically including *Pseudomonas aeruginosa*, *Acinetobacter* ssp., *Klebsiella pneumoniae* and *Aeromonas* ssp. are capable of growing in drinking water and are regularly identified therein. Opportunistic pathogens can cause disease in a small part of the population with a malfunctioning immune system; the immunocompromised people (van der Wielen et al. 2014). However, there is no evidence of the previously mentioned strains to cause disease through the waterborne route among the general population (WHO 2003a). For *Aeromonas* ssp. for example, the strains isolated from patients are different from these isolated from drinking water. For other species like nontuberculous mycobacteria and *Legionella pneumophila* on the other hand, the strains isolated in patients are genotypically identical as the ones isolated in drinking water (van der Wielen et al. 2014). *L. pneumophila* for example grows in drinking water at elevated temperatures and is transmitted through aerosols (Baskerville et al. 1981). This bacteria causes Legionnaires’ disease, a life-threatening disease, with a reported 10% mortality rate for the European hospitalized cases (ECDC 2012,
van der Kooij 2014). Overall, the epidemiological role of some opportunistic pathogens that can grow in DWDS is proven, while for others, there is few or no evidence (van der Wielen et al. 2014).

### 1.3.2 Network integrity and hydrology

Bacteria grow in biofilms on pipe surfaces in DWDS and induce biocorrosion of metal pipes, leading to increased iron and copper concentrations in the drinking water and damage to the network (Féron and Neumann 2014, van der Kooij and van der Wielen 2014a). Damage and leakage can also be caused by actinomycetes, deteriorating the rubber sealing in pipe joints (van der Kooij and van der Wielen 2014a). Next to the possible health risks as discussed in §1.3.1, damaged and leaking pipes are also a major economic concern: the annual corrosion cost in DWDS is estimated at $22 billion in the US (Edwards 2004). Finally, biofilms increase the pipe surface roughness, leading to increased friction and decreased flow capacity (Lambert et al. 2008). For example, a 1.6 mm thick biofilm has been shown to decrease the flow capacity of a 13 km long 900 mm water pipe with as much as 23% (Picologlou et al. 1980).

### 1.3.3 Dissatisfaction and customer complaints

Bacteria in drinking water can be the cause of discoloured water or induce taste and odour problems. Discolouration is one of the major customer complaints (Vreeburg and Boxall 2007) and is partly caused by iron-oxidising bacteria like *Gallionella*, leading to elevated iron(hydr)oxide concentrations and rust-coloured particles in the water phase (van der Kooij and van der Wielen 2014a). Cyanobacteria can be another cause of microbial discolouration since they can impede coagulation and filtration. Besides, just like Actinomycetes, they can produce geosmin and 2-methyl isoborneol, which can be tasted in drinking water at nanogram per litre level (WHO 2011). By-products of chlorination can be converted microbially into trichloroanisoles, which as well have odour thresholds in the nanogram per liter range and are identified as main cause of off-flavour in chlorinated systems (Piriou et al. 2001, van der Kooij and van der Wielen 2014a). Finally, microbial activity in anaerobic regions like deposits and biofilms can lead to the production of the highly odorous compound dimethyl-trisulphide (van der Kooij and van der Wielen 2014a).

Consumers have no means of judging over hygienic safety of their drinking water, but aspects like discolouration or a distinct taste or smell can be perceived with their own senses. This will
be regarded as suspicious and undermine their trust towards piped drinking water (Payment and Robertson 2004, WHO 2011).

2. What affects bacterial growth in DWDS?

In this section, a discussion is provided on nutrients, bacterial competition and other factors affecting bacterial growth, and finally, on the control strategies to limit bacterial growth.

2.1 Presence of nutrients

The first, obvious requisite for bacterial growth is the presence of food: the right macro- and micronutrients. Organic carbon is the first and most studied nutrient as limiting factor (Lu and Zhang 2005, Polanska et al. 2005a, van der Kooij et al. 1982). Usually, the dissolved organic carbon (DOC) is measured although only a small fraction (0.1-44%) consists of organic molecules which are readily available for bacterial growth (Kaplan et al. 1993, Vital et al. 2010). To determine this fraction, biological tests such as the biodegradable dissolved organic carbon (BDOC) test (Servais et al. 1987) or assimilable organic carbon (AOC) test were developed (Hammes and Egli 2005, van der Kooij et al. 1982). Usually, a correlation is seen between AOC concentrations and bacterial cell concentrations (LeChevallier et al. 1996, Torvinen et al. 2004, Vital et al. 2007), although this might be limited (Vital et al. 2010).

Next to carbon, phosphorus has been mentioned regularly as possible limiting nutrient for bacterial growth in drinking water (Haas et al. 1988, Polanska et al. 2005b, Sathasivan et al. 1998, Vrouwenvelder et al. 2010). This is especially true for boreal regions, where natural waters usually harbour high carbon concentrations (Lehtola et al. 2002, Miettinen et al. 1997). As a result, extensive phosphorus limitation can be an alternative method for limiting bacterial growth (Polanska et al. 2005b, Vrouwenvelder et al. 2010).

Other nutrients than carbon and phosphorus are rarely reported to correlate with bacterial growth, although some correlations are not surprisingly seen for ammonium and nitrite with specific groups like ammonia oxidizing bacteria (Lipponen et al. 2002, Wolfe et al. 1990).
2.2 Bacterial competition

Drinking water nutrient levels are generally extremely oligotrophic, with AOC levels usually below 100 µg C.L\(^{-1}\) (Charnock and Kjonno 2000, Egli 2010, Polanska et al. 2005a, Yang et al. 2011). This forces bacteria to search for competitive nutrient collection strategies, e.g. occupying niches by using carbon sources neglected by others (Hibbing et al. 2010) or growing at very low substrate concentrations (Fuchslin et al. 2012). An overview of these strategies is offered by Egli (2010). The presence of indigenous bacteria has been reported by some as having a supportive effect (Kerr et al. 1999, Moreira et al. 1994). Others stated an antagonistic effect, based on nutrient competition and the release of inhibitory substances (Chandran and Hatha 2005, De Roy et al. 2013, Ducluzeau et al. 1984, Moreira et al. 1994).

2.3 Other conditions

Even when competitive bacteria survive disinfection, and the present nutrients allow them to proliferate, there are more factors affecting bacterial growth and survival in drinking water, of which the most important ones will be discussed here: (i) temperature (ii) attachment surfaces for biofilms and (iii) flow velocity.

(i) Temperature. In temperate regions, like Belgium, winter drinking water temperatures can go as low as a few degrees, while summer temperatures usually go until 20°C, with some higher peaks. This discrepancy is expected to be more pronounced for water deriving from surface water sources compared to ground water, since surface water shows stronger seasonal temperature variations (Kurylyk et al. 2013). Not surprisingly, concomitantly with water temperatures, seasonal variations in the bacterial community are reported, as discussed before in §1.2 (Pinto et al. 2014). Bacterial growth is generally low at temperatures below 5°C, while growth rates initially double per 10°C temperature increase (van der Kooij and van der Wielen 2014a). To the contrary, survival of pathogens in drinking water usually lasts longer at 5°C compared to higher temperatures like 25°C, due to higher nutrient demand at elevated temperatures (Cools et al. 2003, Maalej et al. 2004, Tatchou-Nyamsi-Koenig et al. 2007). Household plumbing systems deserve a special note over here; these last meters before the tap provide stagnation conditions at elevated temperatures, seriously affecting bacterial water quality right before consumption (Flemming et al. 2014, Lautenschlager et al. 2010, Lipphaus et al. 2014).
(ii) Attachment surfaces for biofilms. The formation of biofilms is strongly related to the surface material. For example, the corrosion of iron piping increases the surface area, providing more binding sites for both bacteria and nutrients, resulting in increased biofilm growth (Camper 2004, Sarin et al. 2004). More generally, corroding materials will bear more biomass than noncorrosive materials, while plastics such as PVC and PE will support least biofilm development (Niquette et al. 2000, van der Kooij and van der Wielen 2014a). On the other hand, organic compounds leaching from pipe materials, especially from plastics or rubber sealings, can enhance microbial growth (Hambsch et al. 2014).

By the use of adhesins and the formation of a conditioning film, bacteria can eventually adhere to and grow on any type of surface, independently of surface charge and characteristics (Donlan 2002, Klemm et al. 2010). Surface coatings of materials with specialised molecules such as α-tropomyosin are known to counteract biofilm formation (Klemm et al. 2010), but do not seem relevant for large-scale applications as drinking water piping. Once the conditioning and initial colonization of a surface took place, bacteria can easily further cohere to the biofilm and grow (Garrett et al. 2008). From that moment on, the initial surface properties are of fewer importance, while leaching of nutrients is still going on, with large differences between different types of materials. Therefore, several tests are in use to assess the leaching of piping materials: biomass production potential (BPP, the Netherlands) (van der Kooij et al. 2006), the mean dissolved oxygen difference (MDOD, United Kingdom) (BSI 2000), biomass volume method (Germany) (DVGW 2007), biomigration test (BioMig) (Kotzsch et al. 2010), etc.

A discussion on a possible promoter of biofilm growth is provided in Box 1.

(iii) Flow velocity. Bacteria in the bulk water phase are under influence of water flow velocities, since these are directly determining their retention times in the DWDS. Yet, biofilm growth is as well affected. Firstly, by physical processes as shear forces, sloughing biofilm (Choi and Morgenroth 2003, Lehtola et al. 2006, Lehtola et al. 2004) and the flushing out of loose particles (Lehtola et al. 2004). Secondly, also by chemical processes: typical flow velocities (< 10 cm.s\(^{-1}\) to 50 cm.s\(^{-1}\)) usually result in turbulent conditions, enhancing the nutrient diffusion in biofilms (van der Kooij and van der Wielen 2014a).
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**Box 1-1: The possible role of transparent exopolymer particles (TEP) as biofilm initiator.**

There has been plenty of research over the past years on factors influencing biofilm formation (Hinsa et al. 2003, Pratt and Kolter 1999, Stoodley et al. 2002, Sutherland 1983). However, one new parameter that particularly drew our attention were the TEP, which will be subject of study in **Chapter 2**. They are sticky gel-like particles consisting mainly of surface-active polysaccharides with both hydrophilic and hydrophobic zones (Mopper et al. 1995). They are excreted by algae and bacteria and have been found in significant abundances in oceans as well as fresh water, wastewater and even groundwater (Berman and Parparova 2010, Kennedy et al. 2009, Passow 2002). Initially they received much attention in oceanography (for an extended review, the reader is referred to Passow, 2002). More recently, it was stated that they could induce biofouling on membranes (Berman 2005) and follow-up research indicated the potentially important role of TEP in this field (Bar-Zeev et al. 2009, Berman et al. 2011, Villacorte et al. 2009a, b, Villacorte et al. 2010). TEP adheres quickly to submerged surfaces in the water phase, even before single bacteria do so (Bar-Zeev et al. 2009). They can be pre-colonised with bacteria and once attached to a surface, they can serve as nutrient pools and be a jump start for biofilm formation (Bar-Zeev et al. 2012, Berman 2005, Berman and Passow 2007). Combined with their ubiquitous presence in the water phase and their limited removal by several types of water filtration (e.g. sand and membrane filtration) (Bar-Zeev et al. 2009, Kennedy et al. 2009, Villacorte et al. 2009a, b, Villacorte et al. 2010), this has led us to the hypothesis of TEP, playing a significant role in biofilm formation in DWDS. TEP, abundant in raw water and partly passing a water treatment, could adhere to pipe wall surfaces, and play a role in biofilm formation. The biofilm formation hypothesis was later on supported by Bar-Zeev et al. (2012), showing the fast TEP attachment to surfaces and demonstrating the inhibition of biofilm development after TEP removal from seawater.
2.4 Measures to limit bacterial growth

Drinking water utilities try to limit the growth in DWDS, due to the previously mentioned reasons (§1.3). There are two pathways to achieve this goal: (i) the use of a disinfection process with a disinfectant residual, or (ii) the production of a biostable water (Hammes et al. 2010a, van der Kooij et al. 1999).

The first option is most common, as a residual can effectively limit regrowth, as long as it reaches the tap (van der Kooij and van der Wielen 2014a). Disinfection based on chlorination was first applied continuously in 1902 in Middelkerke, Belgium (Baker 1948). Due to its effectiveness, low cost and easy application, chlorination became by far the most widespread disinfectant (Brown et al. 2011, Freese and Nozaic 2004, Sadiq and Rodriguez 2004). A disinfectant residual, present in the drinking water distribution system, is preferred to limit bacterial growth in DWDS. Chlorine as disinfectant and residual remained unquestioned until 1974, when researchers showed the presence of chloroform in chlorinated drinking water (Bellar et al. 1974, Rook 1974, Sedlak 2014). The reaction of chlorine with organic matter has been found to result in the formation of hundreds of species of disinfection by-products (Brown et al. 2011, Sadiq and Rodriguez 2004). Among these, trihalomethanes and others appear to be carcinogenic or cause adverse effects on the reproduction of laboratory animals. Associations have been found between the ingestion of chlorinated drinking water and the induction of several cancers in laboratory animals (Nieuwenhuijsen et al. 2000, Villanueva et al. 2007, WHO 2005). Monochloramine (NH₂Cl) is a more stable residual and penetrates deeper in biofilms but the decay will add a nutrient, ammonia, to the system and even support bacterial growth when present in lower concentrations (Chang et al. 2010, LeChevallier et al. 1990, Skadsen 1993). More recently, chlorine dioxide (ClO₂) is increasingly used as an alternative disinfectant with a low formation of disinfection byproducts and high efficiency against various microbial pathogens over a wide pH range (Lin et al. 2014). However, in this case unwanted inorganic breakdown products are formed; chlorite and chlorate (WHO 2004), which have mainly been reported to cause oxidative stress resulting in changes in the red blood cells (WHO 2011). The use of chlorinated disinfection products in general entails concomitant negative consumer opinions regarding taste and odour (Bryan et al. 1973, Hambsch 1999).
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As an alternative to chlorinated products, ozone is an efficient disinfectant but is insufficient as residual since it dissipates rapidly and can induce the formation of other carcinogenic DBPs (Freese and Nozaic 2004). UV radiation finally, does not provide a residual. The final balance whether disinfection residuals have a positive or negative effect for limiting regrowth will be dependent on concentrations of disinfectant and organic carbon, temperature, pipe material, water residence time, etc. (van der Kooij and van der Wielen 2014a).

The alternative option is producing a biostable water by maintaining an indigenous bacterial population combined with extensive nutrient limitation (Hammes et al. 2010a, van der Kooij et al. 1999), achieved by an appropriate combination of potent treatment processes such as slow sand filtration, membrane filtration, activated carbon filtration, etc. Since the discovery of disinfection by-product, this option gained importance in countries in northern and central Europe, like the Netherlands and Switzerland (van der Kooij et al. 1999). This water treatment aims at an extensive nutrient removal resulting in the inability for bacteria to proliferate in the produced drinking water. This is mainly monitored by focussing on organic carbon as main nutrient, measured by the assimilable organic carbon (AOC) protocol (Hammes and Egli 2005, van der Kooij et al. 1982) and the biofilm formation rate (BFR) of the water which can be tested in a flow through test (van der Kooij 2000, van der Kooij and Veenendaal 1993).

Disinfection should not go without nutrient limitation and the present nutrient levels dictate the necessity for disinfectant presence needed for having a biostable water. Van der Kooij et al. (1992) suggested a limit of 10 µg.L⁻¹ as an upper limit for AOC concentration, in order for a water to be biologically stable without disinfectant residual. Others proposed a higher and less strict limit of 30 µg.L⁻¹ to take the variability of biological treatment processes and the sensitivity of the AOC assessment into account (Nitisoravut et al. 1997). On the other hand, 50 or 100 µg.L⁻¹ were proposed as upper limits to prevent bacterial regrowth in systems containing a disinfectant residual (LeChevallier et al. 1991, LeChevallier et al. 1996). Independently of meeting these criteria, the likelihood of bacterial regrowth will still be affected as well by other, previously discussed factors such as phosphorus concentrations, temperature, etc.

It should be noted that the discussed pathways solely aim at limiting regrowth of bacteria in the DWDS. However, already the mere survival of certain pathogens can pose a public health
effect (WHO 2011). Therefore, next to the measures taken to limit the regrowth of bacteria in the DWDS, other precautions have to be taken to prevent the entrance of pathogens in the DWDS, such as ensuring raw water quality, point disinfection before distribution, maintaining network integrity, etc (Medema et al. 2014).
3. Non-selective detection of bacteria in DWDS

In order to study bacteria in DWDS, performant methods need to be available. Heterotrophic plate counts (HPC) are the gold standard for enumeration of bacteria in drinking water, while flow cytometric cell counts could be a serious competitor and will be an important part of this research; both will be discussed below. Measurement of adenosine tri phosphate (ATP) can serve as another noteworthy alternative.

3.1 Enumeration of bacteria by HPC: still the gold standard

3.1.1 130 years of development

Around 1850, John Snow showed the relation between cholera prevalence and water consumption from certain wells. He concluded drinking water was the transmitter of diseases, albeit Louis Pasteur was the first to link bacteria with disease (Sedlak 2014). At that time, smell, appearance, taste and early chemical analysis were the only tools the water utilities had to assess drinking water quality (Payment et al. 2003). This changed when Robert Koch published his gelatine plate method in 1881, for the first time offering the possibility to grow pure bacterial colonies and to enumerate bacteria, starting from the principle that one bacterial cell develops into one colony (Koch 1881). One year later, the gelatine plate method was improved by replacing gelatine with agar, which stays solid at a higher incubation temperature of 37°C (Payment et al. 2003, Reasoner 2004). In 1885, Percy Frankland started to apply this method to count the numbers of bacteria in London drinking water routinely for assessing filter efficiency and water quality (Frankland and Frankland 1894). In the meantime, Koch assessed the case of the Altona waterworks, treating highly contaminated Elbe water with slow sand filtration. Whenever bacterial concentrations remained below 100 colony forming units (cfu) per mL, waterborne cholera and/or typhoid fever epidemics were prevented, while a failing filtration leading to 1000 cfu per mL was followed by a cholera outbreak. Therefore, Koch proposed a limit of 100 cfu per mL to prevent outbreaks. Further systematic following observations indeed confirmed that sand filtration could prevent outbreaks of waterborne diseases whenever this criterion was met (Exner et al. 2003, Koch 1893). Today, more than 130 years after the first publication, the method underwent updates with different media compositions (low and high nutrient), incubation times and incubation temperatures (Reasoner 2004), but essentially Koch’s method is still present in drinking water.
legislation worldwide, sometimes even including the same 100 cfu.mL\(^{-1}\) limit (Table 1-2). This type of culture based tests, intended to recover a wide range of microorganisms from water on non-selective agar plates, is commonly referred to as ‘Heterotrophic Plate Count’ (HPC). Standardized HPC methods cover a wide range of conditions, including different media formulations like plate count agar, nutrient agar or R2a-agar, different incubation temperatures ranging from 20°C to 40°C and incubation times ranging from hours to weeks (Allen et al. 2004, WHO 2003a). It should be of no surprise that this leads to a wide quantitative and qualitative variety in results which are difficult to compare, unless the same method is used. Although branded ‘non-selective’, these media and methods are clearly selective for bacteria growing under the specific conditions, including nutrients, temperatures and incubation times (Allen et al. 2004).

### 3.1.2 What is HPC showing?

HPC indicates the number of bacteria capable of growing on a specific agar plate under specific conditions. It is an undeniable indicator of viability for the cells that did grow. If used in a routine manner, it was suggested that HPC is a sensible process indicator. In 2002, an international symposium and expert meeting discussion was organised to provide a consensus report on the actual value of this parameter (WHO 2003a). They agreed that (i) HPC can be used to indicate the effectiveness of disinfection (Ridgway and Olson 1982) or filtration treatment processes for bacterial removal (Fuller 1893); (ii) HPC responds to and is a general indication of the current conditions in drinking water networks, including stagnation, temperature, disinfectant residual, organic carbon and other nutrients (thus a good indicator for biological stability). In this regard, it is argued that the detection of bacterial regrowth in the network is possible when suitable HPC methods are chosen (Maul et al. 1985, Uhl and Schaule 2004); (iii) There is no evidence that HPC values alone directly relate to health risk; virulence characteristics are rarely detected in HPC bacteria (Edberg and Allen 2004, Edberg et al. 1997, Stelma Jr et al. 2004).

Operational limits for HPC are still regularly incorporated in drinking water legislation (Table 1-2). Maximum values range from 20 to 500 cfu.mL\(^{-1}\) depending on sampling location, but are in any case fallacious and only based on the assessment of specific systems, which should not be generalised (Allen et al. 2004). Not surprisingly, they are increasingly replaced by the
guideline ‘no abnormal change’, open for interpretation by the water supplier. For example, the European Union, Canada and Australia have excluded their HPC upper limit in drinking water guidelines compared to a decade ago (Radcliff 2003).

3.1.3 What is HPC missing?

After comparing microscopic counts and plate counts, some of the first microbiologists already realised in the late 19th century that the number of colonies growing on agar plates was not reflecting the real number of bacteria present in the water sample (Winterberg 1898). Early comparisons already showed that microscopic counts were revealing up to 150 times more bacterial cells than detected with HPC counts (Amaan 1911). By mid-century, this was universally recognised (Lewis et al. 2010) and later on branded ‘The Great Plate Count Anomaly’ (Staley and Konopka 1985). Newer estimations of which fraction of the total bacterial community is detected by HPC are usually lower than a few percent in drinking water with fractions of 0.01% (WHO 2003b), 0.001-6.5% (Hammes et al. 2008a) and 0.05-8.3% (Burtscher et al. 2009), depending on sample source.

Multiple studies have examined the so-called “unseen majority”, referring to the major part of the bacteria detected by microscopy or flow cytometry (FCM) but neglected by conventional HPC methods. Seminal reviews on the topic are available for more detailed information (Bogosian and Bourneuf 2001, Epstein 2013, Green and Keller 2006, Kell et al. 1998, Oliver 2005), but the general conclusion is that apart from dead bacteria, this “unseen majority” consists of two basic groups: (1) bacteria belonging to strains which are cultivable but for some reason in a state of being viable-but-not-cultivable (VBNC) and (2) those which are simply not cultivable by conventional HPC methods.

The cultivable strains can remain undetected by HPC due to the presence of (i) injured cells, which can potentially regain cultivability, or (ii) dormant cells that can get activated at more favourable conditions or even at random moments (Bogosian and Bourneuf 2001, Epstein 2013, Kell et al. 1998, Oliver 2005).

Bacteria that are uncultivable under conventional HPC conditions have been the subject of considerable discussion. One possible explanation for uncultivability is the presence of excessive nutrient concentrations in any type of HPC medium compared to drinking water. For
example R2a agar, often declared ‘low in nutrients’ and therefore more suitable for drinking water bacteria (Reasoner and Geldreich 1985), has a DOC concentration which can be 800 times higher than drinking water (Hammes et al. 2008a). Efforts for optimizing growth conditions, including the adaptation of macro- and micronutrients, the use of synthetic natural environments and new cultivation techniques enabled the growth of some previously uncultivable species (D’Onofrio et al. 2010, Kaeberlein et al. 2002, Rappe et al. 2002, Sangwan et al. 2005, Wang et al. 2009). However, millions of microbial species are thought to exist and over 99% have never been cultivated (Achtman and Wagner 2008, D’Onofrio et al. 2010).

Due to the lack of information on the majority of cells, HPCs are considered absolutely inadequate to enumerate the actual bacterial concentrations in the water (Allen et al. 2004), neither do they represent the level of bacterial growth in water supplies (van der Kooij and van der Wielen 2014a).
Table 1-2: An overview of drinking water legislations with regard to HPC values. *: All countries within the European Union have to comply with these standard but can impose additional, stricter rules. **: Indicator parameters are obligatory to measure, but are non-restrictive guidelines. N.a.c.: no abnormal change. ***: In Switzerland, natural water is regularly distributed without any treatment and limits are depending on the presence or absence of a water treatment.

<table>
<thead>
<tr>
<th>Region</th>
<th>2014 limit</th>
<th>Remark</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>&lt; 500 cfu.mL⁻¹</td>
<td>For surface water or ground water under influence of surface water, without filtration</td>
<td>(USEPA 2009)</td>
</tr>
<tr>
<td>European Union*</td>
<td>n.a.c.</td>
<td></td>
<td>(Anonymous 1998)</td>
</tr>
<tr>
<td>-UK</td>
<td>n.a.c.</td>
<td></td>
<td>(Anonymous 2007)</td>
</tr>
<tr>
<td>-France</td>
<td>Max 10 cfu.mL⁻¹ variation compared to ‘usual’ value</td>
<td></td>
<td>(Ministère de la santé et des solidarités 2007)</td>
</tr>
<tr>
<td>-The Netherlands</td>
<td>Geometric mean (year) &lt; 100 cfu.mL⁻¹</td>
<td></td>
<td>(Infrastructuur en Milieu 2011)</td>
</tr>
<tr>
<td>-Germany</td>
<td>n.a.c.</td>
<td></td>
<td>(Bundesministerium der Justiz und für Verbraucherschutz 2013)</td>
</tr>
<tr>
<td>-Flanders (Belgium)</td>
<td>n.a.c.</td>
<td></td>
<td>(VMM 2014)</td>
</tr>
<tr>
<td>Switzerland (***</td>
<td>300 cfu.mL⁻¹ in network Untreated: 100 cfu.mL⁻¹ at source Treated: 20 cfu.mL⁻¹ after treatment</td>
<td></td>
<td>(EDI 2014)</td>
</tr>
<tr>
<td>Canada</td>
<td>No specification</td>
<td>‘Useful operational tool’</td>
<td>(Health Canada 2012)</td>
</tr>
<tr>
<td>Australia</td>
<td>As low as possible</td>
<td>‘For operational or distribution system monitoring’</td>
<td>(NHMRC and NRMMC 2011)</td>
</tr>
</tbody>
</table>
3.2 Flow cytometric cell counts

As an alternative for HPC we propose flow cytometric cell counts, since these are based on the entire bacterial community. FCM is a fast, accurate, precise, quantitative and reproducible technique for counting the total number of bacteria, when combined with a general nucleic acid stain (Hammes et al. 2008a, Prest et al. 2013, Wang et al. 2010b), the intact cell count when with a viability stain (Berney et al. 2008, Helmi et al. 2014b), and more recently for assessing the flow cytometric fingerprint of bacterial communities (De Roy et al. 2012, Koch et al. 2014, Prest et al. 2013). An extensive information box on the basic principle of flow cytometry and bacterial counting is given after this section (Box 1-2), followed by an information box on flow cytometric fingerprinting (Box 1-3).

FCM was initially applied for analysis of mammalian cells, but since 1977 FCM is also used by microbiologists to characterise bacteria (Bailey et al. 1977, Paau et al. 1977). Initially, it was not broadly used in microbiology due to expensive instrumentation and technical difficulties stemming from the small size of bacteria compared to mammalian cells (Wang et al. 2010b). Further developments on the hardware and the emergence of specific stains for bacterial features finally made FCM more popular in microbiology from the 1990s onwards (Hammes and Egli 2010). In 2002, Appenzeller used FCM for the first time in drinking water, detecting the sorption of *E. coli* on FeOOH (Appenzeller et al. 2002). One year later, Hoefel and colleagues applied it for detecting the physiologically active bacterial community in drinking water and highlighted the difference in orders of magnitude between HPC and flow cytometry data (Hoefel et al. 2003). Ever since, the number of studies including FCM for studying drinking water bacteria has been rising yearly. Research applying FCM in drinking water now includes studies on the characterisation of water treatment processes (Hammes et al. 2008a, Helmi et al. 2014b, Ho et al. 2012, Van Nevel et al. 2012, Vital et al. 2012a), more specific disinfection processes (Phe et al. 2005, Ramseier et al. 2011, Wert et al. 2013) or viability assessment (Berney et al. 2008, Pianetti et al. 2005). Regrowth and biological stability are studied in drinking water distribution networks (Gillespie et al. 2014, Hoefel et al. 2005, Lautenschlager et al. 2013, Liu et al. 2013a, Nescerecka et al. 2014, Prest et al. 2013, Vital et al. 2012a, Wen et al. 2014) and household situations (Lautenschlager et al. 2010, Lipphaus et al. 2014). Measurements of assimilable organic carbon are performed with FCM (Hammes and Egli 2005,
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Monfort compared epifluorescence microscopy with FCM cell counts and stated that FCM is reliable to enumerate bacteria in aquatic samples (Monfort and Baleux 1992). For this purpose, various general nucleic acid stains have been used (Wang et al. 2010b) whereby SYBR Green I (SG), SYBR Green II and SYTO 9 were found to deliver high quality results for cell counting in fresh water environments, with respect to fluorescence intensity, ease of use and reproducibility (Hammes et al. 2008a, Lebaron et al. 1998). After more than 20 additional years of development and a recent extensive standardisation of the total cell count (TCC) method based on SG I, this method was accepted as a guideline method into Swiss drinking water legislation (Prest et al. 2013, SLMB 2012). As major advantages, the method was shown to be fast (< 1 min/sample), reproducible (< 3% relative standard deviation on all parameters) and automatable (Prest et al. 2013, SLMB 2012, Van Nevel et al. 2013b).

3.3 ATP-quantification

ATP-quantification allows an easy and fast semi-direct assessment of the active bacterial biomass (van der Wielen and van der Kooij 2010). The correlation with actual biomass can be further improved by filtration of samples, enabling the discrimination between cellular and free ATP (Hammes et al. 2010b). The method is relatively cheap and easy and has been used to assess biological activity and regrowth in research applications (Lehtola et al. 2002, Vital et al. 2012a), but is also routinely used in practice, for example in the Netherlands. A further discussion in the advantages and disadvantages of the ATP assay will be discussed in Chapter 8, §3.5.
Box 1-2: Flow cytometry (FCM) – Understanding cell counting

This box aims at explaining the basic principle of flow cytometry for use in microbiology. Further elaboration is done on general cell counting methods and their accuracy. Partly after Shapiro (2003) and Hammes and Egli (2010).

A. Basic principle

The basic principle of FCM is schematically represented by Figure 1-1.

![Figure 1-1: Schematic representation of a basic flow cytometer. Republished from De Roy (2014).](image)

A liquid sample is guided as a dispersed stream of particles through a light source, usually a laser beam. Due to ‘hydrodynamic focusing’, the particles are passed individually through the light beam, allowing one-by-one analysis of the particles. Fluorescent particles (fluorochromes) are excited by the light source and emit scattered light at another wavelength. Particles of interest can either be autofluorescent, e.g. algae which contain chlorophyll, or be made fluorescent, such as bacteria after staining with fluorescent dyes. The emitted light is detected by a forward and side scatter detector (FSC and SCC), and several fluorescence detectors with bandpass filters selecting for specific wavelength ranges (FL1, FL2,
FL3, etc.). The amount of light scattered at small angles (detected by FSC) gives an estimation of cell size, but is affected by other factors such as refractive index of the cellular material. The amount of light scattered at larger angles (partly detected by SSC) increases with cells’ internal granularity and surface roughness. The information obtained from the fluorescence detectors is dependent on the present fluorochromes emitting fluorescent light. The use of different detectors and possibly different lasers allows the user to extract a quantitative and multiparametric analysis from the gathered data.

B. Fluorescence and cell staining

A common approach to detect bacteria as particles of interest is the use of a general nucleic acid dye, staining all bacterial cells containing nucleic acids, regardless of their intact or damaged state. Alternatively, more specific DNA- or RNA-selective dyes are as well in use for cell counts and finally, strain-specific antibodies or viability dyes can be used to assess metabolic activity and membrane potential or integrity, but will not be discussed in depth.

The dye of choice will depend firstly on the available apparatus, since (i) the dye has to get excited by the wavelength of the light source and (ii) the emitted fluorescent light has to be detectable by the fluorescence detectors. Commonly used light sources are UV (325 - 355 nm), violet (395 - 415 nm), blue (488 nm) and red (633 - 640 nm) lasers, of which the Accuri C6 flow cytometer (BD Biosciences), used for most of this research, has a blue and red laser. Detectors are available over wide UV/VIS wavelength ranges but the commonly used green fluorescence on the FL1 detector (533/30 nm; 30 nm is the width of the wavelength range which is detected) and red fluorescence on the FL3 detector (> 670 nm) were mostly used for this research. A list of widely used dyes for general cell counting, including their absorption and emission maxima, is presented in Table 1-3.
Table 1-3: Commonly used fluorescent dyes for total cell counting with flow cytometry.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Laser</th>
<th>Absorbance max (nm)</th>
<th>Emission max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>UV</td>
<td>300 and 360</td>
<td>590</td>
</tr>
<tr>
<td>Hoechst 33258/33342</td>
<td>UV/violet</td>
<td>352</td>
<td>461</td>
</tr>
<tr>
<td>4’,6-diamidino-2-phenylindole (DAPI)</td>
<td>UV/violet</td>
<td>358</td>
<td>461</td>
</tr>
<tr>
<td>Pico Green</td>
<td>Blue</td>
<td>502</td>
<td>523</td>
</tr>
<tr>
<td>SYTO-9</td>
<td>Blue</td>
<td>480</td>
<td>500</td>
</tr>
<tr>
<td>SYTO-13</td>
<td>Blue</td>
<td>488-491</td>
<td>509-514</td>
</tr>
<tr>
<td>SYBR Green I</td>
<td>Blue</td>
<td>494</td>
<td>521</td>
</tr>
<tr>
<td>SYBR Green II</td>
<td>Blue</td>
<td>494</td>
<td>521</td>
</tr>
<tr>
<td>TOTO-3</td>
<td>Red</td>
<td>642</td>
<td>660</td>
</tr>
<tr>
<td>SYTO 60</td>
<td>Red</td>
<td>652</td>
<td>678</td>
</tr>
</tbody>
</table>

Specifically in this manuscript, SYBR Green I (SG) is used for total cell counting and the combination of SG and propidium iodide (PI) is applied for discriminating between intact and damaged cells. The wavelength of the blue laser is near the absorption maximum of SG, while the emitted green fluorescence is detected by the FL1 detector (Figure 1-2). PI, which is only able to enter the damaged cells, has an absorption pattern which overlaps with the emission pattern of SG. This results in a so-called ‘fluorescence resonance energy transfer’ (Stocks 2004); PI is excited mainly by the emitted fluorescence of SG when both are bound to the DNA and consequently, the green fluorescence is lowered (Barbesti et al. 2000). The difference between the total and intact cell count is further schematically explained in Figure 1-3.
Figure 1-2: Fluorescence spectra of SYBR Green (SG), propidium iodide (PI) and the blue laser and FL1 and FL3 detectors, used for this research. The blue laser emits light near the absorption maximum of SG, while the emission of SG is ideally detected by the FL1 detector. PI is excited mainly by the fluorescent emission of SG, and is detected by the FL3 detector for minimal overlap with SG emission. Figure based on the SpectraViewer (Life Technologies 2014).

Figure 1-3: The principle of flow cytometric total and intact cell counts. The total cell count is based on SYBR Green (SG), which enters and stains all bacterial cells, independently of membrane integrity. The intact cell count method is based on dual staining with SG and propidium iodide (PI), of which PI is only able to penetrate and stain bacterial cells with damaged cell membranes. Therefore, this method enables the specific detection of bacterial cells with intact membranes, apart from damaged bacterial cells e.g. due to chlorination.
C. Exact cell counting?

FCM is regularly used for absolute cell counting. Therefore, a dye which can be excited and detected by the available instrument has to be selected. Ideally, the analysis is triggered on the parameter which is maximally sensitive for bacterial detection. For example, in case of SG staining, this is green fluorescence (FL1) and triggering on this parameter will select for particles with a certain level of green fluorescence and omit data on low fluorescent or non-fluorescent background particles (more information on this topic is provided in Chapter 4). The electronic signals deriving from the detectors after analysis are typically visualized on histogram plots (1-D) or scatter plots (2-D) of several parameters, followed by ‘gating’ (selecting) of the cells of interest on the plots. Histogram plots have often been used in the past (Lebaron et al. 1998) but the advantage of scatter plots is that these allow to do a more trustworthy gating since the gating strategy can be based on the combined information derived from two parameters. A classic choice is the combination of a fluorescence parameter, responding to the unique fluorescence characteristics of the dye, combined with scatter data, based on specific characteristics of cell size and shape (Koch et al. 2013b). Alternatively, it is possible to combine two fluorescence signals deriving from the same dye (Hammes and Egli 2005), as shown in Figure 1-4.
Figure 1-4: Visualisation of flow cytometric data on different types of plots. An effluent from an activated carbon filter typically contains both small and larger bacteria and was stained with SYBR Green I before FCM analysis. The results are visualised and gated for bacterial cells on the FL1/FL3 plot (centre). In case of bacteria, both FL1 and FL3 signals derive mainly from binding of SG to nucleic acids, resulting in a linear relation between both parameters. The events in the bacterial gate on the FL1/FL3 plot are coloured green on the other plots for visualisation purposes. In this example, a gating on the FL1/FSC plot would be more demanding, while gating would deliver false results when only histogram data would be used. Especially the FL3 and FSC histogram are insufficient to discriminate bacteria from background events.
Gating is usually depicted as a demanding and highly subjective process mainly based on experience (De Roy et al. 2012). However, this experience can be built up by analysis of progressively more complex and less known samples, as illustrated in Figure 1-5 for the analysis of a pure strain, a non-chlorinated drinking water and surface water. While a pure strain in a clean dilution medium is usually easily discriminated from the background, this can get more complex for mixed and environmental samples. In any case, the development of a gating strategy should be based on the analysis of enough well-known control samples and comparison with other techniques such as microscopy. Further information on developing a gating strategy is given in Chapter 4.

**Figure 1-5: Examples of bacterial analysis and their gating on FL1/FL3 plots.** A pure *E. coli* sample (A), non-chlorinated drinking water sample (B) and surface water sample (C) were analysed by FCM based on SG staining. In the first two cases, there was a good discrimination of bacteria and background, but this got more demanding in case C. However, when only 10% of the surface water sample was displayed (D) or a zoom was done on the scatter plot (E), the gating strategy seemed valid. In natural samples, a good discrimination between low nucleic acid (LNA) bacteria and high nucleic acid (HNA) bacteria is often seen, as shown in panel B.
Several researchers compared FCM cell counts with epifluorescence microscopic (EFM) enumerations as a reference. As early as 1992, Monfort and Baleux did this comparison based on DAPI-staining and concluded FCM methods to be reliable for counting bacteria (Monfort and Baleux 1992). Comparisons of both methods using an *E. coli* and a lake sample showed that FCM results had smaller standard deviations (3.5% and 7% versus 11.5% and 21.8%), while the difference between the means of both methods was as low as 5.5% and 1.3%, for *E. coli* and lake samples respectively. Several other authors repeated this type of test, using various nucleic acid dyes (TOTO, TO-PRO, SYTO 13 and Picogreen) for FCM analysis and DAPI for EFM analysis. They reported strong linear relationships of 0.76 to 1.05 for FCM counts versus EFM counts (Lebaron et al. 1998, Li et al. 1995, Troussellier et al. 1999, Yanada et al. 2000). One study reported a FCM versus EFM counts ratio of 1.7 for marine samples, but accounted this to the difference in fluorescence intensity between dyes (Kamiya et al. 2007). SG was used for FCM counting and emits much brighter fluorescence than DAPI (Weinbauer et al. 1998), used for EFM counting, but SG is as well brighter than the other dyes used in the previously discussed studies (Lebaron et al. 1998). Therefore, smaller cells or cells with lower nucleic acid content, which were neglected before by both FCM and EFM, could be detected as well. These LNA-cells, as shown in Figure 1-5, panel B, can be subject of debate whether they are bacterial cells, but have been shown to include cultivable bacteria (Wang et al. 2009). Weinbauer et al. (1998) concluded that SG is superior over DAPI for assessing the total number of bacteria by EFM, and whenever this was the stain of choice for both FCM and EFM, the cell counts were seen to be the same as shown in Figure 1-6 (Kamiya et al. 2007).

![Figure 1-6: Number of cells detected after DAPI or SYBR Green I (SG) staining. More bacteria are detect when SG is used, independently of detection method. Both FCM and EFM yield similar results when the same stain is used.](image-url)
Box 1-3: Flow cytometric (FCM) fingerprinting

This box aims at explaining the basic principle of flow cytometric fingerprinting as an analysis technique for FCM data. The FlowFDA method, used in this manuscript, will be explained conceptually and not in full detail, since this work was not focussed on the development of these methods, but on finding applications. Partly after De Roy (2014).

A. Definition and available methods

The FCM fingerprint of a sample is an image or dataset which is unique for that sample. This can either be raw data from an FCM measurement, or being based on further data processing. This ‘processing’ can be very diverse and range from a visualisation of the data in a histogram or scatter plot, the ratio between different clusters appointed on these plots, or be a more complex statistical processing of this data.

Different methods are in use for FCM fingerprinting: (i) methods based on image analysis software such as the Dalmatian plot (Bombach et al. 2011) and the cytometric histogram image comparison (CHIC) (Koch et al. 2013a) (ii) methods based on statistical processing in the statistical programming language R such as cytometric barcoding (CyBar) (Koch et al. 2013b) and FlowFP (Rogers and Holyst 2009) (iii) other methods such as the HNA method, based on basic processing of the data in FCM software (Prest et al. 2013).

For more information on each of these methods, the reader is referred to the cited literature and the review by Koch et al. (2014), comparing the Dalmatian plot, CHIC, CyBar and FlowFP. In this work, the custom-made flow cytometry functional data analysis method (FlowFDA) was used (Clement and Thas 2014). This is a package written in the R programming language and can be seen as a further development of the flowFP method (De Roy et al. 2012, Rogers and Holyst 2009).
B. FlowFDA

FCM fingerprinting based on FlowFDA consists of four steps: (i) selection of parameters of interest for analysis, (ii) processing of the raw FCM data to create the FCM fingerprint, (iii) reduction of the data and (iv) further statistical analysis of choice. More explanation is given based on the way FlowFDA is applied in this manuscript.

(i) Selection of parameters. To the contrary of most other fingerprinting methods, FlowFDA does not rely on a manual and subjective gating step, but the raw data of all analysed events (particles; being bacteria as well as background) is used. However, in order to limit the size of the dataset, the most relevant analysis parameters can be chosen. In our case, the FL1, FL3, FSC and SSC channels were selected. FL1 and FL3 were of special interest due to the use of SYBR Green I and propidium iodide (Box 1-2).
(ii) Processing of the raw FCM data to create the fingerprint. It is advised to accompany the reading of this section with the visualisation in Figure 1-7. For every selected parameter, the data range is divided in 256 equally spaced bins. For every combination of parameters (6 combinations; FL1/FL3, FL1/FSC, FL1/SSC, FL3/FSC, FL3/SSC and FSC/SSC), a virtual scatter plot is created. Since all of the parameters were divided in 256 equally sized bins, each scatter plot contains a grid with $256^2$ (65,536) equally sized rectangular bins. For every bin of the grid, the density is calculated. This resulted in a list, containing the density in each bin, on each plot, for each of the analysed samples. For example, when 20 samples are analysed, this results in a matrix with 20 rows and $6 \times 256^2 = 393,216$ columns. This matrix is the flow cytometric fingerprint, as created by FlowFDA.

**Figure 1-7: The creation of a flow cytometric fingerprint using FlowFDA.** For all parameters of choice (FL1/FL3/FSC/SSC in this case), a virtual scatter plot is made for each combination of two parameters. This results in 6 parameter combinations of each $256^2$ equally spaced bins. The number of events in each of these bins is listed; this list is the FCM fingerprint of the sample.
(iii) **Reduction of the data.** Principal component analysis (PCA) is used to reduce the amount of data in the fingerprint. By using this tool, the data is transformed in such a way that the original variables (393,216 variables) are transformed to ‘principal components’ (PCs) as shown in Figure 1-8. The transformation ensures that a minimal number of PCs can be selected that contain the largest possible amount of variance within the analysed dataset. In this way, the size of the dataset is seriously reduced but mainly redundant data, which cannot be used to discriminate samples, is omitted. In this manuscript, the amount of PCs was based on a cut-off value of 95%; meaning that at least 95% variation in the data was retained. In each case, PC1 explains the largest amount of variance in the data, PC2 the second largest amount of variance, etc. In our analyses, usually 5-50 PCs were retained.

![Graphical representation of a rotation of the data in two dimensions.](image)

**Figure 1-8: Principal component analysis.** Graphical representation of a rotation of the data in two dimensions. After De Roy (2014).

(iv) **Further statistical processing.** Based on the simplified dataset which is obtained after PCA, any further statistical processing can be done, depending on the experimental question which is asked. A typical tool to be used is Fisher discriminant analysis (FDA). Just as PCA, this is a data reduction tool, but with supervised information such as classification in different treatment groups. The PCA data from the previous step is further transformed in discriminants instead of PCs (raw data has variables, after PCA these are called principal components, after
FDA these are called discriminants). This FDA transformation aims at maximising the amount of variance between different treatment groups, while minimising the amount of variance within each treatment group. While PCA is used to omit the similarities, FDA is specifically emphasizing the differences between different samples. Just as for PCA, the first discriminant will explain the largest amount of variance between two treatment groups, followed by the second discriminant, etc. Just as for the original variables or as for PCAs, every sample gets a value (a score) for each discriminant. In order to visualise the data, several discriminants can be plotted in different plots on a linear axis, or the scores for two discriminants can be plotted in a scatter plot (Figure 1-9).

**Figure 1-9: Visualisation of fingerprinting data after Fisher discriminant analysis (FDA).** Three drinking water samples were analysed on FCM with SYBR Green I (A). The discriminant scores can either be visualised on a linear scale (B) or two discriminants can be displayed on a scatter plot (C). Samples with the highest similarity will have similar discriminant scores, whereby the first discriminant is of most importance. The distance between samples on both types of plots indicate their relative similarity; in this example, samples 1 and 2 are most similar, as could be seen from the initial FCM plots.
4. Selective detection

While HPC or flow cytometric cell counts can serve as process indicators and assess the amount of growth in drinking water environments, other analyses are necessary to ensure the hygienic safety of the water, i.e. the presence or absence of pathogenic microbiota.

4.1 Cultivation-dependent methods

In the late 1800s, the golden age of microbiology, many of the primary human pathogens got identified and in the 1890s, microbiologists started to consider whether one should monitor specific pathogens or only indicator bacteria for ensuring the safety of drinking water. Exactly this question returned a century later, and in both cases it was concluded that monitoring for indicators of faecal pollution delivered a higher level of safety, simply because there are too many, still unknown waterborne pathogens (Edberg et al. 2000). A good indicator should (i) be always and only present whenever pathogens are present, in higher concentrations than the pathogens; (ii) not proliferate in the aqueous environment; (iii) be more resistant to disinfection than pathogens; (iv) grow readily on simple media and be easily and unambiguously identified (Bonde 1966, Leclerc et al. 2001, Yates 2007).

*Bacillus coli*, later renamed to *Escherichia coli*, was found to be dominant in faeces and therefore Schardinger proposed in 1892 that its presence in water is 'an indication of the presence of faecal pollution and therefore of the potential presence of enteric pathogens' (Medema et al. 2003). Unfortunately, at that time, there was no specific and easy test for *E. coli* available and the total coliform test (testing for Gram-negative, non-spore-forming, facultative anaerobic bacilli that ferment lactose with production of acid and gas within 48 hours at 35°C) was used as surrogate (Edberg et al. 2000, Eijkman 1904, Medema et al. 2003). This choice was soon criticized since the total coliform test was lacking faecal specificity (Frost 1915). However, an easy test which could be performed on a regular base was considered most important and it was reasoned that most contamination events of drinking water were of faecal origin (Edberg et al. 2000). In the 1920s, the multiple tube fermentation method (MTF) was introduced for routine use. Ten millilitres of sample were added to each of five test tubes with a protein base and lactose. After 48 hours of incubation, the most probable number (MPN) of present bacteria was calculated from positive number of tubes (Edberg and Edberg...
General introduction on bacteriology in DWDS

1988). In the meanwhile, several tests emerged for confirming coliform present and characterising bacteria in the coliform group (Kabler et al. 1964). Parr (1938) combined these in the 'IMViC test, containing the indole, methyl red, Voges-Proskauer and citrate test. When each of these tests were performed, this delivered at that moment the most reliable classification for coliforms, including the detection of *E. coli* (Kabler et al. 1964). In the 1950s, the membrane filtration (MF) method was introduced as alternative for the MFT method (Shipe and Cameron 1954). A certain volume of sample, usually 100 mL, is filtered over a membrane, which is afterwards incubated on an agar containing protein base, lactose and a pH indicator. Coliforms appeared coloured, because of acid production, although additional confirmation tests are still necessary (Edberg and Edberg 1988). In the 1970s, scientists realised that most detected coliforms were actually others than *E. coli* and the surrogate test with total coliforms was no longer regarded valid for *E. coli* detection (Edberg et al. 2000). The faecal coliform test, with an increased incubation temperature, was more selective for thermotolerant coliforms but also gained criticism since it still resulted in many false negatives and positives. The test includes *Klebsiella, Citrobacter, Serratia* and *Enterobacter*, all of which are found in lower concentrations in faeces but are ubiquitous in the environment and do not necessary imply any health risk (Edberg et al. 2000, Leclerc et al. 2001). In the 1980s, the defined substrate technology was developed: samples are inoculated in a medium, containing specific enzyme substrates which change colour after contact with a particular microorganism (Edberg et al. 1990). *E. coli* is for example able to cleave methylumbelliferyl-β-glucuronide (MUG) to form 4-methylumbelliferone, which can be detected due to its fluorescence. This substrate, as well as a substrate specific for general coliforms, is contained in the widely used Colilert-test (IDEXX Laboratories), which therefore selectively detects both coliforms and *E. coli*. The emerging of these possibilities for more specific *E. coli* detection led to the insertion of specific *E. coli* testing in drinking water regulations. Nowadays, a range of media and confirmation tests is in use for coliform and specific *E. coli* testing. The MTF test is used with for example lauryl tryptose broth for coliforms, followed by confirmation in brilliant green lactose bile broth. Faecal or thermotolerant coliforms can be discriminated with the MTF test at 44°C in EC-broth. The MF method is used for coliform detection on for example LES-endo agar or simultaneous coliform and *E. coli* detection based on fluorescence formation on MI-agar (Rice et al. 2012).
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In the meanwhile, a discrimination is made between process indicators, faecal indicators and index organisms. Process indicators demonstrate the efficacy of a process, such as HPC bacteria or total coliforms (Ashbolt et al. 2001). Faecal indicators are an indication of the presence of faecal contamination, such as thermotolerant coliforms or E. coli, which indicate that pathogen presence is possible. Finally, index organisms are species indicative that pathogen presence is likely, such as E. coli as an index organism for Salmonella. When opting for one single indicator organism, it seemed clear to choose E. coli due to its high concentrations in faeces and strong connection with faecal contamination (Edberg et al. 2000, WHO 2011). However, the need for additional indicators or index organisms next to E. coli has been demonstrated by disease outbreaks in the absence of E. coli, including the 1993 Milwaukee cryptosporidiosis outbreak. Cryptosporidium oocysts, more resistant to disinfection than E. coli, could pass the water treatment plant without detection. This resulted in an estimated 400,000 diseases and 54 deaths, mostly among the immunocompromised population (Craun et al. 1997, Hoxie et al. 1997, Mac Kenzie et al. 1994). Other microbial parameters are now regularly incorporated in legislation, as can be seen in Table 1-3. Clostridium perfringens spores are regularly used as index organism for protozoa like Cryptosporidium and Giardia, because of the similar high resistance to disinfection processes and long persistence in the environment (Yates 2007). Enterococci are an addition to E. coli detection, because they are another good indicator of faecal pollution and are more resistant to environmental stress, especially higher salt concentrations (Cabral 2010, Yates 2007). Coliphages have been considered as index organism for enteric viruses, since they have similar properties like human viruses and they show a similar behaviour in aqueous environments and response to disinfection techniques (WHO 2011). Some other indicators or index organisms have been under consideration (e.g. Pseudomonas aeruginosa, Bacteroides fragilis phages, enteric viruses, etc.) but will not be discussed because of their current infrequent use in practice (WHO 2011). More information on the use of indicator and index organisms can be found in the available literature on this topic (Cabral 2010, Edberg et al. 2000, Leclerc et al. 2001, WHO 2011, Yates 2007).

As can be seen in Table 1-3, some guidelines are presented as removal percentages of pathogens or indicators. It is clear that 99.9% removals, as asked, are hard to measure in practice when raw water concentrations were not exceptionally high. Therefore, assessment
of the removal efficiency of a water treatment system is usually based on lab-testing for removal of high concentrations of pathogens or relevant model strains. The effective control of the pathogens in general is ensured by taking into account the level of microbial inactivation needed for the more resistant strains (WHO 2011). More information on quantitative microbial risk assessment, used to assess the needed treatment efficiency and to set drinking water guidelines, is provided in information Box 1-4.
Table 1-3: An overview of drinking water legislation with regard to indicator organisms.

*: Countries within the European Union have to comply with EU-guidelines but can impose additional, stricter legislation. The mentioned values are deviating from EU-guidelines. 

**Indicator parameters are obligatory to measure, but are non-restrictive guidelines, asking for further examination of the non-compliance. References are identical to Table 1-2.

<table>
<thead>
<tr>
<th>Region</th>
<th>Parameter</th>
<th>Value (test volume)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
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<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>( E. coli )</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( Giardia lamblia )</td>
<td>99.9% removal</td>
<td>Optional but recommended</td>
</tr>
<tr>
<td></td>
<td>( Cryptosporidium )</td>
<td>99.9% removal</td>
<td>Optional but recommended</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>0 (100 mL)</td>
<td>Indicator**</td>
</tr>
<tr>
<td>European Union</td>
<td>( E. coli )</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C. perfringens )</td>
<td>0 (100 mL)</td>
<td>In case of surface water. Index for ( Cryptosporidium ) Indicator**</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>0 (100 mL)</td>
<td>Indicator**</td>
</tr>
<tr>
<td>-Germany*</td>
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<td>0 (250 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>0 (250 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( P. aeruginosa )</td>
<td>0 (250 mL)</td>
<td></td>
</tr>
<tr>
<td>-UK*</td>
<td>( E. coli )</td>
<td>0 (100 mL)</td>
<td>Measurement in households</td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>0 (100 mL)</td>
<td>Measurement in households</td>
</tr>
<tr>
<td></td>
<td>Total coliforms</td>
<td>0 (100 mL)</td>
<td>95 % of the samples/month have to comply</td>
</tr>
<tr>
<td>-France*</td>
<td>Sulphate-reducing sporeforming bacteria</td>
<td>0 (100 mL)</td>
<td>Instead of ( C. perfringens )</td>
</tr>
<tr>
<td>USA</td>
<td>( Cryptosporidium )</td>
<td>99% removal</td>
<td>Surface water</td>
</tr>
<tr>
<td></td>
<td>( Giardia lamblia )</td>
<td>99.9% removal</td>
<td>Surface water</td>
</tr>
<tr>
<td></td>
<td>Faecal &amp; total coli</td>
<td>0 (100 mL)</td>
<td>Indicator**, max 5% total coli positive</td>
</tr>
<tr>
<td></td>
<td>( E. coli )</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Viruses</td>
<td>99.99% removal</td>
<td></td>
</tr>
<tr>
<td>Switzerland</td>
<td>( E. coli )</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>0 (100 mL)</td>
<td></td>
</tr>
</tbody>
</table>
**Box 1-4: Quantitative Microbial Risk Assessment (QMRA)**

There is a trade-off between more strict guidelines, resulting in a safer drinking water, and too strict guidelines, resulting in only minor health benefits to the population while there is a major cost to comply with the stricter guidelines. Therefore, in order to set up health-based performance targets and to decide on prioritised water management issues and legislation, quantitative microbial risk assessment (QMRA) is used. QMRA consists of a combination of pathogen exposure information (raw water concentrations, prevalence, etc.) and dose-response models to estimate the probability of infection. Epidemic data on severity and duration is added to calculate the overall disease burden in the population. A four-step-model is described, after the WHO Guidelines (WHO 2011). More information is as well available in the extensive standard work by Haas et al. (2014). The model consists of (A) problem formulation and hazard identification, (B) exposure assessment, (C) dose-response assessment and (D) risk characterisation.

**A. Problem formulation and hazard identification**

This first step includes the identification of possible hazards, sources and events leading to pathogen exposure, as well as their pathways from source to consumer. This can be information on point sources (e.g. sewage leaks), diffuse sources (e.g. livestock farming), extreme events (e.g. severe drought or flooding), etc. Since this is impossible to do for every single pathogen, several organisms are usually selected as models for bacteria (e.g. *Campylobacter*), viruses (e.g. rotavirus) and protozoa (e.g. *Cryptosporidium*).

**B. Exposure assessment**

The second step is an assessment of the total number of pathogens an individual is exposed to (the dose), mostly via ingestion. Therefore, the volume of unheated water ingested per person and the concentration of pathogens in the specific water is needed, and variability over time of both factors has to be taken into account. Since it is often impossible to measure low concentrations of pathogens directly in drinking water, usually the information on raw water concentrations and removal efficiencies by the specific water treatment are combined. The latter is assessed and follow-up can be done by the use of index organisms such as *E. coli*. 
C. Dose-response assessment

In a third step, the effect of exposure to certain concentrations of pathogens is studied and dose-response models are used. What is the probability of infection after ingestion of a certain number of pathogens? This is very pathogen-dependent; while Cryptosporidium parvum already causes infection after the ingestion of one single cyst and enterohemorrhagic strains of E. coli require only about ten cells to be infective, this can be as much as $10^3$ to $10^8$ for other pathogens such as Vibrio cholerae (Schmid-Hempel and Frank 2007). It has to be taken into account that these models especially exist for the average, healthy population and are an underestimation for the young, the elderly and immunocompromised population. For example, whereas the general population can suffer from diarrhoea for a few days in case of a cryptosporidiosis outbreak, HIV-patients may be untreatable and die and create a more significant burden when taken into account (OECD and WHO 2003, Perz et al. 1998).

D. Risk characterisation

In the final step, all previous information is combined to do an overall risk characterisation. Therefore, the DALY system is used (disability-adjusted life year), expressing the amount of years an individual is withdrawn from a good health, either by light or severe illness, or death. The DALY per case of illness is calculated from the severity score of the illness (some are mild, such as diarrhoea), the duration (mild or long lasting, or even death), and the percentage of the population affected by these conditions. In case of illness, the amount of days (expressed as fraction of a year) is taken into account, in case of death, the amount of estimated lost life years is taken into account. For example, for a rotavirus illness in developed countries, the DALY is 0.0125:

- mild diarrhoea (severity 0.1), lasting 7 days in 97.5% of the cases
- severe diarrhoeae (severity 0.23), lasting 7 days in 2.5% of the cases
- death (severity 1) of very young children in 0.015% of the cases

\[
DALY = \left(0.1 \times \frac{7}{365} \times 0.975\right) + \left(0.23 \times \frac{7}{365} \times 0.025\right) + (1 \times 70 \times 0.00015)
\]

\[
= 0.0125
\]
It should be noted that such a calculation is very dependent on location and corresponding health standards.

Starting from the DALY per case and the previous combined information, the total health outcome can be calculated, expressed as DALY per year, as visualised in Figure 1-10. A guideline value of $10^{-6}$ DALY per person per year is advised, although nations are supposed to impose national regulations. The Netherlands for example aims at a maximal infection risk of 1 out of 10,000 persons per year (van der Kooij and van der Wielen 2014a). This health outcome calculation can be used to verify which treatment efficiency and legislation guidelines are necessary to protect the population. Overall, this is part of a general safe drinking water framework, which will further be explained in Chapter 8, § 4.4.

*Figure 1-10: Schematic calculation of the risk assessment calculation of the health outcome target (WHO 2011).*
4.2 Cultivation-independent methods

Other techniques than growth-based methods are rarely routinely applied in drinking water monitoring. Some flow cytometry procedures are under development but will be discussed in Chapter 8, §4.2. Alternative methods for specific bacterial detection and identification are established or emerging: analytical profile index (API, Biomerieux), fluorescent in situ hybridization (FISH) (Wagner et al. 1993), catalysed reporter deposition (CARD)-FISH (Pernthaler et al. 2002), enzyme-linked immunosorbent assay (ELISA) (Ferguson et al. 2000), matrix-assisted laser desorption ionization time-of-flight analysis (MALDI-TOF) (Karas et al. 1985), biosensors based on surface plasmon resonance (SPR) (Dudak and Boyacı 2009, Lu et al. 2009), etc. It has to be noted that several of these technologies need higher bacterial concentrations or pure colonies, the identification steps are however cultivation-independent.

Next to previous technologies, it leaves no doubt that the actual future identification methods are molecular based. Currently, these are mainly used for research but the applicability for routine applications is increasing fast. A recent and extensive overview on molecular methods, currently in use or offering future possibilities in drinking water research, is offered by Douterelo et al. (2014a). A summary will be given below.

The start for any of these methods is nucleic acid extraction, followed by the polymerase chain reaction (PCR) for the amplification of targeted genes (Mullis et al. 1986). Real-time quantitative PCR (qPCR) is an extension, allowing direct monitoring of the amplification of genes, using fluorescent probes (Aw and Rose 2012, Heid et al. 1996). Hereby, fast quantitative information is received on the presence of general genes (allowing quantification of bacteria), a specific targeted gene (allowing detection of a certain species or group, functional or virulence gene) (Kim et al. 2013), or multiple genes at once by multiplex qPCR (Weller et al. 2000). Specific screening for genes in viable cells is possible by combining qPCR with a propidium monazide-pretreatment (Li et al. 2014). These methods are occasionally used by drinking water utilities, beyond the regulations, for the specific detection of pathogens such as Legionella ssp., Helicobacter pylori, etc. (Casasola-Rodriguez et al. 2013, Dusserre et al. 2008, Sen et al. 2007).
Molecular fingerprinting techniques use PCR products (amplicons) to create a pattern, specific for that sample. Examples are denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), temperature gradient gel electrophoresis (TGGE) (Rosenbaum and Riesner 1987) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997). These methods are especially useful for comparing samples, but are generally laborious and identification of target species is challenging, making the applicability for drinking water utilities limited.

Sequencing techniques offer the possibility to receive more detailed and accurate phylogenetic information. Sanger-sequencing (Sanger et al. 1977) has been widely applied for years for identification of bacteria (Douterelo et al. 2014a). The next step was sequencing a whole genome of an environmental sample: metagenomic analysis. This was initially done by shotgun sequencing, fragmenting the genomes to sequence and reassemble them. This method received most fame by the sequencing of the human genome and the global ocean sampling project by Craig Venter, but has not been applied to drinking water systems due to its complexity and high cost (Venter et al. 1998, Venter et al. 2004). Next-generation sequencing (NGS) methods are now fast emerging to bring a change. They offer the possibility for high-throughput screening and detecting less abundant members of microbial communities (Douterelo et al. 2014a, Metzker 2010). Illumina and Roche 454 are nowadays the most frequently used platforms and several authors recently published a metagenomic analysis of drinking water and distribution systems based on these platforms (Douterelo et al. 2014b, Gomez-Alvarez et al. 2012, Liu et al. 2014, Pinto et al. 2014, Prest et al. 2014). Prices are decreasing fast, making these methods promising for future routine applications.

Finally, the active bacteria can be studied more selectively, using metatranscriptomics; the study of actively transcribed RNA. Isolation of RNA is challenging, due to the instability of RNA outside of the bacterial cell. Once isolated, reverse transcription PCR (RT-PCR) and functional microarrays enable the measurement of specific gene expression in the bacterial community. This type of approaches is especially used for assessing active metabolic pathways in the system, e.g. nitrification and iron oxidation (Douterelo et al. 2014a, Li et al. 2010).

For a more extensive description on the above mentioned and other methods for a profound study on microbial ecology in DWDS, the reader is referred to the previously mentioned review (Douterelo et al. 2014a).
5. Objectives and overview of the research

From the previous discussions, it is clear that growth of bacteria in drinking water is a complex process, governed by many factors. Nowadays, the main methods to assess this growth are heterotrophic plate counts and indicator bacteria, while especially the value of the first one is subject of debate and performant alternatives such as FCM are available.

In this work, we will examine two possible important factors for bacterial growth (PART A), overcome possible difficulties encountered using FCM methods (PART B), and apply FCM in drinking water applications (PART C).

A schematic overview of the research is given in Figure 1-11, a more detailed overview is given below.

PART A is a study on factors, possibly affecting bacterial growth.

Biofilms are one of the main pools of bacterial life in DWDS, containing up to 95% of the bacteria in drinking water according to some (Flemming 2002, Servais et al. 2004). They are the hiding place for pathogens, providing protection against disinfectants. For of these reasons, characterisation and understanding of biofilm growth is of utmost importance. Transparent exopolymer particles gained recent interest, as a new factor in the biofouling and biofilm research. These particles are ubiquitous, pass water filtrations and have correlations with biofouling. In Chapter 2, we will examine their presence and removal in three model drinking water systems, and thereby evaluate their possible role as biofilm inducer.

In Chapter 3, the outcome of bacterial invasion is studied in different water types, whereby ‘invasion’ is defined as: the entrance of a novel specie in an existing community. A bacterium (‘the invader’) is spiked in three model waters, containing various and well-characterised levels of nutrients next to their own indigenous bacterial community. Pseudomonas putida, known for its broad substrate utilisation, is used as a model invader. The experimental temperature was fixed at 27°C. This combination served as a worst-case situation for invasion, allowing to search for the parameters dictating the outcome of bacterial invasion in drinking water.
**PART B is an optimization of flow cytometric measurements for routine applications**

We want to propose flow cytometric measurements as a new tool for routine analysis in drinking water utilities. Therefore, two studies will be presented for fully understanding and optimizing flow cytometric measurements.

Know your enemy. FCM background is usually avoided, neglected, minimized, hidden, but has never been studied before. However, it is of major importance, since it is part of the samples which are analysed by FCM. It has to be understood in order to apply fixed gating, discriminating bacteria and particles. Finally, it has an effect on fingerprinting techniques which are incorporating background in their analysis and are therefore heavily affected by this part of the data. **Chapter 4** will be a study on this overlooked part of information, both on the understanding of it and the discrimination from bacteria or other target organisms.

A method for routine applications gets more valuable when options for automation are offered. Autoloaders are available for flow cytometry, however the resulting long waiting times between staining and sample analysis are conflicting with good lab practice. **Chapter 5** will be a study of the effect of automation on flow cytometric analysis of bacteria, for example in drinking water samples. Analysis will be performed in 96-well multititer plates and the final goal is to develop a procedure for a fast, stable and precise measurement of 96 samples.

**PART C is presenting new applications, offered by flow cytometry**

Automated flow cytometry is offering high-throughput results and almost immediate information. This opens up opportunities, which were not or less feasible before using culture-based methods.

Maintenance on drinking water piping often involves the exposure of the inside of the pipe to external influences. After maintenance, the pipes are chlorinated and heavily cleaned by flushing for ensuring the hygienic quality of the distributed drinking water. Quality control with culture-based methods is however slow and in the search for faster information, the use of flow cytometry will be tested in **Chapter 6**. Two types of samples were taken: (i) reference samples consisting of clean drinking water used for pipe flushing, and (ii) flushing samples, taken after flushing the opened pipe section. Both were immediately analysed by flow
cytometry. A comparison of the water quality will be made, based on both cell counts and on the ‘fingerprint’ of the samples.

(Re)growth of bacteria can happen in a drinking water network, but examination with culture-based methods is entangled by low accuracy and sensitivity. Chapter 7 will present a short, one-day study of bacterial growth in a local drinking water network. A large amount of samples is collected spread over the local network. An evaluation on regrowth in the local network will be made based on flow cytometric measurements (cell counts and the fingerprint) and geographic location of the sampling spots.

Finally, Chapter 8 will provide an integrated discussion of the gathered knowledge and serve a future perspective on the characterisation of bacteria and their growth in drinking water.
**GROWTH AND FLOW CYTOMETRIC MONITORING OF BACTERIA IN DRINKING WATER**

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Part A</th>
<th>Chapter 2</th>
<th>Part B</th>
<th>Chapter 3</th>
<th>Part C</th>
<th>Chapter 4</th>
<th>Chapter 5</th>
<th>Chapter 6</th>
<th>Chapter 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characterisation of Growth</td>
<td>Factors affecting bacterial growth</td>
<td>Transparent exopolymer particles as biofilm inducers</td>
<td>Optimization of flow cytometry for routine measurement</td>
<td>Effect of nutrients and the indigenous community on invasion</td>
<td>New applications with flow cytometry</td>
<td>Understanding the overlooked background</td>
<td>Automation as the way to high-throughput</td>
<td>Follow-up of the cleaning of drinking water piping after maintenance</td>
<td>Fast assessment of regrowth in the drinking water network</td>
</tr>
</tbody>
</table>

**Figure 1-11: Schematic research overview with localisation of the different chapters**
PART A
FACTORS AFFECTING BACTERIAL GROWTH
‘When the well is dry, we know the worth of water.’

Benjamin Franklin

Water tower of Ghent (Belgium)
Chapter redrafted after:

Abstract

Transparent exopolymer particles (TEP) have recently gained interest in relation to membrane fouling. These sticky, gel-like particles consist of acidic polysaccharides excreted by bacteria and algae. The concentrations, expressed as xanthan gum equivalents \( \mu g \text{ Xeq.L}^{-1} \), usually reach hundreds up to thousands \( \mu g \text{ Xeq.L}^{-1} \) in natural waters. However, little research was performed on the occurrence and fate of TEP in drinking water, this far. This study examined three different drinking water production centers, taking in effluent of a sewage treatment plant (STP), surface water and groundwater, respectively. Each treatment step was evaluated on TEP removal and on 11 other chemical parameters. An assessment on TEP removal efficiency of a diverse range of water treatment methods and about on correlations between TEP and other parameters was performed. Significant correlations between particulate TEP (> 0.4 \( \mu m \)) and viable cell concentrations were found, as well as between colloidal TEP (0.05-0.4 \( \mu m \)) and total COD, TOC, total cell or viable cell concentrations. TEP concentrations were very dependent on the raw water source; no TEP was detected in groundwater but the STP effluent contained 1572 \( \mu g \text{ Xeq.L}^{-1} \) and the surface water 699 \( \mu g \text{ Xeq.L}^{-1} \). Over 94% of total TEP in both plants was colloidal TEP, a fraction neglected in nearly every other TEP study. The combination of coagulation and sand filtration was effective to decrease the TEP levels by 67%, while the combination of ultrafiltration and reverse osmosis provided a total TEP removal. Finally, in
none of the installations TEP reached the final drinking water distribution system at significant concentrations. Overall, this study described the presence and removal of TEP in drinking water systems.

Graphical abstract: TEP was found to be present in both surface water and waste water effluent, but was retained nearly 100% by the both treatment trains producing drinking water.

1. Introduction

Transparent exopolymer particles (TEP) are gel-like sticky particles consisting mainly of acidic mucopolysaccharides, ubiquitous in natural waters and measuring up to 100s of micrometers (Passow 2002). They are predominantly formed out of algal exudates, bacterial mucus and particular material from the gelatinous envelopes surrounding phytoplankton. Hence they are found abundantly in oceans as well as in fresh water, wastewater and groundwater (Berman and Parparova 2010, Kennedy et al. 2009, Passow 2002). Some examples of measured concentrations in previous studies are given in Table 2-1. These studies only comprised particular TEP (pTEP > 0.4 µm) but recently, colloidal TEP (cTEP) was described (Villacorte et al. 2009b). This fraction is similar to pTEP but passes 0.4 µm membranes and is retained on 0.05 µm. In these studies, cTEP contributed for up to 90% of total TEP concentrations (Villacorte et al. 2009a, Villacorte et al. 2010).
Table 2-1: Overview of TEP concentrations in literature and raw water TEP concentrations in this study.

<table>
<thead>
<tr>
<th>Sampling place</th>
<th>pTEP concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface water (Israel)</td>
<td>759-2385 µg X_{eq}.L^{-1}</td>
<td>(Berman and Parparova 2010)</td>
</tr>
<tr>
<td>Seawater (Israel)</td>
<td>80-1003 µg X_{eq}.L^{-1}</td>
<td>(Berman and Parparova 2010)</td>
</tr>
<tr>
<td>Seawater (Norway)</td>
<td>193 µg X_{eq}.L^{-1}</td>
<td>(Riebesell et al. 1995)</td>
</tr>
<tr>
<td>Secondary wastewater effluent (the Netherlands)</td>
<td>270 µg X_{eq}.L^{-1}</td>
<td>(Kennedy et al. 2009)</td>
</tr>
<tr>
<td>Secondary wastewater effluent (Israel)</td>
<td>746-4,157 µg X_{eq}.L^{-1}</td>
<td>(Berman and Parparova 2010)</td>
</tr>
<tr>
<td>Saline groundwater (Israel)</td>
<td>132-417 µg X_{eq}.L^{-1}</td>
<td>(Berman and Parparova 2010)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling place</th>
<th>pTEP concentration</th>
<th>cTEP concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary wastewater effluent</td>
<td>102 ± 20 µg X_{eq}.L^{-1}</td>
<td>1470 ± 189 µg X_{eq}.L^{-1}</td>
</tr>
<tr>
<td>Surface water</td>
<td>14.8 ± 14.0 µg X_{eq}.L^{-1}</td>
<td>684 ± 94 µg X_{eq}.L^{-1}</td>
</tr>
<tr>
<td>Groundwater</td>
<td>&lt; 5 µg X_{eq}.L^{-1}</td>
<td>&lt; 50 µg X_{eq}.L^{-1}</td>
</tr>
</tbody>
</table>

Since the staining method was developed to visualize these hitherto overlooked particles (Alldredge et al. 1993), they have mostly drawn the attention of oceanographers in relation to organic carbon cycling (Passow 2002). More recently, their importance in membrane technology was recognized. Berman (2005) introduced the concept that TEP can induce biofouling on surfaces. Once attached to a membrane, these particles possibly serve as attachment site and nutritious substrate for microbial growth. Furthermore, the efficiency of RO-pretreatment systems in preventing TEP from reaching the sensitive membranes was verified since up to 70% of all TEP in influent water is seen to deposit on reverse osmosis (RO) membranes (Villacorte et al. 2009a), which is in the range of deposition rates reported for
polysaccharides on cross-flow RO systems (Herzberg et al. 2009). Several combinations of sand and membrane filtration were able to remove pTEP for 30 up to 100%. In contrast, cTEP, which is most abundant, was rarely removed for more than 50% (Bar-Zeev et al. 2009, Kennedy et al. 2009, Villacorte et al. 2009a, b, Villacorte et al. 2010). Moreover, this fraction can easily transform to new pTEP and hereby block pores that would not be blocked by the smaller cTEP. Recently, the positive correlation between TEP concentrations and capillary suction time, a common fouling indicator, was shown in a full-scale MBR (membrane bioreactor) treating domestic wastewater (de la Torre et al. 2008). In a similar system, multivariate data analysis related 95% of the variation of critical flux values to TEP, nitrate and temperature (de la Torre et al. 2010). Besides, Berman et al. (2011) stated that early deposition of extracellular polymeric substances (EPS) on membranes only originated from TEP in the feed water instead of being excreted by active bacteria developing a biofilm. There has already been massive research on attachment of bacteria to surfaces (Donlan 2002), but TEP can be an additional important factor aggravating and speeding up biofilm formation. Therefore, it could be an important characteristic of the incoming water in relation to this topic (Bar-Zeev et al. 2009, Berman et al. 2011).

Meanwhile, many studies have reported the abundance of TEP in marine water, surface water, waste- and groundwater. In contrast, their occurrence in drinking water treatment systems has, to our knowledge, only been described once so far (Villacorte et al. 2009a). In this study, the final RO-treatment could remove the last part of the total TEP. However, the limited TEP removal efficiencies in several RO pretreatment systems like combinations of sand filtration, coagulation and ultrafiltration (UF) suggest the possibility of TEP reaching the drinking water in systems where no RO is present in the end of the treatment line. Furthermore, the putative role of TEP in biofilm formation could thus have safety implications for the drinking water distribution network. Waterborne pathogens like Legionella species use biofilms both for growth and protection against biocides (Szewzyk et al. 2000, Williams and Braun-Howland 2003). TEP occurrence in drinking water would ask for a further assessment of biofilm formation properties of these molecules, which could possibly provide us new insights about biofilm prevention strategies.

This paper evaluated the fate of TEP in a drinking water facility, from raw water to drinking water. The main objectives of this paper were (i) to evaluate TEP concentrations throughout
different drinking water production trains, (ii) to evaluate common water treatment methods on TEP removal and (iii) to evaluate physicochemical parameters and their relation to TEP occurrence.

2. Materials and methods

2.1 Sampling

Sampling was performed in 2011 at drinking water production centers of 3 Belgian drinking water companies: IWVA (‘Plant A’ in Wulpen/Oostduinkerke), VMW (‘Plant B’ in Kluizen) and Pidpa (‘Plant C’ in Grobbendonk) at respectively February 17th, March 11th and January 17th. Each time, a raw (influent) sample was taken, followed by a sample after every single step of the treatment process, until the final drinking water. Plant A uses effluent from a sewage treatment plant (STP), Plant B surface water and Plant C groundwater. For the complete configuration of the plants and the sampling points, the reader is referred to Figure 2-1, 2-2 and 2-3. Additional information about Plant A can be found in Van Houtte et al. (2008) and Dewettinck et al. (2001). Samples were taken in duplicate in plastic 10 L vessels and were stored at 4°C for maximum 5 days until further analysis.

2.2 TEP measurement

TEP measurements were based on the conventional spectrophotometric method as described by Passow and Alldredge (1995) and adapted by Villacorte et al. (2009a). At least three subsamples of each water sample (20mL-2L, depending on the concentration) were successively filtered over polycarbonate membranes with pore sizes 0.4 and 0.05µm (respectively It4ip and Sterlitech). This was done using an adjustable vacuum pump (Knf lab pumps) set at 200 millibar of vacuum and polysulfone filter holders (Nalgene). Subsequently, the membranes were stained with alcian blue (see further) and rinsed and the stain was extracted in 80% sulphuric acid according to Villacorte et al. (2009a). Finally, the absorbance of this acid solution was measured at 787 nm with a spectrophotometer (WPA Lightwave II, Biochrom). Xanthan Gum, a commercial available polysaccharide, was used as a model for TEP in the measurements and therefore TEP concentrations were expressed as µg.L⁻¹ xanthan gum-equivalents (Xeq). The calibration for this staining method was performed by TOC
measurements, as explained by Kennedy et al. (2009) and yielded a calibration factor $f_x$ of 235 µg X$_{eq}$ per unit absorbance at 787 nm. Based on this calibration factor, blank assessments and the filtration of at maximum 2 L samples (for pTEP) or 250 mL samples (cTEP), a limit of detection (LOD) of 11 µg.L$^{-1}$ X$_{eq}$ for pTEP and 46 µg.L$^{-1}$ X$_{eq}$ was calculated.

**Figure 2-1:** Plant A. Configuration of plant A and evolution of colloidal TEP (cTEP) and particular TEP (pTEP) concentrations. Concentrations are expressed as µg.L$^{-1}$ xanthan gum-equivalent ($n \geq 3$). Mind the different axes for pTEP and cTEP. LOD: limit of detection.
**Figure 2-2: Plant B.** Configuration of plant B and evolution of colloidal TEP (cTEP) and particular TEP (pTEP) concentrations. Concentrations are expressed as µg.L⁻¹ xanthan gum-equivalent (n ≥ 3). LOD: limit of detection.
The staining solution, needed for TEP detection, was made by dissolving 150-200 mg of the alcian blue dye (8GX, Standard Fluka) in 100 mL of deionised water and was set at pH 2.5 by addition of acetic acid. This solution was filtered over 0.05 µm membranes to remove coagulated and non-dissolving stain particles. The alcian blue-concentration of this filtered solution is determined by measuring copper concentrations by flame atomic absorbance spectrophotometry (AAS, AA-6300, Shimadzu) and incorporating the proportional mass of copper in alcian blue (4.89 wt%). Subsequently, the solution is diluted with deionized water to set the alcian blue-concentration at 78.5±2 mg.L⁻¹. By doing this, the staining capacity of the staining solution was considered as constant and a fixed calibration factor was used. However, it should be noted that this staining method especially quantifies the amount of negative charges present on the TEP particles. All results should therefore be regarded as relative and the quantifications expressed as xanthan gum are only used as visualization of the results. When not in use, the solution is conserved at 4°C for maximum one week before renewing.

2.3 Analytical methods

The concentrations of sodium, potassium, iron, calcium and magnesium were measured since Passow (2002) reported about the formation of gels like TEP, being promoted and stabilized by cations, which are able to neutralize the negative charges of TEP-precursors and induce cationic bridge formation. All of these cations were measured using flame-AAS. All samples were acidified with either HCl or HNO₃ before measurement and 2% of a 1g.L⁻¹ lanthanum
standard solution (Chem-Lab) was added for the measurement of calcium or magnesium to suppress chemical interference with other elements. Nitrate, nitrite, sulfate, phosphate and chloride ions were determined with a 761 compact ion chromatograph equipped with a conductivity detector (Metrohm). Total organic carbon (TOC) was determined using a TOC-VCPN (Shimadzu). Total chemical oxygen demand (CODt) measurement was performed according to manufacturer’s guidelines with the ‘HI 93754A-25 LR’ kit (Hanna Instruments). Results for chlorophyll concentrations in Plant B were obtained from VMW.

2.4 Statistical analysis

The relationship between either pTEP or cTEP and the other variables was tested for significance using the Spearman’s rank correlation coefficient. This coefficient is obtained by ranking the values of each of the two variables and subsequently calculating Pearson’s product-moment coefficient of the ranks. Resulting p-values below the significance level (p ≤ 0.05) indicate a monotonic association between the two tested variables. All statistical analyses were conducted using version 2.12.1 of the statistical software package R (R Development Core Team).
3. Results

3.1 TEP in plant A

TEP concentrations of Plant A are shown in Figure 2-1. The raw water source of this plant is effluent from a sewage treatment plant (STP). The pTEP concentration measured in this raw water was 102 μg Xeq.L⁻¹, while cTEP yielded 1470 μg Xeq.L⁻¹, accounting for 94% of the total TEP concentrations. Pre-chlorination resulted in an increase in pTEP and cTEP concentrations to respectively 136 μg Xeq.L⁻¹ and 2070 μg Xeq.L⁻¹, while the UF removed more than 91% of pTEP (below LOD) and 97% of the cTEP entering the UF-system. The RO-system accomplished a total TEP removal but 71 μg Xeq.L⁻¹ pTEP and 149 μg Xeq.L⁻¹ cTEP reappeared in the infiltration pond. These newly formed TEP were again efficiently removed by the dune infiltration to a depth of 8 to 12 m.

3.2 Other parameters in plant A

The chemical and microbiological parameters measured in plant A are presented in Table 2-2. Before RO, no noteworthy changes of metal concentrations (Na, K, Ca, Mg) or anions (Cl⁻, NO₃⁻, SO₄²⁻), conductivity, CODₜ and TOC were detected. RO decreased all metals and anions by 99% and minimized CODₜ, TOC and conductivity. The open air infiltration pond resulted in a slight increase of most parameters, while the infiltration caused a 12-13 fold increase of total metal and anion concentrations.

The pH changed after the UF treatment: due to aeration the pH slightly increased but prior to RO, the pH is adjusted using sulfuric acid dosing for scaling prevention. RO results in a pH decrease, partly counteracted by sodium hydroxide dosing.

3.3 TEP in plant B

The raw influent of plant B showed a very low pTEP concentration of 14.8 μg Xeq.L⁻¹. On the contrary, cTEP was more abundant and accounted to 684 μg Xeq.L⁻¹, 98% of total TEP concentrations (Figure 2-2). This influent was split and treated over parallel decantation (42%) or flotation (58%) treatments which joined again in the final treatment steps. The decantation step resulted in a 37-fold pTEP concentration increase while the amount of cTEP, to the contrary, decreased with 74%. However, total TEP concentrations did not show any significant
changes. Combined hydroantracite and sand filtration removed pTEP to minimal levels while cTEP was only removed for 20%. The combination of nitrification, flotation and sand filtration removed the small pTEP fraction while 70% of cTEP was retained. ‘The final treatment steps (ozonation, activated carbon and chlorination) resulted in TEP concentrations below detection limit in the drinking water.’

3.4 Other parameters in plant B

All other measured parameters in plant B are shown in Table 2-3. Few noteworthy changes were detected, except for the pH changes after sulfuric acid or sodium hydroxide dosing and the concomitant sulfate or sodium concentration increase.
Table 2-2: Chemical parameters plant A. Number of sampling points (SP): Figure 2-1.

<table>
<thead>
<tr>
<th>SP</th>
<th>pH</th>
<th>Conductivity (µS.cm⁻¹)</th>
<th>CODₜ (mg.L⁻¹)</th>
<th>TOC (mg.L⁻¹)</th>
<th>Na (mg.L⁻¹)</th>
<th>K (mg.L⁻¹)</th>
<th>Ca (mg.L⁻¹)</th>
<th>Mg (mg.L⁻¹)</th>
<th>Cl⁻ (mg.L⁻¹)</th>
<th>NO₃⁻ (mg.L⁻¹)</th>
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Table 2-3: Chemical parameters plant B. Number of sampling points (SP): Figure 2-2.

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<th>Conductivity (μS.cm⁻¹)</th>
<th>COD (mg.L⁻¹)</th>
<th>TOC (mg.L⁻¹)</th>
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<th>K (mg.L⁻¹)</th>
<th>Ca (mg.L⁻¹)</th>
<th>Mg (mg.L⁻¹)</th>
<th>Cl⁻ (mg.L⁻¹)</th>
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<td>69.0</td>
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<td></td>
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</tr>
</tbody>
</table>
3.5 Plant C

No TEP concentrations above the detection limit (set at 5 µg X_{eq} L^{-1} for pTEP and 50 µg X_{eq} L^{-1} for cTEP) were detected in the ground water entering this plant or in any other water sample taken in the drinking water production train. For this reason, no detailed results were presented for this plant.

3.6 Statistical analysis

The correlation between either pTEP or cTEP and other variables in all samples was tested for significance using the Spearman's rank correlation coefficient. The results are shown in Table 2-4. A significant monotonic association (p ≤ 0.05) was found for pTEP versus cTEP (p = 0.025). cTEP showed monotonic associations with COD_t (p = 0.0066), TOC (0.0070) and pTEP (0.025).

Table 2-4: Statistical associations. P-values for the statistical associations between either pTEP or cTEP concentrations and the other parameters, based on the Spearman's rank correlation coefficient. All p-values ≤ 0.05 are indicated in bold and indicate significant associations between two parameters.

<table>
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<th>Parameter</th>
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<th>cTEP</th>
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<tbody>
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<td>Conductivity</td>
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</tr>
<tr>
<td>cTEP</td>
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</tr>
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</tr>
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</tr>
<tr>
<td>NO_3^{-}</td>
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<td>0.140</td>
</tr>
</tbody>
</table>
4. Discussion

4.1 Raw water TEP concentrations

In this paper, the fate of TEP in drinking water production centers was regarded in three very different plants. It was verified whether TEP is present in several drinking water raw sources and the TEP removal efficiency of a broad range of treatment methods was examined. Recent literature revealed that cTEP is a very abundant fraction of total TEP so in contrast to most previous studies this fraction was enclosed in our measurements (Villacorte et al. 2009a).

Plant A uses effluent of a STP with a pTEP concentration of 102 µg \( X_{\text{eq}} \cdot \text{L}^{-1} \). Only a few studies have already addressed the pTEP contents of STP effluents; the ranges vary from about 270 to 746-4157 µg \( X_{\text{eq}} \cdot \text{L}^{-1} \) in studies in the Netherlands and Israel, respectively (Table 2-1). These notably higher values compared to our results can be due to: (1) seasonal or climate differences, affecting the temperature and hereby the bacterial and algal activity and TEP production or (2) differences in influent water and plant configuration. Besides pTEP, the cTEP concentration was also assessed in our study. This fraction reached concentrations up to 1470 µg \( X_{\text{eq}} \cdot \text{L}^{-1} \), which accounted for 94% of the total TEP. Our results confirmed that cTEP is an important fraction in the TEP measurement. The presence of TEP in this STP effluent could be attributed to the bacterial activity and concomitant polysaccharide production in the biological wastewater treatment, as discussed by Kennedy (2009).

Plant B, with surface water as influent, showed a low pTEP concentration of 14.8 µg \( X_{\text{eq}} \cdot \text{L}^{-1} \), while literature values for surface water are usually higher (Table 2-1). cTEP was more abundant with a measured concentration of 684 µg \( X_{\text{eq}} \cdot \text{L}^{-1} \). Our samples were taken in a water basin in which the raw water is stored with a residence time of one year. During this time, self-purification processes take place, resulting in an oligotrophic environment with low TEP concentrations. Furthermore, the sampling took place in the beginning of March, just during the beginning of a phytoplankton bloom. Peak TEP concentrations are usually associated with phytoplankton blooms (Passow 2002). The higher concentration of cTEP in our study is in accordance with the beginning of the detected bloom (9.99 mg chlorophyll.m\(^{-3}\)), when TEP-precursors, measured as cTEP, are being exudated by algae. The coagulation of these precursors to pTEP did not take place yet, so the pTEP concentrations in our study remained
very low compared to cTEP concentrations. Since cTEP accounted for 94% of total TEP in the raw water sample in plant A and for 98% in the raw water of plant B, this fraction should consequently be enclosed in TEP measurements.

The last sampled plant C is based on groundwater extracted from a depth of 60m. Berman et al. (2010) already reported measurable TEP concentration in the range of 132-417 µg X<sub>eq</sub>.L<sup>-1</sup> in saline groundwater. These TEP were attributed to sulfur and iron bacteria present in that specific well. No notable TEP concentrations could however be found in this extremely oligotrophic water. To our knowledge, nothing is known about the specific bacterial species contributing to the TEP production so a relation with the bacterial species present was not established. Since our study was focused on TEP, this plant was not further considered.

### 4.2 Plant A

The first step in the treatment process consisted of pre-chlorination, which increased cTEP and pTEP concentrations with respectively 34 and 41%. However, note that the absolute increase in cTEP concentrations (600 ± 241 µg X<sub>eq</sub>.L<sup>-1</sup>) is much larger than the increase in pTEP concentrations (34 ± 24 µg X<sub>eq</sub>.L<sup>-1</sup>). Ortega-Retuerta et al. (2009) showed that the UVB fraction of sunlight promoted the production of pTEP with 17% per day in the presence of microorganisms, starting from a similar concentration of ± 120 µg.L<sup>-1</sup> X<sub>eq</sub> for pTEP. Although the oxidative stress caused by UVB cannot be compared to the high stress caused by chlorination, it can be anticipated that both will result in similar effects on TEP release. The release after such treatments can likely be explained by induced surface mucus lysis and cell disintegration.

The following step in the treatment train was UF. The efficient pTEP removal by this step was expected since UF have been reported to remove pTEP with efficiencies varying from 27 up to 100% (Kennedy et al. 2009, Villacorte et al. 2009a, b). However, the membranes in the previous studies all had a nominal pore size of 30 nm, while this study comprised UF membranes with a pore size as big as 100 nm. Since TEP are known to be highly flexible, they can pass through membranes with a nominal pore size smaller than their own diameter, especially when a high pressure is applied. For this reason, the possibility that pTEP are passing the membrane cannot be excluded. Yet, it was surprising that cTEP, with diameters varying
from 0.05 to 0.4 µm were retained successfully by this membrane. This would suggest that the majority of this fraction were particles with diameters above 0.1 µm and below 0.4 µm.

A total TEP removal and efficient mineral retention by RO was observed. This total TEP removal has also been observed by Villacorte (2009a) and can be explained by the non-porosity of RO membranes and the osmosis based separation process. The next step in the treatment process is the remineralization by infiltration in the dunes. In this step, this TEP-free and oligotrophic RO permeate is exposed to the open air in the infiltration pond. A limited supply of nutrients allowed microbical and algal regrowth in this pond, giving the possibility for fresh TEP formation. The following dune infiltration remineralized this RO water (Van Houtte and Verbauwhede 2008) but also comprised a removal below detection limit of the newly formed TEP, likely due to a combination of filtration/adsorption effects and bacterial consumption which were newly formed in the pond.

4.3 Plant B

Plant B consists of two parallel treatment lines. In the first line, a decantation step consisting of coagulation and sedimentation resulted in both a decrease of cTEP amount and an increase of pTEP weight, while total TEP concentrations did not change significantly. This can be explained by the coagulation process with Al\(^{3+}\). This cation enabled the small cTEP to coagulate and form the larger pTEP, which have estimated densities between 0.7 and 0.84 g.cm\(^{-3}\) (Azetsu-Scott and Passow 2004). These light particles could not be removed by sedimentation. The following hydroantracite + sand filtration appeared to be a good option to remove these coagulated pTEP but was a too rough method to abate the smaller cTEP. Other systems with coagulation and sand filtration were reported to remove only 20 up to 68% of total TEP (Kennedy et al. 2009, Villacorte et al. 2009a) while single sand filtration only showed a few percent up to 12% efficiency (Bar-Zeev et al. 2009, Berman 2005). Overall, it can be concluded that the combination of coagulation and sand filtration was able to coagulate cTEP and remove two thirds of total TEP. In the parallel flotation line, the incoming pTEP amount stayed minimal and the cTEP concentration decreased by 70%, probably due to good flotation of these light particles (Azetsu-Scott and Passow 2004), which are in addition known to be highly surface-active and adhere easily to air bubbled through a liquid (Mopper et al. 1995), which is basically the same as flotation.
After the confluence of both streams ozonation removed cTEP below detection limit. The overall TEP-removal was good in both plants and few or no TEP could reach the final drinking waters. No TEP-related technical problems were expected in any of the systems at time of sampling. A second sampling during the expected algal bloom period is advised to confirm whether these water treatment systems have the same TEP-removal efficiency in case of elevated TEP-concentrations, which can be expected in this fresh water system.

### 4.4 Correlations with other parameters

Possible relations between TEP and other chemical and biological parameters were examined. The correlation found between pTEP and cTEP could be explained by the fact that both are by definition retainable by physical methods like filtration. Besides, cTEP is a precursor of pTEP so they have a similar chemical structure, resulting in a similar response to chemical treatment methods like chlorination.

A significant association between cTEP and COD\textsubscript{i} (p = 0.0066) and TOC (p = 0.0070) was partly expected since TEP can be seen as a fraction of both COD\textsubscript{i} and TOC. On the other hand, while cTEP is partly retained by physical filtration systems such as ultrafiltration, this is less the case for TOC and COD. For example, infiltration dropped the cTEP concentrations but increased the TOC concentrations. The association between pTEP and COD\textsubscript{i} or TOC was not significant, which might be explained by the lower pTEP concentrations compared to cTEP, making it a much smaller fraction of COD\textsubscript{i} and TOC. In general, TEP concentrations (usually below 1 mg.L\textsuperscript{-1} X\textsubscript{eq}) only accounted for a small fraction of COD\textsubscript{i} concentrations (0-36 mg.L\textsuperscript{-1}) and TOC concentrations (0.6-10.7 mg.L\textsuperscript{-1}).
5. Conclusions

- Influent TEP concentrations varied a lot depending on the raw water source; ground water contained no TEP while secondary effluent from a STP and surface water respectively contained 1572 and 699 \( \mu g \ X_{eq.} L^{-1} \) of TEP. A follow-up study comparing these values during an algal bloom period, when more TEP is expected, is advised.
- cTEP accounted in both systems for 94-98% of total TEP, while this extensive fraction is usually neglected. In later studies, this fraction should still be included in the measurements.
- A wide range of different water treatment methods was scored on TEP removal efficiency. The combination of coagulation and sand filtration proved effective for a high TEP removal (67% of total TEP) while the combination of ultrafiltration and reverse osmosis resulted in a total TEP removal.
- TEP was below detection limit in the final drinking waters at time of sampling.
- Statistical analyses indicated that especially the cTEP removal was significantly associated with the removal COD\(_t\) and TOC. In general, treatment methods able to lower these fractions will likely also be able to lower TEP concentrations.

Acknowledgements

This work was supported by the project grant no. G.0808.10N of the FWO Flanders, Tom Hennebel is supported by Ghent University Multidisciplinary Research Partnership (MRP) – Biotechnology for a sustainable economy (01 MRA 510W). We thank Koen Huysman, Emmanuel Van Houtte and Jan Cromphout for the support and access to the installations, Joachim Neri, Bart Heyman and Joris Meys for the useful suggestions and Simon De Corte, Karen De Roy and Siegfried E. Vlaeminck for critically reading the manuscript.
Chapter 3

Bacterial invasion potential in water is determined by nutrient availability and the indigenous community

Chapter redrafted after:


Abstract

In drinking water and the distribution systems, bacterial growth and biofilm formation has to be controlled both for limiting taste or odour development and preventing clogging or bio-corrosion problems. After a contamination with undesired bacteria, factors like nutrient availability and temperature will influence the survival of these invaders. Understanding the conditions enabling invaders to proliferate is essential for a holistic approach towards microbial risk assessment in drinking water. Pseudomonas putida was used as a model invader since this easy growing bacterium can use a wide range of substrates. Invasion experiments in oligo- to eutrophic waters whereby P. putida was spiked in similar concentrations as the indigenous community showed the requirement of both a carbon and phosphate source for survival in drinking water. Addition of C, N and P enabled P. putida to grow in drinking water from 5.80 x 10⁴ cells.mL⁻¹ to 1.84 x 10⁸ cells.mL⁻¹ and survive for at least 12 days. However, in surface water with similar nutrient concentrations, P. putida only initially proliferated but was below detection limit (2 x 10³ cells.mL⁻¹) within two days, indicating the concomitant importance of the present indigenous microbial community of the specific water sample. Either extensive carbon or phosphate limitation can be used in water treatment design in order to obtain a drinking water which is not susceptible for unwanted bacterial growth.
1. Introduction

According to the European Environment Agency, 2.4% of the 6493 sampled European inland bathing waters had too high concentrations of faecal coliforms according to bathing water standards, possibly affecting human health (EEA 2012). On the other hand, in drinking water and the distribution systems, bacterial growth and biofilm formation has to be controlled to avoid the concomitant development of undesired tastes, odours, colours and prevent problems like clogging or bio-corrosion of pipes.

After a contamination of drinking or surface water with non-native bacteria - the invaders - factors like nutrient availability, temperature, predators and sedimentation influence the survival and die-off patterns of these invaders (Hellweger et al. 2009). Fast growing bacteria like E. coli or Pseudomonas can be competitive with the indigenous community if the proper conditions are present, while slow growing bacteria need other strategies. Examples are the ability to occupy available ecological niches by accessing carbon sources which are not degradable by the indigenous community (Hibbing et al. 2010), or being able to grow at lower minimal substrate concentrations (Fuchsli et al. 2012).

Numerous studies investigated the survival of an E. coli invasion in drinking and surface water and discussed either a simple first-order or a biphasic decay (Bogosian et al. 1996, Chick 1908, Easton et al. 2005, Finkel 2006, Hellweger et al. 2009, Lewis 2007, Medema et al. 1997, Rhodes and Kator 1988, Xu et al. 1982, Zambrano et al. 1993). In general, the survival of pathogens in these environments lasts longer at 5°C compared to higher temperatures like 25°C (Cools et al. 2003, Maalej et al. 2004, Tatchou-Nyamsi-Koenig et al. 2007). The availability of nutrients can either slow down the decay rate or have no influence at all (Tatchou-Nyamsi-Koenig et al. 2007, Thomas et al. 1999, Warburton et al. 1998). In most cases, organic carbon is considered to be the limiting nutrient for microbial growth in drinking water (van der Kooij et al. 1982, Vrouwenvelder et al. 2010). However, specific for E. Coli O157, Vibrio cholera and Pseudomonas putida, Vital et al. showed that growth did not correlate with dissolved organic carbon and only weakly with the concentration of Assimilable Organic Carbon (AOC) (Vital et al. 2010). Vrouwenvelder et al. (2010) and references therein reported phosphate concentrations in surface and drinking water as limitation for general biological growth. To the contrary, phosphate addition has been seen to decrease the culturability of
Mycobacterium avium due to increased competition (Torvinen et al. 2007). Finally, the presence of an indigenous microbial community can have an antagonistic effect on the survival and growth of an invader, which means they limit the survival of the invader. This can be done by the release of inhibitory substances accumulating in the water (Chandran and Hatha 2005, Ducluzeau et al. 1984, Moreira et al. 1994) or by a niche-overlap for carbon source consumption between the invader and the present community (De Roy et al. 2013). On the other hand, the present community can also have a protagonistic effect and stimulate the growth of the invading bacteria, like was seen for the enhanced survival of E. coli O157:H7 or Klebsiella pneumoniae in mineral water (Kerr et al. 1999, Moreira et al. 1994).

Control strategies to avoid pathogenic contaminations consist of preventing the invaders from reaching the receiving waters (US EPA 2002). There are two main pathways for controlling microbial growth in drinking water; either a disinfection process in which a disinfectant residual is preferably used, or a biologically stable water is created by maintaining an indigenous bacterial population combined with extensive nutrient limitation (Hammes et al. 2010a). The first option is most common, using free chlorine, chloramine or UV radiation. Chlorination is by far the most widespread method but the discovery of possible carcinogenic disinfection by-products has questioned this approach (Brown et al. 2011, Freese and Nozaic 2004, Sadiq and Rodriguez 2004, WHO 2005). Chloramine is a possible alternative, but can support bacterial growth when present in low concentrations, while UV or ozone do not provide disinfection residuals (Chang et al. 2010, Freese and Nozaic 2004, LeChevallier et al. 1990, Skadsen 1993). Consequently, the alternative of distributing a biostable water without disinfection gained importance (van der Kooij et al. 1999). This water treatment aims at an extensive nutrient removal, resulting in the inability for bacteria to proliferate in the produced drinking water. This is mainly monitored by focussing on organic carbon as the main nutrient, measured by the AOC protocol (Hammes and Egli 2005, van der Kooij et al. 1982). In addition, the biofilm formation rate of a specific sample can be tested in a flow through test (van der Kooij 2000, van der Kooij and Veenendaal 1993). However, these tests are only a limited indicator for the ability of the present bacteria to grow in the water or in a biofilm, while for drinking water safety, the ability of pathogen growth after a water contamination is of major importance. Vital et al. (2010) developed the pathogen growth potential bioassay, which combines the conventional AOC assay with quantification of bacterial growth of the model
pathogens *Escherichia coli* O157, *Vibrio cholerae* O1, and *Pseudomonas aeruginosa*. This bioassay indicates if a pathogen can grow in a specific water sample, but does not elaborate on why the bacteria can grow.

The growth of pathogens in drinking or surface water used for recreational purposes has always been a big concern and the parameters influencing growth and survival are poorly understood. Numerous studies have dealt with this subject in drinking water, although few focussed on different nutrients and the indigenous community and pathogen growth and survival remained difficult to predict (Bogosian *et al.* 1996, Easton *et al.* 2005, Finkel 2006, Hellweger *et al.* 2009, Lewis 2007, Medema *et al.* 1997, Rhodes and Kator 1988, Xu *et al.* 1982, Zambrano *et al.* 1993) In this paper, *Pseudomonas putida* - a model invader - was spiked in different water samples in order to examine the effect of (i) an oligo- to eutrophic situation, (ii) different concentrations of C, N and P, and (iii) the indigenous microbial community on the growth and survival of *P. putida*.

### 2. Materials and methods

#### 2.1 Sample collection

Three types of water were used during the experiments. Evian (drinking water or DW) was used as a model for a non-chlorinated drinking water and oligotrophic water. Water from the channel ‘Coupure’ in Ghent, Belgium was used as a model for surface water (SW) and oligo- to mesotrophic water. It was collected at several days prior to analysis. Effluent (ED) from an anaerobic digester treating highly loaded brewery wastewater was used as a model for wastewater treatment plant effluent and eutrophic water with a high bacterial load. All samples were stored at 4°C prior to use. The chemical and biological properties of these waters are shown in Table 3-1.

#### 2.2 Chemical analysis

Nitrate, nitrite, sulphate, phosphate and chloride ions were determined with a 761 compact ion chromatograph equipped with a conductivity detector (Metrohm). Total chemical oxygen demand (COD$_t$) measurement was performed according to manufacturer’s guidelines with the
‘HI 93754A-25 LR’ kit (Hannah Instruments). Ammonium was determined colorimetrically with Nessler reagent according to standard methods (Greenberg et al. 1993).

**Table 3-1: Chemical and biological properties of the samples.** – unknown value.

<table>
<thead>
<tr>
<th></th>
<th>Drinking water</th>
<th>Surface water</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td>6.88</td>
<td>7.72</td>
<td>7.42</td>
</tr>
<tr>
<td><strong>Intact cells.mL⁻¹</strong></td>
<td>4.4 x 10⁴</td>
<td>1.4 x 10⁷</td>
<td>4.6 x 10⁸</td>
</tr>
<tr>
<td><strong>Damaged cells.mL⁻¹</strong></td>
<td>4.3 x 10⁴</td>
<td>3.7 x 10⁶</td>
<td>1.9 x 10⁸</td>
</tr>
<tr>
<td><strong>AOC (µg C.L⁻¹)</strong></td>
<td>57 ± 33</td>
<td>616 ± 77</td>
<td>3,687 ± 637</td>
</tr>
<tr>
<td><strong>Conductivity (µS.cm⁻¹)</strong></td>
<td>417</td>
<td>666</td>
<td>2,460</td>
</tr>
<tr>
<td><strong>COD (mg.L⁻¹)</strong></td>
<td>&lt; 15</td>
<td>25</td>
<td>348</td>
</tr>
<tr>
<td><strong>NH₄⁺ (mg.L⁻¹)</strong></td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cl⁻ (mg.L⁻¹)</strong></td>
<td>8.95</td>
<td>78.2</td>
<td>178</td>
</tr>
<tr>
<td><strong>NO₂⁻ (mg.L⁻¹)</strong></td>
<td>&lt; 0.05</td>
<td>0.713</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>NO₃⁻ (mg.L⁻¹)</strong></td>
<td>4.24</td>
<td>21.0</td>
<td>1.70</td>
</tr>
<tr>
<td><strong>PO₄³⁻ (mg.L⁻¹)</strong></td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>SO₄²⁻ (mg.L⁻¹)</strong></td>
<td>13.5</td>
<td>9.49</td>
<td>3.38</td>
</tr>
</tbody>
</table>

**2.3 Cultivation and harvesting of Pseudomonas putida**

*Pseudomonas putida* SM1699 (Sternberg et al. 1999), with the rrnBP1-gfpmut3b cassette randomly inserted in the chromosome of *Pseudomonas putida* JB156, was grown overnight at 27°C in 50 times diluted Tryptic Soy Broth (BD) with 50 mg.L⁻¹ kanamycin. It was supposed that the gfp-tagged *P. putida* the same growth rate as the wild type strain, as shown before for both this strain and different *E. coli* strains (Allison and Sattenstall 2007, Webb et al. 2014). The transfer of *P. putida*, grown in undiluted Tryptic Soy Broth, to drinking or surface water would result in an osmotic shock and immediate die-off of the bacteria. Diluted medium was used with a conductivity of 307 µS.cm⁻¹, which was in the range of drinking or surface water. The bacteria were harvested by 4 min centrifugation at 3000 g and washed twice in 0.22 µm filtered drinking water to remove traces of medium and kanamycin.
2.4 Flow cytometry

For the counting of intact and damaged bacterial cells, two fluorescent dyes, SYBR® Green I (SG) and propidium iodide (PI) were used as a live/dead staining (Wang et al. 2010a). Samples were diluted in 0.22 µm filtered drinking water prior to staining, as required. The staining solution was prepared as follows: PI (20 mM in dimethyl sulfoxide (DMSO), Invitrogen) was diluted 50 times and SG (commercial solution with unknown concentration in DMSO, Invitrogen) was diluted 100 times in 0.22 µm-filtered DMSO. In case of total cell count analysis, only SG was used in the same concentration without PI. All water samples were stained with 10 µL mL⁻¹ staining solution and 10 µL mL⁻¹ EDTA (pH 8, 800 mM) for outer membrane permeabilization. 10 µL mL⁻¹ CytoCount counting beads (Dako) were added for concentration determination. The stained samples were incubated for 5 min in the dark at 37°C prior to flow cytometric analysis. For the analysis of gfp-labeled P. putida cells, no stain, nor EDTA were added to the samples, only the CytoCount counting beads. Flow cytometry finally was performed using a Cyan™ ADP LX flow cytometer as described by Boon et al. (2006). Cells of all sizes were measured and the threshold was fixed on green fluorescence. Under optimal conditions, the detection limit could go as low as 2 x 10³ cells mL⁻¹, both for gfp-labeled cells or for live/dead staining. However, present autofluorescent (an)organic particles could induce a background on the measurements, which could elevate the detection limit in case of measuring gfp-cells, which were low in fluorescence compared to stained cells.

2.5 AOC measurement

AOC measurement, as developed originally by Van der Kooij et al. (1982), was performed using flow cytometry according to the adaptations of Hammes et al. (2006, 2005). In short, 10 mL samples were filter sterilized over sterile 0.22 µm filters (Millex GV, Millipore), pasteurized for 30 min at 60°C and inoculated with 1% (0.1 mL) of the non-sterilized sample. The total cell count was determined with flow cytometry, according to 2.4. The samples were then incubated in the dark for 3 days at 28°C. Finally, the total cell count was measured again. The AOC concentration was calculated using a conversion factor of 10⁷ bacteria.µg⁻¹ C and the following equation:

\[
AOC \ [\mu g \ C \ L^{-1}] = \frac{(total \ bacteria \ (day \ 3) - total \ bacteria \ (day \ 0)).L^{-1}}{10^7 \ bacteria.\mu g^{-1} \ C}
\]
Carbon-free, ultra pure glassware was used and prepared according to Greenberg et al. (1993) and Hammes et al. (Hammes and Egli 2005).

2.6 Pseudomonas putida survival tests

For the survival tests with *Pseudomonas putida*, 10 mL samples were prepared in 40 mL carbon-free, ultrapure glass vials. *P. putida* was spiked from a concentrated harvested solution, of which the concentration was determined by flow cytometry. The desired final concentration was half of the concentration of the initial microbial community of the specific sample. Acetate (as sodium acetate solution) was added to the samples for C-source, ammonium chloride solution was added for the N-source, and disodium hydrogen phosphate solution for the P-source.

The bacterial communities of drinking water and surface water were switched when testing the influence of the bacterial community: the drinking water bacteria were suspended in sterile surface water and surface water bacteria in sterile drinking water. This was done by the filtration of aliquots of both samples over a 0.22 µm filter membrane on a filter holder (Nalgene). Subsequently, the retained bacteria were resuspended by vortexing the filter membrane for 10s in 5 mL of 0.22 µm filtered water. The concentrations of these harvested bacteria were measured by flow cytometry and these bacteria suspensions were finally used to spike in the sterilized water samples until the desired concentrations. M9-medium was used with different concentrations of Na-acetate to evaluate the yield of *P. putida*-growth on acetate. This medium contained per litre: 8.5 g Na$_2$HPO$_4$.7H$_2$O, 3 g KH$_2$PO$_4$, 0.5g NaCl, 1 g NH$_4$Cl, 0.24 g MgSO$_4$, 0.11 g CaCl$_2$. All tests were performed in triplicate and all samples were consequently incubated in the dark on a shaker at 27°C. After the experiments were performed in 3.4 and 3.5, selective plating using Tryptic Soy Agar (BD) with 50 mg.L$^{-1}$ of kanamycin was performed. Visual inspection of these plates under blue light confirmed the presence or absence of the *gfp*-labelled *P. putida* in the samples. A short overview of all *P. putida* survival tests, including the findings, is given in Table 3-2.
2.7 Bacterial community analysis

Prior to PCR-DGGE (Denaturing Gradient Gel Electrophoresis), the leftovers of the triplicate samples (3 x 5-7 mL, depending on the used dilution for flow cytometric sampling) were merged and were concentrated by filtering the water samples over a 0.2 µm filter (Pall Life Sciences). DNA extraction from the filter was carried out using the UltraClean® Water DNA Isolation Kit (Mo Bio Laboratories, Inc) and subsequently concentrated 100 times. PCR was performed with 30 cycles as described previously (Boon et al. 2002) with primers P388f and P518r, targeting all bacterial 16S rRNA genes (Ovreas et al. 1997). According to Muyzer et al. (1993), DGGE analyses were performed on an Ingeny PhorU apparatus (Ingeny International). Further analysis was carried out using BioNumerics software (Applied Maths) and richness and evenness calculations were done based on Marzorati et al. (2008).
3. Results

3.1 Yield of P. putida growth on acetate

Acetate was used as carbon source for the growth of P. putida. The growth of P. putida growing with acetate as sole carbon source was verified and yielded \((2.80 \pm 0.42) \times 10^6\) cells.µg C\(^{-1}\) (data not shown).

3.2 Influence of different types of water on the growth of P. putida

Three water samples with a different biological and chemical composition were selected: bottled drinking water, surface water and effluent from an anaerobic digester of a brewery. The bottled drinking water is oligotrophic water, low in C, N and P but containing a range of micro-nutrients. In this water, a low AOC-value of around 60 µg C.L\(^{-1}\) has been measured. Concentrations of NH\(_4^+\), NO\(_2^-\) and PO\(_4^{3-}\) were below detection limit (respectively 0.1, 0.05 and 0.05 mg.L\(^{-1}\)) and nitrate was as low as 4.24 mg.L\(^{-1}\). The present number of bacteria was in the order of \(10^5\) cells.mL\(^{-1}\). The surface water and effluent water represented waters with higher nutrient and bacterial contents. The AOC values were respectively 10 and 60 times higher compared to the bottled drinking water. N was present in 21 mg.L\(^{-1}\) nitrate in the surface water but phosphate was still below detection limit. The effluent was low in nitrate but had 11,4 mg.L\(^{-1}\) phosphate present. The bacterial cell count for surface water and effluent were in the ranges between \(10^7\) and \(5 \times 10^8\) cells.mL\(^{-1}\).

In all of these waters, a spike with Pseudomonas putida in similar concentrations as the initially present bacteria was done. The goal was to study the growth and survival of a competitive bacterial contamination in either drinking or surface water which can be used for recreational purposes. The digester effluent was a eutrophic counterexample, helping to evaluate the most decisive factors for the growth of a contaminant (Figure 3-1). A summary of the results of all P. putida survival tests is displayed in Table 3-2. After spiking \((4.37 \pm 0.73) \times 10^4\) P. putida cells.mL\(^{-1}\) in drinking water, the number of P. putida cells tripled within one day and they survived for two days, but were not quantifiable afterwards. In surface water, the spike of \((5.26 \pm 1.78) \times 10^6\) P. putida cells was already decreased with 65% after one day. After two and more days, few or no P. putida could be detected. In effluent water, the bacterium could proliferate for two days and reach seven times the initial concentration of \((1.99 \pm 1.13) \times 10^8\) cells.mL\(^{-1}\).
cells, followed by some die-off and a steady-state concentration after 5 days, comparable to the initial level of spike. It could be concluded that a *P. putida* spike in these waters could only survive and proliferate in effluent water for more than 2 days.

![Diagram of P. putida growth in raw water](image)

**Figure 3-1: P. putida growth in raw water.** The growth and survival of *P. putida*, spiked in drinking water (DW), surface water (SW) and effluent of an anaerobic digester (ED) (n=3). The initial bacterial concentration of the indigenous community (IC) is indicated. After two days, *P. putida* was below detection limit in surface water, while after more than two and five days respectively, it was not quantifiable against the background in drinking water or effluent.

### 3.3 Influence of carbon concentrations on the growth of *P. putida*

The importance of carbon concentrations for the growth or survival of *P. putida* was tested by repeating the previous survival tests with additional acetate added to the samples. For drinking water, 500, 3600 and 37000 µg C.L⁻¹ was added to the samples so they reached the AOC-concentrations of surface water, effluent, or a 10-fold of this last concentration respectively (Table 3-1). For surface water, the effluent concentration and the 10-fold of this concentration were selected and for effluent, the 10-fold of the effluent concentration was selected. In drinking water higher carbon additions initially promoted the growth of *P. putida* and after one day, in the samples with 37 mg of carbon, *P. putida* reached $(5.87 \pm 2.17) \times 10^5$ cells.mL⁻¹, which was 5 times the concentration of the samples without added carbon (Figure 3-2A).
Figure 3-2: *P. putida growth in water + acetate.* The growth and survival of *P. putida,* spiked in (A) drinking water, (B) surface water and (C) effluent of an anaerobic digester (n=3). Carbon was added to samples as indicated in the legend. After two days, *P. putida* was below detection limit in surface water, while after more than two and five days respectively, it was not quantifiable against the background in drinking water or effluent.

However, after two days this maximal concentration lowered and no clear differences could be detected anymore between the samples. In surface water, a similar trend could be detected after one day (Figure 3-2B). The samples with the highest carbon concentration promoted the initial growth until a *P. putida* concentration of \((2.45 \pm 1.02) \times 10^8\) cells.mL\(^{-1}\), which was 130 times the concentration in the initial sample, but after two days, *P. putida* could not be distinguished in any sample. In effluent, carbon addition only had a limited positive effect on the growth of *P. putida* (Figure 3-2C), and no clear influences could be observed between the effluent without and with carbon addition. Plating confirmed that the spiked *P.*
*P. putida* could survive for at least 8 days in both sample types although they were not quantifiable anymore by flow cytometry. Overall, carbon addition could possibly promote the initial growth of *P. putida* to higher concentrations but could not elongate the limited survival time in surface water.

### 3.4 Influence of C, N and P on the growth of *P. putida*

After testing the importance of available carbon concentrations on *P. putida* survival, this section will verify whether nitrogen and phosphate could be limiting survival. In a new set of experiments, *P. putida* was spiked in drinking water and surface water which was enriched with each of these nutrients in the following concentrations: 3.7 mg.L\(^{-1}\) C, 3.7 mg.L\(^{-1}\) N and 11.4 mg.L\(^{-1}\) PO\(_4^{3-}\), using Na-acetate, NH\(_4\)Cl and Na\(_2\)HPO\(_4\) (Figure 3-3). In drinking water, *P. putida* concentrations increased over 3 orders of magnitude until a maximum concentration of (1.84 ± 1.49) \(\times\) 10\(^8\) cells.mL\(^{-1}\) after five days. This was followed by a die-off the next days (one order of magnitude) after which concentrations kept stable. In surface water, *P. putida* concentrations initially increased twentyfold over two days until a maximum concentration of (1.13 ± 0.58) \(\times\) 10\(^8\) cells.mL\(^{-1}\), but dropped below detection limit after five days. However, selective plating indicated *P. putida* presence in these samples after 7 days.

In a next experiment, the individual or combined importance of both N and P was tested. Therefore, nitrogen and/or phosphorus was each time dosed in drinking water in the same concentrations as mentioned before, however without carbon dosed. When only nitrogen was added to drinking water, a die-off of *P. putida* was seen, without any growth. When phosphorus was added, there was a limited growth for the first two days (1 order of magnitude), followed by a 1.9 log die-off over the next two days. When nitrogen and phosphorus were added together, a similar initial growth was seen the first day, followed by a limited die-off (0.35 log). At day 7, *P. putida* could not be detected anymore by flow cytometry in any of the samples, which was confirmed at day 8 by plating. In summary, adding only nitrogen did not influence the survival of *P. putida* in drinking water, but adding phosphorus prolonged the survival for a limited period of time.
Figure 3-3: *P. putida* concentrations in different waters + C, N and P. In DW, the concentration of *P. putida* stabilized and the measurement was stopped after 14 days. In SW, *P. putida* was below detection limit after 5 days and in ED, the bacterium could not be quantified anymore against background.

Figure 3-4: *P. putida* growth in water + N and/or P. *P. putida* does not grow in drinking water with added N, but did in drinking water with P or N and P added (n=4). Later than 4 days, *Pseudomonas putida* was not quantifiable anymore against background.
3.5 The indigenous community has influence on the growth of *P. putida*

The previous test showed a large difference in the survival of *P. putida* in drinking versus surface water, even after adding sufficient C, N and P in both cases. In this next step, the influence of the indigenous community of both waters was studied. Both drinking water and surface water were filtered to harvest their respective indigenous microbial community. The drinking water microbial community was suspended in the surface water and vice versa. In drinking water, the surface water community was added in concentrations of either $(1.41 \pm 0.13) \times 10^5$ bacterial cells.mL$^{-1}$ (‘DW$_{SWC \text{ low}}$’) or $(9.63 \pm 0.59) \times 10^5$ bacterial cells.mL$^{-1}$ (‘DW$_{SWC \text{ high}}$’) resembling the concentrations of bacteria initially present in the drinking water and surface water samples at that moment. In surface water, $(5.91 \pm 0.82) \times 10^4$ cells.mL$^{-1}$ were spiked from the drinking water community (‘SW$_{DWC}$’), resembled the initial drinking water concentration. C, N and P were added in all samples, until the same concentrations as tested in §3.4. After spiking *P. putida* in SW$_{DWC}$, proliferation of this strain with more than 3 orders of magnitude of growth could be seen within 2-4 days (Figure 3-5). A slightly lower steady-state concentration of about $2 \times 10^7$ cells.mL$^{-1}$ for *P. putida* was maintained for at least 14 days. In DW$_{SWC \text{ low}}$, *P. putida* increased almost 2 orders of magnitude within 4 days. Over the following two weeks, this high concentration only slightly decreased to $8 \times 10^6$ cells.mL$^{-1}$. In DW$_{SWC \text{ high}}$ however, the initial spike concentration of $(3.13 \pm 0.78) \times 10^6$ cells of *P. putida* per mL was maintained with only limited concentration shifts. A bacterial community analysis by DGGE, performed after the end of the experiment showed a stronger presence of *P. putida* in the SW$_{DWC}$ and DW$_{SWC \text{ low}}$, as suggested in Figure 3-5. The richness (number of bands present) of 13 in raw surface water was was higher than drinking water at 11 bands (Figure 3-6). Furthermore, analysis of these results according to Marzorati *et al.* (2008) showed a Gini coefficient of 0.38 for raw drinking water and 0.29 for surface water, indicating that a higher degree of evenness characterized the surface water. In summary, the lower concentration of surface water bacteria in drinking water, or drinking water bacteria in surface water, did not prevent *P. putida* from growing several orders of magnitude and maintaining a high steady-state concentration. The higher surface water bacteria concentration in drinking water prevented *P. putida* from growing, although the initially spiked *P. putida* survived.
**Figure 3-5:** *P. putida* concentrations after switching indigenous communities. *P. putida* was spiked in DW with SW bacteria in low concentrations (DW$_{SWC \text{ low}}$), DW with SW bacteria in high concentrations (DW$_{SWC \text{ high}}$), surface water with DW bacteria (SW$_{DWC}$) (n=3) and nutrients were added in the same concentrations as in § 3.3. After more than 13 days, *P. putida* was below detection limit in DW$_{SWC \text{ high}}$ while DW$_{SWC \text{ low}}$ and SW$_{DWC}$ showed no new trends and the measurement was stopped.

**Figure 3-6:** Bacterial community analysis by DGGE after the community switch experiment. The strongest presence of *P. putida* in the DW$_{SWC \text{ low}}$ and SW$_{DWC}$ is confirmed.
Table 3-2: Overview of the different survival test conditions and outcomes. *P. putida* was in each of the experiments spiked at a target concentration of 50% of the indigenous community. Sodium acetate, ammonium chloride and disodium hydrogen phosphate were used for C, N and P additions, which are expressed per litre. DW: drinking water. SW: surface water. ED: effluent of anaerobic digester. LOD: limit of detection.

**Experiment 3.2: *P. putida* in different raw water types**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW + IC</td>
<td>: &gt; 2 d survival</td>
</tr>
<tr>
<td>SW + IC</td>
<td>: below LOD after 2d</td>
</tr>
<tr>
<td>ED + IC</td>
<td>: limited growth, &gt; 5d survival</td>
</tr>
</tbody>
</table>

**Conclusion:** ED supports the growth/survival, DW supports survival, SW does not support.

**Experiment 3.3: Influence of carbon concentrations on *P. putida***

<table>
<thead>
<tr>
<th>Condition</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW + IC + 500/3700/37,000 µg C</td>
<td>: Limited growth, &gt; 2d survival</td>
</tr>
<tr>
<td>SW + IC + 3,000/37,000 µg C</td>
<td>: 1 d growth, below LOD after 2d</td>
</tr>
<tr>
<td>EW + IC + 33,000 µg C</td>
<td>: Growth, &gt; 5d survival</td>
</tr>
</tbody>
</table>

**Conclusion:** Increasing carbon concentrations help to support growth to some extent, SW however prevents growth.

**Experiment 3.4: Influence of carbon, nitrogen and phosphorus concentrations on *P. putida***

<table>
<thead>
<tr>
<th>Condition</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW + IC + 3.66 mg NH₄⁺</td>
<td>: No growth, survival &gt; 4d</td>
</tr>
<tr>
<td>DW + IC + 11.4 mg PO₄³⁻</td>
<td>: Growth, survival &gt; 4d</td>
</tr>
<tr>
<td>DW + IC + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Growth, survival &gt; 4d</td>
</tr>
<tr>
<td>DW + IC + 3,700 µg C + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Strong growth, high steady-state</td>
</tr>
<tr>
<td>SW + IC + 3,600 µg C + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Growth, below LOD after 5d</td>
</tr>
</tbody>
</table>

**Conclusion:** *P* is the first limiting nutrient for growth in DW, followed by C. Adding C, N and P allows a high steady state concentration in DW, but *P. putida* still gets below LOD after 5d in SW.

**Experiment 3.5: Influence of indigenous community on *P. putida***

The SW community was added to the DW community in low concentrations (*: 1.41 x 10⁵ cells.mL⁻¹) or high concentrations (**: 9.63 x 10⁵ cells.mL⁻¹). The DW community was added to the SW at 5.91 x 10⁴ cells.mL⁻¹.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW + IC SW* + 3,700 µg C + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Strong growth, high steady-state</td>
</tr>
<tr>
<td>DW + IC SW**+3,700 µg C + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Initial strong growth, below LOD after &gt; 14d</td>
</tr>
<tr>
<td>SW + IC DW + 3,600 µg C + 3.66 mg NH₄⁺ + 11.4 mg PO₄³⁻</td>
<td>: Strong growth, high steady-state</td>
</tr>
</tbody>
</table>

**Conclusion:** The SW community is more resistant to invasion than the DW community.
4. Discussion

In this study, *P. putida* was spiked in several waters, ranging from oligotrophic to eutrophic. Bottled drinking water and surface water represented the oligotrophic situation where bacterial and pathogen regrowth must be avoided because of the drinking and recreational purposes of these waters. Bottled drinking water was preferred instead of tap water for several reasons; the microbial composition in tap water is unstable due to stagnation (Lautenschlager *et al.* 2010), the available tap water in Belgium is chlorinated and the composition is similar to ground water based tap waters. The third water type, digester effluent, was selected as a eutrophic counterexample with totally different bacterial and nutrient concentrations, mainly helping to point out the most decisive factors for contaminant growth.

This effluent offered in each test an environment suitable for growth and long-term survival of *P. putida*. Addition of acetate only induced a minor increase in the maximum reached cell concentrations of *P. putida*, the survival lasted each time for at least five days. Later on, *P. putida* became unquantifiable against the background bacterial signal (Heim *et al.* 1994). The easy growth of *P. putida* in digester effluent was however expected since it was rich in acetate and diverse nutrients.

Yet, in raw drinking or especially surface water, *P. putida* could hardly grow and soon decayed. These raw waters appeared to be much depleted in nutrients for the growth of *P. putida*, as only survival at the same concentration level was seen. This can be seen as an example of the ‘opportunist’ versus ‘gleaner’ relationship (Grover 1990, Veldkamp and Jannasch 1972, Vital *et al.* 2012b). For example, Vital (2012) showed for example that *E. coli*, the ‘opportunist’ grew much slower at low carbon concentrations compared to an indigenous drinking water community, the ‘gleaner,’ which was specialised for growth at low nutrient concentrations. Under higher carbon concentrations, the opportunist could easily overrule the gleaner (Vital *et al.* 2012b). It was indeed seen that the indigenous community in our examples was overruling the opportunist, *P. putida*. When we dosed an easily degradable carbon source, acetate, one would expect this to boost the growth of the opportunist, yet this only elevated the *P. putida* concentrations after one day, but could not prevent the following fast decay of the bacteria. Carbon source availability, an important factor according to many authors...
Chapter 3

(LeChevallier et al. 1987, van der Kooij 1992), is not fully explaining biological stability and regrowth, while it can still be a descriptive parameter in the assessment, as some suggest (Hammes et al. 2010a, Miettinen et al. 1997).

The addition of extra phosphate and nitrogen next to acetate permitted *P. putida* to grow above $10^8$ cells.mL$^{-1}$ in drinking water and survive for an extended period of time. In surface water on the other hand, the dosing of these nutrients only enhanced the growth and survival of *P. putida* to a limited extent. Additional testing with only phosphate and/or nitrogen addition indicated the importance of phosphate for growth in drinking water. After two days, the *P. putida* concentrations were very similar when either only phosphate or carbon was dosed. It was concluded that both phosphate and carbon were limited in this system. Next to the more classic approach of AOC or general carbon limitation, ortho-phosphate limitation should be considered more often as pathway to achieve regrowth limitation in drinking water systems, as suggested by some (Kasahara et al. 2004, Miettinen et al. 1997). Vrouwenvelder et al. (2010) for example mention that a phosphate concentration of 0.3 µg P.L$^{-1}$ could limit biofouling on RO membranes, even at high carbon concentrations. Others mention 1-3 µg P L$^{-1}$ as growth-limiting concentrations in drinking water distribution networks, making 1 µg.L$^{-1}$ PO$_4$-P a possible guideline value (Miettinen et al. 1997, Sathasivan and Ohgaki 1999). In comparison, a survey in different Flemish drinking water plants showed concentrations ranging from 0.3 to 15.2 µg.L$^{-1}$ PO$_4$-P, measured as microbially available phosphorus (Polanska et al. 2005b). Each of these concentrations are well below the detection limit of 50 µg.L$^{-1}$ PO$_4$-P. We consequently aim at ortho-phosphate, instead of total phosphorus, since it is most readily available for biological utilization (Maher and Woo 1998). Other soluble phosphorus forms like inorganic polyphosphates and dissolved organic phosphorus are not directly available for microbial growth (Holtan et al. 1988, Maher and Woo 1998).

The limited growth of *P. putida* and fast die-off was consistently seen in surface water in previous tests, independently of the dosage of C, N and P. However, regarding the macro-nutrients carbon, nitrogen and phosphate, the composition of this water was supposed to be the same like the drinking water with C, N, and P dosed. Yet in surface water, no long-term survival was possible while in drinking water, and major growth and long-term survival was observed. This difference could be explained by the different indigenous community of both waters, either supporting or suppressing growth. To examine this
Bacterial invasion in drinking water

hypothesis, the bacterial communities of drinking and surface water were switched by means of filtration and resuspension. C, N and P were added in the same concentrations as in previous tests. After spiking *P. putida*, these could proliferate and survive in the surface water with a drinking water community for a long time, while this was not possible without a switch of the microbial communities. On the other hand, *P. putida* could also survive in drinking water with surface water microbiota, but not proliferate when a high initial concentration of surface water community was present. Previous authors already reported the antagonistic effect of indigenous bacteria on the survival of *E. coli* and *Pseudomonas aeruginosa* (Ducluzeau *et al.* 1984, Moreira *et al.* 1994). Ducluzeau *et al.* (Ducluzeau *et al.* 1984) hypothesized that this effect could be due to inhibitory substances, released into the bulk water by the indigenous bacteria. Yet, this hypothesis does not seem to be applicable since the indigenous surface water bacteria had been living in the surface water before and possible released inhibitory substances would not be removed by filtration. Alternatively, bacterial or protozoan predators could be grazing in the surface water and hereby induce the antagonistic effect. Another option is that the drinking water community was having a protagonistic effect on the growth of *P. putida*, while the effect of the surface water community was limited, as suggested in other studies (Kerr *et al.* 1999, Moreira *et al.* 1994). However, this could still not explain why *P. putida* did not survive the surface water samples with indigenous community and C, N and P added. In search for an alternative explanation, a microbial community analysis with DGGE was performed and species richness was measured. The diversity-invasibility hypothesis states that a higher species richness results in a higher invasion resistance (Kennedy *et al.* 2002), as can be explained by a greater niche-overlap for available substrates. The surface water indeed had a slightly higher richness but richness estimations based on DGGE are only an indication and have limited value in this case of minimal difference. In addition, other authors even state the contrary; more diversity would lead to more invasion (Jiang and Morin 2004). Alternatively, at fixed richness a higher initial community evenness has been shown to result in a more diverse carbon source utilisation and a higher invasion resistance (De Roy *et al.* 2013). This is also in accordance with our results, where the invasion-resistant surface water was shown to have a higher evenness compared to drinking water. Since PCR-DGGE is a semi-quantitative method (Boon *et al.* 2002), these results should be handled with care and it should be understand that they are only tested in this specific situation.
In this study, a worst-case situation was chosen as starting point. Several types of water were spiked with high concentrations of *Pseudomonas putida* and incubated at 27°C. This species is ubiquitous, can use a vast range of substrates, grows fast at an optimum of 25-30°C and tends to overgrow other bacteria (Monteiro *et al.* 2000, Timmis 2002). It is closely related to *Pseudomonas aeruginosa*, a major cause of hospital-acquired infections and skin infections for swimmers, but lacks some of the virulence genes of *P. aeruginosa* (ECDC 2011, Nelson *et al.* 2002). Therefore, *P. putida* was selected as a safe model for a competitive pathogenic bacterium. First, it was verified what the yield was of *P. putida* growing on acetate, since acetate was used as easily degradable carbon source in the following experiments. *P. putida* was grown in a medium where carbon was the only limiting nutrient, so a yield for growth on acetate could be calculated. Concomitantly, this test verified whether the guide number of $10^7$ cells growth per µg of AOC provided (Hammes and Egli 2005) was in a good order of magnitude for characterising the growth of *P. putida* on acetate. *P. putida* proved to be able to grow on acetate and yielded $(2.80 \pm 0.42) \times 10^6$ cells.µg C$^{-1}$, which is near the previously reported value of $10^7$ cells.µg C$^{-1}$ (Figure 3-7).

Finally, it should be noted that the results in this study could change, depending on the initial spike concentration of the invader. This ‘density effect’ is already subject of debate since years (Phelps 1944) and gained new attention more recently, especially since mechanisms as quorum sensing are now well-known (Hellweger *et al.* 2009). While some note a decay pattern which is independent of initial spike concentrations (Hellweger *et al.* 2009), others noted that the effect of a high enough initial spike concentration ruled out the effect of the presence of an indigenous community (Kerr *et al.* 1999).

In conclusion, taking into account the results of earlier studies, the antagonistic (Ducluzeau *et al.* 1984, Moreira *et al.* 1994) or protagonistic (Kerr *et al.* 1999, Moreira *et al.* 1994) effect of indigenous water communities on growth of invaders is hard to predict. This depends on the bacterial community structure and each water source, surface water or drinking water produced from either surface or ground water, has its own unique bacterial community. Bacteria present in one water inducing an antagonistic or protagonistic effect may be absent in another water. It will remain difficult to draw general conclusions about these complex effects without testing for the specific bacteria in the specific water, but the main conclusion should be that the indigenous community can as well have an effect on invasion. Furthermore,
these experiments confirmed the combined importance of carbon and phosphate; extensive limitation of one or both can prevent the long-term growth or survival of an invader. A drinking water production process can consequently aim at less strict AOC limitation, combined with extended phosphate removal, as a pathway for providing safe water, not susceptible to invasion. Once a contaminating invader actually reaches drinking water without disinfectant residual, it could however survive for several days irrespective of nutrient concentrations, and could thereby reach consumers.

Figure 3-7: The growth of Pseudomonas putida on a medium where carbon is the only limiting nutrient yields \((2.80 \pm 0.42) \times 10^6\) cells.µg C\(^{-1}\) after linear regression through the origin \((n = 3)\).

Acknowledgements

This work was supported by the project grants no. G.0808.10N and G.0700.10N of the FWO Flanders, and the Inter-University Attraction Pole (IUAP) “µ-manager” funded by the Belgian Science Policy (BELSPO, 305 P7/25). We thank Simon De Corte, Massimo Marzorati and Stephen Andersen for critically reading the manuscript.
PART B

OPTIMIZATION OF FLOW CYTOMETRY FOR ROUTINE MEASUREMENT
‘Water is the driving force of all nature’

Leonardo da Vinci
Chapter redrafted after:

Van Nevel S., de los Reyes III F., Hammes F., Boon, N. The importance of background during flow cytometric analysis of aquatic microorganisms. In preparation for *Journal of Microbiological Methods*.

Abstract

A flow cytometric (FCM) analysis in microbiology is usually followed by a gating procedure to discriminate cells from background. Such FCM background is subsequently nearly always neglected. However, here we argue that FCM background should in fact be properly considered to (i) characterise and understand samples better, (ii) optimize and fix gating and (iii) understand the possible influence of background on FCM fingerprinting techniques. This study distinguishes between ‘instrument background’ (originating from electronic noise and improper maintenance), ‘sample background’ (originating from sample and staining procedures) and ‘artefact background’ (accidently added to the analysis). We characterised typical features of each background category. Moreover, we present a guideline for discriminating between background and cells of interest, allowing the use of FCM for a wide range of samples.
1. Introduction

Flow cytometry (FCM) has become a widely accepted and used method for screening a range of microbiological parameters in diverse aquatic samples (Diaz et al. 2010, Lebaron et al. 1998, Wang et al. 2010b). The basic principle of FCM is the analysis of particles in a liquid sample by guiding them one by one through a light source, usually a laser beam (Shapiro 2003). The technique has been used for the detection and enumeration of bacteria in drinking water (Hammes et al. 2008a), wastewater (Ma et al. 2013), surface water (Besmer et al. 2014), marine water (Pan et al. 2005), groundwater (Wang et al. 2008), bioprocess samples (Diaz et al. 2010) and more. Notably, all these samples can contain a wide range of microorganisms including viruses, bacteria, algae, yeasts and protozoa. Usually the target of analysis is only one of these microbial groups, or several groups but discriminated from each other. However, these samples all contain abiotic particles, organic matter, etc. Therefore, following an FCM measurement, regions-of-interest in the data are usually selected based on manual gating on either 1D or 2D-plots of the measured parameters, e.g., fluorescence intensity in certain wavelengths or forward and side scatter signals. Approximated gates are drawn manually to separate several groups of microbial cells and (what is usually deemed as irrelevant) background signals, originating from instrument noise and sample medium components (Diaz et al. 2010, Prest et al. 2013). Different literature examples of flow cytometric plots, including indicated bacterial gates and surrounding background, are illustrated in Figure 4-1.

Figure 4-1: Literature examples of flow cytometric background. The above examples illustrate flow cytometric background, displayed previously in literature. In each case, the gates are indicating bacteria while the surrounding dots were depicted as background. Figures after Gregori et al. (2001), Hammes et al. (2010) and Wang et al. (2010b).
There are three main reasons to strive for a better understanding of FCM background: (i) to characterise and understand the sample better, (ii) to optimize gating strategies and (iii) to understand and quantify the effect of background on FCM fingerprinting analyses. These three points are further discussed in detail. (i) The sample background is the result of present organic matter such as cell fragments, free DNA, other microorganisms, etc. and abiotic particles such as salt crystals, soil particles etc. As a result, the background actually contains additional sample information, which is for now only limitedly understood. A better characterisation of background would thus result in a higher level of information obtained from FCM analysis. (ii) Optimizing gating strategies. Gates are drawn to the best of the researcher’s abilities but, depending on sample type and contents, can offer real challenges. Pronounced background in samples can severely complicate a clear distinction between background and cells (Hammes and Egli 2010). This can result in gating being an extremely arbitrary and subjective process, seriously affecting the results and concomitant conclusions in case of poor gating. Routine FCM analysis methods, like the standardized method for total cell count in the Swiss food handbook rely on fixed gating (Prest et al. 2013, SLMB 2012). Therefore, every possible sample to be measured by such a protocol, needs to fit in a pre-defined gating, discriminating bacteria from the background. This seems reliable for clean and low-background samples such as drinking water, but could be more demanding for other samples such as surface or wastewater. Therefore, it is of utmost importance to understand the behaviour and appearance of background, in order to do proper gating. (iii) Finally, FCM fingerprinting methods for data analysis, like the flowFP-method (De Roy et al. 2012) and Cytometric Histogram Image Comparison-method (Koch et al. 2013a), incorporate all raw data, including background. This makes the methods arguably more sensitive and allows detection of both biological and non-biological differences between samples. However, this also makes the methods potentially more prone to errors due to varying background. In this case, it is of crucial importance that background is deriving from samples and not induced by artefacts in sample handling or measurement.

There has to the best of our knowledge never been a dedicated study to understand FCM background in microbial analyses. In the present study, several factors leading to background were examined, including organic and inorganic medium compounds and measurement
2. Materials and methods

2.1 Analysis by flow cytometry and microscopy

All samples were prepared and stained according to Prest et al. (2013) and Van Nevel et al. (2013b) and measured on a BD Accuri C6 instrument with autoloader (BD Biosciences) unless mentioned otherwise. In short, samples were stained with SG (10,000x diluted from stock, Invitrogen) and incubated for 13 min at 37°C. Unless otherwise stated, sample dilution is done in filtered bottled mineral water (0.1 µm, Millipore Millex-VV), a stable buffered solution yielding minimal additional background. The plots displayed are drawn on a logarithmic scale on FL1 (range 300-8,000,000 plotted) versus FSC (10-10,000,000 plotted) for yeast, FL4 (range 300-10,000,000 plotted) versus FSC (10-10,000,000 plotted) for algae and FL1 (range 300-4,000,000 plotted) versus FL3 (range 10-2,000,000 plotted) for all other samples. These ranges were selected to provide an optimal visualisation and zoom of the analysis, without losing any data outside of the displayed ranges. The FL4 detector was fitted on the red laser (640 nm) while all other detectors were fitted on the blue laser (488 nm). Fingerprinting of flow cytometric data was performed as described with the custom-made flowFDA-method (Clement and Thas 2014), based on De Roy et al. (2012). Microscopic analysis was performed with an Olympus IX81 microscope (Olympus K. K.), *E. coli* was therefore stained according to the FCM procedure.

2.2 Biotic samples

*E. coli* (K12 MG1655) was grown overnight in Lysogeny broth (LB-Lennox, AppliChem), containing per liter 10 g tryptone, 5 g NaCl and 5g yeast extract. The algae (unialgal *Chlamydomonas reinhardtii*) were grown photoheterotrophically in petri dishes on TAP medium (1.5% agar) at 20°C and were suspended in 0.9% NaCl prior to analysis. Yeast originated from a dry baker’s yeast sample (*Saccharomyces cerevisae*), suspended in ultrapure water by 30 min stirring. Pure DNA originated from salmon sperm standards (Sigma-Aldrich). Wastewater effluent was collected from the secondary effluent of a small-sized wastewater
treatment plant. Bottled mineral water was Evian. Tap water was sampled from a drinking water fountain in Dübendorf, Switzerland. Surface water was sampled from the stream Chriesbach, Dübendorf, Switzerland. The final concentrations and used dilution media of the measured samples are displayed in Table 4-1.

Table 4-1: Dilution media and final concentrations of the biotic samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution medium</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>0.1 µm filtered Evian</td>
<td>1.4 x 10^5 cells.mL^-1</td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>Ultrapure water</td>
<td>8.0 x 10^5 cells.mL^-1</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisae</em></td>
<td>Ultrapure water</td>
<td>1.6 x 10^5 cells.mL^-1</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>Ultrapure water</td>
<td>10 ng.mL^-1 DNA</td>
</tr>
<tr>
<td>Wastewater effluent</td>
<td>0.1 µm filtered Evian</td>
<td>4.4 x 10^4 cells.mL^-1</td>
</tr>
<tr>
<td>Tap water</td>
<td>0.1 µm filtered Evian</td>
<td>1.7 x 10^5 cells.mL^-1</td>
</tr>
<tr>
<td>Surface water</td>
<td>0.1 µm filtered Evian</td>
<td>2.0 x 10^5 cells.mL^-1</td>
</tr>
</tbody>
</table>

2.3 Abiotic samples

Abiotic samples were created to study background, deriving from a sole pure compound. Each of them had ultrapure water as medium, originating from either from a Milli-Q (Millipore) or Nanopure (Barnstead) system, both equipped with 0.22 µm filters. Humic acids, NaCl, Na₂S₂O₃, Na₂SO₄ (pure crystal stocks, Sigma-aldrich) and NaOCl (10-15% available chlorine stock, Sigma-aldrich) were dissolved or diluted to working concentrations in ultrapure water. In case of NaOCl, an initial concentration of 10% available chlorine was assumed.

2.4 Other specific samples

An absolute control sample was analyzed as blank before the measurements. For this analysis, ultrapure water was analyzed and all sample preparation, staining and incubation took place in ultraclean glassware, including a Pasteur pipette for staining and a glass 96-well plate (Zinnser) for analysis. The preparation of this glassware consisted of an acid wash, rinsing with ultrapure water and a muffling step (6 hours at 550°C) according to Greenberg *et al.* (1993). All other samples were analyzed in plastic 96-well plates.
In order to induce artefact background in samples, ‘polluted stain’ and ‘polluted pipet tips’ were used. The stain was polluted due to filtration of the dimethylsulfoxide (DMSO, the stain solvent) with a PVDF-filter (Millipore Millex VV), which cannot withstand DMSO. Normally, a PTFE-filter (Millipore Millex IC) was used for DMSO filtration. The ‘polluted pipet tips’ were polluted by dust on the ground and were wiped visually clean afterwards.
3. Results and discussion

In the analysis of FCM background, we differentiate between instrument background, sample background and artefact background. The background discussion is based mainly on analysis of samples for bacteria, stained with SYBR Green I (SG) which is a general nucleic acid stain to detect all microorganisms (Wang et al. 2010b). SG is selected since it is a well-established and standardized method (Prest et al. 2013), widely used to detect microbiota in different matrices (Hammes et al. 2008a, Ma et al. 2013, Qin et al. 2008). Although this discussion is based on a single staining procedure and instrument, the principles discussed are widely applicable and independent of procedure and instrument.

3.1 Instrument background

Instrument background results mainly from (i) electronic noise resulting from sub-optimal instrument settings and (ii) sub-optimal maintenance and cleaning.

Electronic noise or white noise appears dependent on detector settings like thresholds, gains and voltages. The latter are instrument-dependent nomenclature, which will be referred to as the ‘threshold’ from here onwards. When thresholds are set for a higher sensitivity, an increasing amount of noise will be detected, especially in the low scatter and low fluorescence region. This electronic noise is instrument-specific, and has to be assessed when taking a new instrument in use. Figure 4-2 is illustrating the measurement of both ultrapure, unstained water and a diluted and stained surface water sample, with varying fluorescence threshold values. The use of lowering threshold values led to exponentially growing electronic background, which ultimately suppressed the signal of the target organism; in the illustrated example, nearly 90% of the bacteria stayed undetected due to this effect. Alternatively, a too high fluorescence threshold will neglect bacteria emitting a lower fluorescence emission. Instrument background can be minimized by good maintenance, stringent cleaning procedures and well-chosen measurement settings.
**Figure 4-2: The impact of a threshold and electronic noise on sample analysis.** Unstained ultrapure water (top) or ten times diluted and SG stained surface water samples (bottom) were measured repeatedly with a decreasing detection threshold for the FL1-detector (left to right). Approximated polygons are drawn in order to count present bacteria. The number of background particles is indicated, as well as the number of bacteria and their fraction on the total analysis (in the gate). A lowering threshold results in exponentially growing background, which finally results in suppression of the detection of the bacteria (bottom right sample). Contrarily, a too high threshold can as well neglect bacteria with weaker fluorescence emissions, if these are under the threshold limit (bottom left).

Sub-optimal instrument maintenance and cleaning can result in the detection of particles, not originating from the analysed sample. This can be induced for example by withered tubing which is shedding particles or from malfunctioning filters for sheath fluid, which can contain detectable particles. Sub-optimal cleaning and disinfection after measurement can result in biofilm formation in the fluidic system of the apparatus, shedding microorganisms. An optimal cleaning strategy is instrument- and sample-dependent, e.g. the analysis of low fluorescent particles ask for a cleaner instrument. For microbial samples, this protocol should however at least contain a daily and thorough disinfection and rinsing with ultrapure water. A cleaning strategy has to be combined with a stringent use of standardised daily quality controls such as unstained blanks and standardized beads. Daily logging of the amount of detected particles in blanks and standardized samples allows for detecting trends of increased instrument...
The importance of FCM background

Background. It should be noted that flow cytometers were primarily designed for the analysis of mammalian cells (Givan 2001), which are typically 3 orders of magnitude higher in cell volume and DNA content than for example *E. coli* (Steen *et al.* 1994). Therefore, analysis of mammalian cells usually results in far higher scatter and fluorescence signals which are more easily distinguished from low scatter and fluorescence background.

### 3.2 Sample background

Sample background results from the combination of the sample with the selected measurement procedure, including staining and instrumental settings (thresholds, gains, voltages, measurement speed, *etc.*). The sample background can be the combined effect of several factors, which may result in no or less background when present alone. Sample background includes: (i) Organics present in the sample: DNA-containing material such as cell fragments, free DNA or other organisms than the ones of interest (bacteria, viruses, yeast, fungi, eukaryotic cells, *etc.*) but also humic acids and more. (ii) Inorganics present in the sample: diverse salt crystals, soil particles, *etc.*

In order to assess the behaviour of previously mentioned compounds, a set of samples, containing each time one of these compounds diluted in ultrapure water, was measured (Figure 4-3). A range of pure compounds (NaCl, Na₂SO₄) and more complex organic compounds (LB-broth, humic acids, DNA), typically encountered in microbiological samples, were selected. Instrument settings were chosen to minimize instrument background, in order to study the specific sample background caused by these compounds. It can be seen from the different NaCl concentrations that sample dilution is not necessary leading to proportional background dilution. NaCl was far below solubility thresholds in these samples, so NaCl-crystals are expected to be absent (Koutsoukos *et al.* 2007). Therefore, it was suggested that the detected events were either a result of the reaction of salt with stain (Zipper *et al.* 2003) or impurities in the NaCl-stock. The samples with chlorine and Na₂S₂O₃ illustrate the formation of additional background when both are present together, potentially due to the formation of sulphate crystals. Finally, a generalising scheme locating sample background and bacteria is drafted, as a guideline to locate bacteria based on SG-staining. The sample background does not have to be avoided but can be controlled by a well-chosen measurement.
procedure, especially allowing the discrimination between background and cells, and supressing electronic noise.

Figure 4-3: Examples of sample background. The scheme in the bottom summarizes where which background typically is to be seen at an FL1 versus FL3 plot. (*) In the ultrapure, absolute control, ultrapure water was stained with SG in an ultrapure glass 96-well plate, prepared according to Greenberg et al. (1993). (**) Cl is expressed as active chlorine and added as NaClO.
The importance of FCM background

The additional information, contained in the sample background can be useful for a more complete characterisation of a sample. It is for example possible to discriminate different samples, based on their background by sensitive FCM fingerprinting techniques which providing a statistical analysis of the data (De Roy et al. 2012, Koch et al. 2014). The presence or absence of 1 g.L\(^{-1}\) CaCO\(_3\) in samples containing \(E.\) coli was detected easily by fingerprinting, as shown in Figure 4-4. This illustrates the power of fingerprinting techniques, in being able to discriminate between samples with an identical microbial community.

**Figure 4-4: The effect of background on fingerprinting techniques.** Aliquots of one batch of \(E.\) coli with (B) and without (A) added CaCO\(_3\) were stained with normal and polluted stain (C). FCM analysis results were processed with a fingerprinting technique, based on Fischer discriminant analysis. In short, differences between samples get detected and visualized on a discriminant plot. The distance between samples indicates relative similarity of samples. Both the effect of sample background (CaCO\(_3\)) and artefact background (polluted stain) affected the fingerprint of the samples.

Real examples of the analysis of microorganisms, including inherent sample and instrument background, are given in Figure 4-5. The microorganism discrimination is shown based on manual gating on 2D-plots. In case of surface water, the background got heavily increased due to rainfall, illustrating how environmental factors can influence background over time and entangle discrimination of target organisms.
Figure 4-5: Examples of various micro-organisms on flow cytometric plots. The bacteria and yeast are stained with SG. The algae were not stained, since these contain chlorophyll, making them autofluorescent and detectable by FCM. The surface water examples indicate how both bacteria and background can change over time due to factors such as environmental influences. In this case, gating is more complicated and is performed based on the sample before the rainfall.

A poor choice of staining procedure can lead to poor staining and severe background, complicating the distinction between bacteria and background. It is however still considered sample background since it results from the staining procedure. An example, where different incubation times and temperatures lead to the incorrect detection of a lower number of cells is shown in Figure 4-6.
3.3 Artefact background

Artefact background results from other causes not included in sample and inherent background. It is induced by artefacts such as (1) impurities in the stain or dilution medium, (2) unclean sampling materials (sample storage materials, pipet tips, etc.), (3) certain events, such as the analysis of air due to air bubbles in the flow cell. In flow cytometers where the flow cell is visible, air bubbles can sometimes be seen by the naked eye. These typically result in heavily altered scatter and fluorescence patterns detected, unlike good sample. Examples on artefact background are explained in Figure 4-7, illustrating that background can wrongly be interpreted as bacteria in some cases, or even totally suppress the measurement of bacteria. Furthermore, it can wrongly affect the result of fingerprinting techniques. For example, in Figure 4-4, the use of polluted stain induced serious artefact background. This resulted in a sharp discrimination from other samples with an identical bacterial community, but stained...
with a clean batch of stain. While the incorporation of sample background was an illustration of the power of fingerprinting methods, this example with artefact background illustrates the weakness of fingerprinting; it is prone to errors induced by artefact background. In general, artefact background should be avoided at all times by precautious work and overall good laboratory practice.

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**Figure 4-7: Examples of artefact background.** In all plots, a standardized gate for detecting bacteria in drinking water is added and the number of events detected in 25 µL of sample are shown. The number of bacteria refers to (artefact) background seen as bacteria, since all samples were sterile. Physiological solution (8.5 g/L NaCl) is often used as dilution medium, but results in additional background when not properly filtered. Similarly, unclean pipet tips or poorly prepared staining solutions can cause background. In the above example, the dimethyl sulphoxide used to dilute the SG stock solution was run through a filter, inappropriate for this solvent.
3.4 Discriminating target organisms from background

A scheme on how to distinguish between targeted micro-organisms and background is given in Figure 4-8. It should however be considered that the physical differences and thereby the appearances of the microorganisms on flow cytometric plots are enormous and in addition, different staining protocols yield totally different results. Therefore, it is impossible to cover every single situation in one scheme. When one applies flow cytometry on a new type of sample, care has to be taken and a situation-dependent set of controls has to be measured with flow cytometry to learn to discriminate between target organisms and background.

Finally, a general illustration of the different types of background and microorganisms is given in Figure 4-9, showing the typical resulting range for each on the FL1 (green fluorescence) detector. It is clear that a single parameter histogram is only in rare cases sufficient for discriminating target organisms and background. Ideally, several 2-parameter plots are drawn to find the best plot for discrimination for every specific type of sample. One should however stay realistic that in some situations with heavy background, a sharp discrimination will remain impossible with every single protocol or plot, e.g. between large viruses, bacterial cell debris with DNA fragments, small bacteria, etc.
Figure 4-8: Flowchart on how to discriminate microorganisms from background. In samples with low background, microorganisms are often easy to discriminate. Alternatively, dilution can facilitate this. Additional controls generally consist of comparison with sterile broth if this is available, comparison with filtered samples if the microorganisms are expected to be the only particles, or centrifugation and resuspension in a clean dilution medium if the microorganisms are large enough to be centrifuged. Other sample specific options can be applied or finally, comparison with other established techniques like microscopy is possible for a few model samples.
Figure 4-9: Green fluorescence (FL1) results for typical samples with microorganisms. A single parameter histogram will only be sufficient in rare situations for discriminating between target organisms and background.

4. Conclusions

Different situations yield different background and a custom, sample dependent approach can be necessary and consists of selecting relevant model samples for running control experiments. These can include broth samples, filtered samples, washed samples, etc. Alternatively, comparison with other methods is possible. When one understands how to discriminate target organisms and background in the model samples, this approach can be applied to all samples and FCM can be used as high throughput method.

Acknowledgements

SVN was supported by the project grant no. G.0808.10 N and travel grant no. V424114N of the FWO Flanders and the Inter-University Attraction Pole (IUAP) “μ-manager” funded by the Belgian Science Policy (BELSPO, 305 P7/25). FdlR was funded by the Erasmus Mundus Transatlantic Partnership for Excellence in Engineering Programs. The authors want to thank Synthia Maes for critically reading the manuscript.
Chapter 5

Routine Bacterial Analysis with Automated Flow Cytometry

Chapter redrafted after:


Abstract

Flow cytometry (FCM) has become a widespread method for the microbial analysis of aquatic samples. An important advantage is the speed and possibility for high-throughput use when an autoloader is available. However, automated processing of large batches of samples entails extended waiting times for stained samples, possibly affecting sample stability and measurement reproducibility. Therefore, the impact of automated FCM measurement on analysis results was investigated in detail. Different aquatic samples were selected as a model, including surface, ground and drinking water, ultrapure water and pure strain samples. Single samples were stained with either SYBR Green I (SG) or both SG and propidium iodide (PI), followed by incubation in batch to ensure homogeneous sample preparation. These samples were aliquoted in 96-well multititer plates and measured under different conditions, such as measurement speed and volume, light conditions, etc. Cell concentrations in up to 96 samples could be measured with high precision (relative standard deviations < 2.5%), as long as a stringent staining protocol and an analysis time of below 80 minutes were used. Fluorescence distribution in the samples could display variability, and additional analysis steps such as sample agitation or washing steps may not have the expected effect, or even induce an adverse effect. Carry-over between different samples was limited (0.5%), although this still has an impact if high and low concentrated samples are alternating. In conclusion, a reliable measurement protocol could be prepared, but extensive controls are necessary for protocol validation whenever sample types, staining procedures or the used instrument are changed.
1. Introduction

Flow cytometry (FCM) has become an established method for the microbial analysis of aquatic samples (Diaz et al. 2010, Lebaron et al. 1998, Wang et al. 2010b). One of the advantages of this method is the potential for rapid, high throughput processing of multiple samples. The development of multi-well autoloaders for flow cytometry enables researchers to take this to a next level, where physical presence of the operator is no longer required during measurement of large sample batches. However, when many samples are stained simultaneously, there will be a substantial delay between the measurement of the first and last stained sample. Such a delay clearly conflicts with the logic of immediate sample processing after preparation and identical preparation of all samples. A delay prior to measurement may impact on both the staining reaction and the biology of the sample. With respect to the biology, a delay may result in changes to cell physiology or sedimentation of large cells. With respect to staining dynamics, it is possible that either more stain enters cells over time, stain leaks from cells, or stains deteriorate (e.g. fading (Suzuki et al. 1997)). Such changes can affect FCM results profoundly. For example, during viability staining this may alter the number of viable cells (Berney et al. 2007, Hammes et al. 2012). Additionally, sensitive FCM fingerprinting methods, which compare samples by the analyses of fluorescence patterns (De Roy et al. 2012), will be compromised by changes in the fluorescence distribution after staining. However, until now only very limited information is available on the stability of stained samples (Hammes et al. 2012, Lebaron et al. 1998).

In this study, the impact of automated high throughput sample loading on measurement precision was investigated in detail, using both bacterial pure cultures and indigenous aquatic microbial communities and focussing on reproducibility with respect to total cell concentrations, viability assessment, and fluorescence distribution.
2. Materials and methods

A recently standardized staining protocol was used to ensure reproducible staining of all samples (SLMB 2012). Water samples containing approximately $10^3$ cells$\mu$L$^{-1}$ of either a pure *E. coli* K12 culture or indigenous surface water bacteria (subject to daily natural variation) were preheated at 37 °C, stained with either SYBR Green I (SG) (10,000x diluted from stock; Invitrogen) or SG combined with propidium iodide (PI, 3 µM final concentration; Invitrogen) (Barbesti *et al.* 2000), followed by 10 min incubation in the dark at 37 °C. Unless otherwise stated, subsamples of a stained batch were loaded on a 96-well plate and measured at different instrumental settings (see below) on an Accuri C6 flow cytometer (BD Biosciences), equipped with an autoloader. Cell counts were done by measuring the number of particles in a set volume after gating on green vs. red fluorescence plots in the BD CSampler software as described elsewhere (SLMB 2012). Quality control with respect to continuity of absolute cell counting was done on a daily basis with standardized beads with an unknown concentration (Partec).
3. Results and discussion

The FCM flow rate and sample volume are critical parameters, as this will impact directly on the time required to process a batch of samples. These are instrument specific parameters and according to manufactures’ guidelines ‘medium’ (35 µL.min⁻¹) and ‘fast’ (66 µL.min⁻¹) measurements yield the most accurate results for the Accuri C6. A detailed comparison of measurement precision at different measurement rates and volumes showed negligible variations at any selected flow rate (relative standard deviation (RSD) below 2.5%), but clearly showed that a ‘slow’ speed (14 µL.min⁻¹) measured approximately 10% lower cell concentrations than medium or fast rates (Figure 5-1 and 5-2).

![Figure 5-1: Precision comparison of different measurement speeds on surface water. A total cell count was done on surface water and 25 µL was measured at 66 µL.min⁻¹, 35 µL.min⁻¹ or 14 µL.min⁻¹. Only minimal statistical differences in cell concentration and green fluorescence precision were detected for each different measurement speed, respectively; the RSD on the cell concentration were 2.4%, 2.0% and 2.0% respectively. However, the cell concentration at the slow measurement was almost 10% lower compared to medium or fast measurements (n=3x12)
Figure 5-2: Accuracy comparison of different measurement speeds and sample volumes on control beads. A count was done on a 3.0 µm calibration bead solution with unknown concentrations (Partec) and 25, 50 or 100 µL was measured at 66 µL.min⁻¹, 35 µL.min⁻¹ or 14 µL.min⁻¹. The bead concentration at 14 µL.min⁻¹ was 11% lower compared to the 35 or 66 µL.min⁻¹ measurements. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=3x12).

A fast screening protocol was subsequently compared with an extended measurement protocol on SG-stained surface water samples. For the fast screening, 25 µL of surface water samples was measured on a fast speed requiring 74 minutes for processing a 96-well plate. In the extended measurement protocol, 100 µL was measured on medium speed, requiring 310 minutes for completion. The fast screening protocol showed an RSD of 1.8% on the cell concentration, but for the extended protocol this was 11% (Figure 5-3, Table 5-1). A trend was calculated as the relative difference between the first and last ten measured samples. The trend in cell concentration from the fast screening was -3.9%, while for the extended protocol it was -30%. The median green fluorescence of the cells (representing the fluorescence distribution) showed trends of +1.8% and +12% respectively. In the case of the extended protocol, the overall decrease in cell concentration was caused by the extensive waiting time between the first and last measurement. This is ascribed to sedimentation and attachment of bacteria to the surface of the plate and bleaching effects on the stained cells.
Figure 5-3: Comparison of a fast screening to an extended measurement. A total cell count was done on surface water and either 25 µL was measured at 66 µL.min⁻¹ (A, 74 min) or 100 µL at 35 µL.min⁻¹ (B, 310 min). The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).
### Table 5-1: Overview of measurement conditions and stability of the results

(*) The trend is expressed as the difference in average between the first and last 10 measured samples. (**) A standard measurement is carried out in a white 96-well plate and 25 µL of a surface water sample, stained with SYBR Green I is measured at 66 µL.min⁻¹, without any agitation or washing steps in between. Any deviation from these conditions is mentioned in the table below. NA: not applicable

<table>
<thead>
<tr>
<th>Measurement conditions</th>
<th>Cell concentration (cells.µL⁻¹)</th>
<th>Relative standard deviation</th>
<th>Trend*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard**</td>
<td>1386 ± 25</td>
<td>1.8%</td>
<td>-3.9%</td>
</tr>
<tr>
<td>35 µL.min⁻¹, 100 µL</td>
<td>1249 ± 136</td>
<td>11%</td>
<td>-30%</td>
</tr>
<tr>
<td>Mineral water</td>
<td>57.1 ± 1.6</td>
<td>2.8%</td>
<td>+3.6%</td>
</tr>
<tr>
<td>Mineral water, 34 µL.min⁻¹, 100 µL</td>
<td>48.8 ± 4.0</td>
<td>8.2%</td>
<td>-23%</td>
</tr>
<tr>
<td>Adding stain to sample in well</td>
<td>814 ± 29</td>
<td>3.5%</td>
<td>-7.0%</td>
</tr>
<tr>
<td>Adding sample to stain in well</td>
<td>881 ± 18</td>
<td>2.0%</td>
<td>+0.31%</td>
</tr>
<tr>
<td>Transparent 96-well plate</td>
<td>849 ± 28</td>
<td>3.3%</td>
<td>-8.2%</td>
</tr>
<tr>
<td>Black 96-well plate</td>
<td>1721 ± 26</td>
<td>1.5%</td>
<td>-0.60%</td>
</tr>
<tr>
<td>Transparent 96-well plate, 35 µL.min⁻¹, 100 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Light conditions</td>
<td>795 ± 71</td>
<td>9.0%</td>
<td>-21%</td>
</tr>
<tr>
<td>-Dark conditions</td>
<td>773 ± 33</td>
<td>4.2%</td>
<td>-8.3%</td>
</tr>
<tr>
<td>Agitation every 6 wells</td>
<td>776 ± 59</td>
<td>7.6%</td>
<td>-20%</td>
</tr>
<tr>
<td>Transparent plate, agitation every 6 wells</td>
<td>626 ± 68</td>
<td>11%</td>
<td>-29%</td>
</tr>
<tr>
<td>E. coli, intact cells, SGPI</td>
<td>196 ± 6</td>
<td>2.9%</td>
<td>+5.1%</td>
</tr>
<tr>
<td>E. coli, damaged cells (chlorination), SGPI</td>
<td>188 ± 5</td>
<td>2.4%</td>
<td>-5.7%</td>
</tr>
<tr>
<td>E. coli, slightly chlorinated: intact, SGPI</td>
<td>156 ± 7</td>
<td>4.6%</td>
<td>-5.7%</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random spread samples: mineral water</td>
<td>57.6 ± 1.5</td>
<td>2.6%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>surface water</td>
<td>895 ± 28</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>fountain water</td>
<td>256 ± 5</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>groundwater</td>
<td>405 ± 6</td>
<td>NA</td>
</tr>
<tr>
<td>E. coli</td>
<td>213 ± 6</td>
<td>2.7%</td>
<td>NA</td>
</tr>
</tbody>
</table>
Similar results were obtained in repetition experiments with different waters, with a pure strain, or with individual staining of wells instead of staining single sample batches which are divided into the 96-well plates (Figure 5-4 to 5-6). Additional control experiments with shaking steps or different light conditions suggested attachment and light effects rather than sedimentation as the cause of the decrease (Figure 5-7 to 5-9). It was concluded that cell concentrations retrieved from SG staining remained very stable during 75 minutes. Therefore, the fast screening protocol was chosen for all further experiments.

**Figure 5-4:** Comparison of a fast screening to an extended measurement with natural mineral water. A total cell count was done on mineral water and either 25 µL was measured at 66 µL.min⁻¹ (A) or 100 µL at 35 µL.min⁻¹ (B). In case A, the measurement took 74 min and the trend throughout the measurement was minor with a 3.6% increase, while in case (B), it took 310 min and a 23% decrease was seen. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).
**Figure 5-5: The stability of E. coli analysis at a fast measurement rate.** A fast screening was done with a diluted E. coli sample. An RSD of 1.8% and a trend of 0.0% on the cell concentration was calculated, while the median green fluorescence showed an RSD of 13.6% and an upwards trend of 37%. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=96).

Although cell concentrations remained stable with the fast screening protocol, the fluorescence intensity could vary. For *E. coli*, a strong effect was already seen with the fast screening protocol; median green fluorescence intensities rose by 37% over the course of the analysis. For the other samples, a clear trend was only seen using the extended screening protocol, where the fluorescence intensity of surface water rose by 12%, while the natural mineral water showed a downwards trend of 19% over the course of the experiment. The rising of fluorescence intensity with increasing incubation temperatures has been documented before ([Hammes et al. 2012, Prest et al. 2013](#)). This can likely be attributed to cell membrane permeabilisation, leading to more stain entering the cells and binding to the nucleic acids, an effect which can as well be induced by adding EDTA or small doses of chlorine (non-published data). The opposite effect, lowering of fluorescence intensity, is however less known to occur and is usually only seen in case of cell damage. In the experiment studying light-induced effects (Figure 5-8), a lowering of the green fluorescence intensity was seen as well.
Figure 5-6: Comparison of real-life staining-procedures of individual samples. A total cell count was done on surface water with the fast screening protocol. However, instead of staining a single large batch which is subsequently divided into the 96 wells, the SG-stain was either added by pipetting to a sample which was already present in a multiwell plate (n=47) or vice versa, the sample was added to stain which was already present in the multiwell plate (n=93). In the first case, a single agitation step was used to accomplish mixing in the wells while in the second case, the addition of the sample to the small volume of stain was assumed to guarantee good mixing. After staining in the 96-well plate, a standard 10 minutes incubation at 37 °C was done. In the first case, when adding stain to the sample, an RSD of 3.5% and a downwards trend of 7.0% was calculated. In the second case, when adding sample to the stain, the RSD and the trend were as low as 2.0% and +0.31% respectively. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses).
Figure 5-7: The use of plate agitation during the measurement. A total cell count was performed on two batches of surface water samples loaded in either a white or transparent plate. After every six measured wells, an agitation step was programmed during the measurement. A trend of -20% and -29% on the cell concentration were seen in the white and transparent plates respectively. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).

Figure 5-8: The effect of light and sample container on sample stability. Subsamples of a SG-stained batch of surface water were stored in either a plastic tube (Greiner) or a glass tube and placed open in direct sunlight or in the dark. Samples were taken from the surface of the sample with a minimal disturbance of the sample. In both glass and plastic, there was almost a 40% decrease in cell concentration after 30 in the sun. The stable results for the samples stored in the dark suggest the limited importance of sedimentation.
Figure 5-9: The adverse effect of light in case of an extended measurement time. A total cell count was done on surface water and 100 µL was each time measured at ‘medium’ speed (the ‘extended measurement protocol’) in a transparent plate. The total measurement time was 310 minutes and the measurement was run twice. The first time at our normal lab conditions where the system is standing with its back facing the window, with a limited amount of sunlight entering the lab, and the lab lights turned on. The second time, the system was covered and the measurement was run in absolute darkness. Under light conditions, the trend throughout the measurement was -21%, while in dark conditions, this was limited to -8.3%. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).

Viability staining consisting of SG combined with propidium iodide (PI) is commonly used to differentiate between intact and damaged cells (Berney et al. 2007, Gregori et al. 2001). A critical point of viability staining is whether increased incubation times would result in more cells labelled as damaged. This was investigated for short incubation periods previously (Hammes et al. 2012) but not for the extended periods required for processing a 96-well plate. Stability of staining with SGPI was tested using an E. coli culture. Intact cells (case A), slightly damaged cells (case B) and heavily damaged cells (case C) were prepared by chlorination as described elsewhere (Ramseier et al. 2011). After treatment, the residual chlorine was quenched with sodium-thiosulphate. The cell concentrations of the subgroups of ‘intact’ and ‘damaged’ cells stayed stable in all cases (Figure 5-10). However, the green fluorescence of the intact cells clearly changed over time (Figure 5-11). This was ascribed to more SG entering
the cells over time, resulting in increased fluorescence. In the case of slightly damaged cells, the outer membrane of the Gram-negative *E. coli* was already partly permeabilized by the chlorine, resulting in an immediate high SG concentration inside the cell. Longer incubation times did not increase this SG concentration, resulting in a stable staining which did not alter over time. A similar effect had been described previously for cells damaged by either UV-A-radiation, EDTA or chlorine (Berney *et al.* 2007, Hammes *et al.* 2011). The fluorescence of PI-stained damaged cells also changed over time, which was caused by progressively more PI entering the cells, causing the quenching of SG-fluorescence (Barbesti *et al.* 2000, Berney *et al.* 2007). It could be concluded that a SGPI staining of *E. coli* cells resulted in mostly stable intact cell concentrations, but with a fluorescence varying over time. Further testing with *Salmonella*, surface water or tap water supported these results as long as a final PI-concentration of 3 µM was used (Table 5-2). Light upwards trends in cell concentrations were seen in case of pure strains (*E. coli* +5.1% and *Salmonella* +5.2%) during the course of the measurement, and light downwards trends for other samples (-2.8% to -5.7%). Yet, more intact cells shifted to the damaged region during measurement when 6 µM was used as final PI-concentration. This increasing PI concentration resulted in a decrease in intact cell concentrations of 10 to 45% over time during the processing of a plate. In conclusion, the stability of SGPI-staining should be treated with care and appears to be dependent on the sample or strain and the PI-concentration. In addition, variation over time needs to be considered for the calibration and interpretation of fingerprinting techniques as described by De Roy *et al.* (2012).

**Table 5-2: Trends in intact cell concentrations for different SGPI measurements.** The stability over time was both sample and PI-concentration dependent, but generally, 3 µM as final PI-concentration yielded stable results while higher concentrations resulted in a decrease of intact cell concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trend on the intact cell concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 µM final PI-concentration</td>
</tr>
<tr>
<td><em>Salmonella</em>, intact</td>
<td>+5.2</td>
</tr>
<tr>
<td><em>Salmonella</em>, slightly chlorinated</td>
<td>-3.5%</td>
</tr>
<tr>
<td>Surface water</td>
<td>-2.8%</td>
</tr>
<tr>
<td>Tap water</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em>, intact</td>
<td>+5.1%</td>
</tr>
<tr>
<td><em>E. coli</em>, slightly chlorinated</td>
<td>-5.7%</td>
</tr>
</tbody>
</table>
Figure 5-10: Staining of intact and damaged E. coli with Sybr Green (SG) and Propidium Iodide (PI). An intact (case A), slightly chlorinated sample (case B) and heavily chlorinated and damaged sample (case C) were stained with SG and PI. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).
**Figure 5-11**: Green fluorescence intensity over time for *E. coli* stained with SGPI. Case A: Intact cells, no chlorination. Case B: Slightly chlorinated cells, with intact cells and damaged cells. Case C: Damaged cells, chlorinated. After SGPI staining, the green fluorescence intensity of intact cells in case A was increasing over time. In case B, the intact cells showed a stable fluorescence which did not alter over time. However, the damaged cells in both case B and C lost fluorescence over time.

A practical problem with multiple sample processing is unintentional carry-over of bacteria between the samples from adjacent wells. A worst-case scenario was simulated where a plate was loaded with each time six nanopure water samples, containing no cells, alternating with six surface water samples, containing $862 \pm 15 \text{ cells.}\mu\text{L}^{-1}$ (Figure 5-12A). The bacterial carry-over between the surface water and each first nanopure water sample measured after surface water samples was 0.53%, which translated to $4.53 \pm 0.32 \text{ cells.}\mu\text{L}^{-1}$. An instrument specific washing step did not lower the carry-over, while it added considerable time to the analysis (data not shown). A realistic application was tested by loading a plate with five totally
different samples, spread in a random order over the plate (n=5x18) (Figure 5-12B). For every sample the RSD on cell concentrations was <3.1%. The fluorescence intensity of both *E. coli* and mineral water increased over time, in contrast to previous data (above). Surface water showed a negative trend in cell concentration. It was concluded that a series of random samples could be measured with high precision without extra cleaning procedures. Additional information on how to deal with this carry-over is given in Appendix 5-1 of this chapter, other operational parameters that can affect the measurement precision and accuracy are described in Appendix 5-2.
Fig 5-12: The carry-over between different samples minimal. (A) When measuring alternating sets of 6 samples with nanopure water and surface water with a heavy bacterial load, the carry-over in the first nanopure samples after a set of surface water samples, was by average only 0.53%, and consequently decreasing over the next samples. (B) When measuring five totally different samples in 18-fold and randomly spread over a plate, the precision of measurement was similar as when only one type of sample was measured.
4. Conclusions

In summary, we showed that 96 samples stained with either SG or SGPI can be measured automatically yielding stable cell concentrations and viability assessments. FCM fingerprinting patterns to the contrary, may be subject to variability over time and need proper calibration.

In order to obtain optimal results on any flow cytometer equipped with an autosampler, five rules are given:

I. Use or develop a highly optimized staining and measurement protocol and apply in a stringent way.

II. Limit the total measurement time by adapting measurement speed and time. Verify sample stability within this time, especially staining procedures which rely on selective penetration of dyes through cell membranes are expected to be vulnerable.

III. Prevent excessive light and whenever possible, have your analysis running in the dark for optimal sample stability.

IV. If additional steps such as agitation or washing steps are applied, verify the effect introduced by these procedures.

V. Choose a smart sample order and avoid the measurement of low concentrated samples after highly concentrated samples whenever possible. If concentration differences can be large and unpredictable, consider an additional washing step.

Acknowledgements

This work was supported by the project grant no. G.0808.10N and the travel grant V402813N of the FWO Flanders and the Inter-University Attraction Pole (IUAP) “μ-manager” funded by the Belgian Science Policy (BELSPO, 305 P7/25). We thank Simon De Corte and Karen De Roy for critically reading the manuscript.
Part C
New applications with flow cytometry
'You and I may not live to see the day, and my name may be forgotten when it comes, but the time will arrive when great outbreaks of cholera will be things of the past; and it is the knowledge of the way in which the disease is propagated which will cause them to disappear.'

*John Snow, 1854*
FLOW CYTOMETRY FOR IMMEDIATE FOLLOW-UP OF DRINKING WATER NETWORKS AFTER MAINTENANCE

Chapter redrafted after:


Abstract

Drinking water networks need maintenance every once in a while, either planned interventions or emergency repairs. When this involves opening of the water pipes, precautionary measures need to be taken to avoid contamination of the drinking water at all time. Drinking water suppliers routinely apply plating for faecal indicator organisms as quality control in such a situation. However, this takes at least 21 hours of waiting time, which can be crucial when dealing with major supply pipes. A combination of flow cytometric (FCM) bacterial cell counts with FCM fingerprinting techniques is proposed in this study as a fast and sensitive additional technique. In three full scale situations, major supply pipes with 400-1050 mm diameter were emptied for maintenance, shock-chlorinated and flushed with large amounts of clean drinking water before taking back in operation. FCM measurements of the discharged flushing water revealed fast lowering and stabilizing cell concentrations once flushing is initiated. Immediate comparison with clean reference drinking water used for flushing was done, and the moment when both waters had similar cell concentrations was considered as the endpoint of the necessary flushing works. This was usually after 2-4 hours of flushing. FCM fingerprinting, based on both bacteria and FCM background, was used as additional method to verify how similar flushing and reference samples were and yielded similar results. The FCM approved samples were several hours later also approved by the
drinking water supplier after routine analysis for total Coliforms and Enterococci, which were used as decisive control to set the pipes back in operation. FCM proved to be a more conservative test than plating, yet it yielded immediate results. Application of these FCM methods can therefore avoid long unnecessary waiting times and large drinking water losses.

1. Introduction

Drinking water networks need maintenance every once in a while, either planned interventions or emergency repairs. Both involve pressure loss or opening of a pipe, exposing the drinking water to risks of contamination with pathogenic microorganisms and increasing the risk of gastrointestinal illness among water recipients (Ainsworth and Holt 2004, Nygard et al. 2007). Several cases of contamination and disease outbreaks after construction and repair activities were reported (Craun and Calderon 2001). Possible causes include - but are not limited to - contaminations from soil and trench water exposure, biofilm sloughing due to sudden changes in flow velocity and stagnant, unsanitary water from adjacent piping sections (AWWA 2002).

Organisations like the American Water Works Association (AWWA), UK Water and the Environmental Protection Agency (EPA) are therefore advising cleaning protocols after opening water pipes, all of them which are based on shock chlorination and flushing (AWWA 2005, EPA 2010, Water UK 2011). The European EPA guideline consists of:

- close and empty the specific pipe section
- keep the trench surrounding the reparation zone dry;
- disinfect the pipe with 50 mg.L\(^{-1}\) of chlorine for 30 minutes, or alternatively, disinfect surfaces in contact with water with 1000 mg.L\(^{-1}\) of chlorine;
- flush the pipe with clean water and take microbiological samples for coliforms;
- return the pipe to service after flushing, provided there is no reason to suspect that contamination had entered the pipe, otherwise, wait for the results to declare the pipe hygienically safe (EPA 2010).

When drinking water pipes are emptied, disinfected and filled again, chemical and physical processes take place (Ainsworth and Holt 2004). The chemical disinfection with high concentrations of chlorine containing compounds (flash chlorination) not only kills bacteria
but also oxidises extracellular materials at the periphery of the biofilm clusters (Davison et al. 2010). This leads to detachment of biomass and consequently contamination of bulk water by organic matter (Fass et al. 2003). Physical processes arise from the changing shear forces when the emptied pipes are filled again or stagnant water flows again. These changing forces detach biofilms, damaged by the chlorine, even more into the bulk water (Douterelo et al. 2013, Mathieu et al. 2014, Mesquita et al. 2013, Volk et al. 2000). Loose deposits, ubiquitously present in the drinking water network, get partly resuspended (Lehtola et al. 2004, Liu et al. 2014). These consist of non-removed particles from the raw water, precipitation of metal oxides or calcium carbonate, external contaminations introduced during maintenance, etc. (Prevost et al. 2014) and are heavily loaded with bacteria (Liu et al. 2014).

Multiple studies are stressing the hygienic importance of these biofilms and loose deposits in drinking water networks, containing an estimated 95% of the total amount of present bacteria in the drinking water pipes (Flemming et al. 2002, Liu et al. 2014, Servais et al. 2004). In addition, biofilms have an increased chlorine resistance resulting in extended bacterial survival after this procedure (Berry et al. 2006, Menaia and Mesquita 2004). Loose deposits on the other hand frequently contain opportunistic pathogens like *Mycobacteria* (Torvinen et al. 2004), which have as well high chlorine resistances (Le Dantec et al. 2002). Therefore, it is important to supplement chlorine disinfection with rigorousflushing for the physical removal of biofilm debris and loose deposits (Ainsworth and Holt 2004). This combination, taken to ensure the hygiene in repaired pipes, results however temporarily in high bacterial loads in the bulk water, originating both from biofilm sloughing and loose deposits.

Consequently, there is a need for reliable measurement methods, quickly providing the drinking water supplier with information about current bacterial densities in the flushing water. Based on this information, the supplier has to conclude when the quality of the flushing water has stabilized and is similar to the drinking water quality of the influent. The conventional Coliform count is now the advised method but is only supplying limited information and takes 21 hours up to several days. In the meanwhile, large volumes of clean drinking water are unnecessarily discharged. Moreover, valuable time gets lost because important pipes are closed for a longer than necessary time, thus stressing drinking water supply in the subsequent sections of the drinking water network.
In the search for fast and reliable information on flushing procedures, we applied flow cytometry (FCM) for immediate comparison of flushing water with the influent water of the emptied pipes. In three full scale cases, the end of the flushing procedure was suggested based on FCM bacterial cell counts and FCM fingerprinting. The *E. coli* and *Enterococci* counts were performed as a benchmark to make the final decision of returning the pipes to service and for the evaluation of the FCM measurements as alternative method.

2. Materials and methods

2.1 Flushing procedure and case information

The sequence of maintenance and cleaning generally consisted of (1) closing a pipe section and depending on geography, emptying this partly or totally for maintenance works; (2) after completion of the works, loading a large dose of concentrated sodium hypochlorite solution (15%) at the beginning of the emptied section; (3) flushing with clean drinking water until the increased chlorine concentration is flushed out and the microbial analysis complies to legal standards; (4) finally taking the pipes back in service. Samples were taken upstream of the emptied sections as a reference (reference point) and downstream from the flushing water (flushing point). Since these pipes were temporarily out of use, stagnating water could be present between the reference point and the pipe closure, and within the closed pipe section (respectively section C and D on Figure 6-1 and in Table 6-1). The moment water reaches the flushing point continuously is in each case regarded as time 0. All pipes involved were made of high-pressure sidero-cement (PN16 grade). More information about pipe diameters, length of emptied sections, chlorine dosing and sampling points is given in Figure 6-1 and Table 6-1.

The filling water velocity and normal water velocity were obtained from the SCADA-software (supervisory control and data acquisition) of the water supplier, which is used to control flow rates and reservoir water levels.
Flow cytometric follow-up of network flushing

Figure 6-1: Schematic representation of the drinking water mains and sampling points during the cleaning procedures. The exact lengths of all sections are given in Table 6-1. (A) pipe diameter; (B) closed pipe section; (C) length of standing water, not emptied due to the geographic height-profile; (D) length of standing water before pipe closure.

Table 6-1: Pipe characteristics, chlorine dosing while flushing and operational parameters for the different pipes. REF: reference point. (*) Stagnant water in the closed pipe section. (**) The NaOCl-solution is injected at once in the pipe for shock chlorination. This dose is adapted to several parameters such as pipe diameter.

<table>
<thead>
<tr>
<th>Location</th>
<th>(A) Pipe diameter [mm]</th>
<th>(B) Closed section [m]/[m³]</th>
<th>(C) REF-closure [m]/[m³]</th>
<th>(D) Stagnant water(*) [m]/[m³]</th>
<th>Cl dose(**) [g NaOCl]</th>
<th>Filling water velocity [m/s]</th>
<th>Normal water velocity [m/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Brakel</td>
<td>1050</td>
<td>2525/2186</td>
<td>2940/2546</td>
<td>0/0</td>
<td>4500</td>
<td>0.13</td>
<td>0.94</td>
</tr>
<tr>
<td>2: Zuienkerke</td>
<td>400</td>
<td>5820/731</td>
<td>438/55</td>
<td>1520/191</td>
<td>300</td>
<td>0.20</td>
<td>0.55</td>
</tr>
<tr>
<td>3: Aalst</td>
<td>900</td>
<td>4490/2856</td>
<td>0/0</td>
<td>1900/1209</td>
<td>2250</td>
<td>0.11</td>
<td>0.62</td>
</tr>
</tbody>
</table>

2.2 Flow cytometric measurements

In case 1, samples were stored in sterile, carbon-free, ultra-pure 40 mL glass vials (Hammes and Egli 2005). These contained 400 µL of a 100 mM Na₂S₂O₃-solution to quench the chlorine activity. In case 2 and 3, samples were stored...

In case 1, samples were stored in sterile, carbon-free, ultra-pure 40 mL glass vials (Hammes and Egli 2005). These contained 400 µL of a 100 mM Na₂S₂O₃-solution to quench the chlorine activity. In case 2 and 3, samples were stored in sterile 10 mL plastic vials without Na₂S₂O₃.
The reason for this change is explained in the discussion. Sampling taps remained open at low flow during the whole flushing procedure. Samples were stored at 4°C and were usually analyzed by FCM within 2 hours after sampling. In case 3, this could be at maximum 5 hours for flushing samples and 9 hours for reference samples. FCM analysis was performed with an Accuri C6 with autoloader (BD Biosciences) as described before (Van Nevel et al. 2013b). In short, samples were either stained with SYBR Green I (SG, 10000x diluted from stock, Invitrogen) for total cell concentrations or SG combined with propidium iodide as a viability measurement, for assessing intact cell concentrations (PI, final concentration 4 µM, Invitrogen). Cell counts were done by measuring the number of particles in a set volume after gating on green vs. red fluorescence (FL1 vs. FL3) plots in the BD Csampler software as described elsewhere (SLMB 2012). All samples were measured in triplicate, unless otherwise stated. Quality control with respect to absolute cell counting was done with standardised beads (Partec). The limit of detection was 250 cells.mL⁻¹, the limit of quantification 650 cells.mL⁻¹, which was far below measured concentrations.

2.3 Flow cytometric fingerprinting

After flow cytometric measurements, the data was further analyzed using fingerprinting methods (De Roy et al. 2012, Koch et al. 2014). The analysis was performed in statistical language R with the custom-made package flowFDA (Clement and Thas 2014), based on the previously published work of De Roy et al. (2012). The workflow consisted of three steps: (i) creating a fingerprint of the flow cytometry data, (ii) dimension reduction based on principal component analysis (PCA) and (iii) Fisher discriminant analysis (FDA) to assess the differences between samples. To construct the fingerprint, the forward scatter, sideward scatter, FL1 (533/30 nm) and FL3 (>670 nm) channels were selected because of their biological relevance in this experiment. For every channel the data was divided in 256 equally sized bins in order to form an n-dimensional equally spaced grid. For every bin of the grid the density of events was calculated. Data dimensions were reduced by using PCA with a cut-off of 95%. PCA rotates the data in such a way that the information retained by q PCs is higher than any combination of q dimensions of the original data set. The dimension reduction of PCA prior to FDA has been shown to be beneficial by reducing the influence of noise in the fingerprint (De Roy et al. 2012). The FDA transforms the data in order to characterize the differences between k different groups by projecting the data onto discriminants. The discriminants are linear
combinations of the features that maximize the between class variance compared to the within class variance. Thus we can detect and compare the differences between reference and flushing samples collected in this study.

2.4 Sample analysis by other microbiological and chemical methods

Free chlorine concentrations were measured on-site with a Hach Pocket Colorimeter II, using DPD reagent (N,N-diethyl-p-phenylenediamine). Samples above detection limit (2.2 mg/L) were diluted in Case 1 and 2. For microbiological analyses by plating, separate samples were taken by the water supplier in sterile plastic 500 mL bottles, containing 1 mL of a 250 mM Na$_2$S$_2$O$_3$-solution as a quenching agent. Samples were analysed for total Coliforms and *E. coli* (ISO 9308-1), Enterococci (7899-2) and heterotrophic plate count (HPC, ISO 6222) by the appropriate accredited methods for drinking water analysis. For total Coliforms, *E. coli* and Enterococci, each time 100 mL of sample was filtered over sterile 0.45 µm membrane filters. For total Coliforms, this filter was incubated for 21 hours at 36°C on Tergitol-7 agar. After incubation, yellow-orange colonies were regarded as Coliforms, which has to be confirmed after an oxidase test. For *E. coli*, the procedure was similar, although incubation took place at 44°C and the confirmation included both an oxidase and indol test. For Enterococci, the membrane filters were incubated for 44 hours at 36°C on Slanetz & Bartley agar. After incubation, pink-red colonies were regarded as Enterococci, which has to be confirmed using plating on selective Enterococcus agar. Since no samples have been declared to contain Coliforms, *E. coli* or Enterococci, the confirmation procedures will not be explained. For HPC, 1 mL samples were incubated on glucose-free Water Plate Count agar using the pour-plate method. Colonies were counted after 68 hours of incubation at 22°C.
3. Results

3.1 Case 1: Brakel

In case 1, a 2525 m pipe section with diameter 1050 mm was emptied and flushed. The reference point was located 2940 m before the closed pipe section. This long distance had as a consequence that the reference water only reached the flushing point an estimated 16.9 hours after the sampling campaign began or 11.5 hours after the first water had reached the flushing point. An absolute comparison of two water samples could not be made in this case. This estimated delay is taken into account in all of the displayed figures, the calculations for the delays in the three cases are shown in Appendix 6-1.

Both the intact and total cell concentrations in the reference water heavily increased after 2 hours (intact cells: $1.3 \times 10^3$ cells.mL$^{-1}$ to $10.5 \times 10^3$ cells.mL$^{-1}$) to stay at this increased level for several hours (Figure 6-2). After 10 hours of sampling, the cell concentrations suddenly decreased again to the initial level. Similar fluctuations in the cell concentrations were seen when sampling the reference water the weeks before (data not shown). The flushing water showed stable cell concentrations around $2.3 \times 10^3$ intact cells.mL$^{-1}$ after two hours of sampling. The FCM fingerprinting based on FDA analysis shows how similar or different samples are, based on their relative distance in the plots. In this case, the plot showed large differences between the first two flushing samples (0 and 2 minutes of flushing, respectively 0F and 0.03F on Figure 6-2) and all later samples. While the 2 hour sample (2F) is close to the reference samples, no distinction is possible between flushing and reference samples after 4.25 hours of flushing.
Figure 6-2: Cell concentrations (A) and SGPI fingerprinting analysis (B) of the flushing and reference samples of case 1 (n=4). The reference samples are shifted according to their delay at the flushing point, as explained in Appendix 6-1. Several flushing (F) and reference (R) samples are indicated with their timing (hours), the timing of sample collecting is shown in the lower bars. After two hours, the flushing sample cell concentrations are stabilizing. The fingerprinting analysis, shows that the flushing and reference samples are similar after 4.25 h, as seen from their small distance in the graph.
Free chlorine concentrations were measured and kept high (10-20 mg.L\(^{-1}\)) for 30 minutes, to reach the legal drinking water limit (max. 0.25 mg.L\(^{-1}\) free chlorine) after 60 minutes (Figure 6-3). Microbial sample analysis of the flushing water on Coliforms, *E. coli*, Enterococci and HPC showed that none of these were present after two hours of flushing, so the water was approved.

**Figure 6-3: Chlorine concentrations in the flushing samples for each case.** After 1.7, 0.5 and 2 hours, the concentrations were lower than the legal drinking water limit. In case 3, samples were never diluted for measurements, resulting in an upper detection limit of 2.2 mg.L\(^{-1}\).
3.2 Case 2: Zuienkerke

In case 2, the closed pipe section measured 5820 m with 400 mm diameter, the reference point was situated 438 m before this section. Compared to case 1, more overlap could be seen on the timing of the reference samples, as a result from the reference point being closer located to the closed pipe section. The reference samples showed stable intact cell concentrations around $2.1 \times 10^3$ cells.mL$^{-1}$ and total cell concentrations fluctuated slightly higher than this (Figure 6-4). The flushing samples initially fluctuated above $10^5$ intact cells.mL$^{-1}$ and lowered under $10^4$ intact cells.mL$^{-1}$ after less than two hours of flushing. After 2.5 hours, flushing cell concentrations kept stable in the range of reference cell concentrations. The fingerprinting analysis of the SGPI samples is plotted based on the first two discriminants of the FDA. This showed a stabilization of the flushing samples in the region of the reference samples after 2 hours of flushing (2.1F). The free chlorine appeared after 20 min as a sharp peak at the flushing point (peak width < 20 minutes, maximal measured concentration 21.3 mg.L$^{-1}$) before and after which the drinking water limit was reached (Figure 6-3). The analysis on E. coli, total Coliforms, Enterococcus and HPC showed none of them present after two hours of flushing and approved the water.
Figure 6-4: Cell concentrations (A) and SGPI fingerprinting analysis (B) of the flushing and reference samples of case 2 (n=3). The reference samples are shifted according to their delay at the flushing point, as explained in Appendix 6-1. Several flushing (F) and reference (R) samples are indicated with their timing (hours), the timing of sample collecting is shown in the lower bars. After 2.5 hours, the flushing sample cell concentrations remain stable in the range of the reference sample cell concentrations. The reference samples cluster locally in the FDA analysis, which indicates their high relative similarity. The flushing samples are stabilizing towards this region to cluster with the reference samples after two hours of flushing.
3.3 Case 3: Aalst

In case 3, a pipe section of 4490 m was closed and the reference point was located at the closing valve. The amount of samples taken was decreased to what is absolutely necessary for the water supplier to get basic understanding of the flushing procedure. Sample analysis was limited to SGPI staining in duplicate. After an initial sample, flushing samples were taken only after 2 hours, when free chlorine concentrations were lower than the drinking water limit (Figure 6-3). This limited experimental set-up was chosen in order to search for a practically feasible technique for future non-research applications. As in case 1, there was a large amount of stagnant water between the reference point and the sampling point, resulting in the absence of an overlap in sample timing of the reference and flush samples. While reference samples remained stable around $6.1 \times 10^3$ cells.mL$^{-1}$, flush samples started around $42.5 \times 10^3$ cells.mL$^{-1}$ and lowered steadily, except for one small peak, until $13.6 \times 10^3$ cells.mL$^{-1}$ after 6.3 hours of flushing (Figure 6-5). The fingerprinting analysis shows that, although the cell concentrations were still higher, the samples after 2 hours (2F) clustered already together with the reference samples. The analysis on *E. coli*, total Coliforms, *Enterococcus* and HPC showed none of them present after four hours of flushing and approved the water.
Figure 6-5: Cell concentrations (A) and SGPI fingerprinting analysis (B) of the flushing and reference samples of case 3 (n=2). The reference samples are shifted according to their delay at the flushing point, as explained in Appendix 6-1. Several flushing (F) and reference (R) samples are indicated with their timing (hours), the timing of sample collecting is shown in the lower bars. A gradual decrease of the flush sample cell concentrations can be seen, although after 6.3 hours, these are still higher than the reference sample cell concentrations. The fingerprinting analysis shows that variance between the reference samples mutually is similar to the variance between reference and flushing samples, as can be seen from their relative distance in the graph.
4. Discussion

This study examined three full scale cases where drinking water pipes were disinfected and flushed after maintenance works. Samples were taken at the reference and flushing points before and after the maintenance works. Based on FCM measurements and FCM fingerprinting, the flushing-out of bacteria and other particles was measured. Since drinking water networks are well maintained and extensively controlled systems, the hygienic quality of the reference water can be assumed as good. Consequently, the moment when the reference and flushing samples are the same can serve as an indication for the end of the necessity for flushing procedure.

4.1 Effect on cell concentrations

In each case, cell concentrations in the flushing samples lowered over time. In case 1 and 2, cell concentrations in the flushing samples decreased and stabilized within 2-3 hours (Figure 6-2 and 6-3). In case 3, flushing sample concentrations were not yet stabilized after 6 hours of flushing (Figure 6-4). This is expected to be caused by the large amount of stagnant water in the closed pipe section, which was not refreshed during time of the maintenance works (± 36 hours). During pipe filling operations, this stagnant water is pushed through the pipe by the flow of fresh water coming in. As such, the batch of stagnant water will keep at the waterfront and be the first water to be discharged, once the pipe is full and the flushing and discharging is starting. However, depending on local geography, extensive mixing of the stagnant water with the fresh water can to be expected in practice, resulting in a gradual decrease in the fraction of stagnant water in the discharged flushing water. The calculations Appendix 6-1 show that stagnant water mainly affected case 3, where this stagnant water discharge takes 4.8 hours, assuming no mixing with fresh water. It is well known that stagnant water shows increased cell concentrations (LeChevallier et al. 1987, Lehtola et al. 2007, WHO 2011), so the higher initial concentrations in the flushing samples compared to the reference are likely to result both from the flushing operation (biofilm and particle resuspension) and bacterial growth due to water stagnation (LeChevallier et al. 1987, Mathieu et al. 2014). The gradually decreasing cell concentrations in the flushing samples are likely to be explained by the strong mixing of this initial stagnant water with the fresh water front. This would as well explain why these increased concentrations lasted for more than 4.8 hours.
Case 2 reached notably high maximal cell concentrations: the intact cell concentrations reached after 10 min of flushing \((326 \pm 4) \times 10^3 \text{ cells.mL}^{-1}\), which is 2 orders of magnitude higher than the stable concentrations after flushing. For other cases, this initial increase is limited to less than one order of magnitude. This is most likely to be explained by the pipe section being situated at the end of the network, at large distances of the water treatment plants, which possibly resulted in decreased chlorine residual concentrations, increased biofilm growth and concomitantly, more possible biofilm dispersion in the bulk water (Lee and Kim 2003). In addition, the smaller pipe diameter leads to a higher water velocity of 0.2 m.s\(^{-1}\) compared to the larger pipes (± 0.1 m.s\(^{-1}\)) (Table 6-1). The higher shear stresses can initially slough more biofilm and resuspend more loose deposits (Choi and Morgenroth 2003, Lehtola et al. 2006), although it should be noted that velocity criteria for general pipe flushing are higher, even 1.5 m.s\(^{-1}\) in the Netherlands (Vreeburg and Boxall 2007). An additional consequence of the smaller diameter is the higher surface to volume ratio of 10, compared to 3.8 or 4.4 in case 1 and 3. This leads to a larger influence of pipe surfaces, discharging biofilm cells and deposits in the bulk water.

In case 2, it was possible to see when reference and flushing samples were similar based on cell concentrations, but in case 1 and 3, only assumptions were possible due to the time shift in sampling, as explained in Appendix 6-1. The fluctuating cell concentrations in case 1 could be explained by a varying water source and show the importance of having good reference sample measurements, while the gradually decreasing concentrations in case 3 show the problem when a lot of stagnant water is present after the reference. Ideally, when the situation allows this, the amount of stagnant water should be limited.

### 4.2 Effect on FCM fingerprint

FCM fingerprinting tools are statistical analysis methods which help to detect and quantify the differences between samples (Koch et al. 2014). The use is ideally suitable for monitoring community stability and drinking water quality, where a fast analysis procedure is required (De Roy et al. 2012). In this study, the flowFDA package (Clement and Thas 2014) was used for making a fingerprint of samples and compare these with Fisher discriminant analysis (FDA). The results are shown in scatter plots based on the first and the second discriminant of the
samples. The relative distance between the samples can serve as an indication how similar or different the samples are.

Based on the SGPI analysis, the FDA shows for each case gradual changes in the flushing samples, which are moving in the proximity of the reference samples within 2-4 hours of flushing. In case 1, it is remarkable that the reference cell concentrations show strong variations while this is limitedly affecting the fingerprint. This FCM fingerprinting technique is based on the raw data without gating for cells. As a result, the background in the sample can play an important role in the fingerprint. This should in this case be seen as an advantage since the FCM background is mainly caused by organic particles (cell fragments, viruses, free DNA, humic acids, etc.) or inorganic particles (salt crystals, metal oxides, etc.) present in the samples. While the assessment of the present bacteria remains the main measurement and most important for the hygienic quality of the water, the incorporation of the background in the fingerprinting is expected to make this technique more conservative to judge when reference and flushing samples are similar. The consequence of background in the water can be discolouration, a common water quality issue (Husband and Boxall 2011) but more important, it might also contain the suspended particles and loose deposits, colonised by opportunistic pathogenic bacteria like Mycobacteria (Torvinen et al. 2004). Examples on how background is affecting the fingerprinting analysis is given in Appendix 6-2.

After case 1, it was concluded and verified by additional tests that the added sodium thiosulphate, especially in combination with the chlorine, produced disproportionate amounts of background (Figure 6-6). This background was not sample inherent but induced by the measurement procedure and heavily affecting the fingerprinting analysis. Since time between sampling and analysis was short, avoiding this background was regarded more important than the chlorine quenching. Therefore, thiosulfate addition was omitted from case 2 onwards. For similar reasons, the focus was put on the fingerprinting analysis of SGPI-measurements and not of SG-measurements. The SGPI-measurements yield a slightly higher fraction of cells in the total number of analysed particles, in case 2 for example 9.3 ± 4.6% compared to 6.6 ± 2.4% for SG measurements. While background is truly inherent to the sample when thiosulphate is omitted, the large fraction of background still results in a fingerprint focussed almost solely on the background. Nevertheless, the fingerprints based on SG-measurements are shown in Appendix 6-3 at the end of this chapter. Ideally, the
measurement procedure should undergo further optimization to balance the relative importance of background and cells better.

**Figure 6-6:** The effect of sodium thiosulphate addition on the background of flow cytometric measurements. The addition of 1 mM $\text{Na}_2\text{S}_2\text{O}_3$, even in ultrapure water as in this example, results in a strong background formation. This is most pronounced in case of a SG staining, although it is still clear without staining. The presence of 1 mg.L$^{-1}$ Cl$_2$, which is a realistic concentration in this context, intensifies this effect.

### 4.3 Different methods

While cell concentrations give you clear and straightforward information, the fingerprinting takes all data into account. In practice, fingerprinting appears to be more conservative in case 1 but cell concentrations are more conservative in case 3. In case 2, both methods seem to stabilize and be similar as the reference samples at the same time. It is concluded that the relative importance of the background decreased the fingerprinting sensitivity for bacterial measurements. Therefore, both methods should be combined.

FCM has been shown before to be a valuable method for monitoring drinking water processes and network biostability (Hammes *et al.* 2008a, Liu *et al.* 2013a). The moment when reference and flushing samples are similar according to FCM, should consequently be a good indication
Flow cytometric follow-up of network flushing for hygienic water quality. However, care should be taken and until FCM is more widely used in comparable applications and the interpretation of the results is better understood, it is ideally backed up by other methods. EPA guidelines suggest to plate for *E. coli* (EPA 2010), which is together with other faecal indicators and heterotrophic plate count the standard method in legislation for microbial drinking water quality (Anonymous 1998, Cabral 2010, Edberg *et al.* 2000). The water supplier approved the hygienic quality of the flushing samples in this study mainly based on heterotrophic plate counts and faecal indicator bacteria. In each of the three studied cases, FCM showed to be more conservative since it took at least as much or more flushing before samples were approved by FCM, compared to the classic methods.

General FCM staining procedures like SG and SGPI stain the whole bacterial community (Wang *et al.* 2010b) and are hardly or not correlating with heterotrophic plate counts (Liu *et al.* 2013a, Siebel *et al.* 2008) or giving info about faecal indicator organisms. This should however not be seen as a disadvantage, since heterotrophic plate count bacteria are as well only a process indicator without health information also irrelevant as a health risk indicator (Allen *et al.* 2004, Sartory 2004). Furthermore the lack of a correlation between the presence of indicator organisms and real pathogens is shown (Horman *et al.* 2004). Finally, plate counts do not give results fast (Mesquita *et al.* 2013). An alternative, faster and more exact detection method could be qPCR. It can give within the same day results for general cell concentrations or can search for a wide range of pathogenic bacteria and viruses (Aw and Rose 2012). It provides more information than FCM, although it is more complicated. ATP analysis is both fast, easy and is shown to correlate well with intact cell concentrations, based on SGPI-staining (Hammes *et al.* 2010b). It gives however no information about the composition of the microbial community and the similarity of flushing and reference samples. Finally, particle counters or turbidity measurements give fast information about particle resuspension and discolouration of water (Verberk *et al.* 2008) but have to be combined with microbial methods.
5. Conclusions for practical use

Flow cytometry showed to be able to deliver fast and reliable information on pipe flushing operations. As a conservative guideline, the flushing can be regarded as finalised and the pipes can be set back in operation, when both FCM cell concentrations and FCM fingerprints of reference and flushing samples are similar. In order to use this method, it should be taken into account that:

- A good reference and flushing sampling point are installed;
- The amount of stagnant water between the reference point and flushing point is minimal, in order to enable fast sample comparison between reference and flushing samples (as seen from case 1 and 3);
- Thiosulphate should not be added for chlorine quenching, however, this stresses the importance of fast sample measurement (as seen from case 1);

Since no specific information about the present strains is given, this method is advised to be combined with assessment of indicators of hygienic quality, such as coliforms or \textit{E. coli}, for final approval.

When executed well, large amounts of water and time can be saved by shortening the flushing procedure, thus helping drinking water providers to safeguard both the quality and the supply of the water to their customers.

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Chapter 7

Flow cytometric study of bacterial regrowth in a local drinking water distribution network

Chapter partially redrafted after:


Abstract

Bacterial growth in drinking water networks can result in health-based implications, affect network integrity and hydrology and induce customer complaints. Legislation appoints cultivation-based analysis for assessing bacterial growth, although these methods yield incomplete, inaccurate and slow information. In this study, cultivation-based methods were compared to flow cytometric methods for the study of growth in a local drinking water network. Flow cytometric cell concentrations indicated growth in several samples, and suggested a geographic pattern. However, further data analysis with flow cytometric fingerprinting algorithms, showed the different origin of this growth; low similarity between samples with elevated cell counts indicated that growth occurred in the sampled household plumbing installations and not in the central drinking water network. This thorough analysis was possible based on fast obtained flow cytometric data, while cultivation-based methods were not yielding any information: nearly none of the plates showed any colonies after 72 hours of incubation. Flow cytometry showed to be far superior over cultivation-based methods for a network growth study.
1. Introduction

Bacterial growth in drinking water distribution systems (DWDS) can lead to various types of problems, dangers and discomfort (van der Kooij and van der Wielen 2014a). Next to possible health-based implications due to the growth of pathogens (van der Wielen et al. 2014), bacteria can also affect network integrity by inducing biocorrosion (Féron and Neumann 2014), or affect the network hydrology. Finally, bacteria can be the cause of taste and odour formation, which leads to discomfort and customer dissatisfaction (Piriou et al. 2001). A more extensive description of these factors, caused by bacterial regrowth in a drinking water network, is given in Chapter 1, §1.3.

The use of disinfection with disinfectant residual, usually chlorine or chloramine, is the main method to limit regrowth in DWDS (Freese and Nozaic 2004). Alternatively, in many countries in northern Europe, water utilities aim at the production of biostable water without disinfectant residual, in combination with a stringent maintenance of the network quality (Medema et al. 2014). Regrowth is routinely assessed by water utilities using heterotrophic plate counts (HPC) due to regulations, although the method is not sensitive. For a more extended discussion on this topic, the reader is referred to Chapter 1, §3.1. Next to HPC, ATP-analysis is regularly used as a more sensitive and faster measurement of bacterial concentrations (Hammes et al. 2010b, van der Wielen and van der Kooij 2010).

In this study, flow cytometric (FCM) cell counts were used for a fast assessment of the bacterial presence in a local, non-chlorinated drinking water network. FCM cell counts have been regularly used in the recent past as a descriptive parameter for water treatment processes (Hammes et al. 2008a, Helmi et al. 2014b) and regrowth in networks (Gillespie et al. 2014, Nescerecka et al. 2014). Flow cytometric fingerprinting will be used as a sensitive method for further data analysis to characterise the regrowth (De Roy et al. 2012, Prest et al. 2013).
2. Materials and methods

2.1 Description of the studied installation and network

The studied network was part of the distribution network of water utility TMVW (FARYS), and had a yearly distribution of 800,000 m³ of water. A schematic overview of the treatment installation is presented in Figure 7-1. In short, groundwater was extracted (± 500,000 m³ per year) and treated solely by activated carbon filtration, in order to remove pesticide residuals. Before distribution, this treated water was stored on average for 7.6 hours together with chlorinated drinking water, originating from another water source (± 300,000 m³ per year). Although a chlorination installation was present, it is generally not in use. Therefore, the possibly present disinfection residual in the added water served as the only means of disinfection. At the day of sampling, the free chlorine concentration measured in the network was 80 µg.L⁻¹.

This particular network was sampled because of the low connectivity with the surrounding network; while usually, it is hard to determine the origin of distributed waters, it was well characterized in which parts of the network water was received from this central water production plant.

2.2 Sampling

Sample collection was conducted at 26 different household taps, with a maximal geographical spread over the network, and at the water treatment installation (raw water, filtered water before storage, distributed water). Samples (500 mL) were stored cold in clean glass bottles until analysis (cooling box, not temperature controlled). All sampled taps underwent exterior disinfection with 70% ethanol and were opened at moderate flow rate 5-10 minutes prior to sampling.
Figure 7-1: Representation of the studied drinking water system. The taps which were sampled in the drinking water production plant (as described in 2.2) are indicated in circles.

2.3 Flow cytometric measurement

FCM measurements were conducted within 3 hours after sampling. Measurements were performed in triplicate, using two independent staining procedures for the assessment of total and intact cell counts. Measurements were performed as described by Van Nevel et al. (2013b) and Prest et al. (2013). In short, 200 µL aliquots were pipetted in a 96-well multititer plate and stained with 2 µL of SYBR Green I (SG, Invitrogen, 100x diluted in dimethylsulphoxide from stock) for total cell counting. For intact cell counts, propidium iodide (PI, Invitrogen, final concentration 4 µM) was added together with SG. After 13 minutes of incubation at 37°C, sample measurement was done with an Accuri C6 (BD Biosciences) equipped with an autoloader. Cell counting was done by enumeration of the number of particles in a set volume, after gating on green versus red fluorescence (FL1/FL3) plots in the BD Csampler software, as described elsewhere (SLMB 2012).
2.4 Flow cytometric fingerprinting

After flow cytometric measurements, the data was further analyzed using fingerprinting methods (De Roy et al. 2012, Koch et al. 2014). The data analysis pipeline was similar as described in Chapter 6, § 2.3. However, in a last step, no FDA analysis was performed but a cluster analysis was done based on Gaussian mixture models, included in flowFDA (Clement and Thas 2014).

Maps were plotted in R based on the RgoogleMaps-package and the coordinates of the sampling sites, extracted from Google Maps.

2.5 Bacterial analysis by agar-plating

Samples were plated less than 24 hours after sampling for heterotrophic plate counts (HPC), using both water plate count agar (WPC agar) and R2a agar, and for total coliforms, on tergitol 7 agar. Agar compositions are displayed in Table 7-1. For HPC, 1 mL of sample was added to liquid agars (the pour-plate method), which could solidify and incubate at 22°C for 72 hours. For total coliform analysis, 100 mL aliquots were vacuum-filtered over 0.45 µm sterile membranes. The filter was applied on tergitol 7-agar and incubated at 36°C for 21 ± 3 hours. Coliforms are able to convert lactose to acids, using β-galactosidase and therefore, bromothymol blue, present in tergitol 7-agar, changes color to yellow. Coliforms are thus detected as yellow colonies.
Table 7-1: Composition of the used agar types.

<table>
<thead>
<tr>
<th>Component</th>
<th>WPC-agar (g.L⁻¹)</th>
<th>R2a-agar (g.L⁻¹)</th>
<th>Tergitol-7 agar (g.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Tryptone</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Peptone</td>
<td></td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>K₃PO₄</td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td></td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td></td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Sodium heptadecyl sulphate</td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
</tbody>
</table>
3. Results and discussion

3.1 The bacterial community in the water treatment process

Samples were taken within the water treatment process in order to examine the effect of the treatment process on the indigenous bacteria. The activated carbon filtration increased the cell concentrations heavily; from \((21 \pm 2) \times 10^3\) to \((132 \pm 3) \times 10^3\) intact cells.mL\(^{-1}\). A clear pattern could be observed as well in the flow cytometric plots (Figure 7-2). This ‘bacterial inoculation’ by the community residing on biologically colonised filtration media has been described before (Hammes et al. 2008a, Pinto et al. 2012).

More remarkably, intact cell concentrations dropped after storage (this sample will further be regarded as the reference sample for comparison with network samples). Nearly the whole previously intact community (96%) had now damaged membranes or was fully disintegrated by the chlorine. This indicated the strong effect of the present chlorine residual in the added chlorinated drinking water, although this was regarded as negligible before by the water supplier. The presence of a chlorine effect also stressed the importance of using of a staining protocol, including a viability indicator. Propidium iodide, used in this study in combination with SYBR Green, is ideally suitable since it detects membrane integrity, a parameter typically affected by chlorination (Berney et al. 2008, Ramseier et al. 2011).
Figure 7-2: Flow cytometric analysis of the bacterial community through the treatment process. The bacteria are indicated in the plots with approximated polygons. It can be seen that the activated carbon filtration is shaping the bacterial community. After storage, especially the intact cell concentrations drop sharply, while total cell concentrations only lower slightly. In the SG-plot of this distribution sample, a distinct pattern with three groups of bacteria can be seen (n = 3 x 3).

3.2 Bacteria in the network

During the sample round, 26 samples were collected in public places in the network; in supermarkets, restaurants, etc. However, there was uncertainty about five of those samples since the local employees were not sure about the use of piped drinking water or an own water source, or it was unclear if the distributed water originated from the studied water production center. For further analysis, these five samples were taken out of the dataset. The total cell concentrations in the network were lower compared to the reference sample from the water production plant in 15 out of 21 cases (Figure 7-3). Intact cell concentrations were
lower in 9 out of 21 cases, although mostly, these were too low to provide a reliable comparison. On average, the water was stored in the buffer for 7.6 hours, but the network residence time can as well be up to several days. These lower cell concentrations were a second indication of the effect of the chlorine residual, which was apparently further damaging cells during this additional storage time in the network.

As an indicator of bacterial regrowth, the ratio of intact to total bacteria was introduced. Initially, this value was low (0.05) since the chlorination damaged the cell membranes, but left most damaged bacteria detectable. In 7 cases, this ratio was at least doubled in the network samples, although in one case (17) this was clearly due to lowered total cell concentrations.

Flow cytometric plots typically showed the same distinct pattern as previously seen in the reference sample, although novel patterns appeared in some samples (Figure 7-4), indicating regrowth in these samples.

**Figure 7-3: Absolute cell concentrations of the bacterial community in the network.** Samples 1-21 were network samples, ‘R’ indicates the reference sample at the treatment plant. Inlay box plots show the median (horizontal line), 25 and 75 percentiles (box) and 10 and 90 percentile (lines). Sample 1, 2, 10, 11, 13, 17 and 19 have at least a doubled intact/total cell ratio, although in case of 17, this is due to lower total cell concentration (n = 22 x 3).
Figure 7-4: Flow cytometric plots of network samples. The bacteria are indicated in the plots with approximated polygons; most samples still hardly have any intact cells present. The distinct 3-group-pattern from the reference water (Figure 7-2) is usually easily recognisable. Some samples however (e.g. sample 1) show a strong regrowth, as can be seen from both intact and total cell analysis.

3.3 Geographic analysis

From the previous discussion, it became clear that bacterial concentrations remained low in most samples, although there were some exceptions. These deviations could have two different origins; either real regrowth of bacteria in the network, or being the result of bacterial growth in the household installation. Both situations are well-known occurring problems (Lautenschlager et al. 2010, Lipphaus et al. 2014, 2014b), although only network regrowth can fully be attributed to and controlled by the water supplier, and was the real goal of this study. The origin of regrowth was searched by a geographic analysis, looking for a geographic pattern correlated with cell concentrations. Two pathways were followed: the use of a geographic heath map of relative cell concentrations and a fingerprinting-based clustering of the samples, which searched in an objective way for differences between samples.

In the geographic heath map, the relative cell concentrations of all samples were plotted on a map, at their actual geographic sampling location. A white-to-red colour scale indicated the relative low or higher cell concentrations. The result for intact cells is shown in Figure 7-5. It could be seen that four of the samples with elevated cell concentrations and elevated
intact/total ratios, were situated in the north-east corner of the sampled area, which could be an indication of local regrowth in the network, but no conclusive proof.

The fingerprinting-based clustering of samples consisted of an algorithm, searching for similarities between the samples, based on the raw data of flow cytometric analysis. Next, an automated clustering is done, whereby the algorithm decides on number of groups and number of samples in each group. The results, based on the intact cell staining, are shown in Figure 7-6. In this case, samples were clustered in 8 groups of a varying amount of samples. Each of the four samples with increased cell concentrations in the north-east corner of the area was clustered in another group, indicating limited similarity between these samples. This was an indication that regrowth in these samples originated from different sources, more specifically the household plumbing installations, and not from the drinking water network.

As a final assessment, a visual comparison of the flow cytometric plots of these four samples was made (Figure 7-7). Low similarity between the present bacterial communities could be seen in these samples, confirming the earlier made conclusions about different origins of regrowth.
Figure 7-5: Geographic health map of intact cell concentrations. The intact cells were plotted on their sampling location in the drinking water network, the dot surface represents the relative intact cell concentrations. The reference sample (R) at the central installation showed relatively low concentrations, while four samples in the north-east area harboured the highest intact cell concentrations.
Figure 7-6: Geographic map of fingerprinting-based clustered samples. A statistical analysis automatically clustered the samples in eight groups of one or more samples, which are depicted by the A-H on the network map. No geographic patterns emerged for samples with increased cell concentrations, the grouping appeared to be independent of location for these samples.
Figure 7-7: Visual comparison of four samples showing bacterial regrowth. These four flow cytometric plots of intact cell staining originate from samples with elevated cell concentrations and were sampled geographically close to each other. The low relative similarity of the bacterial communities in the plots however suggests that regrowth in these samples happened independent from each other.

3.4 Comparison with other methods

All samples were also analyzed by the routinely used plate counting methods, the basis for microbial monitoring of drinking water and incorporated in drinking water legislation worldwide (Edberg et al. 2000, WHO 2003b). Water plate count agar was used for heterotrophic plate counts, since this method was routinely used by the water utility. R2a agar was used in addition, since this agar is known to result in a higher recovery of the present bacteria and is more useful for detecting regrowth in a drinking water network (Reasoner and Geldreich 1985, Uhl and Schaule 2004). Finally, coliforms presence was verified in 100 mL water samples. The obtained data from these culture-based method was disappointingly scarce. Only one sample resulted in the detection of HPC-bacteria after 72 hours: sample 2, which was seen by FCM to have second highest intact cell concentrations, containing 14 CFU.mL⁻¹ (WPC agar) or 6 CFU.mL⁻¹ (R2a agar). Two samples, 13 and 14, both had one coliform detected in 100 mL. The scarce data provided by culture-based methods could not serve as a basis for a regrowth study in case of this drinking water network. Even if elevated cell concentrations would have been measured in several samples, it would remain unclear
whether the origin of regrowth is to be situated in household plumbing installations or in the central drinking water network.

4. Conclusions

In this study, flow cytometric methods were tested for their usefulness for characterising bacterial growth in a drinking water network, and tested against the routinely used cultivation-based methods. The flow cytometric analysis revealed increased bacterial presence in some samples, but this was fully attributed to bacterial regrowth in the household plumbing and not in the network. It has to be noted that, while these methods can indicate the absence of bacterial regrowth, the opposite has to be verified further and confirmed to exclude the occurrence of single events being detected. While a thorough analysis was possible based on the data obtained with flow cytometry, the cultivation-based methods did not yield any significant or useful data. It is suggested that the future use of cultivation-based methods has to be reconsidered for this type of applications. While the detection of indicator organisms can deliver important health-based information, it is debatable how relevant general counting methods like heterotrophic plate counts are, while more performant methods are available. For this type of applications, flow cytometry showed to be far superior in both speed and obtained information.

Acknowledgements

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GENERAL DISCUSSION
AND FUTURE PERSPECTIVES
'Recently there has been a flurry of reports suggesting that bacteria may exist in an eclipsed state, escaping detection by standard methods. The ensuing debate has been unusually energetic for the normally cultured community of microbiologists.'

Gregg Bogosian, A matter of bacterial life and death, 2000
CHAPTER 8

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Parts of this chapter are redrafted after:


1. Main outcome and positioning of this work

1.1 Positioning of this work

The growth of bacteria in drinking water can induce serious problems as well as nuisance; health-related dangers, taste and odour formation, biocorrosion, hydrological problems, etc. (Chapter 1). Therefore, it is of utmost importance to understand and characterise this growth and two factors have been chosen for a more fundamental study on bacterial growth: induction of biofilm growth and bacterial invasion. Over 95% of the bacterial biomass in DWDS is present in biofilms (Flemming 2002) and transparent exopolymer particles are hypothesised as a new factor possibly inducing biofilm growth (Bar-Zeev et al. 2012). Chapter 2 examines the plausibility of this hypothesis, in the context of Belgian drinking water systems. Chapter 3 is a more general study on the effect of nutrient availability and the indigenous community on invasion of an invading species in an existing bacterial community in drinking water.

In order to understand and assess bacterial growth, performant tools need to be available. In routine applications, heterotrophic plate counts are still the gold standard (WHO 2003b), while this work argues on the feasibility of FCM cell counts to replace HPC as an monitoring parameter (Hammes et al. 2008a). The practical interest of the water utilities in this type of tool is demonstrated by the acceptance of an FCM method in the Swiss drinking water guidelines (SLMB 2012). Chapter 4 and 5 contain a more fundamental study on the
The main outcomes of this work are (i) the growth of invaders in drinking water is heavily affected by the indigenous community, (ii) flow cytometry is a fast and precise analytical tool, which can be used to replace routine analysis of drinking water by heterotrophic plate counts and (iii) next to routine applications, FCM can also be used for various other drinking water analyses, which were before not feasible based on growth-based methods.

Transparent exopolymer particles recently gained interest as new factor in biofouling and as possible biofilm inducer (Bar-Zeev et al. 2012, Berman 2005, Villacorte et al. 2009a). Chapter 2 describes the presence and removal of these TEP in three model drinking water production plants as a first step to assess their possible role as biofilm inducer in DWDS. The low TEP-concentrations measured and good removal efficiencies in the sampled drinking water systems could lead to the conclusion that their importance in the studied DWDS is questionable. Therefore, we concluded that TEP should not be a major topic of research as a biofilm inducing factor in DWDS.

Chapter 3 presents a worst case scenario whereby an invading bacterium was added to an existing bacterial community in different water types. In this case the invader was Pseudomonas putida, a species closely related to Pseudomonas aeruginosa and equipped with a very versatile metabolic system (Timmis 2002). Whenever enough carbon and phosphorus was available, this invader could proliferate to high concentrations. However, after a first phase of exponential growth in surface water, a fast decay could be seen. This was not the case in drinking water samples, harbouring the same concentrations of added nutrients. A system was proposed, whereby the indigenous surface water community was more resistant to invasion compared to drinking water, even when tested at the same initial concentrations. This study hereby stressed the importance of the bacteria present in water, as a decisive factor for biostability and invasion.

Chapter 4 & 5 show a study of FCM as a possible tool for routine assessment of bacteria in drinking water. Staining procedures have been optimized and standardised before (Prest et
General discussion and future perspectives

*al. 2013*) but background had always been considered as something to be avoided. However, **Chapter 4** is guiding towards a deeper understanding of flow cytometric background. Only in this way, it is possible to apply fixed gating for routine applications and understand when this technique is not applicable. In addition, it has been shown that background consists of useful information. After getting this more complete understanding of flow cytometric results, including sample background, **Chapter 5** is turning FCM in a high-throughput process. Automation of measurements is an absolute advantage for any technique used for routine applications (*e.g.* in drinking water analysis). A procedure could be developed allowing to measure 96 samples in an precise (RSD < 3%) and fast way (76 minutes), as long as a highly standardised staining procedure was used (*Prest et al. 2013*).

Finally, **Chapter 6 & 7** study real practical applications of FCM. In **Chapter 6**, drinking water pipes were cleaned after maintenance and an immediate follow-up of the water quality was done with FCM. Both absolute cell counts and sensitive FCM fingerprinting (*De Roy et al. 2012*) were used for immediate comparison of clean reference water (drinking water used to flush the pipe) and flushing water, used for the pipe flushing. Cell counts of the flushing water were in each case lowering towards the ones measured in the reference water. This was confirmed by the flow cytometric fingerprints of the samples, being increasingly similar to each other over time. Flow cytometric based methods were concluded to be a rapid and valid addition to culture-based methods for immediate follow-up of network maintenance.

In **Chapter 7**, a one-day study of bacterial growth in a drinking water network is discussed. Fast FCM analysis of samples, collected in a local drinking water network, provided the possibility to do an immediate, on-site assessment of bacterial regrowth in the network. Absolute cell concentrations were low and few samples indicated network regrowth. To the contrary, the samples with elevated cell concentrations showed a clearly different fingerprint, indicating a different origin of regrowth. It was concluded that the measured regrowth originated from the household plumbing installations and not from the distribution network.
2. Review on overlooked factors affecting growth in DWDS

Two overlooked factors, possibly affecting the bacterial presence in DWDS, have been studied in Chapter 2 and Chapter 3. A short evaluation on their actual relevance is given below.

2.1 Transparent exopolymer particles

Transparent exopolymer particles, or TEP, were regarded as a possible biofilm inducer in drinking water. The sticky properties, their possible role in biofilm formation and the limited removal efficiencies of filtration processes were reported in previous studies and discussed in Chapter 1 §1.2 and Chapter 2 §1. Therefore, as a first step in the assessment of their biofilm formation properties in DWDS, their presence was examined throughout drinking water production plants, based on either surface, waste- or groundwater. The raw water TEP concentrations were already remarkably low compared to literature (pTEP-concentrations <5 - 102 µg X eq.L^-1 in our study, versus 36 - 9038 µg X eq.L^-1). The differences were attributed to (i) different raw water sources: none of these were supportive for abundant algae growth; (ii) climate differences: most other TEP research is situated in the Mediterranean climate zone, where elevated temperatures support algae growth and TEP production (Passow 2002)); (iii) seasonal differences: although algae blooms do occur during spring and summer in the pond which was the surface water source in this study, no real bloom was present at time of sampling.

The studied plants were considered as relevant models to cover the most frequently used water treatment techniques in Belgium. A majority of 63.7% of the Belgian drinking water originates from ground water (Belgaqua 2014), where no influence of TEP is expected based on our results. Surface water, the source for the rest of the drinking water, can be under stronger influence of algae blooms and TEP. However, both treatment trains in this study removed the majority of the TEP-fraction. (Removal efficiencies were never calculated due to high relative standard deviations on the measurement of low TEP-concentrations. Recently, two authors independently criticised the unreliable and non-standardised TEP-measurements (Discart et al. 2014b, Villacorte 2014)). The occurrence of algae blooms could possibly result in higher TEP concentrations and breakthrough situations, where significant amounts of TEP reach the final drinking water. However, the relative importance of these events is assumed
to be low in Belgium. This conclusion can be extrapolated towards other countries, using similar water treatment trains and raw water sources with limited susceptibility for algae blooms.

In a follow-up study, the actual role of TEP as biofouling initiator on membranes used for drinking water treatment was examined (Discart et al. 2014a). A full scale ultrafiltration system was monitored for 8 months for TEP, membrane pressures and performance and over 25 raw water parameters. Correlations between different parameters were examined and a full membrane autopsy was conducted before and after a full cleaning-in-place with oxalic acid. TEP-concentrations were found to be low and correlations between TEP and membrane performance parameters were less pronounced than correlations between general algae parameters and membrane performance. A complex fouling mechanism without a universal fouling parameter was suggested. Humic substances, bound to the membrane with iron, were the main fouling substances found on the membranes.

Although TEP created some commotion as a new fouling and biofilm formation parameter (as demonstrated by the 172 publications, published on this topic in the period 2006-2012 (Discart et al. 2014b)), we want to argue the importance is insignificant for drinking water production based on surface and groundwater, unless serious algae blooms are to be expected.

2.2 The indigenous community

2.2.1 The antagonistic or supportive effect

Chapter 3 concludes that the right nutrients and temperature, in the absence of disinfection residual, were not necessarily enough for determining whether an invading species could grow or survive in a certain water sample. The importance of the present bacterial community was shown, and it appeared that the absolute bacterial concentrations were not the only factor impacting the survival or growth of an invader; even with similar concentrations of a different bacterial community, the resistance to invasion was different. The importance of the indigenous community was already previously reported. Several authors showed for example a longer survival of E. coli in non-sterilised drinking water (Kerr et al. 1999, Moreira et al. 1994). Others mentioned a more widespread idea: the presence of an indigenous community
counteracts the growth of an invader, for example by the release of antagonistic substances (Ducluzeau et al. 1984, Jousset et al. 2011, Vachee et al. 1997), or by the production of siderophores and thereby competition for the present iron (Vachee et al. 1997). In the last case, it was shown that the addition of iron counteracted this effect. It is indeed easy to understand the competition between the invader and the indigenous community from the nutrient point of view. The present community can be expected as being most adapted for assimilating the present range of substrates. Competitive strategies can either exist of faster growth rates, being able to consume diverse carbon sources and hereby occupying certain niches, or on the other hand, having a higher substrate-affinity and being able to grow at lower substrate concentration (Egli 2010, Fuchslin et al. 2012, Vital et al. 2012b). In addition, it is not surprising that a richer indigenous community exhibits higher invasion resistance, as initially hypothesised by Elton (Cook et al. 2006, Elton and Miller 1954). Indeed, especially if richness translates into a high functional diversity, a wider range of carbon sources can be consumed, leaving few niches for the invader (Eisenhauer et al. 2012, Hodgson et al. 2002). A higher genotypic diversity will result in the presence of a wider range of (bacterio)toxins, with a maximal invasion resistance at medium genotypic richness (Inglis et al. 2009, Jousset et al. 2011). Some studies however disagree with the richness-invasibility hypothesis and state that in large-scale experiments a higher richness leads to a higher invasion susceptibility (Zeiter and Stampfli 2012). However, these studies have been mostly studying plant invasion and it is of doubt if this kind of ecological theories is truly transferrable to microbiology. Finally, De Roy et al. (2013) were the first to study the effect of evenness on invasion, at a fixed richness (a fixed number of species in varying proportions). Again, the ability to assimilate diverse carbon sources appeared to be crucial: more even communities were able to degrade a wider range of carbon sources, and were seen to have higher invasion resistance.

2.2.2 Consequences for disinfection strategies

It is easy to argue, for any of the above mentioned reasons, that a drinking water with a strong bacterial community present would be more resistant to invasion and therefore less likely to have pathogen proliferation after a contamination event. This conflicts with the widespread idea that a hygienically clean and safe water harbours low bacteria concentrations. In addition, it stresses the importance of the maintenance of a disinfectant residual until the tap, in case disinfection is applied. A disinfected water, without residual, will harbour only a limited
bacterial community and can be supposed to show a high susceptibility for invasion. This argument is in favour of either applying high disinfectant concentrations, or not disinfecting at all, whenever the source water can be regarded as absolutely safe and stable. In this case, extensive nutrient limitation has to be applied to create a biostable water with a strong indigenous community present, resistant to invasion. However, absolutely safe and stable source waters are scarce so usually, there is a disinfection strategy present. An alternative strategy could be to apply a biological ‘probiotic’ inoculation, to supply the water with a safe, stable and highly invasion resistant bacterial community. It leaves no doubt that truly dosing a bacterial culture into drinking water would be unacceptable for both legislation and public opinion. The bacterial dosing could therefore be disguised as a final biological filtration step after disinfection, e.g. biological sand filtration or activated carbon filtration, which have already been shown to inoculate drinking water (Hammes et al., 2008, Pinto et al., 2012, Chapter 7).

2.2.3 Future research on bacterial invasion

A final remark to be made for future invasion studies is the initial set-up of this type of studies. Most of them, including Chapter 3, focus on one pure strain, which invades a certain type of water in high concentrations (for example $10^3$-$10^6$ cells.mL$^{-1}$ (Kerr et al. 1999)). Potentially, the invader is accompanied by some pure and easily assimilable nutrients (Lu and Chu 2005), or exceptionally, a mixture of natural organic matter (Vital et al. 2012b). These are nicely controlled lab-experiment which are perfectly suitable for introducing some ecological theories. It is however debatable how close theory can be to practice in this case, where invasion in drinking water for example would typically be caused by intrusion of leaking sewage or groundwater entering the DWDS. How transferable are these single-species and pure-carbon-source studies? More relevant would be the use of actual contaminating streams, e.g. sewage, harbouring a diverse indigenous community and a complex natural mixture of organic matter. Cooperation in bacterial populations, for example by using siderophores or enzymes secreted by other bacteria in the community, has been shown before to affect growth (D’Onofrio et al. 2010, Hibbing et al. 2010) and can have its implications in invasion studies. For the sake of theory building, simplification could be done until the level of having a well-defined group of previously co-cultured bacteria as invading community, possibly with additional low concentrations of a complex broth.
3. FCM challenges HPC for routine detection in DWDS

In Chapter 1, we discussed already on the disadvantages of HPC and advantages of FCM as an operational parameter in drinking water analysis. Further optimization of FCM methods improved the understanding of the results, including background (Chapter 4), and a protocol was developed for high-throughput measurements (Chapter 5). Finally, Chapter 6 and 7 proved the advantage of FCM methods over HPC in non-routine situations. The information provided by HPC would in both cases either be too slow or too incomplete.

3.1 Replacing HPC with FCM cell counts as process parameter?

It is imperative to question whether HPC is still the way to go for routine water analysis in the 21st century. It is argued here that FCM cell counts can replace HPC due to the (i) level of information, (ii) precision and reproducibility, (iii) speed and (iv) price.

3.1.1 Level of information

The consensus value of HPC as an operational parameter is the enumeration of bacteria, either for evaluating treatment processes (e.g., filtration or disinfection efficiency) or for assessing the general microbial quality of raw and treated water. From a decision making perspective, it is clear that any process evaluation based on a variable fraction of 0.001 up to a few percent of concerned events (in this case bacteria) is highly questionable since it concealing all information on the other > 99%.

FCM assessments consider 100% of the present bacteria and deliver additional information, next to pure enumeration as done by HPC. The fluorescence and scatter detectors deliver information on cell size and DNA content, which can be used for a more detailed study by creating a microbial fingerprint of the water. These methods are especially sensitive for detecting small changes and shifts within the bacterial community, overlooked by pure enumeration (De Roy et al. 2012, Koch et al. 2014, Prest et al. 2014, Prest et al. 2013). For example, Prest (2013) detected the contamination of drinking water by 4% wastewater effluent, based on the combination of cell concentrations and a basic fingerprinting method. This 4% could be regarded as a serious contamination, but it should be noted that the effluent bacterial concentrations were as low as $4.8 \times 10^5$ cells.mL$^{-1}$, compared to high drinking water concentrations of $1.8 \times 10^5$ cells.mL$^{-1}$ at a household tap. In a more relevant example, raw
wastewater could contaminate drinking water in the distribution network, due to leaking piping. Raw wastewater contains around $10^8$ bacteria.mL$^{-1}$ (Ma et al. 2013), while drinking water in a chlorinated network would contain around $10^4$ bacteria.mL$^{-1}$, based on Chapter 6 and 7. In this case, a 0.01 % contamination would already lead to a doubling in cell concentration, while Prest et al. detected an increase as small as 5% in cell concentrations, using basic fingerprinting methods. In addition, in ongoing research in our own lab, a 5% contamination of one pure *Lactobacillus* strain in another one with identical concentrations can be detected.

### 3.1.2 Precision and reproducibility

From a statistical perspective, HPC only counts between 0 and 300 colonies in a well-chosen dilution, while FCM analysis usually collects between 50 and 20,000 data points for drinking water analysis (Hammes et al. 2008a). HPC shows relative standard deviations of $> 30$ up to 100% (Hammes et al. 2008a, Prest et al. 2013) while the inter lab- and instrument variability stays $< 7\%$ for FCM (SLMB 2012), or even $< 2.5\%$ for a single operator and instrument. An example displaying this large discrepancy between HPC and FCM sensitivity and precision is given in Figure 8-1. Two raw waters (groundwater and spring water), a finished drinking water and drinking water from a household tap have been measured independently in triplicate by three different routine laboratories, both by a fixed FCM TCC and HPC method. The results showed that only 0.005% of the cells were detected by HPC methods, while the absolute numbers were partly below quantification level (10 colonies detected) and no clear differences between the samples could be detected. The TCC had a RSD as low as 6.9%, allowing easy discrimination between any of the samples.

Finally, FCM results are mutually comparable when measured by the standardized guideline, while HPC methods already have heavily differentiated over the years, hindering comparison between labs (Reasoner 2004, SLMB 2012).
Figure 8-1: Comparison of HPC and FCM sensitivity and precision. Four samples were analysed in triplicate by three routine laboratories by both HPC and FCM TCC. Only 0.005% of the cells were detected by HPC, which made discrimination between the samples impossible. Based on FCM, clear differences could be detected. Figure redrafted from (Koetzsch et al. 2012).

3.1.3 Speed and automation

The usefulness of an operational parameter is further influenced by the moment when results are available. HPC incubation usually takes 3-7 days, depending on the method, but by that time, suspicious water is spread throughout the drinking water network and consumed widely. FCM results can be available within 10 - 20 minutes after sampling, enabling immediate action and identification of suspicious samples for further investigation. The high level of automation easily allows to measure up to 500 samples within a day (Van Nevel et al. 2013b) or even independent online use for several weeks (Besmer et al. 2014, Brognaux et al. 2013, Hammes et al. 2012).

3.1.4 Costs

A final argument for choosing any method for application in routine laboratories is incontrovertibly the cost. A previous comparison for HPC versus FCM is available (Helmi et al. 2014b) and for the purpose of this review, we compiled a new one based on own
instrumentation and protocols for FCM and information from a routine laboratory for HPC. Both were based on the analysis of 100 samples per day, relevant for a routine laboratory in drinking water industry (Helmi et al. 2014b). All amortization, consumables, quality control and labour costs were included. An overview of the results is given in Table 8-1, more detailed information is available in Appendix 8-1.

FCM costs range from $214 - $865 per day, depending on instrumentation cost, procedure choice and level of automation. The initial investment is considerable, but automation and low consumable use can keep sample dependent cost minimal, asking few additional cost for larger amounts of samples. HPC was estimated at $420 - $1030 per day, depending on automation level. The initial investment is lower, but the procedure is labour-intensive making the price increasing almost linearly per sample. Based on our estimations, both methods would cost $ 155 per day for the daily processing of 15 samples, FCM becomes more profitable for any higher number.

It has to be noted however that the price comparison is based entirely on high-income countries, where labour costs are decisive. For low-income countries however, the high initial investment for FCM will become more demanding.

Table 8-1: Comparison of daily cost for HPC and FCM. While FCM methods ask a high initial investment cost, it is less labour intensive than HPC. Therefore, FCM becomes more profitable whenever larger amounts of samples are processed. The cut-off is dependent on the used protocols and level of automation.

<table>
<thead>
<tr>
<th>Daily cost ($)</th>
<th>Helmi et al. (2014a)</th>
<th>New comparison</th>
<th>New comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>100 samples per day</strong></td>
<td><strong>15 samples per day</strong></td>
<td><strong>100 samples per day</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Instrument</strong></td>
<td>HPC</td>
<td>FCM</td>
<td>HPC</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td><strong>Consumables</strong></td>
<td>645</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td><strong>Labour</strong></td>
<td>90</td>
<td>65</td>
<td>114</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>420</td>
<td>865</td>
<td>155</td>
</tr>
</tbody>
</table>
Chapter 8

3.2 Counter-arguments against FCM methods

3.2.1 Disinfection: how dead is dead?

A critical counter-argument against FCM total cell counts is the inclusion of dead cells in the enumeration. Disinfection (e.g., chlorination) is common in drinking water treatment worldwide and in such a case the use of TCC provides limited information and can lead to erroneous conclusions. Viability staining (e.g., for membrane integrity, membrane potential or metabolic activity) combined with FCM can serve as an alternative (Berney et al. 2008, Helmi et al. 2014b). Each of these types of information is regarded as an indicator of bacterial viability. Unfortunately, there is not one single straightforward method to judge viability, since bacteria show many intermediate and hard to interpret stages between being clearly alive and clearly dead (Hammes et al. 2011).

We propose the use of membrane integrity, since it measures a cellular property which is targeted by conventional disinfection based on chlorine and ozone (Ramseier et al. 2011). As a conservative indicator of cell death (Lisle et al. 1999) (Berney et al. 2008) it is arguably a safe indicator for disinfection efficiency (Ramseier et al. 2011). Combined use of propidium iodide with SG I offers the possibility for FCM assessment of membrane integrity, allowing to discriminate cells with intact and damaged membranes (Hammes et al. 2008b, Vital et al. 2012a). This intact cell count (ICC) is advised instead of or as supplement for TCC whenever disinfection is applied. Recently, a successful application of this combination showed the instability of a chlorinated drinking water network, showing a strong increasing ICC, despite a lowering TCC (Nescerecka et al. 2014).

One specific challenge to the use of ICC is the evaluation of UV-C disinfection. The primary mode of action of UV-C is nucleic acids damage; the formation of pyrimidine dimers and other photoproducts of the nucleic acids inhibits replication and transcription and thereby prevents the bacteria from multiplication (Hijnen et al. 2006, Villarino et al. 2003). The membrane remains intact however (Nocker et al. 2007), making this damage undetectable by propidium iodide. In this case HPC will regard these cells as dead and has the advantage over FCM for now.
3.2.2 Operator demands

An often mentioned critique towards FCM cell counts for routine use is the need for manual gating to separate the present bacteria from the background (Aghaeepour et al. 2013, De Roy et al. 2012, Prest et al. 2013). This strategy was until recently mainly based on personal experience of the operator, which is a serious disadvantage for standard methods. However, several strategies emerged recently to overcome this issue. Firstly, Prest et al. (2013) optimized a staining protocol and proved to acquire absolutely stable results based on a fixed gate, widely applicable for drinking water samples. Nevertheless, aberrant samples might conflict with this fixed gate, but will presumably be easily distinguishable and can indicate an event, e.g. filter malfunctioning. Secondly, an increasing amount of researchers tried to circumvent manual gating, by doing a gating-independent statistical processing of the data (Aghaeepour et al. 2013, De Roy et al. 2012, Koch et al. 2014). However, these advanced methods might rather complicate the analysis.

3.2.3 Cell clustering

One should realise that FCM detects single cells or bacterial aggregates, but does not necessarily discriminate between both, a disadvantage which is shared with HPC (Shapiro 2003). As a consequence, the presence of clumps of sloughed biofilm or colonised suspended particles, will lead to incorrect enumeration of bacteria (van der Kooij and van der Wielen 2014a). Some authors applied mild sonication to break up bacterial aggregates in wastewater and validated their procedure using microscopic techniques (Foladori et al. 2010, Ma et al. 2013). Care should be taken, since this method has to be validated to find the optimal sonication dose every specific matrix. For example, an activated sludge sample asks a higher sonication dose compared to a settled wastewater sample (Ma et al. 2013), while excess sonication damages bacterial cells (Buesing and Gessner 2002) and hereby affects viability measurements. Standard application of a sonication step would nevertheless extend the FCM procedure and partly counteract the advantages of FCM. Bulk analysis methods such as ATP-analysis or molecular methods do not face this problem.

3.3 Operational experience with FCM cell counts

FCM cell counts are finding their way towards routine application in drinking water industry. A standardized guideline for drinking water analysis was incorporated in the Swiss food
handbook in 2012 (SLMB 2012), a milestone for this research. This reflects the interest of drinking water utilities for FCM as an accurate, precise and rapid alternative monitoring tool next to or in replacement of HPC (Prest et al. 2013). The Zürich water utility for example stated that the legal recognition of FCM would significantly increase water safety (Sicher 2012). Several Swiss and one Dutch accredited drinking water analysis laboratory are using FCM cell counts now for routine measurements while two Belgian drinking water labs have it in a test phase. In France, Veolia is testing flow cytometry for process control and comparison with HPC. They concluded that FCM is superior over culture methods and a powerful method for treatment efficiency assessment and active management of drinking water production (Helmi et al. 2014a, Helmi et al. 2014b).

After one decade of FCM research in drinking water, large amounts of data are available for making a strong comparison between what is detected by HPC and FCM methods. A large dataset was compiled for comparing the both: the detection of bacteria by several HPC methods versus absolute or intact cell counts, measured by FCM. The dataset consists of ground and surface waters used for the production of drinking water, samples collected during water treatment, finished drinking water, samples of drinking water networks and household installations, from both chlorinated and non-chlorinated waters, from Switzerland, the Netherlands, Belgium and Latvia. HPC values were measured using the methods as described by the different local drinking water legislations, flow cytometric TCC was based on a general nucleic acid stain as described by Hammes et al. (2008a) or Prest et al. (2013), flow cytometric ICC was based on an additional viability staining for membrane integrity, as described by Nescerecka et al. (2014) or Van Nevel et al. (2013b). The data was collected from accredited drinking water analysis labs as well as published and ongoing research projects.

The comparison between TCC or ICC and HPC can be seen in Figure 8-2, which comprises several years of data. According to the Kendall correlation test, there is a correlation between HPC and TCC or ICC ($p < 0.001$, $\tau = 0.20$ for TCC, $\tau = 0.25$ for ICC), however it is clear from the graph and $R^2$-values below 0.1 that the correlation is extremely weak. For a certain number of bacteria present and enumerated by FCM, any number of colonies will be displayed by HPC. By average, less than 1 out of 3000 cells is detected by HPC.
Figure 8-2: The comparison of flow cytometric cell counts with HPC results in water samples. Samples originate from different (un)chlorinated treatments, including source, treatment and distribution network samples (TCC: n = 2713, ICC: n = 1027). It is clear that the correlation between HPC and TCC or ICC is very weak ($R^2 < 0.1$ for linear regression in both cases). For a certain amount of (intact) bacteria present in a sample, any number of colonies can be obtained by HPC.

3.4 How to apply flow cytometry for routine drinking water monitoring

We opine that FCM cell counts can be used, similar to HPC, as a process parameter without a direct link with hygienic risk. Therefore, the operational limit is likewise to be set to ‘no abnormal change’ without an absolute upper limit. However, this opens the question to what should be interpreted as an abnormal change. As explained by Sartory for the use of HPC in the UK, suppliers have to set up their own local risk assessment approach, specified for their drinking water system (Sartory 2004).

Long term monitoring allows for the setting of a baseline on how many cells are to be expected in normal situations (Besmer et al. 2014). This value will be dependent on (i) raw water source: surface water has usually considerably higher cell counts than spring- and groundwater (Leclerc 2003), (ii) treatment plant design, e.g. activated carbon filtration is known for elevating cell counts (Hammes et al. 2008a), (iii) use of final disinfection and a disinfectant residual, which lowers (intact) cell counts, (iv) seasonal variations, whereby groundwater
tends to be more stable over time than spring- and groundwater (Pinto et al. 2014, Richardson et al. 2009). Baseline setting allows us for easy detection of deviations, which are to be evaluated both in relative and absolute numbers. An increase from 5,000 to 55,000 cells.mL\(^{-1}\) for example can be considered as more alarming than 100,000 to 150,000 cells.mL\(^{-1}\), while the absolute increase with 50,000 cells.mL\(^{-1}\) is identical. On the other hand, a regrowth in the network from 5,000 to 10,000 cells.mL\(^{-1}\), is the same relative increase as going from 100,000 to 200,000 cells.mL\(^{-1}\). However, 20 times more available nutrients were needed for the latter situation, depicting this as more alarming. Finally, it seems sensible to be alarmed in any situation when a value of \(10^6\) cells.mL\(^{-1}\) is reached, which is higher than 99% of the drinking water samples included in Figure 8-2 (the raw water samples are omitted for this comparison).

Whenever an abnormal change or an exceeding of guideline values is detected, this should lead to increased attention or further examination, just as is the case for HPC measurements. The reaction will be dependent on severity of the deviation and the sampling location, for example a sudden increase in cell concentrations by 100% in the treatment plant will ask for a more severe action than a small locally detected increase, e.g. in a household installation. Possible reactions can include an adaptation of the disinfection treatment, further assessments based on other microbial indicator and index organisms, inspection of treatment systems, etc.

### 3.5 Alternative methods for non-selective assessment of cell numbers

Since Robert Koch’s establishment of HPC, plenty of other methods next to FCM have been developed to study disinfection efficiency, bacterial water quality and network regrowth. Microscopy is well established and can use the same (viability) stains, but is in general too labour-intensive for routine application. Molecular assays, like 16S-based real-time PCR (qPCR) methods can serve as indirect measurements of absolute bacterial counting by measuring the abundance of 16S gene copies (Hoefel et al. 2005, Nadkarni et al. 2002). A drawback of this method is the detection of DNA in damaged cells, or naked DNA which can persist in the environment after cell death (Masters et al. 1994, Nocker et al. 2007). In order to prevent these genes to be detected, a pre-treatment with propidium monazide (PMA) is possible (Nocker et al. 2007). Just as propidium iodide, this molecule only binds to nucleic acids in damaged cells or to naked DNA, and therefore withdraws these genes from detection.
The technique has been used in drinking water for general viable bacterial detection (Chiao et al. 2014) or specific detection of viable Nitrosomonas (Wahman et al. 2009) or Salmonella (Singh et al. 2013) and is regarded as conservative (Wahman et al. 2009). For now, qPCR is mostly used for research, but fast progress is being made in this field.

Measurement of adenosine triphosphate (ATP) is another noteworthy alternative for FCM, since this is a direct assessment of present active bacteria and non-labour intensive (Hammes et al. 2010b, van der Wielen and van der Kooij 2010). It has been shown to have a strong correlation with especially ICC (Hammes et al. 2010b), although it is less sensitive than FCM to small changes (Liu et al. 2013a). It was for example demonstrated that small increases in bacterial abundance, as detected by FCM-TCC after a softening or activated carbon reactor, could not be detected by ATP analysis. Several other differences between supply areas in a drinking water network were significant based on FCM-TCC but not significant based on ATP-analysis (Liu et al. 2013a, Vital et al. 2012a). There is as well some discussion on whether a discrimination should be made between total, free and cellular ATP, whereby total ATP is the sum of the latter two. It is stated by some that free ATP is almost absent in their samples and this can be a filtration artefact (van der Kooij 1992, van der Wielen and van der Kooij 2010), while others showed that 16% of a large batch of fresh- and drinking water samples contained more than 50% free ATP (Hammes et al. 2010b). In addition, samples after a harsh treatment such as ozonation have been seen to contain remarkably high ATP-concentrations, of which 83-100% was free ATP, while both HPC and FCM cell counts dropped (Hammes et al. 2008a, Vital et al. 2012a). Standard incorporation of the filtration and double analysis method to discriminate free and cellular ATP however increases labour and consumables cost and induces an additional potential error (Hammes et al. 2008a). Several of the previously mentioned studies combined ATP and FCM and recommend the use of these supplementary assays to have a more solid understanding of growth and viability (Berney et al. 2008, Nescerecka et al. 2014, Vital et al. 2012a).

A new comparison between ICC and ATP measurements is made, based the dataset introduced in §3.3 (Figure 8-3). The dataset included chlorinated as well as non-chlorinated raw, processed and distributed water and originates from different countries and routine laboratories. All ATP analyses were done according to (Hammes et al. 2010b). The statistical
analys
is confirmed the strong correlation between ATP and ICC ($p < 0.001$ according to the Kendall correlation test, $\tau = 0.60; R^2 = 0.73$ for linear regression).

Figure 8-3: The comparison of FCM intact cell counts with bacterial ATP-concentrations in (raw) drinking water. Samples originate from different (un)chlorinated treatments, including source, treatment and distribution network samples ($n = 1441$). There is a clear correlation between both parameters, which is of no surprise since both methods are a direct assessment of viable cells, either intact or active.

3.6 Conclusions on FCM cell counts as an operational parameter

HPC played an undeniably important role in both drinking water management and general microbiology over the past century. In the absence of any other method, HPC has been of great value, but FCM cell counting now appears ready to take over for routine drinking water analysis for the following reasons:

- The information provided by HPC is absolutely obsolete, neglecting the majority of the bacteria, while FCM provides an assessment based on 100% of the cells with standard deviations on cell counts below 2.5%
- FCM cell counts are in various studies proven to be a sensible process parameter
- Next to absolute cell numbers, FCM can provide information on viability based on various viability indicators. The information can be used to create a fingerprint of the bacterial community, which enhances detection of small changes
- The information can be available within 15 minutes, while HPC incubation usually takes 3-7 days

- FCM offers easy automation options, allowing economic benefit over HPC from at least 15 samples onwards in high income countries.

Depending on the presence of disinfection techniques, one can either choose to enumerate the number of total or intact cells, or a combination of both. Ideally, FCM is backed up with additional methods like ATP measurements, just as is the case for HPC.

There is no reason left to conserve HPC measurements for routine drinking water analysis and FCM is an ideal candidate for providing a better, faster and more representative characterisation of bacterial presence in drinking water.
4. Future perspectives on drinking water monitoring: searching for an indicator needle in a haystack or going straight for the target?

4.1 Evaluation of indicator organisms

In §3, the applicability of flow cytometry as an operational parameter in drinking water analysis was discussed. While operational parameters are of importance for the management of drinking water production and distribution, there is an additional need for health-based monitoring. Nowadays, this is performed based on culture-based screening for indicator and index organisms, as discussed in Chapter 1, §3.2.1. Are there, with the current development of knowledge and techniques, options for a serious update of these parameters? A shift to flow cytometric assessments? The use of molecular tools?

*E. coli* might still be regarded as the best faecal indicator and index organism (Edberg et al. 2000), although the use of a single index organism might not be sufficient anymore. Parasitic protozoa such as *Cryptosporidium* or *Giardia* and viruses such as *Noroviruses* are more resistant to disinfection than *E. coli* and can persist much longer in the environment outside of a host organism, compared to *E. coli* (WHO 2011). In addition, the use of coliforms and *E. coli* to detect faecal contamination and the concomitant risk of pathogenic micro-organisms has been postulated in the 1890s, when faecal contamination was the single most important source of pathogens (Edberg et al. 2000). Nowadays, this risk is still considerable due to new causes of faecal contamination, such as aging sewage and drinking water piping and storm sewer overflows (Sedlak 2014), but the overall faecal contamination risk decreased due to better sanitation. Other potential dangers, like the growth of (opportunistic) pathogens in drinking water, such as *Legionella* or certain *Mycobacteria* gained importance now. Importantly, these do not originate from faeces, which renders them unnoticeable by monitoring for faecal indicator organisms. Several authors have in the meanwhile discussed the bad correlation between indicator organisms and the presence of real pathogens (Carter et al. 1987, Horman et al. 2004, Leclerc et al. 2001, Tillett et al. 2001). However, some others disagreed and saw a good correlation (Arvanitidou et al. 1997, Payment et al. 2000) or attributed the absence of correlation to a lack of data (Wu et al. 2011). In conclusion, it seems
clear that the origin of pathogen contamination is very case-dependent and only in case of faecal origin one should expect a good correlation with *E. coli* presence. This might indicate the need to compile a more extended set of indicator and index organisms, adapted according to the raw water source, type of water treatment, *etc.* Ideally, an index organism is present for every relevant class of microorganisms (bacteria, viruses and protozoa), all relevant ecological niches (faecal contaminations, pathogens growing in drinking water, *etc.*) and the most survival and treatment resistant groups of microorganisms (*e.g.* spores resistant to chlorination, *etc.*).

### 4.2 Flow cytometry as a tool for rapid and specific screening

In §3 of this chapter, we concluded on the suitability of flow cytometric cell counts as an operational parameter, but how suitable would flow cytometry be for the selective detection of specific bacteria and pathogens?

Already in the early 1980s, in the early years of flow cytometry for bacterial detection, flow cytometry was combined with fluorescent antibodies for the specific detection of *Legionella*, *e.g.* in cooling towers (immunofluorescent detection). It was declared to be much faster than detection by microscopy, but had detection limits above $10^4$ cells.mL$^{-1}$ (Ingram *et al.* 1982, Tyndall *et al.* 1985). Other researchers soon developed other immunofluorescence-based methods for the selective detection of *Streptococcus* ssp. (Sahar *et al.* 1983), *Listeria* ssp. (Donnelly and Baigent 1986), *Salmonella* spp. (McClelland and Pinder 1994), *Vibrio cholera* 01 (Alvarado-Aleman *et al.* 1994), *Staphylococcus aureus* (Diaper and Edwards 1994), *Cryptosporidium* (Vesey *et al.* 1997), *etc.* However, detection limits stayed high.

More recently, researchers focussed on increasing the sensitivity of strain specific detection methods. One possible approach was the use of antibodies combined with quantum-dots, because of their superior fluorescence intensity over normal fluorophores, allowing for better background separation (Hahn *et al.* 2008, Su and Li 2004). Other authors proposed a customised flow cytometer to use lanthanide-based labelling for the selective and background-free detection of low-concentrated target organisms, for example *Cryptosporidium* (Jin 2011). Yang *et al.* (2010) used this method to detect *E. coli* 0157:H7 with a lab-build FCM, with a detection limit of 100 cells.mL$^{-1}$. Keserue *et al.* (2012) combined filtration, immunomagnetic separation and fluorescent staining for detecting 50 *Legionella*
cells per mL or, more remarkably, 40 *Giardia* cysts per litre. Finally, Edgar *et al.* (2006) developed an assay for specific detection, based on phage binding and quantum-dots, allowing a specific *E. coli* detection of 10 cells.mL$^{-1}$. However, although many of these methods show major improvements, they are generally extensive, laborious and highly specialized techniques with a detection limit which is far above 1 cell per 100 mL$^{-1}$, as usually specified for common indicator organisms in multiple drinking water guidelines worldwide (Chapter 1, Table 1-2). One possible way to go for a further decrease of detection limits could be the use of more extensive filtration, possibly dialysis methods, combined with highly specific staining and background suppression. However, the use of more extensive and laborious pretreatment methods would make these methods less attractive.

In conclusion, developments are still ongoing for specific detection of indicator organisms or pathogens with flow cytometry. Detection limits are slowly decreasing, although the technical difficulty and labour time needed for the pre-treatment techniques seems to be increasing concomitantly. Flow cytometric methods for specific strain detection are probably not the way to go for routine monitoring of drinking water. However, the fast information offered by these methods can make the difference in urgency situations, e.g. serious contaminations in the DWDS.

### 4.3 The molecular highway towards in-depth screening

The massive amount of progress being made with next-generation sequencing methods over the last decade is impressive. It seems clear that their importance in drinking water research will take a sharp increase over the next few years, just as everywhere in microbiology. These methods, including microarrays, qPCR and next-generation sequencing (NGS) will most likely soon lead to the identification of novel pathogens and virulence genes (Aw and Rose 2012), especially in the uncultured part of the community. A deeper understanding of processes like biofilm formation or unculturability will emerge, new insights will be given on how the community and the water quality influence each other; future applications seem endless.

Microarrays can be used to simultaneous detect thousands of genes in a single assay via nucleic acid hybridisation (Straub 2011). Promising microarrays such as the PhyloChip (DeSantis *et al.* 2007) and ViroChip (Wang *et al.* 2002) are able to simultaneously identify thousands of bacterial taxa present in environmental samples or the presence of any virus,
known to infect humans. However, the use for detecting pathogens in drinking water is for now hindered by the lack of sequence information for many pathogens, non-specific binding and low sensitivity (Straub 2011). These high-density microarrays therefore need proper sample filtration, purification and perhaps amplification techniques to be used for drinking water monitoring (Aw and Rose 2012).

Quantitative real-time PCR (qPCR) measures fluorescence increase as quantification of amplification products during PCR cycling (Bustin 2010). Currently, qPCR offers high sensitivity and specificity, a fast rate of detection and quantitative results (Aw and Rose 2012). The detection limit of bacterial, viral and protozoan pathogens goes down to 80 gene copies.L⁻¹ for *Legionella pneumophila* for example (Merault *et al.* 2011), or even 2 gene copies.L⁻¹ for Noro- and Enteroviruses (USEPA 2012). The use in drinking water applications is slowly getting more attention.

NGS methods took a sharp flight since the introduction of the Roche 454 sequencing platform (Rothberg and Leamon 2008). NGS methods offer serious advantages over classing Sanger sequencing, including simplified sample preparation and massive parallelisation allowing high throughput (Aw and Rose 2012). Nevertheless, for more routinely application of NGS methods and more general, molecular methods, there are however still some major challenges left. Firstly, there is a major challenge on the bioinformatics side: the development of improved and powerful, user-friendly platforms for assessing and visualising the data (Aw and Rose 2012). This entails practical implementation for routine monitoring, asking for specially trained personnel. Secondly, sampling strategies are at this moment developed for culture-based methods and no specific standards are available yet for molecular methods (Douterelo *et al.* 2014a). How much water has to be sampled for capturing the bulk water microbiome, for example? The same amount like for standard indicator screening, a 100 mL sample? Or should this rather be up to 100 L to incorporate more rare species? Which sample containers are appropriate and what is the maximum storage time before analysis? Thirdly, these methods detect any gene copy present in the sample, although naked DNA can persist in the environment, without posing any infectivity risk (Masters *et al.* 1994). Methods such as PMA pre-treatment before qPCR offer more information on the presence of viable cells (Nocker *et al.* 2007), but need more study for routine applications. The fourth and last issue is the high capital investment costs and overall price. The fast development and increasingly
widespread use have heavily affected this aspect. As can be seen in Figure 8-4, sequencing costs dropped over the previous years. The comparison is made with Moore’s law, stating that the number of transistors on a computer chip would double every second year (Moore 1965). This law is more widely applied in technology and it is generally accepted that technologies, doubling efficiency or cutting costs by 50% every second year, are doing exceedingly well.

**Figure 8-4: The evolution of sequencing costs.** In comparison, the price decrease is much faster than predicted by Moore’s law. The sharp drop in January 2008, when the curve deviated from Moore’s law, is caused by replacement of Sanger by next-generation sequencing. Figure redrafted after Wetterstrand (2014).

More and more small, integrated and straightforward to use tools are being developed in this field (Mikheyev and Tin 2014, Nayak et al. 2013). Ideally, an integrated ‘genetic mining assay’ can be developed based on qPCR or by integrating quantitative techniques like flow cytometry with qualitative techniques like NGS in one tool. A sample is analysed in a single run for a wide range of virulence genes, possibly supplemented by other targeted genes, informing on EPS-formation, induction of bio-corrosion, nitrification, etc. Based on the newly gained molecular insights and an automated algorithm, a weighing factor is assigned to every detected gene. The final outcome is a list of scores which can be immediately tested against
guideline values. These scores inform straightforward on the likeliness of pathogen presence, corrosion induction, etc., including the opportunity to compare these scores with guidelines.

4.4 Safe drinking water framework

The previous sections have been a discussion on present and possible future monitoring technologies, but these should only be seen as a small part of providing safe drinking water. A framework for safe drinking water needs to be present, providing a preventive risk-based approach to manage water quality (Figure 8-5). The framework includes (i) health-based targets; how many disease outbreaks per year are acceptable, (ii) water safety plans, describing whether a certain water treatment can convert the raw water source in safe drinking water, how frequently monitoring is advised, and what to do in case of failure and (iii) a solid system of independent surveillance (WHO 2011). What follows, is my adaption of the WHO view on this framework.

Health-based targets set the goal for the compilation of a water safety plan. WHO is advising the use of disability-adjusted life years (DALY); a calculation method for the amount of years a person is ‘detracted from good health’, either by light or severe illness, or death. A guideline value of $10^{-6}$ DALY per person per year is advised, although nations are supposed to impose national regulations (WHO 2011). The Netherlands for example aims at a maximal infection risk of 1 out of 10,000 persons per year (van der Kooij and van der Wielen 2014a).
As the first part of the water safety plan, a system assessment has to be compiled, consisting of a thorough description of the drinking water production processes and the network; how capable is the applied treatment to produce a safe water out of the specific raw water source, including possible variation in raw water quality? How capable is the drinking water network to transport the produced water in an unchanged way to the consumer? Operational choices have to be described, for example on disinfection. Providing a disinfectant residual can lead to a lower pathogen risk, as long as sufficient doses are used. On the other hand, this advantage has to be balanced by the increased carcinogenic risk due to the formation of disinfectant by-products, or the decreased customer satisfaction due to unpleasant taste development. It should be understood that a sterile water, flowing through an impeccably clean pipe, is not a possible target (Douterelo et al. 2014a), and health-risks will never be totally absent. The real target is to comply with realistic health standards, by a good overall management.

The second part of the water safety plan describes monitoring strategies. These have to take into account potential variations of water quality in space and time. Water quality can vary rapidly and all systems are at risk of occasional failure; for example rainfall can greatly increase the levels of microbial contaminations in source waters and waterborne outbreaks are often preceded by rainfall. Therefore, the WHO clearly stresses the importance of frequent monitoring at random moments, but increased at moments of epidemics, flooding,

**Figure 8-5: The framework for providing safe drinking water. Redrafted after WHO (2011).**
interruptions in water supply, repairs in the network, etc. (WHO 2011). High-frequency monitoring, based on simple but well-chosen assessments, is hereby strongly preferred over occasional monitoring based on a wide range of specialized analytical methods. Flow cytometric cell counts could serve as a simple, but high-throughput and possibly even online assessment of bacterial quality. In case of aberrant results, a wider set of parameters and specialised methods can be available in centralised laboratories, ultimately even including next-generation sequencing. Sampling strategies as well have to take into account the size of the network and served population, with an increased monitoring demand within larger networks. For example, a water supply serving 5,000 inhabitants asks for a minimum of only 12 samples per year for faecal indicator testing, while a minimum of 840 samples is demanded when 500,000 people are served (WHO 2011). In the first case, randomisation of sampling moments, with additional focus on possibly disruptive events, is of utmost importance and independent surveillance is an absolute criterion. The Swiss situation for example, where every village has its own small water supply and a responsible ‘Brunnenmeister’ or ‘master well builder’, can serve as an example of bad strategy. These scattered, small supplies are infrequently monitored, at moments chosen by the Brunnenmeister himself. It is questionable if the most relevant sampling moments for this person aim at maximal water safety, or minimal hassle.

The third and final part of water safety plans finally, is a management and communication plan to describe actions which have to be taken in case of both normal as well as deviating events.

As a final part of the framework, an independent surveillance mechanism is responsible as well for setting up regulations and verifying the follow-up.
5. Conclusions

Bacteria in drinking water are no reason for concern, but for study. Processes governing their growth and presence are only partly understood and it remains a question how exactly they affect drinking water quality, and vice versa. TEP was seen as a possible biofilm inducing factor, but was concluded not to be of major importance in the studied model systems. On the other hand, the indigenous community of a water sample clearly affected its susceptibility for invasion by a non-indigenous bacterium. Flow cytometry showed to be a performant tool to study drinking water microbiology; it is suitable for routine analysis by drinking water utilities as well as for research applications. High-throughput, automated measurements showed to be feasible, without precision loss. The short time to result and the high level of information provided, enabled applications which were before hampered by the disadvantages of the routinely used growth-based methods. Finally, the impressive technological progress which is nowadays being made by molecular analysis methods leads us to the dawn of a new golden era in microbiology. It is incontestable that we will gain new fundamental insights in drinking water microbiology, affecting treatment design as well as monitoring strategies of drinking water systems, providing us the safe drinking water of tomorrow.
Bacteria are omnipresent, also in our drinking water. This is unavoidable, but neither should it be a reason to worry. It is better to understand and characterise them, to be able to control and predict their behaviour. Bacteria are present in drinking water both in the bulk water phase as well as in solid phases: on piping surfaces and in suspended solids and loose deposits. For the matter of simplicity, these last three phases will be combined and declared 'the biofilm phase'. Biofilms offer bacteria several opportunities; protection against rinsing out, the presence of higher nutrient concentrations and protection against disinfectant residuals from chlorination or chloramination. Therefore, biofilms have been under study numerous times and various formation mechanisms and biofilm inducing parameters have been proposed. In this study, we added and examined an additional factor: transparent exopolymer particles (TEP). These particles were introduced a decade ago in biofouling research; their stickiness and ubiquitous presence in natural waters made researchers conclude that these particles are affecting biofouling on membranes. Varying correlations have indeed been shown, but meanwhile, it became clear that TEP could easily pass various water filtration systems. In addition, it is known that these particles can be heavily colonised by bacteria and attach easily to surfaces. This combined information led us to the following hypothesis: TEP is able to pass drinking water treatment systems and, once arrived in the drinking water network, will settle down, attach to pipe surfaces and induce biofilm formation.

Chapter 2 is describing a study of TEP-presence and –removal, conducted in three different drinking water production plants: Kluizen (De Watergroep), Grobbendonk (Pidpa) and Torreele (IWVA). These plants served as models for Belgian water treatment and were using surface, ground- and wastewater respectively. It was however clear that TEP-removal efficiencies were very high in any case; TEP was in none of the cases present in significant concentrations in the final drinking water. This sampling was conducted in winter, resulting in relatively low algae concentrations and concomitant low TEP-concentrations. However, it was concluded that TEP-removal was performant enough to prevent a serious role of TEP as a biofilm inducing component. In a follow-up study in drinking water production plant De Gavers
(De Watergroep), it was shown that the role of TEP as a biofoulant was limited as well, at least in this installation.

The present nutrient can as well, next to biofilm growth, seriously affect bacterial growth; especially assimilable organic carbon and phosphate appear to be crucial and limiting nutrients. Chapter 3 describes a study using *Pseudomonas putida*, a bacterium with an extremely versatile metabolic apparatus. What will be the decisive factors impacting growth or survival when this bacterium is spiked, as an invader, in the microbial community in drinking water? Carbon and phosphorus were confirmed as crucial nutrients, although these were not the only decisive factors. Indeed, the indigenous community of surface water for example appeared to be more resistant against *P. putida* invasion, compared to drinking water, independent of present nutrient concentrations. The increased richness and diversity of the surface water community was indicated as causing this effect, although the true underlying mechanisms were not fully understood. It was concluded that the present indigenous community, characterised by concentration as well as composition, is an important factor affecting resistance against invading, possibly pathogenic bacteria. A biostable water, including a stable microbial community, is expected to show a higher invasion resistance compared to a strongly disinfected water, without disinfection residual nor a present bacterial community.

The characterisation of bacterial presence in drinking water is since the 1890s routinely done based on the agar plating method, developed by Robert Koch. Heterotrophic plate counts (HPC) is used for enumeration of the total bacterial community, while selective plating methods are applied for detecting specific indicator organisms, predicting the presence of for example faecal contamination and the concomitant risks of pathogen presence. Therefore, these indicators have a direct hygienic relevance, while HPC is applied as an operational parameter; to monitor filtration and disinfection efficiency, or to measure regrowth in the network. However, HPC shows serious issues, of which the major one is the level of obtained information. Typically, less than 1% of the actual present bacterial community is growing on agar plates, resulting in the absence of information on the leftover 99%. We compiled an extensive dataset, consisting of 2606 drinking water analyses, and estimated that only 1 out of 3000 cells are actually detected by HPC. As a result, small variations are impossibly detected by this method, making it in the end very debatable how relevant this information truly is. As
a 21st century alternative, we propose flow cytometric cell counts. It has already been demonstrated that these result in reliable measurements of the actual present bacterial cell concentrations. Results are obtained nearly immediately (20 minutes) and the technique offers good automation possibilities. Therefore, a deeper methodological study on the use of flow cytometry (FCM) for routine applications was done in Chapter 4 and 5.

An FCM analysis comprises next to information on targeted microorganisms as well FCM background or noise. Typically, this is regarded as disturbing and to be avoided. Nevertheless, this background can actually include relevant sample information. A background characterisation was therefore done to (i) provide a better understanding of background, (ii) to be able to apply fixed gating, (iii) to assess the effect of background on fingerprinting methods, which consist of a statistical analysis of all the data. For this study, background was split in three parts: sample, instrument and artefact background. Sample background is inherent to the sample and is caused by present organic and inorganic components: other microbiota, cell fragments, free DNA, humic acids, salts, soil particles, etc. Consequently, this type of background yields additional information. Instrument background consists of electronic noise or can be the result of sub-optimal instrument maintenance, and should be limited. Artefact background finally is the result of inaccurate sample preparation, contamination of consumables, etc. and has to be avoided at all time. A good and accurate sample preparation, based on good staining protocol, and the use of a good set of control samples will usually result in a good discrimination between background and the targeted microorganisms.

An analysis method for routine applications is extra valuable whenever it allows for automation. The use of autoloaders for FCM is common, but the resulting long waiting times between sample preparation and analysis can negatively affect measurement accuracy. In Chapter 5, it could however be demonstrated that a batch of 96 samples could be measured accurate and fast, whenever stringent procedures were used (< 2.5% relative standard deviation, 75 minutes analysis time).

The discussed advantages (complete information, accuracy and speed) led us to the conclusion that FCM cell counts are ready for routine application in drinking water analysis, which is confirmed by the incorporation of FCM cell counts in the Swiss drinking water
Abstract

legislation. Next, in Chapter 6 and 7 we looked for other applications, deviating from routine measurements, were FCM could as well be valuable for drinking water analysis.

A first situation where FCM methods showed added value was the flushing of main supply pipes after maintenance or construction works. Whenever pipes are opened and therefore exposed to external influences and contaminations, a thorough cleaning and disinfection is necessary. In practice, large amounts of clean drinking water are used to flush the pipe. Water quality validation is typically done based on agar plating and results are only available the next day. In Chapter 6, FCM was implemented as an additional technique. Reference samples (clean drinking water, used for flushing) and flushing samples (the disposed water after flushing) were immediately compared, based on absolute cell counts as well as the bacterial fingerprint of the water. The combination of both methods was tested and approved in three full-case field studies, involving the flushing of main supply pipes (diameters 400-900 and 1050 mm) of water supplier TMVW (FARYS). In each of these situations it was clear that the necessary flushing was completed after 2-4 hours, and the pipes could be taken back in operation. This was consistently approved by official analysis for indicator parameters.

Another application where the added value for FCM methods could be tested, was found in a case-study for assessing bacterial growth in a local drinking water network. In Beersel, a Belgian village, a series of drinking water samples were collected and immediately analysed, based on FCM cell counts and fingerprinting analysis. Bacterial regrowth was demonstrated in several samples based on absolute cell concentrations, but fingerprinting analysis showed the different origin of the regrowth in the different samples. It was therefore concluded that regrowth took place in the household plumbing installations instead of the drinking water network. These subtle changes would be impossible to detect based on HPC.

Flow cytometry overall showed to be a performant tool to study drinking water microbiology; it is suitable for routine analysis by drinking water utilities as well as research applications. High-throughput, automated measurements are be feasible, without accuracy loss. The short time to result and level of information provided enabled applications which were before hampered by the disadvantages of the routinely used agar plating methods.

Finally, next to flow cytometry, other methods are as well emerging as possible candidates for drinking water analysis by the water utilities. Molecular methods such as qPCR and
next-generation sequencing can provide deeper insights on suspicious samples, selected based on more basic methods. In addition, the impressive technological progress which is nowadays being made by molecular analysis methods even leads us to the dawn of a new era in microbiology. It is incontestable that we will gain new fundamental insights in drinking water microbiology, affecting the treatment design as well as monitoring strategies of the drinking water systems, providing us the safe drinking water of tomorrow.
SAMENVATTING


Hoofdstuk 2 beschrijft een studie naar TEP-aanwezigheid en -verwijdering die werd uitgevoerd in drie verschillende drinkwaterproductiecentra: Kluizen (De Watergroep), Grobbendonk (Pidpa) en Torreele (IWVA). Deze stonden model voor de Belgische waterbehandeling en gebruikten respectievelijk oppervlakte-, grond- en behandeld afvalwater als bron. Er werd echter duidelijk geconcludeerd dat de TEP-verwijdering steeds heel efficiënt gebeurde doorheen de verschillende waterbehandelingen: in geen van de systemen waren er significante concentraties meetbaar in het uiteindelijke drinkwater. Hoewel deze staalname in de winter gebeurde en er dan lage algenconcentraties, en daarmee samengaande, lage
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TEP-concentraties konden verwacht worden, werd er besloten dat de verwijdering van TEP doorheen het volledige jaar voldoende zou zijn om te voorkomen dat deze een significante rol zouden kunnen spelen in biofilmvorming in drinkwatersystemen. In een vervolgonderzoek in het drinkwaterproductiecentrum De Gavers (De Watergroep) werd intussen ook het beperkte belang van diezelfde deeltjes aangetoond voor de membraanvervuiling in de ultrafiltratie-installatie.

Naast biofilmgroei hebben ook de aanwezige nutriënten een sterke invloed op bacteriegroei; voornamelijk assimileerbare koolstof en fosfor (in de vorm van fosfaat) blijken hiervoor cruciaal en limiterend te zijn. Hoofdstuk 3 beschrijft een reeks testen met Pseudomonas putida, een bacterie met een erg veelzijdig metabolisch systeem. Als deze bacterie, als invasieve soort, terechtkomt in een bestaande microbiële gemeenschap in drinkwater, wat zal er dan bepalen of deze kan overleven en/of groeien? Koolstof en fosfor bleken hier inderdaad cruciaal te zijn, maar dit waren niet de enige voorwaarden. Zo werd aangetoond dat de microbiële gemeenschap van oppervlaktewater meer resistent en vijandig was tegen deze invasie dan deze van drinkwater, onafhankelijk van de aanwezige nutriënten. De meer diverse gemeenschap, aanwezig in oppervlaktewater, werd als mogelijke reden aanzien voor de verhoogde resistentie, maar de werkelijke mechanismen werden niet volledig begrepen. Er werd alvast geconcludeerd dat de aanwezige bacteriële gemeenschap, zowel gekarakteriseerd door concentratie als samenstelling, een belangrijke factor is om drinkwater resistent te houden tegen de groei van invasieve bacteriën, mogelijks pathogenen. Zo zal een biostabiel water, met een sterke aanwezige microbiële gemeenschap, wellicht meer resistent zijn tegen invasie dan een sterk gedesinfecteerd water, waar er geen residueel desinfectans meer aanwezig is.

Het karakteriseren van de aanwezigheid en groei van bacteriën in drinkwater gebeurt sinds de jaren 1890 routineus op basis van groei op agarplaten, oorspronkelijk ontwikkeld door Robert Koch. Enerzijds wordt er met de heterotrofe plaattelling (HPT) gezocht naar de hoeveelheid aanwezige bacteriën. Anderzijds worden selectieve agarplaatmethodes gebruikt om te zoeken naar indicatorbacteriën, die duiden op de aanwezigheid van bijvoorbeeld fecale vervuiling, wat dan weer een risico voor de aanwezigheid van pathogenen met zich meebrengt. Bijgevolg hebben deze indicatorbacteriën een directe hygiënische betekenis, terwijl de HPT eerder als gebruikt als operationele parameter; om de efficiëntie van filter- en
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desinfectietechnieken te bepalen, of om bacteriegroei in het drinkwaternetwerk te meten. Er stellen zich echter heel wat problemen bij deze HPT-methode, waarvan de eerste en allerbelangrijkste is: de verkregen informatie. Er groeit zelden meer dan 1% van de aanwezige drinkwaterbacteriën op deze agarplaten, waardoor elke informatie over de overige 99% ontbreekt. Eigen schattingen, op basis van een dataset met 2606 drinkwateranalyses, toonden zelfs aan dat er gemiddeld slechts 1 op meer dan 3000 cellen worden gedetecteerd. Kleine variaties kunnen dan ook onmogelijk worden aangetoond met deze methode. Het is dus erg discutabel hoe waardevol deze info nog kan zijn. Als alternatief voor de 21e eeuw stellen we flowcytometrische celtellingen voor. Er werd vroeger reeds aangetoond dat dit toestel een goede meting geeft van de werkelijk aanwezige celconcentraties. De resultaten zijn bovendien bijna direct beschikbaar (20 minuten) en de techniek biedt de mogelijkheid tot verregaande automatisatie. Daarom werd er in hoofdstuk 4 en 5 een diepere methodologische studie uitgevoerd op het gebruik van flowcytometrie (FCM) voor routinetoepassingen.

Een dergelijke FCM-analyse detecteert steeds, naast de eigenlijke gezocht micro-organismen, ook heel wat ‘achtergrondsinaal’ of ruis. Dit wordt algemeen bekeken als iets storend en overbodig, dat vermeden moet worden. Maar anderzijds kan deze achtergrond ook heel wat waardevolle informatie bevatten. Daarom werd er een karakterisering van deze achtergrond gedaan; om (i) achtergrond beter te begrijpen, (ii) vaste gating te kunnen toepassen op FCM grafieken en (iii) te begrijpen wat de invloed is van achtergrond op zogenaamde fingerprinting-methodes, die een statistische verwerking van de data uitvoeren. Achtergrond werd opgesplitst in drie delen: staal, instrument en artefact achtergrond. De staal achtergrond is inherent aan het staal en wordt veroorzaakt door de aanwezige organische en anorganische componenten: andere microbiota, vrij DNA, organisch materiaal, humuszuren, zouten, bodemdeeltjes, etc. Daarom biedt achtergrond extra informatie, naast analyse van de aanwezige micro-organismen Instrument achtergrond bestaat uit elektronische ruis of kan het gevolg zijn van slecht onderhoud, en moet beperkt worden. Artefact achtergrond ten slotte, is het gevolg van onnauwkeurig werk of contaminatie van staalnamenmaterialen, en moet ten allen tijde vermeden worden. Mits gebruik van een goede staalvoorbereidingsprocedure en de juiste controles kan in de meeste gevallen een duidelijk onderscheid gemaakt worden tussen deze achtergrond en de gezocht micro-organismen.
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Een methode voor routine-analyse wordt extra waardevol indien deze verregaande mogelijkheden voor automatisatie kan bieden. Het gebruik van autoloaders voor FCM raakt dan ook ingeburgerd, maar de lange wachttijden tussen de staalbereiding en de eigenlijke analyse die daaruit voortvloeien, kunnen een nefaste invloed hebben op de accuraatheid van de metingen. In hoofdstuk 5 werd echter aangetoond dat het mogelijk is om, mits een goed gestandaardiseerde staalvoorbereiding, een batch van 96 stalen accuraat en snel te analyseren (< 2.5% relatieve standaardafwijking, 75 minuten analyse-tijd).

Omwille van de eerder vernoemde voordelen van de complete informatie, accuraatheid en snelheid, werd geconcludeerd dat FCM bacterietellingen klaar zijn voor toepassing als routinemethode voor drinkwateranalyse, getuige ook de opname van een FCM-methode in de Zwitserse drinkwaterwetgeving. In de hoofdstukken 6 en 7 werd vervolgens op zoek gegaan naar een aantal nieuwe toepassingen voor FCM-methodes in de drinkwaterindustrie, naast de routine-analyses.

Een eerste situatie waar FCM een belangrijke meerwaarde kon hebben werd gevonden in de spoeling van grote drinkwaterleidingen na onderhoud. Als leidingen opengebroken worden en daardoor blootgesteld worden aan externe omstandigheden en eventuele contaminatie, moeten deze achteraf uitvoerig gedesinfecteerd en gereinigd worden. In praktijk wordt er met grote hoeveelheden zuiver drinkwater gespoeld, waarna dit water geloosd wordt. Beoordeling van de waterkwaliteit gebeurt ook hier opnieuw op basis van de agarplaatmethode, die pas de volgende dag resultaten geeft. In hoofdstuk 6 werden FCM metingen als alternatief voorgesteld, en werden referentiestalen (zuiver drinkwater, gebruikt voor de spoeling) en spoelstalen (het gebruikte en geloosde water) direct met elkaar vergeleken, op basis van zowel absolute bacterieconcentraties als de fingerprint van het water. De combinatie van deze FCM methodes werd getest en goedgekeurd op basis van drie volle schaal veldstudies, waarbij waterbedrijf TMVW (FARYS) onderhoudswerken uitvoerde aan hun toevoerleidingen (diameters 400, 900 en 1050 mm). In elk van de situaties was het na 2-4 uur duidelijk dat de spoeling ‘volledig’ was, en de leidingen opnieuw in gebruik genomen konden worden. Dit werd telkens bevestigd door de officiële analyses voor microbiële indicatorparameters.

Een andere toepassing waar de meerwaarde van FCM werd getest, was de studie van bacteriegroei in een lokaal leidingennetwerk. In het Vlaams-Brabantse Beersel werden een reeks
drinkwaterstalen verzameld en ter plaatste geanalyseerd, opnieuw met FCM celbacterieconcentraties en fingerprinting. Op basis van celconcentraties werden er in verschillende stalen inderdaad verhoogde bacterieconcentraties gemeten. De fingerprinting kon echter aantonen dat de veranderingen in deze stalen onafhankelijk van elkaar gebeurden: de hergroei had dus niet plaatsgevonden in het centrale leidingnetwerk, maar in de huishoudinstallaties. Deze subtiele verschillen zouden onmogelijk kunnen opgemerkt worden op basis van de beperkte informatie die verkregen wordt met HPT.

Er werd geconcludeerd dat FCM methodes krachtige tools kunnen zijn om drinkwater microbiologie te bestuderen; het is zowel geschikt voor routineanalyses door drinkwatermaatschappijen als voor gespecialiseerde onderzoek toepassingen. Snelle en geautomatiseerde metingen zijn mogelijk zonder verlies aan accuraatheid. De verkregen informatie is uitgebreid en snel beschikbaar, wat toepassingen mogelijk maakt die voordien bemoeilijkt werden door de nadelen van routineus toegepaste agarplaat methodes.

Tot slot zijn er naast FCM ook andere methodes in opkomst met mogelijkheden voor routine drinkwateranalyses. Moleculaire methodes zoals qPCR en next-generation sequecing kunnen meer duiding geven over verdachte stalen, geselecteerd door eenvoudigere methodes. Bovendien brengt de indrukwekkende technologische vooruitgang van de moleculaire methodes ons zonder momenteel in een nieuw gouden tijdperk in de microbiologie. We zullen ontegensprekelijk nieuwe fundamentele inzichten verkrijgen in drinkwatermicrobiologie, die zowel impact zullen hebben op het ontwerp als de monitoringstrategie van drinkwatersystemen, die ons het veilige drinkwater van de toekomst zullen leveren.
Appendix 5-1: how to deal with carry-over

When measuring a series of different samples, a carry-over of bacteria between the samples from adjacent wells is possible. It should be noticed that this carry-over is extremely instrument specific. In this case, a limited amount of cells were detected within the first blanks after concentrated samples, accounting to 0.53% of the concentration of the latter ones. A possible way to deal with this problem is the introduction of a washing step, in this case available on the instrument. However, when this step was applied, the results did not improve. It was concluded that a washing step is not useful but neither necessary in cases when dealing with similar samples with less than a factor ten difference in concentration. At that moment, only a 5% error on the cell concentration will be induced. Whenever this can be predicted, very different samples should be loaded in increasing order of concentration. Alternatively, other instrument-dependant cleaning procedures can be applied, e.g. loading a decontaminating and cleaning liquid in a well to be measured after a concentrated sample. When processing results, one might decide to re-measure certain low-concentration samples which appear to be preceded by a high-concentration sample, and where significant carry-over is considered possible.

Appendix 5-2: Other factors affecting a stable measurement

More factors and operational choices can impact the stability of the results. For example, the choice for either black or white instead of transparent polystyrene 96-well plates was compared. When measuring SG-stained surface water, the results of black and white plate were regarded as very stable and stable, with trends of 0.60% and 3.9% decrease in cell concentration respectively (Figure Appendix 5-1). The transparent plates however, showed an 8.2% decrease in cell concentration over the plate. This can be a too high deviation, depending on the required precision. This effect was mainly ascribed to more light, affecting the samples in transparent plates compared to white or black plates.
Furthermore, the effect of an agitation step after every six wells was studied. One might want to apply this function, available on the instrument, in order to ensure homogeneity of the samples and prevent sedimentation. However, this step appeared to have a dramatic impact on the cell concentration, which decreased with as much as 20% over the plate (Figure 5-7). This test was repeated in a transparent plate, since this type is most in use in practice and previously, agitation and transparent plates were combined in the author’s lab. The impact appeared to be even more dramatic, showing a 29% decrease in cell concentration. This dramatic decrease was believed to be caused by strong interactions between bacteria and/or surface charges on the plates. Combined with the agitation which is leading to an increased collision rate between the individual bacteria and the surface of the plate, this could lead to increased aggregation and adhesion of bacteria to the plate.

![Figure Appendix 5-1: Comparison of a measurement in a black, white or transparent plate.](image)

*Figure Appendix 5-1: Comparison of a measurement in a black, white or transparent plate.*

A total cell count was performed on surface water samples loaded in either a black, white or transparent plate. The resulting trend was minor for black plates (-0.60%) while for white and transparent plates, this was -3.9% and -8.2% respectively. The inlayed box plots show the median (line), 25 and 75 percentiles (box) and 10 and 90 percentile (whiskers) as well as outliers (black crosses) (n=93).
Appendix 6-1: Calculations

Estimated delay for reference sample to be at the sampling point

Below, a calculation is given for the delay it takes for the water to flow from the reference point to the sampling point. It is important to understand that these are rough estimations of the amounts of standing water and flow volumes.

Case 1:
- It took 6.16 hours to refill the pipe (2186 m³) (timed)
- Estimated refill flow
  - First two hours of flushing after refill: 400 m³/h
  - Afterwards: 200 m³/h
- Pipe volume between reference and flush sampling point: 4732 m³
- Pipe volume to be filled at 200 m³/h:
  \[ \frac{4732 \text{ m}^3 - 2186 \text{ m}^3 - 2h \times 400 \text{ m}^3/h}{200 \text{ m}^3/h} = 1746 \text{ m}^3 \]
- Total estimated delay: \[ 6.16h + 2h + \frac{1746 \text{ m}^3}{200 \text{ m}^3/h} = 16.9 \text{ h} \]

Case 2:
- Refill volume: \[ 731 \text{ m}^3 - 191 \text{ m}^3 = 540 \text{ m}^3 \]
- Refill time: 6 hours
- Refill flow: \[ \frac{540 \text{ m}^3}{6h} = 90.1 \text{ m}^3/h \]
- This flow is assumed to be constant during flushing, by lack of other available information
- Pipe volume between reference and flush sampling point: 786 m³
- Total estimated delay: \[ \frac{786 \text{ m}^3}{90.1 \text{ m}^3/h} = 8.73 \text{ h} \]

Case 3:
- Estimated refill flow: 250 m³/h.
- Pipe volume between reference and flush sampling point: 2856 m³.
- Total estimated delay: \[ \frac{2856 \text{ m}^3}{250 \text{ m}^3/h} = 11.4 \text{ h} \]
Estimated time for the stagnant water to be flushed out

Once the pipe has been refilled, it takes a certain amount of time to flush out the possibly present stagnant water, which is likely to have increased bacterial cell concentrations.

**Case 1:** No stagnant water was present.

**Case 2:** \[ \frac{191 \text{ m}^3 \text{ stagnant water}}{90.1 \text{ m}^3/\text{h refill flow}} = 2.12 \text{ h} \]

**Case 3:** \[ \frac{1209 \text{ m}^3 \text{ stagnant water}}{250 \text{ m}^3/\text{h refill flow}} = 4.84 \text{ h} \]
Appendix 6-2: The influence of background on the fingerprint

As mentioned several times in the discussion, FCM fingerprinting both incorporates bacterial cells and FCM background. To understand what the influence is of this background on the FCM fingerprint, an example of case 1 is given here. As discussed, the sample after 2 h of flushing shows stable cell concentrations, while the FDA results only stabilize at the next sample, after 4.25 h (Figure 6-2). Indeed, when examining the original FCM histograms of flushing samples over time, almost no bacteria are detected anymore after two hours and no change is seen afterwards (Figure Appendix 6-1). The background, to the contrary, is after 2 hours still showing an additional cluster which is high on the FL3 detector. This cluster disappeared after 4.25 h and did not reappear in later analyses. In conclusion, these background particles were flushed out later than the bacteria. The incorporation of the background in the analysis made the fingerprinting more conservative in this case.

Figure Appendix 6-1: FCM histograms for SGPI measurements of flushing samples in case 1. The cells are indicated in green and can be seen to be almost absent after 2 h of flushing. A part of the background, indicated in blue, is only flushed out after 4.25 h of flushing.
Appendices

Appendix 6-3: Fingerprinting analysis based on SG-staining

Figure Appendix 6-2: Fingerprinting analysis of SG-measurements for total cell concentrations of case 1, based on the first two discriminants of the FDA (n=4). Several flushing (F) and reference (R) samples are indicated with their timing (hours), the timing of sample collecting is shown in the lower bars. After two hours, the flushing samples approach the region where they stabilize after 4.25 h, which indicates their high similarity to the reference samples. There is however still a clear difference between all of the flushing and reference samples. This is possibly to be explained by the time delay for the reference samples.
Figure Appendix 6-3: Fingerprinting analysis of SG-measurements of case 2, based on the first two discriminants of the FDA (n=3). Several flushing (F) and reference (R) samples are indicated with their timing (hours), the timing of sample collecting is shown in the lower bars. The trend is similar as for the SGPI-analysis: after 2.6 hours, the samples stabilize within the same region of the reference samples, which indicates their high relative similarity.
Appendix 8-1: Cost calculation for FCM analysis

<table>
<thead>
<tr>
<th>Individual sample cost *Assuming that we use 96 well plates, and we run on average 50 samples (200 µL) per plate</th>
<th>Price</th>
<th>Unit</th>
<th>Use/ month</th>
<th>Cost per sample</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR green</td>
<td>322</td>
<td>500 µL</td>
<td>10,000 x diluted</td>
<td>0.013</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>DMSO</td>
<td>75.2</td>
<td>1 L</td>
<td>2 µL/sample</td>
<td>0.00015</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>96 wells plates</td>
<td>121.12</td>
<td>400</td>
<td>1/50 samples</td>
<td>0.0061</td>
<td>Greiner Bio</td>
</tr>
<tr>
<td>gloves</td>
<td>4.83</td>
<td>100</td>
<td>2/50 samples</td>
<td>0.0019</td>
<td></td>
</tr>
<tr>
<td>1 mL tips</td>
<td>35</td>
<td>1000</td>
<td>1/sample</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>10 µL tips</td>
<td>35</td>
<td>1000</td>
<td>1/sample</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td><strong>PER SAMPLE</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.091</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other consumables</th>
<th>Price</th>
<th>Unit</th>
<th>Use/ month</th>
<th>Cost per month</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ water</td>
<td>5</td>
<td>1 L</td>
<td>20L</td>
<td>100</td>
<td>Millipore system</td>
</tr>
<tr>
<td>Decon liquid</td>
<td>34.4</td>
<td>1 L</td>
<td>0.25L</td>
<td>8.6</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Soap solution</td>
<td>34.4</td>
<td>1 L</td>
<td>0.25L</td>
<td>8.6</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Paper rolls</td>
<td>25.95</td>
<td>6 rolls</td>
<td>0.5</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Desinfecting Spray</td>
<td>21</td>
<td>1 L</td>
<td>1</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Waste vessel + processing</td>
<td>6.49</td>
<td>1 vessel</td>
<td>1</td>
<td>6.49</td>
<td></td>
</tr>
<tr>
<td><strong>SUM</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>149.0</strong></td>
<td></td>
</tr>
<tr>
<td><strong>PER DAY</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>6.8</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Daily start and shutdown, QC</th>
<th>Cost per day</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 peak beads</td>
<td>1.1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>6 peak beads</td>
<td>1.1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Standard control beads</td>
<td>0.098</td>
<td>Partec</td>
</tr>
<tr>
<td>FCM tubes</td>
<td>0.3</td>
<td>Elkay Labs</td>
</tr>
<tr>
<td>0.22 µm filter</td>
<td>0.8</td>
<td>Millipore</td>
</tr>
<tr>
<td><strong>PER DAY</strong></td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Instrumentation reimbursement (5 years)</th>
<th>Price</th>
<th>Cost per day</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuri C6 + Csampler + full warranty</td>
<td>75800</td>
<td>57.4</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Pipettes</td>
<td>1730</td>
<td>1.3</td>
<td>Brand</td>
</tr>
<tr>
<td><strong>PER DAY</strong></td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Labour (€ 44 per hour)</th>
<th>Time (h)</th>
<th>Total time</th>
<th>Labour cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily startup and shutdown</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average maintenance/day</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>100 samples</td>
<td>1.25</td>
<td></td>
<td><strong>SUM 88</strong></td>
</tr>
</tbody>
</table>

**Daily cost for 100 samples:**

100 x € 0.091/sample + € 6.8 (consumables) + € 3.4/day (start-up and QC) + € 59 (amortization) + €88 (labour) = € 166

Using an exchange rate of € 1 = $ 0.777:

**Daily cost for 100 samples = € 166 or $ 214**


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SAM VAN NEVEL

CURRICULUM VITAE

PERSONAL INFORMATION

Surname                  Van Nevel
Name                     Sam
Date of birth            December 9, 1987
Place of birth           Deinze
Address                  Akkerstraat 84a
                         9000 Gent
                         Belgium
Phone                    +32 (0) 484 41 30 12
E-mail                   samvannevel@gmail.com

EDUCATION

- **Ph.D. in Applied Biological Sciences, Environmental Technology** 2011-2014
  Doctoral School of (Bioscience) Engineering
  Laboratory for Microbial Ecology and Technology (LabMET)
  Ghent University, Belgium
  Supervisors: prof. Nico Boon and dr. Frederik Hammes

- **M. Sc. Bioscience Engineering: Environmental Technology** 2005-2010
  Ghent University, Belgium
  Great distinction

- **Master thesis** 2010
  *Self-activating biogenic palladium nanoparticles for pollutant removal*
  Supervisors: prof. Willy Verstraete and prof. Nico Boon
WORKING EXPERIENCE

Scientific collaborator
Laboratory for Microbial Ecology and Technology (LabMET) 2011-2014

- Ph.D. research (FWO project grant)
  Growth and flow cytometric monitoring of bacteria in drinking water
  Study on the growth and detection of bacteria in drinking water
  Applying flow cytometry for fast bacterial monitoring of water processes

- Teaching activities:
  Lab exercises ‘Microbial Ecological Processes’  2012-2013
  Calculation exercises ‘Biotechnological Processes in Water Treatment’  2013

- Tutor of 3 master thesis students and 4 bachelor thesis students

- Flow cytometry expert and responsible at LabMET  2013-present

- Collaboration with TMVW (FARYS) on the use of flow cytometry for bacterial monitoring in drinking water  2013-present

- Collaboration with BD on the applications of flow cytometry  2014

INTERNATIONAL STUDY LEAVES

- Scientific visit to Eawag, Dübendorf, Switzerland (2x)  Jan-March, 2013
  Drinking water microbiology group under Frederik Hammes
  at the Environmental Microbiology Department  July-October, 2014

- Summer school on Environmental Life Sciences Engineering  July 7-23, 2013
  Singapore Centre on Environmental Life Sciences Engineering (Scelse)
  Singapore

- Erasmus study at Danmarks Tekniske Universitet (DTU)  Feb-June, 2009
  Copenhagen, Denmark
ACQUIRED COMPETENCES

- **Leadership**
  ‘Leadership foundation course’ by Doctoral School of (Bioscience) Engineering and Dan Steer
  March 2014

- **Career management**
  ‘Applying for a postdoctoral job’ by Doctoral School of (Bioscience) Engineering
  2013

- **Presentation skills**
  ‘Effective scientific communication’ and ‘Effective graphical displays’ 2011-2013 by Doctoral School of (Bioscience) Engineering and Principiae.
  As participant and as presentation coach

- **Writing skills**
  ‘Advanced academic English: writing skills’ by Doctoral School of (Bioscience) Engineering
  2011

SCIENTIFIC AWARDS

- **Best short presentation award**
  Oct 4, 2013
  3rd IWA BeNeLux Regional Young Water Professionals Conference
  ‘Invasion of pathogens in drinking water: determining factors’

- **Poster award**
  Dec 20, 2010
  16th PhD Symposium on Applied Biological Sciences
  ‘Palladium nanocatalysts produced by fermentative bacteria’

- **Physics Olympiad**
  2005
  17th Flemish Physics Olympiad, Final 15

SCIENTIFIC PUBLICATIONS

A1 papers


Curriculum Vitae


- Callewaert, C. (*), **Van Nevel, S.** (*), Kerckhof, F.-M., Granitsiotis, M. S., Boon, N. Microbial exchange in household laundry machines. Submitted to *Scientific Reports*.

(*) Both authors contributed equally.


A4 Papers:

B2 Chapters in books:

**CONFERENCES, SYMPOSIA AND WORKSHOPS**

**Active participation**
- **Van Nevel, S., Hennebel, T., Verstraete, W., Boon, N.** Occurrence of transparent exopolymer particles (TEP) through drinking water treatment plants. 6th IWA specialist conference on membrane technology for water & wastewater treatment, Aachen, Germany, October 4-7, 2011. *Poster presentation.*

Van Nevel, S., De Roy, K., Boon, N. Bacterial invasion potential in water is determined by nutrient availability and the indigenous community. 3rd IWA BeNeLux Regional Young Water Professionals Conference, Belval, Luxembourg, October 2-4, 2013. *Oral presentation.*

**Passive participation**


**PRESENTATIONS AT OTHER EVENTS**


Het is wellicht deze pagina waar je naartoe wou bladeren.

Net zoals een zware bergtocht maak je een doctoraat liever niet alleen. De ervaring van mensen die het gebied kennen kan onmisbaar zijn, soms heb je elkaar nodig om obstakels te overwinnen of gewoon ervaringen en de zwaarste last lichter te maken.

Gelukkig kon ik op mijn 4-jarige tocht rekenen op twee fantastische berggidsen: prof. Nico Boon en dr. Frederik Hammes. Nico, uw enthousiasme, optimisme en gedrevenheid waren onmisbaar. Ik hoop dat enkele van uw wilde onderzoeksideeën waar we over praatten ook ooit echt toekomst kunnen worden, en uwen ervaringen en de zwaarste last lichter maakt. Frederik, uw tochtbegeleiding is er halfweg bijgekomen, maar heeft nog een wereld van verschil gemaakt. Als ik bij u aanklopte met een idee, had je steeds tijd voor een discussie en legde je uit dat je zo’n experiment al gedaan had. Daarna kwam ik steeds naar buiten met een beter idee. Tot slot, nog een eervolle vermelding voor prof. Willy Verstraete, die 4 jaar op papier mijn promotor was zonder dat 1 van ons beiden dit wist, maar toch een levensinspiratie geweest is. Uw energie zal hier altijd in de gangen blijven hangen!

A next sincere thank you is for my jury members, for the help in redrawing and finalising my manuscript. Dr. Ameet Pinto, Prof. Arne Verliefde, Prof. Mieke Uyttendaele, Sabine Kreps and chairman Prof. Paul Van der Meeren, although you have been roasting me for nearly three hours during my pre-defence, in my opinion, it was a relax and interesting talk, thanks for the useful suggestions and feedback to make my story better. Ameet, too bad we met so late, it would have been nice working together with you, and Arne, if you’re still in for the brainstorm session, just let me know!

Tijdens mijn tocht heb ik de eer gehad om enkele mensen te mogen meenemen onder mijn begeleiding, wat de tocht steeds iets meer leerrijk maakte. Steffie, met plezier ben ik voor u nog even op het BioPAD, volgens sommigen het juiste pad, gebleven. Eva, onze samenwerking is altijd aangenaam geweest, ook al had je een iets andere prioriteit. Elise, bedankt voor de samenwerking, met speciale vermelding voor uw urenlange analyses in ons tijdelijk labo in Brakel die ik enorm heb geapprecieerd! Tot slot in deze rij een eervolle vermelding voor Veerle, Freke, Ynse en Evelien: dankzij jullie enthousiasme hebben we een prachtig project kunnen maken!

Mijn belangrijkste tochtgenoot is er maar het laatste jaar gekomen. Benjamin, gij zijt een perfect voorbeeld van hoe een ‘eerste investering’ al heel snel zijn vruchten afwierp; ge hebt
Dankwoord

snel bijgeleerd en de samenwerking gaf een boost aan mijn onderzoek, en bovendien kon ik eindelijk mensen doorsturen die vroegen over de flowcytometer. Ik ben benieuwd waar uw onderzoek zal eindigen, maar ik geloof er sterk in dat het de moeite gaat zijn! Karen, in dit gebied zijde gij mijn eerste gids geweest, merci voor de tijd die jij dan in mij gestoken hebt. Veel van mijn onderzoek ging er nooit geweest zijn zonder uw hulp, met speciale vermelding voor ons onderzoek met panoramisch zicht over nachtelijk Gent. Francis de los Reyes, it was great working together, and I promise that background publication will get published! Tot slot, Valerie, ook al zat je bij de concullega’s in Leuven, ‘t was leuk om samen met u een project te lopen en uw motivatie heeft mee de doorslag gegeven dat ik de dodentocht heb uitgelopen.

Naast collega’s, heb ik ook veel steun en goeie samenwerking gehad met industrie. De samenwerking met Farys (TMVW in de volksmond) heeft het parcours van mijn tocht op een interessante manier hertekend, een dikke merci aan Bart is hier op zijn plaats. Ooit mijn goeie buur op de Rotonde, daarna mijn ‘feet-on-the-ground’. Willy zou trots zijn op zo’n goeie samenwerking! François, merci voor de sterke verhalen over drinkwater (die bestaan!) en grote jachten in Antibes, en Kristin, voor de vele uitleg & toegang tot het labo. Bij Pidpa wil ik Koen, Paul & collega’s bedanken voor het interessante overleg en bij De Watergroep Jan voor de goeie samenwerking. Tot slot, Manu, ook al zijt ge mij vergeten ophalen aan’t station, bedankt voor de ontvangst op IWVA.

Tijdens mijn tocht heb ik ook altijd een fantastische uitvalsbasis gehad, en intussen besef ik dat ik bij een top generatie ben toegekomen in de Rotonde, onder leiding van voorzitter dr. Hannibal. Tom, merci voor alles tijdens zowel mijn thesis als erna! Ik ben binnengeloodst op LabMET door Simon, en los van het feit als ik u daarvoor moet dankbaar zijn of niet, heb ik toch zeker drie goeie etentjes aan u te danken via de knipquiz. Onzen tocht door Kreta met als hoogtepunt het politiemuseum/gokkantoor, is er ook eentje om te onthouden! Joachim, vanaf nu kom ik met veel plezier weer uw slagen incasseren of uw gewrichten pijn doen op training. Willem (dr. Beton), we lachten wel ne keer met de knie, maar stiekem wil iedereen wel zo nen legendarisen bompa hebben die elke dag het fijnste uit het nieuws verteld! Iemand zei me ooit ‘als uw doctoraat niet over flow cytometrie gaat, dan doctoreerde gij niet’, merci Joeri, om mij tot inzicht te laten komen. Haydee & Loïs (Yeti!), merci om tussen al dat enthousiasme ook wat rust te brengen. Beetje bij beetje kwamen er nieuwe collega’s toe; Emilie, die beloofde spaghetti komt er en samen met Sylvia, draag zorg voor de nieuwe Rotonde, maar ik heb der vol vertrouwen in. Synthia, merci om zovele teksten na te lezen, maar straks kom jij misschien wel nog eens aan bod in deze tekst ;-) . When I entered the Rotonde, we were 8 Belgians and a Frenchy, but now, we’re the most multicultural office: with 8x Belgium, 3x Italy, 2x France, and 1x Australia, Spain, China, Malaysia, India and Greece, no other office beats us in this, but neither do they in office feeling or atmosphere! Keep the Rotonde spirit alive!
Anderen op LabMET wil ik bedanken om allerlei redenen: Kris & Regine, die vanuit de regiekamer alles stuurden en in de gaten hielden, voor de administratieve vereenvoudiging, Tim voor de fantastische designs en het mij ontlasten van moleculair werk, en Jana & de rest van het ATP voor labohulp, en nog zowat iedereen voor de goede sfeer, zowel in LabMET, als aan de overkant van de straat. Keep care of LabMET, the lab where you can pleasure others by donating armpit sweat, urine or faecal material.

I had the great opportunity to continue a part of my journey in Switzerland under the guidance of Frederik, somebody who manages amazingly well in the idea ‘make sure your staff likes their job, and they’ll work well’. It was great to be part of the drinking water group (sometimes abbreviated as ‘drinking group’) for a while. All the best to Stefan (the most rock ’n roll scientist I’ve ever known), Hansueli (the most rock ’n roll lab technician I’ve ever known), Jürg (the lab technician doing most crazy things), as well as Michael and the ladies, Caitlin, Franziska, Romina, Steffie & Alina. Officemates Bastian (Ralphy) & Desiree, happy that I had you around for my finish and Ralphy, all the best with your PhD & start writing on time, then there’s no reason to be stressed. Thanks Bettina for the support, thank you to all of UMIK for the lunches and aperos!

Tijdens de pauzes op een tocht moet een mens eens tot rust kunnen komen en bij voorkeur kunnen lachen, ver weg van al het werk - dat kon nergens beter dan in mijn thuisbasis Zulte. Daan, Hannes, Vozzie, Verhalle, Rop & Bompke, de Ksa-vrienden zijn de vrienden voor het leven, en Lieselot, Lynn, Elke, Claudia & Annelies, de vrouwen erbij maken het enkel maar meer compleet! Daan, kheb nu ook gezien: een boekske maken is niet gemakkelijk en da kan veel tijd kosten en Hannes, ooit doen we dat nog, die bierproeverij.

De zwaarste tochten, letterlijk dan, deed ik met Bunny & Jason. Life begins at the end of the comfort zone, en dat hebde gunder begrepen. Merci om mijn limiet te pushen, en dervoor te zorgen da we reisfoto’s hebben waar iedereen jaloers op kan zijn. Wanneer gaan we samen naar de top van de Mt. Blanc? Om te oefenen kunnen we nog eens op’t strand gaan wandelen, als Jason nog durft natuurlijk... Ter info voor Bart & Reggie: 1 euro is momenteel 9.30 SEK, en die blijft nog wel stabiel voor een paar jaar.

Na de uren kon ik in Gent ook altijd genoeg plaatsen vinden om mijn gedachten te verzetten. Thuis was ik op’t gemak bij Joke, Jasmine, Mathias en nu Tim, die op zich misschien nu wel niet de meest rustige persoon is. Mijn Gentse studietijd heeft mij nog goeie vrienden opgeleverd, hoog tijd om nog eens af te spreken met deze mensen: Jo, merci om mij voor te gaan door de jungle van de doctoraatsprocedures, en Katrijn, als je daarover iets moet weten vraag je’t maar. Ik ben nu al benieuwd naar een doctoraat over lucht in varkensstallen! Als Jo zweeg konden we ook wel eens over iets anders dan doctoreren praten, over actieve kool
bijvoorbeeld (Stefaan!) of afvalverwerking (Alexander!) of misschien gewoon over iets helemaal anders (dankuwel Annelies & Naima!). De vrienden van KSA OVL, blijf dervoor gaan, ge zij in goeie handen bij Lien & Siglin. Openluchtleven nationaal, OL the way, niemand twijfelt eraan, het blijft de beste nationale werkgroep, en vanaf januari kunde weer op mij rekenen! Als ik echt mijn hoofd helemaal leeg wou maken en enkel nog sterrekes zien, was ik bij den budokai-do altijd aan’t juiste adres. Guru Michel, merci voor alle inzet en creatieve voorbereiding, vanaf januari sta ik weer op de mat!

We komen bijna aan het einde van de lange lijst, maar vanaf nu wordt het enkel maar belangrijker. Ik wil een hele grote merci zeggen aan mijn ouders, om mij altijd mijn ding te laten doen, in mij te geloven, mij vrij te laten gaan maar mij ook te steunen waar nodig, tot vandaag toe. Ik ben blij da je de laatste keer in Zwitserland eindelijk goed weer gehad hebt! Mijn broers moet ik ook voor vele dingen bedanken, en om maar iets te zeggen, Tom, merci voor de rit naar Zwitserland en Stijn, merci voor de vele pogingen die je gedaan hebt met Sanne om eindelijk een goeie coverfoto te krijgen!

Na mijn 4-jarige tocht, mijn queeste, neem ik het allerbeste souvenir van LabMET mee naar huis. Synthia, liefje, tis niet om nationale parken te bezoeken dat ik naar de VS ben gevlugt, tis niet om terug naar huis te komen dat ik u naar Zwitserland liet rijden, maar wel omdat ik mijn ideale tochtgenoot in u gevonden heb! Tis ook dankzij mijn fantastisch lief dat ik onder alle omstandigheden zo rustig kon blijven. De Skype-tijd is eindelijk voorbij en we hebben al prachtige tochten samen gedaan and there’s more to come, samen gaan we verder op tocht, te voet, per fiets of op het water, maakt niet uit, maar wel samen. Ik heb u lang genoeg laten wachten – ik heb met u de vrouw van mijn leven gevonden & ik wil de rest van mijn tocht met u verder zetten!

‘Water is to me, I confess, a phenomenon which continually awakens new feelings of wonder as often as I view it’

 * Michael Faraday