Bacterial Community Dynamics in a Biofilter exposed to a Micropollutant

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Acknowledgements

Abstract

Perfluorooctanoic acid (PFOA) is a fluorinated compound used in many industrial and consumer products. It is environmentally persistent, widespread and bioaccumulative, and multiple toxicities have been reported in experimental models and wildlife. Trace amounts of PFOA have been detected in humans, animals and numerous environmental compartments, including drinking water sources.

PFOA can be removed from drinking water by technically complex and energy intensive treatment processes. If the target compound is biodegradable, biological filtration could be a suitable alternative for removal of the contaminant from drinking water. Biofiltration is simple and robust, and represents a "green" treatment technology. Studies have demonstrated potential of microbial degradation of fluorinated compounds, however the published information is very limited.

The short-term impact of environmental occurrence PFOA concentration on biofilm bacterial community dynamics in a biofilter for drinking water treatment was examined. Fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) were applied as molecular methods for characterization of bacterial community dynamics.

Bacterial community structure changed during continuous PFOA exposure, as demonstrated by both FISH and PCR-DGGE. FISH analysis of α-, β- and γ-proteobacteria revealed a significant shift from γ- to β-proteobacterial dominance in the biofilter communities in response to the polluted synthetic surface water.

Sammendrag

Perfluorert oktansyre (PFOA) er et fluorert stoff benyttet i mange industrielle og forbruker produkter. Stoffet er miljømessig vedvarende, utbredt og bioakkumulerende, og flere toksisiteter har blitt rapportert i eksperimentelle modeller og dyreliv. Spormengder av PFOA har blitt detektert i mennesker, dyr og en rekke deler av miljøet, inkludert drikkevannskilder.

PFOA kan fjernes fra drikkevann ved bruk av teknisk kompliserte og energi-intensive behandlingsprosesser. Dersom målforbindelsen er biologisk nedbrytbar kan biologisk filtrering være et egnet alternativ for fjerning av forurensningen fra drikkevann. Biologisk filtrering er enkelt og robust, og representerer en "grønn" renseteknologi. Studier har demonstrert potensiale for mikrobiell nedbrytning av fluorholdige forbindelser, men den publiserte informasjon er svært begrenset.

Den kortsiktige effekten av miljøkonsentrasjoner av PFOA på biofilm bakteriesamfunnsdynamikken i et biofilter for drikkevannsrensing ble undersøkt. Fluorescens *in situ* hybridisering (FISH) og denaturering gradient gel elektroforese (DGGE) ble benyttet som molekylære metoder for karakterisering av det bakterielle samfunnets dynamikk.

Det bakterielle samfunnets struktur viste forandringer under kontinuerlig PFOA eksponering, demonstrert både ved FISH og PCR-DGGE. FISH analyse av α-, β- og γ-proteobacteria avslørte en signifikant skift fra γ- til β-proteobacterial dominans i biofilter samfunnet i respons til det forurensede syntetiske overflatevannet.

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Abbreviations

2D	2 dimensional
ANOVA	Analysis of variance
BDOC	Biodegradable dissolved organic carbon
BOM	Biodegradable organic matter
bp	Base pair
CLSM	Confocal laser scanning microscope
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
dsDNA	Double stranded DNA
DWT	Drinking water treatment
DWTP	Drinking water treatment plant
EBCT	Empty bed contact time
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FISH	Fluorescence *in situ* hybridization
GAC	Granular activated carbon
H'	Shannon diversity index
HS	Humic substances
J'	Pielou's evenness index
K'	Band richness
$NaPP_i$	Tetrasodium pyrophosphate
NM-MDS	Non-metric multidimensional scaling
NOM	Natural organic matter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFASs	Perfluoroalkyl and polyfluoroalkyl substances
PFOA	Perfluorooctanoic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDOM	Standard deviation of the mean
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
TOC	Total organic carbon
WWT	Wastewater treatment

1. Introduction

The quality of water sources is decreasing worldwide due to pollution through industrial processes, agriculture, product usage and disposal. The presence of contaminants in drinking water sources at trace concentrations is often observed in highly populated and industrialized areas, and where indirect water reuse is practiced (Proia et al., 2013).

Fluorinated contaminants are a subgroup of micropollutants. These compounds are capable of being transported through the air, and show a tendency to precipitate in colder areas, i.e. near the North Pole (Dreyer et al., 2009). Fluorinated substances demonstrate high toxicity and persistency, and may cause development delays and cancer (Fei et al., 2008; Kudo and Kawashima, 2003; Staples et al., 1984). If present in drinking water, consumption of these compounds provides a direct route into the body.

The main objective of drinking water treatment (DWT) is to ensure that the drinking water is of a satisfactory quality, including free of odor and color and does not contain pathogens and is otherwise considered safe for the recipients. The second objective of DWT is removal of micropollutants. Current technologies for removal of perfluorinated compounds include technically complex and energy intensive treatment processes, resulting in high operational costs. Biofiltration is a cost-effective, green treatment-technology, and a suitable treatment process for removal of micropollutants if the target compound is biodegradable.

To date, there is limited data available demonstrating a direct correlation between biodegradation and biological activity in drinking water biofilters. This thesis will investigate the effect of micropollutant exposure to biofilter used for drinking water treatment.

1.1 Biological filtration for drinking water treatment

Applications

The different processes and combinations of treatment technologies for DWT depend on the characteristics of the raw water. Typical DWT processes include coagulation, flocculation and sedimentation, aeration, bank filtration, rapid and slow sand filtration, advanced oxidation and disinfection and adsorption onto activated carbon (Benner et al., 2013). In Norway, conventional DWT techniques include membrane filtration or chemical precipitation (coagulation, flocculation and sedimentation), followed by disinfection (UV-radiation or chlorination) (Skjærstad, 2013).

Biological filtration (biofiltration) is a viable DWT technology with widespread use in Europe, where the application of biofiltration processes for the removal of natural organic matter (NOM) has a long tradition (Juhna and Melin, 2006). Drinking water treatment plants (DWTP) are engineered and designed based on the multi-barrier approach, where multiple water treatment processes are combined. This increases the reliability of the overall system. Biofiltration if often combined with other treatment processes, e.g. ozonation. The use of ozonation requires biofilters since ozonation increases the amount of biodegradable organic matter (BOM) in the water. Due to its low maintenance costs and effective removal of BOM, ozonation-biofiltration is becoming an attractive DWT method (Juhna and Melin, 2006). In Norway, biofiltration is combined with ozonation when treating drinking water. In 2011, Norway had 15 ozonation-biofiltration treatment plants (Aasand, 2011).

Biofiltration is expected to become even more common in the future as efforts intensify to decrease the presence of disease-causing microorganisms and disinfection by-products in drinking water, to minimize bacterial regrowth in distribution systems, and where operator skill levels are emphasized. It is regarded a green treatment technology due to simple operation and maintenance of the system and without addition of chemicals (Juhna and Melin, 2006).

General concepts

The main objective of biofiltration is to produce drinking water that is biologically stable and thus does not support significant microbial growth during distribution (Rittmann, 1995). Biofiltration is a process in which water is filtered through filter materials with attached microorganisms originating from the raw water. Biofiltration has shown to be an effective treatment process for removal of substances that causes biological instability in the distribution system, including nitrate, ammonium, BOM, manganese (II), sulfate and iron (Boley et al., 2006). Moreover, biofilters are used for removal of pesticides (Boley et al., 2006), algal metabolites and taste and odor causing substances (Nerenberg et al., 2000).

Water in which bacteria are not multiplying is called biologically stable. The growth of bacteria in distribution networks is not desirable because some of these bacteria can potentially be pathogenic to humans and cause change in water quality. Bacterial growth in networks is mainly occurring in biofilms on surfaces of pipes. Biological instability in the distribution system can lead to odor and taste events, acceleration of corrosion, consumption of dissolved oxygen, increased heterotrophic plate counts, turbidity and bacterial regrowth. The biofilter removes BOM and therefore increase the biological stability of the water and reduces the risk of formation of disinfection by-product (DBP) in the following disinfection process (Rittmann and McCarty, 2001).

Several factors influences biofilter performance, including pH, presence of easily biodegradable compounds and nutrients in the raw water, temperature, the empty bed contact time (EBCT), type of filter media and backwashing procedures (Fonseca et al., 2001; Hallé, 2009; Moll et al., 1999).

Biofilm

Most of the microorganisms present in biofilters are living and growing in biofilms. Most granular media filters, i.e. anthracite and sand, can be converted into biofilters. The development and life cycle of a biofilm is a multistep process, including attachment, growth and dispersal (Figure 1.1). Surfaces often accumulate chemicals from the passing bulk liquid, and serves as substrates for microorganisms from the source water. Planktonic (free-floating) microorganisms encounter a surface, attach and become immobilized on the surface (Hozalski and Bouwer, 1998). The microorganisms move along the surface and associate with one another. Once the nascent microcolony has established, cells of other species are recruited to the biofilm from the passing bulk liquid (Stoodley et al., 2002).

Figure 1.1: Development and life cycle of a biofilm; attachment, growth and dispersal. Adapted from Cunningham et al. (2008).

The microorganisms are embedded in a three-dimensional gel-like structure of extracellular polymeric substances (EPS), which includes polysaccharides, proteins, humic substances (HS), nucleic acids and lipids (Costerton et al., 1995). During the proliferation phase, cells divide and EPS increases by production and debris from

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dead cells (Stoodley et al., 2002). The EPS protects the microorganisms from adverse environmental conditions, often enforced by multivalent cat-ions which cross-link the polymers in the EPS matrix (Flemming and Wingender, 2010). Biofilm formation may therefore be a survival mechanism for microorganisms that thrives in an aquatic environment. In the dispersion phase, the biofilm propagates through detachment of cell-clumps, allowing also individual cells to detach. The microorganisms are then enabled to reattach to surfaces downstream of the original community (Cunningham et al., 2008).

A mature biofilm consist of accumulated dissolved organic molecules and other colloidal- and inorganic particles provided from the passing bulk fluid. This increases the mass and complexity of the biofilm, and may serve as structural elements as well as nutrient substrates (Costerton et al., 1995; Flemming and Wingender, 2010). The microorganisms are surrounded by water channels allowing nutrients to reach biofilm-associated microorganisms and allow toxic metabolites to diffuse out of the biofilm (Stoodley et al., 2002).

Factors influencing biofilter performance

Utilization of substrate, substrate diffusion inside the biofilm, growth, decay and mass transport between the bulk liquid and the biofilm controls biofiltration performance (Rittmann, 1995). The electron donor (primary substrate) for sustaining growth and maintenance of the biomass is BOM (Stratton et al., 1983). The major substances of BOM are carbohydrates, HS, amino acids and by-products from ozonation, if ozonation is applied as a treatment method. Surface waters being used for source of drinking water usually contain low BOM, is heterogeneous and show seasonal variations (Urfer et al., 1997).

The biomass obtains its energy through redox reactions of the primary substrate. However, these reactions are often slow and enzymatic reaction is required to increase

the kinetics of the reaction. Temperature, pH and substrate availability affects the rate of the reaction. Low water temperature may also influence cell permeability, or the ability of nutrients to be transported into the cell (Rittmann and McCarty, 2001). Alkalinity may lead to e.g. denaturation of DNA, instability of the plasma membrane and inactivation of cytosolic enzymes (Higashibata et al., 1998).

To keep the thickness of the biofilm below a certain limit in order to prevent clogging of biofilters, a common procedure is to perform periodical backwash operations, i.e. the liquid flow in the filter is temporarily reversed. A backwash event leads to fractional biofilm removal through a combination of detachment mechanisms (Morgenroth and Wilderer, 2000).

Adaptation of biofilter bacterial communities

The second objective of engineered biofilters is removal of micropollutants. The removal process include adsorption to the biofilter media, bioabsorption (i.e. the compound is accumulated within the organisms), or biodegradation mechanisms by the biofilm microbial community. Many micropollutants are recalcitrant to biodegradation. The compound must be accessible to the organism, concerning physicochemical aspects (i.e. sorption and bioavailability), chemical structure and biochemical aspects (i.e. membrane permeability and adequate enzymes). Excessive toxicity of the primary compound or degradation products, high concentration of the compound, unfavorable pH or temperature, or lack of oxygen or mineral nutrients may also negatively influence the adaptation process (Alexander, 1999).

However, microorganisms have shown an extensive capacity to degrade anthropogenic substances in addition to utilizing natural substances. Biodegradation of organic compounds in biofilters occur either by direct metabolism or by cometabolism. Cometabolism is the fortuitously degradation of a cometabolite by an operating enzyme system (Alexander, 1999). Concentration of micropollutants are

often trace, therefore considered secondary substrates. Secondary substrate utilization is the metabolism of a compound in the presence of primary substrates, which supply the microorganism's primary growth needs (Bouwer and McCarty, 1984).

To increase the rate of transformation of anthropogenic substances as a result of exposure, the microbial community adapt by using one or several mechanisms, including enzyme synthesis regulation, gene transfer and genetic alterations, and selective enrichment. Enzyme synthesis is regulated by environmental stresses, and usually requires short adaptation time, i.e. hours. Exchange of genetic material can occur via transformation, conjugation or transduction and usually happen within hours to days. In this case, the community structure usually stays the same. Inheritable genetic alterations can happen via duplication, mutation or recombination, and require a long adaptation time, i.e. weeks to years (Rittmann and McCarty, 2001). One important mechanism in environmental processes is selective enrichment, generally leading to significant changes in bacterial community composition. It can take days to months for selective enrichment to occur (Garland, 1997).

Moreover, microbes may favor growth of other microorganisms by changing the environment, e.g. change of pH or redox potential, supply deficient substrates or eliminate toxic compounds. One of the most important requirements for biodegradation of a micropollutant is existence and presence of an organism with potential to biodegrade the compound. The "microbial infallibility hypothesis" by microbiologist Dr. Ernest Gale in 1952, suggests that accumulation of organic compounds originally resistant to biodegradation will exert a strong selective pressure on microbes that eventually will evolve to consume them, given that suitable environmental conditions are present.

1.2   Micropollutants in the environment

Water quality is adversely affected by continuous input of anthropogenic chemicals originating from several point and non-point sources including domestic and commercial activities, industrial discharge, wastewater treatment plants or pesticides from agriculture (Hansen et al., 2002; Lin et al., 2010). Anthropogenic contaminants, commonly referred to as micropollutants, are organic compounds occurring at μg/L to pg/L concentrations. Common micropollutants detected in the aquatic environment include pharmaceuticals, personal care products, steroid hormones, surfactants, industrial chemicals and pesticides (Luo et al., 2014). These pollutants are frequently detected in surface waters used as drinking water sources (Focazio et al., 2008; Proia et al., 2013).

Because of the number of chemicals that has been introduced into the environment the last 50 years and new compounds synthesized every year, environmental contamination by organic micropollutants is a problem of increasing complexity. The increased frequency of the detection of these chemicals is both a function of improved analytical capabilities and increased use of the pollutants. Anthropogenic substances contaminating drinking water resources raises important questions related to impact on the ecology and human health.

1.2.1 PFOA

Sources and attributes of PFOA

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a diverse class of anthropogenic fluorinated carboxylic acids in which the hydrogen atoms of the aliphatic carbon backbones have been partially (prefix: poly-) or completely (prefix: per-) replaced with fluorine atoms. PFASs are emerging pollutants of the 21st century (Vierke et al., 2012). These substances have unique chemical properties including extremely high chemical and thermal stability due to their highly polar and strong

carbon-fluorine bonds, and were first produced by the 3M Company in the 1940's by electrochemical fluorination (Simons, 1950). In the 1950's, DuPont started using the chemicals in the manufacturing of fluoropolymers (Emmett et al., 2006).

Perfluorooctanoic acid (PFOA, Figure 1.2) is one of the fluoropolymers that has received particular attention in recent years. PFOA, also known as C8, APFO and perfluorooctanoate, is a surfactant used as a processing aid to manufacture long-chain fluoropolymer high performance materials including Gore-Tex, Teflon, stain-resistant carpets, firefighting foam, nonstick cookware and fast-food packaging (Banks et al., 1994). Moreover, a recent review by Liu and Mejia Avendaño (2013) demonstrated that a number of perfluoroalkyl precursors are biodegraded into PFOA under favorable environmental conditions.
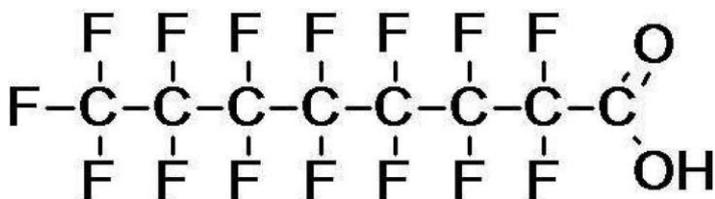
Figure 1.2: Chemical structure of PFOA.

Toxicity of PFOA

There is a growing body of literature on toxicity of PFASs in animal models, however data on the toxicological effects of these compounds on humans are limited (Steenland et al., 2010). PFOA gets absorbed quickly following oral and inhalation exposure (Kudo and Kawashima, 2003), and circulates in the blood of humans and rats mostly in a protein-bound form (Han et al., 2003). Once PFOA enter the body, it is poorly eliminated. In humans the serum half-life of PFOA was reported to approximately 3.5 years (Olsen et al., 2007), thus showing bioaccumulative potential.

A review by Kudo and Kawashima (2003) reported that PFOA is linked to increased incidents of Leydic cell adenoma in rats, liver carcinogenesis in rodents and immunotoxicity in mice. Moreover, PFOA is associated with reduced organ growth during organ development in humans (Fei et al., 2008) and may contribute to reproductive and developmental toxicity in rats (Staples et al., 1984). PFOA was recently included on a list of 'obesogens', compounds that may contribute to obesity (Holtcamp, 2012). A recently published study carried out on Ohio-valley residents who had been working at the local DuPont Washington Works chemical plant or been exposed to polluted drinking water, found a correlation between testicular and kidney cancer and PFOA (Barry et al., 2013).

Environmental persistency of PFOA and regulatory framework

Giesy and Kannan (2001) were among the first to report the extensive distribution of PFASs, which during their industrial production and application are released in the environment. Moreover, their distribution is a result of leaching from, and degradation of consumer products. A study of European groundwater in 2010 reported that PFOA accounted for about 48% of detected PFASs (Loos et al., 2010).

Trace amounts of PFOA have been detected in diverse environmental compartments and biological media including drinking water (Mak et al., 2009), surface waters of lakes (Jin et al., 2009), ocean waters (Yamashita et al., 2008), soil (Strynar et al., 2012), in the blood of the general population (Kannan et al., 2004) and wildlife (Giesy and Kannan, 2001). Median serum concentrations of PFOA in residents from a community exposed to this compound via contaminated drinking water was 27 ng/mL (Steenland et al., 2009). Studies have also demonstrated the occurrence of PFOA in the arctic environment and -biota (Butt et al., 2010), indicating that PFASs can be transported over long distances, as also reported by Dreyer et al. (2009).

Manufacturing and use of PFASs have been restricted or reduced in recent years, due to the wide global distribution and hazardous properties. By 2015, PFOA and its longer chained homologues and precursors will be eliminated from emissions and products produced by eight major manufacturers (U.S.EPA., 2006). From June 1 2014, manufacturing, importing, exporting and selling consumer products and textiles containing PFOA and individual salts and esters of PFOA was prohibited in Norway. The prohibitions do not apply to food packaging, food contact materials and medical devices (Norwegian Environment Agency, 2013).

Although companies that manufacture PFOA will have reduced emissions of this compound into the environment, the exceptional stability to environmental and metabolic degradation caused by the strong carbon-fluorine bond (Liou et al., 2010), ensures that it will have a continued presence in environmental and biological media.

1.3 Treatment technologies for removal of PFOA

Collection and treatment of PFOA is difficult using most conventional treatment technologies due to the trace concentration (Schwarzenbach et al., 2006). Vecitis et al. (2009) reviewed the mass flow of PFOA in the context of wastewater treatment (WWT) and reported that conventional WWT techniques, such as activated sludge, trickling filtration, chlorination and anaerobic digestion, show a generally low removal of this compound. In some cases, PFOA concentrations were significantly greater in the effluent compared to influent (Sinclair and Kannan, 2006), suggesting microbial degradation of PFAS precursors into PFOA, which has been demonstrated in several laboratory experiments (Wang et al., 2009; Wang et al., 2005). Tertiary water treatment technologies, such as adsorption to granular activated carbon (GAC) effectively removes more than 90% of PFOA from WWT effluents. However, a subsequent combustion step is necessary for complete removal of the compound (Vecitis et al., 2009).

A number of less commonly employed treatment options for removal of PFOA have been evaluated in the literature, e.g. persulfate- and hydrogen peroxide photolysis (Kutsuna and Hori, 2007) and UV-KI photolysis (Park et al., 2009). However, these fluorochemical treatment processes are technically complex and energy intensive leading to high operational costs (Vecitis et al., 2009).

Biofiltration for removal of PFASs in DWT

In the context of DWT, Rahman et al. (2014) reported that conventional coagulation, flocculation and sedimentation achieves some removal (less than 20%) of PFASs. Under typical drinking water treatment plant conditions, most long-chained PFASs such as PFOA, will not oxidize under oxidation and advanced oxidation processes, and may also be oxidized to other PFASs. A more suitable DWT option for removal of perfluorinated contaminants from drinking water resources would be an efficient and inexpensive technique such as biological filtration. Perfluorinated contaminants are known to be present in drinking water and in the general population in rural areas and sparsely populated countries like Norway (Gützkow et al., 2012; Haug et al., 2010).

The published information on the biodegradation of PFASs is very limited. A recent review by Rahman et al. (2014) presented the limitations of present day drinking water treatment technologies for removal of PFASs, and concluded that current drinking water treatment processes, including aerobic biofiltration, are unlikely to biodegrade most PFASs. However, studies have demonstrated potential of bacterial degradation of fluorinated compounds by laboratory isolates in aerobic enrichment culture experiments (Key et al., 1998; Thelakkat Kochunarayanan, 2011) and anaerobic sludge batch experiments (Remde and Debus, 1996).

1.4 Molecular methods for microbial community analysis

99% of all microorganisms in nature cannot be cultured with nutrient medium. The primary source of information for these uncultured organisms is therefore their biomolecules, i.e. nucleic acids, proteins, and lipids. The field of microbial ecology has progressed tremendously over the last few decades. Recent advances in methodology has allowed for development of molecular techniques for detecting and characterizing the phylogenetic and functional diversity of microorganisms (Rastogi and Sani, 2011).

rRNA suitable targets for partial microbial community analyses

Partial microbial community approaches generally include polymerase chain reaction (PCR)-based methods, in which DNA or RNA isolated from an environmental sample is used as a template for amplification and characterization of microorganisms. PCR has revealed huge diversity in the microbial world, and made it possible to detect non-culturable microorganisms in virtually any environment (Baker et al., 2003).

Prokaryotic ribosomes consist of three rRNA subunits; 5S, 16S and 23S (Fabrice and Didier, 2009). The gene encoding 16S rRNA has been extensively applied as a marker for microbial taxonomic classification in PCR-based methods because this gene is ubiquitous in all prokaryotes, includes variable and highly conserved regions, and is structurally and functionally conserved (Hugenholtz, 2002). Furthermore, the 16S gene is more easily and rapidly sequenced compared to the 5S and 23S genes (Spiegelman et al., 2005), and a growing number of 16S rRNA sequences are available for comparison in sequence databases.

The regions of the 16S rRNA gene are conserved enough to allow the design of PCR primers that target several taxonomic groups, while other gene regions are variable enough to provide phylogenetic comparisons of bacterial community structure (Woese, 1987). Other conserved genes, e.g. *hsp 60* and *rpoB* encoding heat shock

protein and RNA polymerase beta subunit, respectively, have also been employed for differentiation of bacterial species (Ghebremedhin et al., 2008).

Environmental DNA and its amplified PCR products are primarily analyzed by genetic fingerprinting, DNA microarrays, clone libraries, or by a combination of these methods. Clone library is the most widely used method for analysis of PCR fragments amplified from an environmental sample. Individual gene fragments are cloned, sequenced and then compared to known sequences in a database. DNA microarrays is a method in which PCR fragments are hybridized to molecular probes attached on microarrays, and positive signals are detected by the use of CLSM (Rastogi and Sani, 2011).

Genetic fingerprints generate a profile of microbial communities based on direct analysis of the PCR amplified fragments (Muyzer et al., 1993). Genetic fingerprinting approaches are rapid and allow simultaneous analyses of several samples. These techniques can demonstrate effects on microbial communities or inequalities between microbial communities. However, they do not provide any direct taxonomic identities. The most common genetic fingerprinting techniques include DGGE, TGGE and T-RFLP. Terminal restriction fragment length polymorphism (T-RFLP) involves the use of restriction enzyme(s) which digest PCR products, and the resulting terminal restriction fragments are then detected an separated by an automated DNA sequencer (Rastogi and Sani, 2011).

DGGE

Fischer and Lerman (1983) were the first to describe the theoretical aspects of DGGE, and Muyzer et al. (1993) introduced the method in molecular ecology. The PCR amplicons are electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturants. Temperature gradient gel electrophoresis (TGGE) is based on the same principle as DGGE, with a temperature gradient employed as a denaturing agent instead of a chemical agent.

The PCR products are all the same size, but because of differences in base sequences between different species, their denaturing properties differ. The PCR amplicons stops migrating in the gel at the point where sufficient denaturant is enough to melt the PCR product. A 40 base pair (bp) GC-rich fragment (GC-clamp) is incorporated in the forward (5') primer during the PCR step to prevent complete dissociation when the double-stranded PCR fragments migrate through the electrophoresis gel (Muyzer et al., 1993).

The analysis has high sensitivity for detecting differences in sequences. If 16S rRNA is used as the target gene, the pattern of the DGGE gel immediately reveals number of distinct 16S rRNA genes present in the habitat. Once DGGE has been performed, individual bands can be excised and sequenced for further identification and characterization of phylogenetic relationships (Madigan et al., 2012). Figure 1.3 illustrates the basic steps of PCR-DGGE approach for bacterial community analysis.
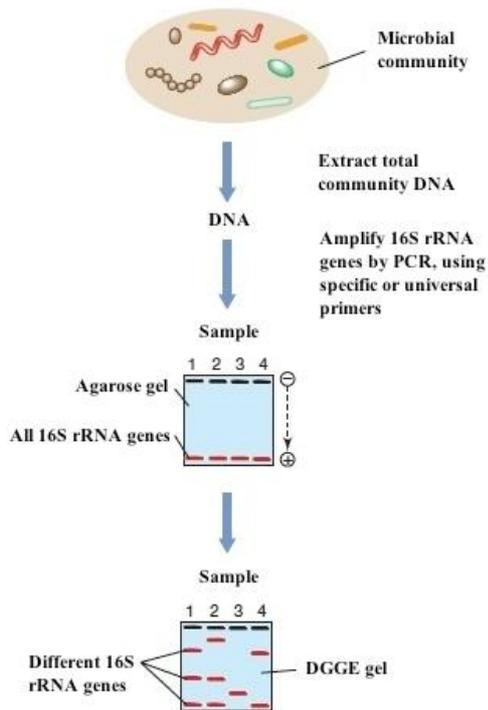
Figure 1.3: The basic steps of PCR-DGGE for bacterial community analysis. Adapted from Madigan et al. (2012).

FISH

Fluorescence *in situ* hybridization (FISH) has become a powerful tool for *in situ* phylogenetic identification and quantification of individual microbial cells (Amann et al., 1995). FISH is based on whole-cell hybridization of fluorescently labeled DNA oligonucleotide probes to cellular rRNA (Figure 1.4). The principle of using fluorescently labeled rRNA-targeted oligonucleotide probes as phylogenetic stains for non-culturable microorganisms was first presented by DeLong et al. in 1989.

A large number of rRNA-targeting probes have been reported at various taxonomic levels (Amann, 1995). The probe is a short DNA sequence, designed to bind their

complementary target sequence on the rRNA, and is typically 15-30 bp in length. The fluorescent dye molecule (fluorophore) is typically bound to the 5'-end of the probe. 6-carboxyfluorescein (6-FAM) and cyanine dyes, such as Cy3 and Cy5, are some of the most widely used fluorophores due to their bright signal and their photo bleaching stability compared to conventional dyes (Dailey et al., 2006; Moter and Göbel, 2000).
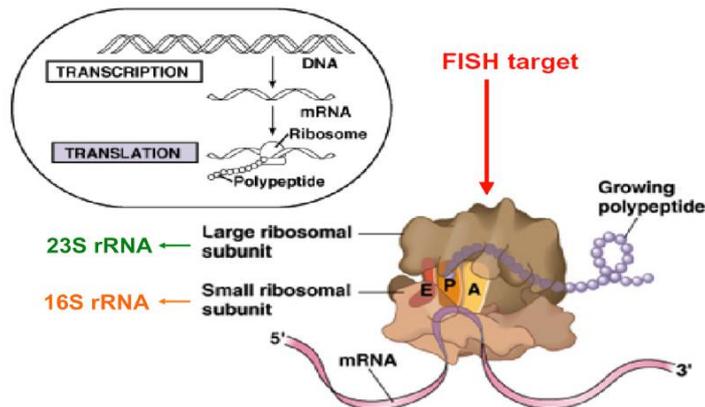
Figure 1.4: FISH probes targeting rRNA (Rogne, 2010).

Each ribosome within a bacterial cell contain one copy each of the subunits 16S and 23S rRNA. The most commonly employed rRNA target is 16S due to its genetic stability, high copy number and its domain structure with conserved and variable regions. During the hybridization procedure, the chosen rRNA probe attaches to the subunit within the ribosome. The intensity of the fluorescent signal is correlated to the amount of ribosomes in a cell (rRNA content) and cellular growth rate. This provides information about the physiological and metabolic state of the bacteria (Moter and Göbel, 2000).

The FISH approach is a potent tool for the examination of multispecies biofilm bacterial community dynamics in a variety of environmental compartments, including oligotrophic environments such as drinking water (Manz et al., 1993). Using molecular oligonucleotide probes targeting major bacterial phylogenetic groups, e.g.

alpha- (α-), beta- (β-) and gamma- (γ-) subpopulations of proteobacteria (Manz et al., 1993), which are known to be dominant in tap water (Williams et al., 2004), the abundance of these species can be quantified.

For quantification, the bacteria of interest is simultaneously hybridized with two probes; one probe targets the population to be quantified (population specific probe), while the other probe targets all bacteria (general probe) (Daims et al., 1999) or to all organisms. The latter probe can either be a RNA- or DNA-binding dye. DNA-binding dyes, e.g. DAPI (4',6-diamino-2-phenylindole), stains all microorganisms (Zimmer and Wähnert, 1986). DAPI fail to differentiate between living and dead cells or between different species of microorganisms, and can therefore not be used to assess cell viability or track species of microorganisms in an environment. A requirement is that the two probes are labeled with fluorophores having different excitation and emission maxima to make their signals distinguishable.

Figure 1.5 illustrates the basic steps of the FISH approach. An epifluorescence microscope or confocal laser-scanning microscope (CLSM) are generally applied for detection and visualizing the fluorescent labeled microbes in a sample. The CLSM can visualize and obtain images of the two probes simultaneously. The *in situ* abundance of specific microbial populations labeled by a specific fluorophore can be calculated by counting the colored pixels (Nielsen et al., 2009), which is performed by a quantification software, such as e.g. *daime* (Daims et al., 2006) or ImageJ (Abràmoff et al., 2004).
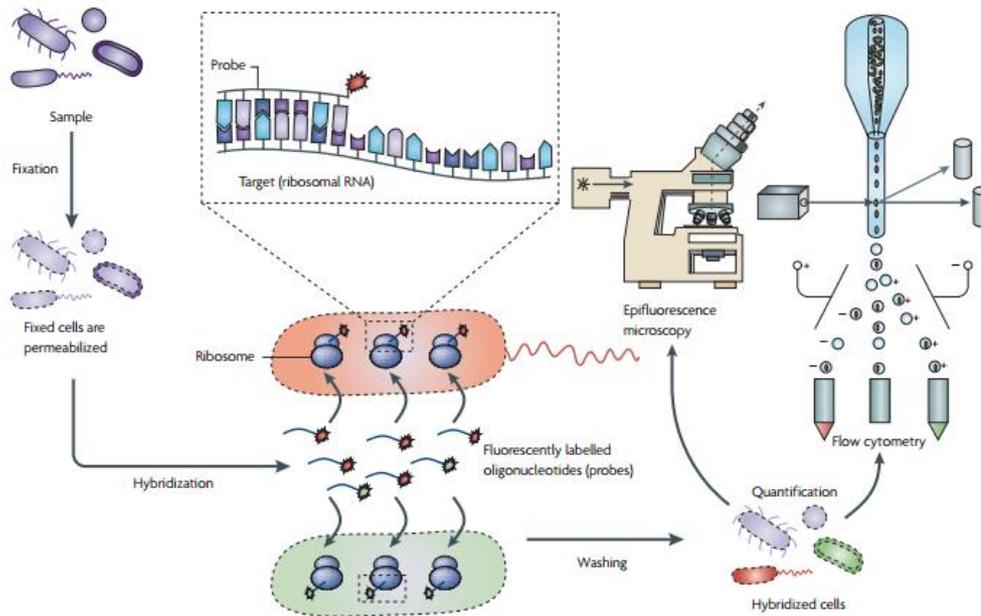
Figure 1.5: The basic steps in FISH (Amann and Fuchs, 2008).

Next-generation techniques for microbial community analysis

Sequence technologies allow us to investigate in the deeper layers of the microbial communities. In most microbial ecological surveys, sequence analysis of genes encoding 16S rRNA is commonly applied. Recent developments in instruments, bioinformatics and new sequencing chemistries have revolutionized the field of microbial ecology and genomics. Pyrosequencing allows massive parallel high-throughput sequencing of hypervariable regions of 16S rRNA, and multiple environmental samples can be combined in a single run (Rastogi and Sani, 2011).

1.5 Objectives

The main aim of this project was to investigate bacterial community dynamics in a drinking water biofilter in response to a micropollutant, PFOA. More specific, the objectives were to:

1. Design a biofilter system for removal of PFOA from drinking water
2. Obtain a steady-state biofilter environment prior to PFOA exposure
3. Investigate influence of pH, temperature and filter bed depth on bacterial community dynamics by a PCR-DGGE approach
4. Establish a protocol for quantification of sand-attached bacterial populations by FISH and CLSM
5. Investigate bacterial community dynamics in response to trace amounts of PFOA by FISH and PCR-DGGE analyses

2. Materials and methods

2.1 Experimental design

This study was part of a project investigating the removal of PFOA by a biofilter for DWT. Pilot-scale biofilters for DWT was in operation for this study; however, the setup was not ready for spiking with PFOA. Bench-scale batch biofilter and continuous flow biofilter systems were therefore designed and evaluated as options for analysis of biofilm bacterial community dynamics. The latter was finally chosen for the analysis of biofilm bacterial community dynamics in response to PFOA. The biofilter performance was monitored to ensure that stable conditions were present prior to PFOA exposure. This study also included optimization and development of a FISH protocol for quantification of bacterial populations.

Figure 2.1 presents the experimental flow chart of activities, processes and experiments presented in this report. Rectangles represent activities and processes concerning the different biofilter columns; dark blue represents the temporary biofilter for maturation of biofilm on biofilter media, grey represents the bench-scale batch biofilter, light blue represents the pilot-scale biofilter columns, green represents the bench-scale continuous flow biofilter. Ellipses represent all laboratory analyses, including water and media sampling as red and yellow colors, respectively, physicochemical analyses in pink and molecular methods in orange. Orange represents microbial community analysis as a whole, where diamonds represent computational and statistical methods. The dashed lines represents experimental pathways that have not been emphasized in this report.
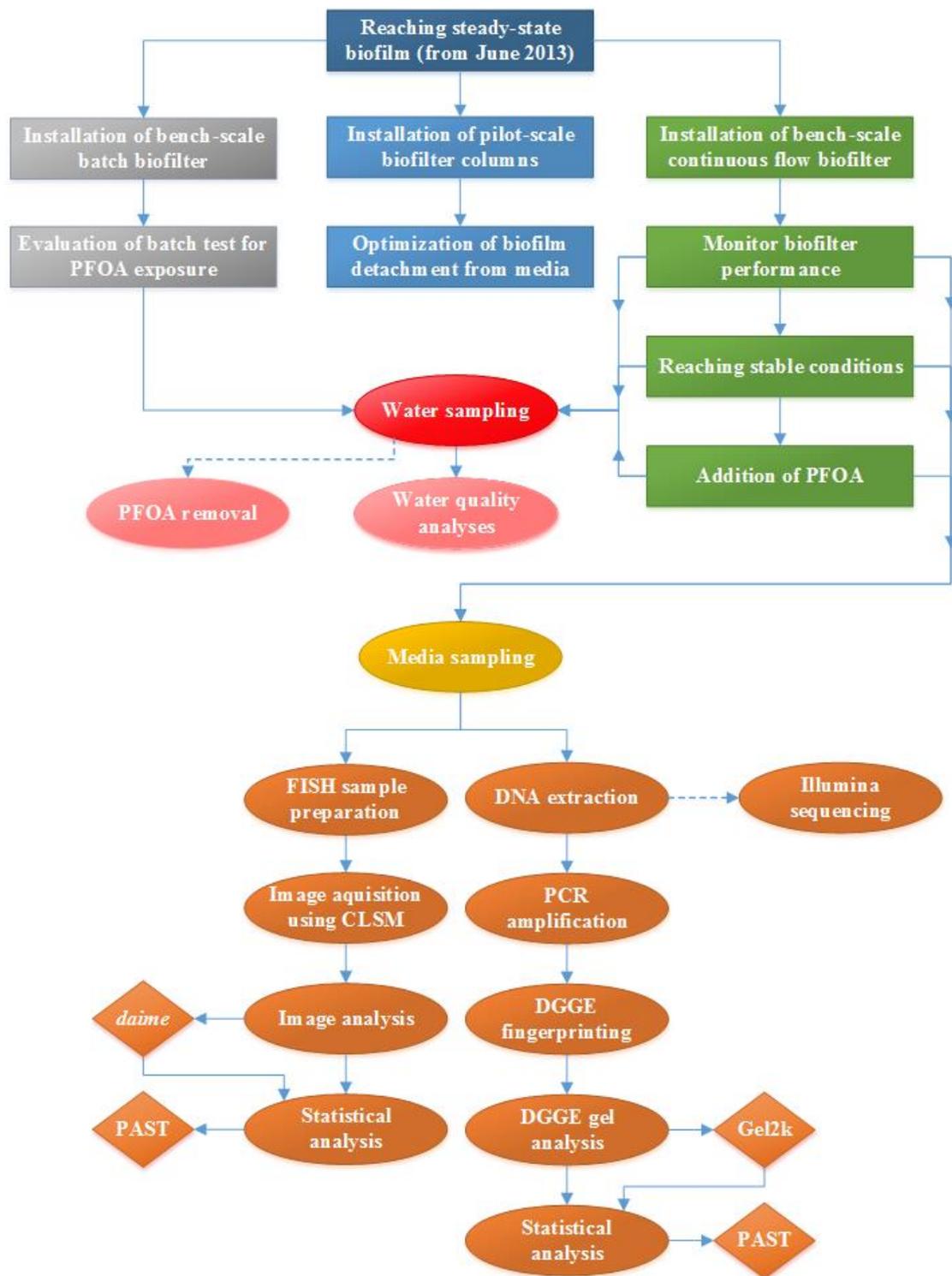
Figure 2.1: Experimental flow chart of activities, processes and experiments presented in this report. See text above the figure for more details about the flow scheme.

2.2 Pilot-scale biofilters

The installation of pilot-scale biofilter columns for DWT and associated equipment was performed in November-December 2013. The pilot is specifically designed for optimal treatment of drinking water, and PFOA removal will subsequently be investigated using the pilot; hence, these data are not included in this report. Since the design of the experimental setup used in this study is based on the pilot-scale biofilters, the detailed operation conditions are included in this chapter.

Biofilter design

The experimental setup of the pilot-scale biofilters consists of four down-flow filters and dosing equipment. Below is a schematic figure of the setup of all the filter columns with general flow directions and all the installations necessary to run the drinking water plant (Figure 2.2). The columns are made of glass to avoid adsorption. The inner diameter of the columns is 2.4 cm, with a total length of 185 cm from the overflow outlet to the bottom of the column to allow a 10% filter bed expansion during backwashing. One of the columns does not contain media and is used as a control column to detect adsorption of PFOA within the system. The second column has a 126 cm single layer bed of non-adsorptive sand media. The two remaining columns consist of dual media filters with a 20 cm anthracite top layer over a 106 cm sand layer. The effective size of the anthracite and sand is 1.4 – 2.5 and 1.0 – 1.6 mm, respectively.

A 5 cm layer of glass beads is situated at the bottom of the columns for support. The columns are jacketed with insulation to prevent phototrophic growth. One of the dual media filters will be filled with carriers containing immobilized fungi to study the effect of fungi enhanced biofilter and their PFOA degradability. Granular activated carbon (GAC) will be used for adsorption of PFOA prior to discharge into municipal sewage, since it has been demonstrated that GAC has adsorption capacities for PFOA (Yu et al., 2009).
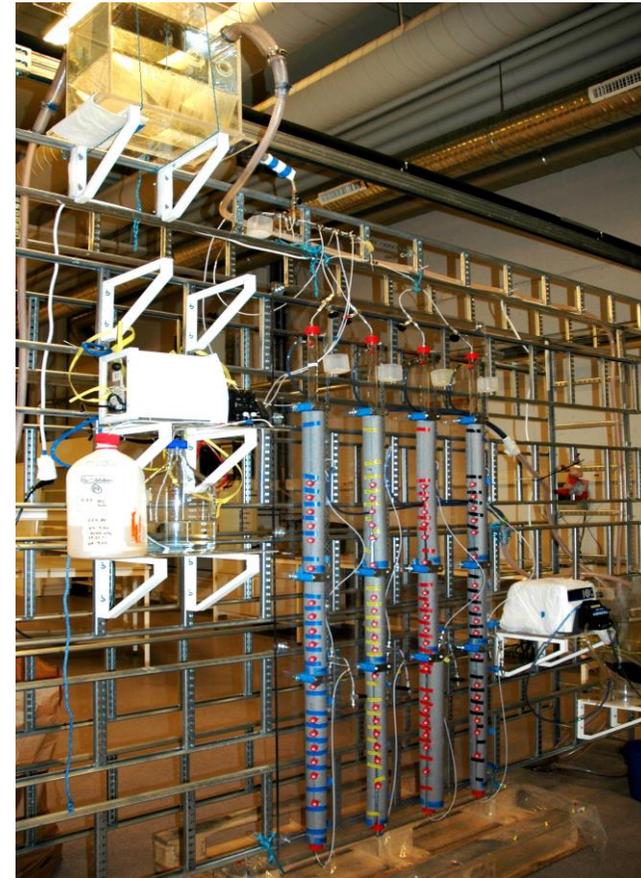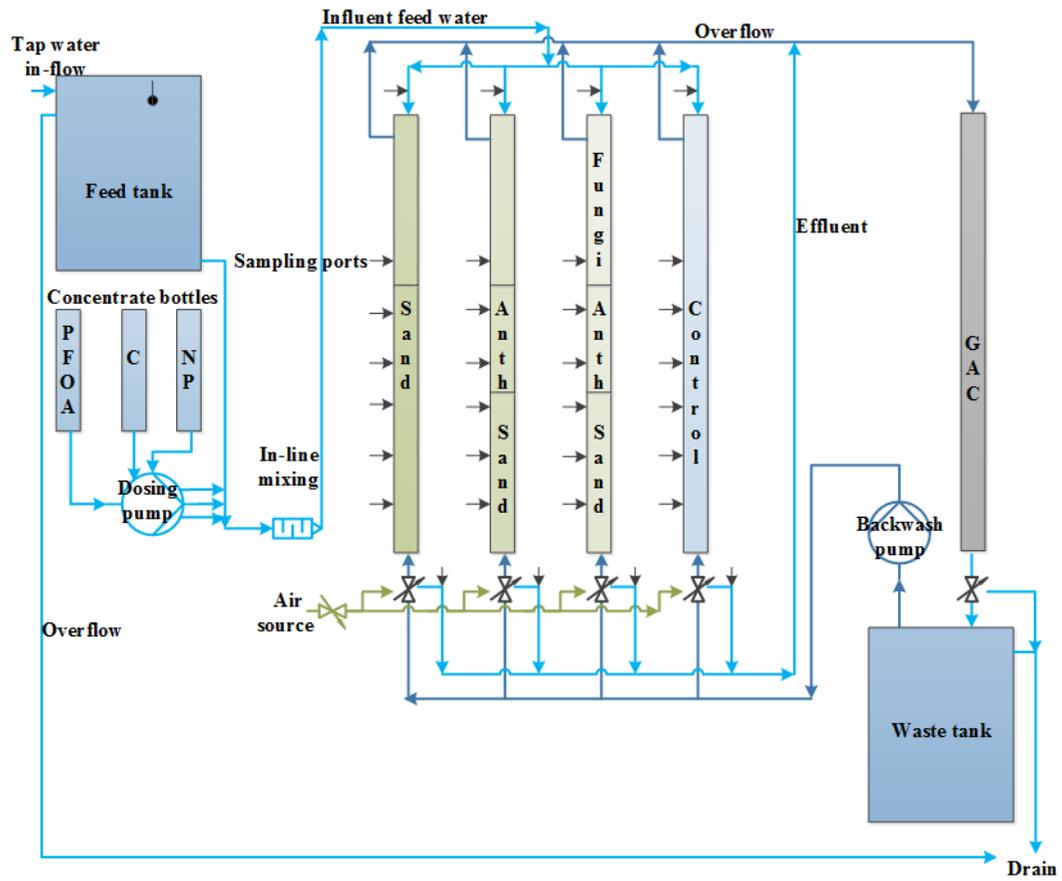
Figure 2.2: Pilot-scale biofilters.

Operating conditions

The down-flow biofilters are operated in parallel at 5 m/h and with an empty bed contact time (EBCT) of 15 min, which is representative for rapid sand filtration. However, the chosen EBCT is much shorter than presented in the literature regarding degradation of fluorinated contaminants (Eschauzier et al., 2011). The water level is kept at approximately 20 cm above the active filter bed to keep a constant pressure on the filter bed of about 0.02 bars.

Backwash of the filters is an important operational practice for filter efficiency. It maintains a constant flow rate by eliminating excess biofilm clogging the system or bubbles caused by bacterial activity or sampling. Effluent is collected in a separate barrel and used as backwash water. During the backwash process, collected effluent from the barrel is flushed throughout the filter from the bottom. Effluent is used as backwash water to prevent the microorganisms in the biofilter from being exposed to substances they have not yet encountered.

The pilot-scale biofilters were seeded with biologically active media (steady-state biofilm) from a temporary biofilter on December 13th 2013 to accelerate the acclimation process. The temporary bench-scale biofilter column containing anthracite and sand was fed tap water and nutrients necessary for establishment of media-attached biofilm from June 2013 and onwards at the Department of Hydraulic and Environmental Engineering, NTNU.

Feed water composition

Synthetic water made from tap water, carbon, nitrogen, and phosphorous was used as feed. Concentrations of nutrient are based on median environmental occurrence concentrations; 400 µg/L nitrogen, 6 µg/L phosphorous and 2.7 mg/L dissolved organic carbon (DOC). Additional 2.5 mg/L DOC is naturally present in tap water, hence only a small fraction of this (about 0.2 mg/L) is biodegradable. The carbon

content of the organic cocktail was composed of a mixture of sodium acetate (22% w/w DOC), sodium formate (28% w/w DOC) and HS (50% w/w DOC). HS were isolated from surface water via ion exchange at Juptjenn Vannverk SA in Nord-Odal. HS are the major components of NOM in water. Sodium nitrate and sodium dihydrogen phosphate are used as nitrogen and phosphorous sources. Other nutrients necessary for biological growth are considered to be available in the tap water. Operation parameters and physical and chemical data of the pilot-scale biofilters are presented in Table 2.1.

Table 2.1: Summary of operational, physical and chemical parameters for the pilot-scale biofilters.

Parameter	Value
Temperature	Room temperature (20 ± 2 °C)
pH	8.2
Hydraulic loading	5 m/h
EBCT	15 min
Flow rate	38 mL/min
Pressure	0.02 bars
Backwashing frequency	Once a week (or after sampling)
DOC feed	5 mg/L
DOC from sodium acetate	0.65 mg/L
DOC from sodium formate	0.85 mg/L
DOC from humic concentrate	1.2 mg/L
Tap DOC	2.5 mg/L
Nitrogen feed	400 µg/L
Phosphorous feed	6.0 µg/L
PFOA feed	250 ng/L

2.3 Batch biofilter

Biofilter design

The bench-scale batch biofilter column was designed as a down-flow filter. A sand filter bed thickness of 5 cm was supported by a 5 cm glass beads layer. Diameter of the glass column was 6 cm and the total length was 40 cm. The batch biofilter is presented in Figure 2.3.

Operating conditions

The column was seeded with biologically active sand media (steady-state biofilm). The flow through the batch biofilter was set to 235 mL/min. The flow was calculated using Equation 2.1, which divide the hydraulic loading by the surface of the filter. A hydraulic loading of 5 m/h was selected to reproduce operating conditions of the pilot-scale biofilters. The feed water used for the batch had the same nutrient composition as for the pilot-scale biofilters (Table 2.1), except tap water was replaced by distilled water. Other parameters and physical and chemical data used for operation of the batch biofilter is presented in Table 2.1. The feed water was recirculated.

$$\text{Total flow through the media} = \frac{\text{Hydraulic loading rate}}{\text{Surface area of filter}} \qquad \text{(Equation 2.1)}$$
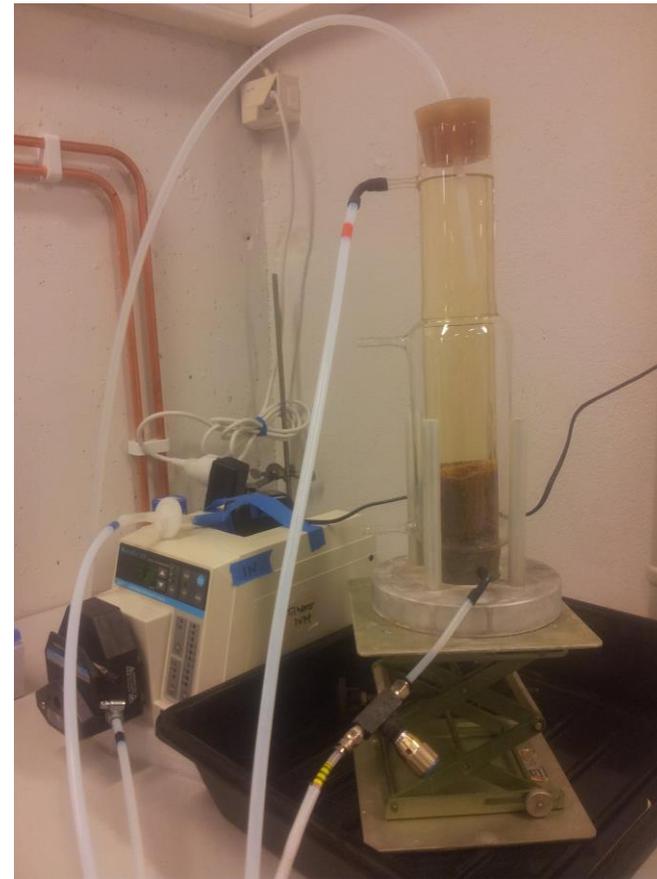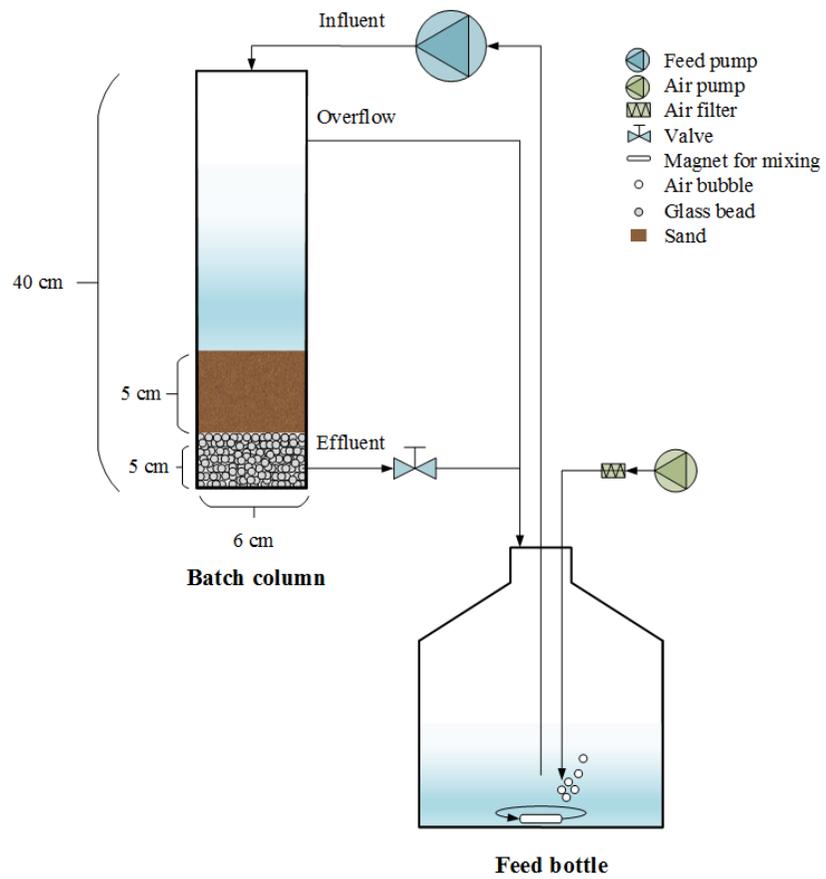
Figure 2.3: Batch biofilter column.

2.4　Bench-scale biofilter with continuous flow

Biofilter design

The column was a replica of the pilot-scale biofilter columns with an inner diameter of 2.4 cm, but a total length of 82 cm. The biofilter column is presented in Figure 2.4. The biofilter was operated as a down-flow filter, where the influent port was 54 cm above the top of the filter bed, and the effluent port was at the bottom of the column. The column was jacketed with insulation to prevent phototrophic growth. The filter had a total depth of 20 cm supported by a 10 cm glass bead layer. Non-adsorptive sand is a commonly used filter medium in full-scale rapid media filters, and was therefore chosen as filter media in this study. The goal was to characterize changes in biofilm bacterial community in response to a micropollutant, thus it was important to limit adsorption of contaminant to the media. Moreover, by limiting the adsorption to the media the removal of BOM in the biofilter could be evaluated.

Operating conditions

Figure 2.5 presents a timeline of important biofilter operational events. The column was seeded with biologically active sand media (steady-state biofilm) on May 6th 2014. The hydraulic loading and total flow through the media was the same as for the pilot-scale biofilters (Table 2.1). The flow varied due to buildup of particles and biomass within the filter and was measured every day and adjusted as needed. A hydraulic loading of 5 m/h was selected to reproduce operating conditions of the pilot-scale biofilters. The EBCT was 2.4 min (Equation 2.2). Rapid sand filtration usually has an EBCT of minimum 5 min to allow for a relatively high removal of biodegradable organic matter.

$$\text{EBCT} = \frac{\text{Volume media}}{\text{Volumetric flow rate} * \text{cross sectional area}} = \frac{\pi\, r^2\, h\ (m^3)}{x\, \frac{m}{h}\, \pi\, r^2\ (m^2)} = y(h)\ \ \text{(Equation 2.2)}$$
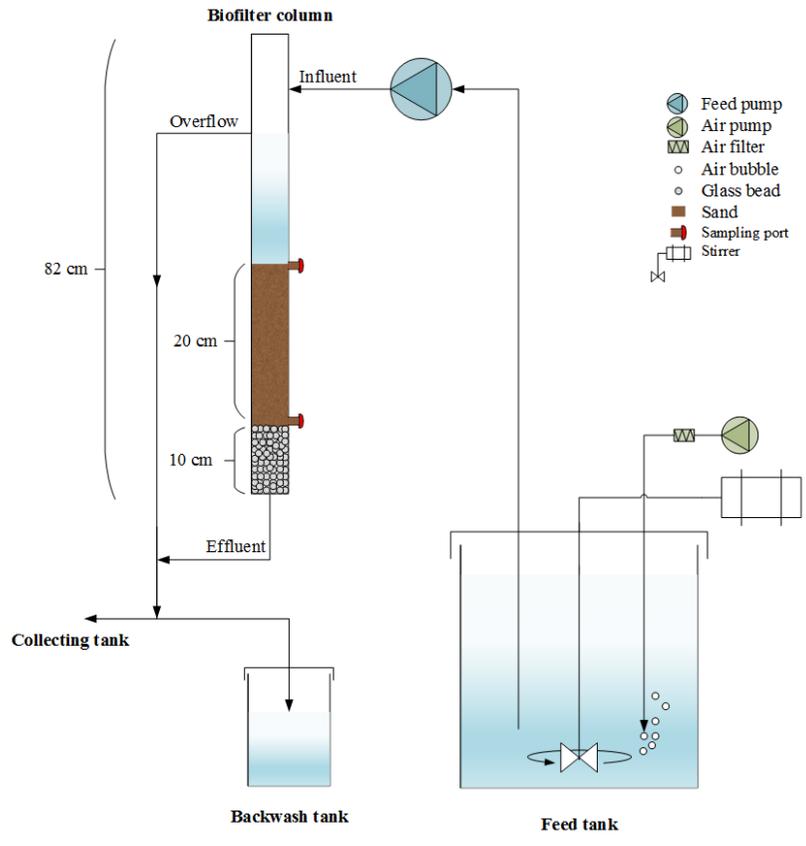
Figure 2.4: Bench-scale continuous flow biofilter.

At stable conditions, the biofilter was operated at a pH of 8.2 and at room temperature. Tap water was collected in a separate barrel for a day to reach room temperature (20 ± 2 °C) before it was used for preparation of the synthetic feed water to keep a stable biofilter environment. The immediate tap water temperature was about 9 °C. Higher temperatures have demonstrated higher percentage removal of micropollutants (Hallé, 2009). Feed water composition is listed in Table 2.1. The feed water was supplied with dissolved oxygen (DO) in the bottom of the feed tank, through an air diffuser connected to an air pump with filter, to prevent anaerobic conditions in the biofilter. The average influent DO was 9.0 ± 0.4 mg/L.

The feed tank was rinsed with chlorine every second day to prevent nutrient consumption and microbial growth, which had previously been observed. The biofilter column was backwashed after each sampling. During backwash procedure, a 30 % bed expansion was performed by reversing the flow of the filter for 15 min.

2.5 Micropollutant selection and analysis

The micropollutant selected for this project was PFOA; a compound that has been detected in surface water and drinking water (Jin et al., 2009; Mak et al., 2009). The target micropollutant influent concentration was based on median environmental occurrence concentrations ranging from sub ng/L to above 100 µg/L (Jin et al., 2009).
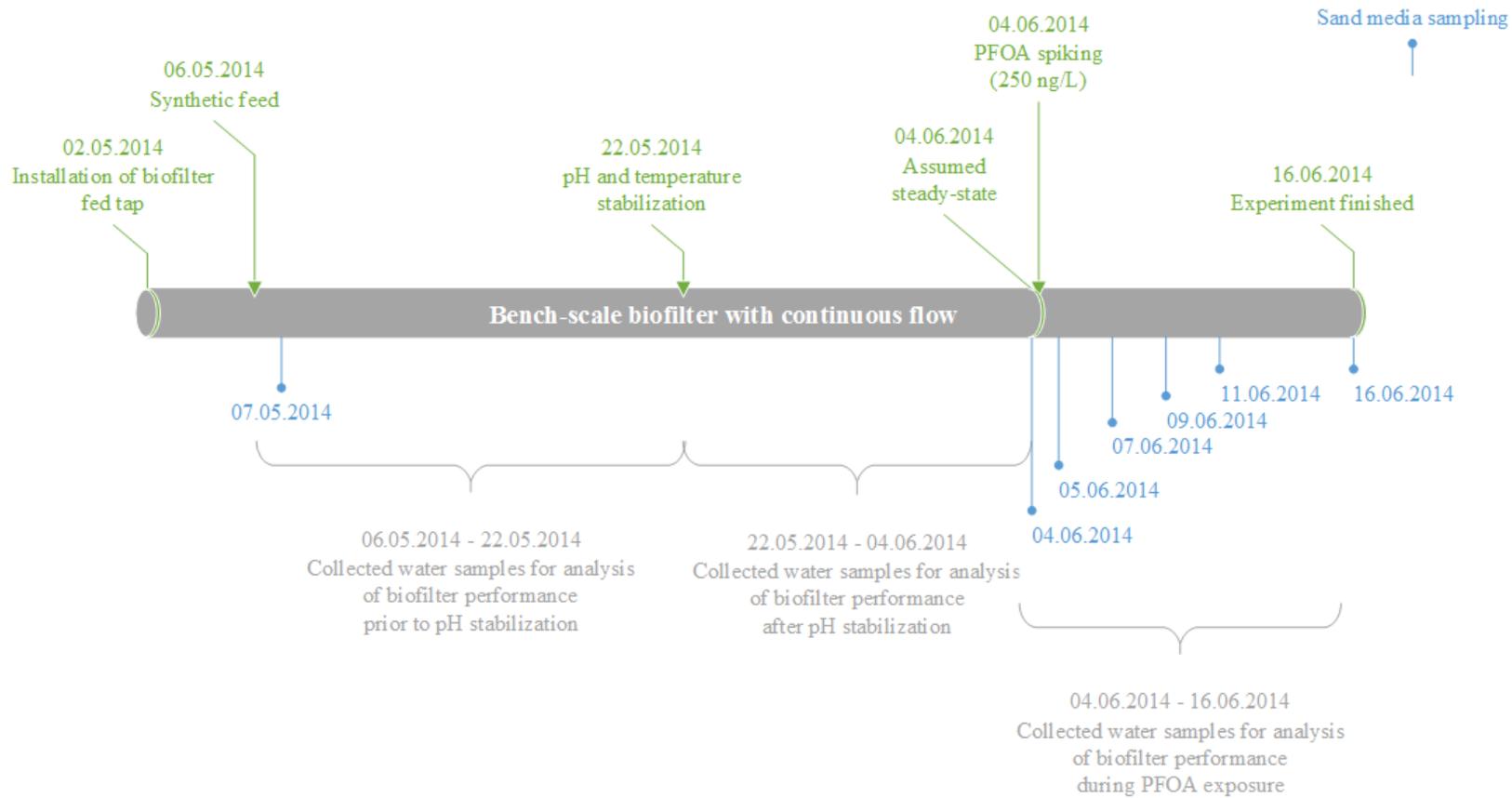
Figure 2.5: Operational timeline of bench-scale biofilter with continuous flow.

2.6 Physicochemical analyses

The water quality of the feed and effluent from the bench-scale continuous flow biofilter was monitored for physicochemical analyses. The methods used in this study have been developed by the Norwegian Standardization Association (NSF), which is a member of the International Organization for Standardization (ISO) and the European Committee for Standardization (CEN). The physicochemical analyses were DOC, color and UV absorbance. The pH, temperature and DO were measured to monitor the feed water quality, ensuring stable conditions in the biofilter. Limited access to DO and major changes in pH and temperature may have an impact on the biofilm bacterial community dynamics in the biofilter.

Organic carbon

Total organic carbon (TOC) analysis measure the content of all organic carbon in a water sample. Dissolved organic carbon (DOC) is the dissolved fraction of organic carbon. DOC samples were filtered through a 0.45 μm cellulose nitrate filter before analysis to collect only the dissolved fraction of the carbon. Ortho-phosphoric acid (85%) was added the DOC samples immediately after collection and filtration to lower the pH below 4 to prevent bacterial decomposition of the carbon. The samples were stored for a maximum of one week at 4 °C before analysis.

Analyses of DOC followed the standard method NS-EN 1484 IR. DOC analyses were performed using a TOC analyzer; Teledyne Tekmar TOC Fusion. The instrument uses free radical oxidation by UV/persulfate to convert organic carbon into CO_2, which is detected by the instrument. The precision of the instrument is $\pm$ 1%. A 20 ppmC calibration solution is used as a standard, which is checked at a daily basis. A precision within $\pm$ 10% is accepted. Distilled water is used as a blank.

UV absorbance

UV$_{254}$ absorbance was analyzed according to standard method NS 9462 A. The UV absorbance was measured at 254 nm with a UV/Visible spectrophotometer (Lambda 650 PerkinElmer) in a 1 cm quartz cell. Unsaturated double bonds absorbs light strongly at this wavelength. Milli-Q water was used as a blank.

Color

Color analyses were performed according to standard method NS-EN ISO 7887:2011 C). The color of the water was measured at a wavelength of 410 nm with a UV/Visible spectrophotometer (Lambda 650 PerkinElmer) in a 1 cm quartz cell. Milli-Q water was used as a blank.

pH

pH analyses were performed according to standard method NS-EN ISO 10523:2012. pH was measured by a portable PC 5000 H meter (VWR® International). The instrument was tested with technical buffers pH 4.01 and 7.00 25 °C (VWR® International) before each measurement, and manually calibrated if the values of the buffers ended up outside >3 % of the expected values pH 4.01 or 7.00.

Conductivity

Conductivity was measured according to standard method NS-ISO 7888. Conductivity was analyzed by a portable PC 5000 H meter (VWR® International) to monitor feed water quality and removal through the biofilter. The instrument was tested with conductivity standard 1413 µS/cm 25 °C (VWR® International) before each measurement, and manually calibrated if the value ended up outside >3 % of the expected value.

Dissolved oxygen

Dissolved oxygen (DO) was analyzed according to standard method NS-EN ISO 5814:2012. DO was measured by a portable oxygen meter ProfiLine Oxi 3315 (WTW GmbH) to make sure that anaerobic conditions were not occurring, and to assess the biological activity by analyzing the oxygen consumption. The instrument was calibrated with air.

Statistical analysis

Paired two-sample t-test was used to estimate significant differences in the mean of physicochemical quality parameters of influent and effluent water samples collected from the bench-scale continuous flow biofilter. Unpaired two-sample t-test was used to estimate significant differences in the mean of the removal efficiency of DOC, conductivity, UV absorbance and color. The null hypothesis (H_0) stated that the mean of different samples compared were significantly equal. H_1 hypotheses stated that there were significant differences between the mean of samples. The H_0 hypotheses were rejected if estimated p-values exceeded a significance level of 0.05. The statistical analyses applied assume normal distribution. The analyses were performed using PAST (Hammer et al., 2001).

2.7 Analysis of the bacterial community dynamics

2.7.1 Biomass sampling procedure

Sand media samples were collected after complete drainage of the filter column. The samples were obtained through a sample port at the bottom of the sand filter bed, where the EBCT was 2.4 min. One sample was collected at the top of the filter bed where the EBCT was approximately 0 min (Figure 2.4). The sand grains were collected into sterile plastic tubes with a sterile spatula. For DGGE profiling of the sand-attached biofilm, approximately 0.4 g (wet weight) of sand was collected from

the column. The samples were stored at -20 °C until further analysis. For FISH analysis of the biofilm, approximately 4 g (wet weight) of sand was collected, and immediately fixed for FISH analysis, as described in section 2.7.3.

2.7.2 PCR-DGGE

DNA extraction and purification

The extraction of DNA from the media samples was done as described by the PowerSoil® DNA Isolation Kit (Cat. No.: 12888-100) Instruction Manual, delivered by MO BIO Laboratories (Appendix E). The extracted DNA was stored at -20 °C.

Primers and PCR amplification

The varable region 3 (v3) of the bacterial 16S rRNA gene was amplified using the forward primer 338F-GC and reverse primer 518R (Table 2.2) in reactions with 2 mM MgCl$_2$, 0.2 mM of each dNTP, 0.3 µM of each primer and Taq DNA polymerase and reaction buffer (VWR International). The primer pair targets two conserved regions encompassing the v3 region of the bacterial 16S rRNA gene, giving amplified sequences of approximately 240 bp. This particular primer pair is widely used for generating PCR products for DGGE analysis (Bakke et al., 2011).

Table 2.2: Overview of PCR primer sequences.

Primer	Specificity	Position (*E.coli*)	Sequence (5'-3')*
338F-GC	Bacteria	~ 320-355	cgcccgccgcgcgcggcgggcggggcggggcacggg gggACTCCTACGGGAGGCAGCAG
518R	Bacteria	~ 505-535	ATTACCGCGGCTGCTGG

* GC-clamp is presented in lowercase

The thermo-cycling parameters for the PCR reaction are presented in Table 2.3. A non-template control to check for potential contaminations was included. The

amplification was carried out by the use of Arktik Thermal Cycler (Thermo Scientific).

Table 2.3: Temperature regimes during the PCR reaction.

Step*	Temperature (°C)	Time (min:sec)	Explanation
1	95	03:00	Initial denaturation
2	95	00:30	Denaturation
3	53	00:30	Annealing
4	72	01:00	Elongation
5	72	10:00	Final step of elongation
6	10	∞	Stop

*Step 2-4 were repeated in cycles of 38 times prior to step 5.

Agarose gel electrophoresis

The size and amounts of the PCR products from the sand biofilter samples were evaluated by the use of agarose gel electrophoresis in an Owl EasyCast Mini Gel System (Thermo Scientific). 1 % agarose gel was prepared by mixing 4 g of SeaKem® LE agarose (Lonza) in 400 mL of 1 x TAE (5.04 g tris-base, 2 mL 0.5 M EDTA and 1.14 mL glacial acetic acid/L). The mixture was heated in a microwave oven until boiling and then slowly down to approximately 65 °C. 20 µL GelRed™ Nucleic Acid Gel Stain (QIAGEN) was added to the gel. The mix was kept at 65 °C until further use. 1 µL of 6x loading dye (Fermentas) was added to each of the PCR products, and the mix was applied in separate wells in the gel. The gel was operated at 140 V for approximately 60 min, before the gel was visualized and photograhped using G:BOX (Syngene) UV table and gel-images were obtained by GeneSnap.

DGGE

The DGGE system Ingeny phorU (Ingeny, the Netherlands) was used for analyzing the PCR products. A detailed DGGE protocol is listed in Appendix F, while reagent preparation is presented in Appendix G. After installing the system according to the manufacturer's instructions, a denaturing gradient had to be chosen to achieve the

optimal separation of the PCR fragments. A 35 – 55 % denaturing gradient was chosen for this experiment, since it has shown to achieve satisfactory band separation. Acrylamide was mixed with tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) to achieve the desired gradient. The selected denaturing acrylamide solution was poured into the gradient maker, which pumps the solution on top of the gel. The gel was polymerized for 2 h.

The gel was then placed in the buffer tank filled with 0.5x TAE (Tris-Acetate-EDTA) buffer, pre-heated to 60 °C. The samples were mixed with loading dye and added in the gel-wells. Gels were run for 17 h at 100 V. After 17 h, the gel was removed from the buffer tank and stained with SYBR® Gold (Invitrogen, Molecular Probes), 50x TAE and MilliQ water and incubated for 60 min in the dark. After staining, the gel was washed with MilliQ water and visualized and photographed using G:BOX (Syngene) UV table, and gel-images were obtained by GeneSnap.

DGGE banding patterns analysis

The Gel2K software (Norland, 2004) was used to analyze the band patterns of the DGGE images. The software uses a band searching algorithm to recognize bands on the gel. Each lane is transformed into a histogram where the bands form peaks. The peak area reflects the fluorescence intensity of the bands on the gel, creating densitometric curves. The peak areas were given a measured value, calculated by the Gel2K software. The values were exported to Excel spread sheets and used for statistical analysis, providing the basis for further computational analysis of the DGGE gel.

The relative abundance of each band, p_i, was calculated using Equation 2.3. The peak areas for each band (n_i), reflecting its abundance, were normalized by dividing on the total peak area (N) in the densitometric curves for all bands in the lane. The fractional

peak area was used to calculate the band richness (S), Shannon diversity index (H') of general diversity and Pielou's evenness index (J').

$$p_i = \frac{n_i}{N} \qquad\qquad \text{(Equation 2.3)}$$

The Shannon diversity index (H') presented in Equation 2.4. is a measurement of the species diversity of a community in a given sample (Peet, 1975). In a gel, it represents the number of bands, and the relative abundances of the bands within a lane. The band richness, S, is the number of bands in a given lane in the gel. A community having only one bacterial species will have a H' value of 0. This is because p_i would equal 1, $\ln p_i$ therefore equals 0, and multiplying these two together gives $H' = 0$. High H' values therefore represents more diverse and even communities (Smith and Wilson, 1996).

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \qquad\qquad \text{(Equation 2.4)}$$

For any given number of species, there is a maximum possible H', $H_{max} = \ln S$, which occurs when all species are present in equal numbers. Pielou's evenness index (J') is a numerical expression of how evenly the individual species are distributed among all the species in the sample. A community with an unequal abundance has a low evenness value, and the opposite for a community with equally abundant species (Smith and Wilson, 1996). The evenness index of species was calculated based on Equation 2.5.

$$J' = \frac{H'}{H_{max}} = \frac{H'}{\ln(S)} \qquad\qquad \text{(Equation 2.5)}$$

Statistical analysis

Peak areas, obtained by Gel2K, were exported to Excel spread sheets. The software PAST (Hammer et al., 2001) was used for statistical analyses of multivariate analysis. The normalized peak area values were then square root transformed, and used for non-metric multidimensional scaling (NM-MDS) ordination with Bray-Curtis similarity measure for comparison of the samples. Untransformed data preserves relative species abundance information, while square root transformed data reduces the effect of abundant groups (high-intensity bands) (Thorne et al., 1999).

NM-MDS is an ordination method based on any distance measure. Distances between samples are converted to ranks, which are plotted in a coordinate system. Similar and dissimilar objects will then appear close or far away from each other in the plot, respectively. The distance between the samples is proportional to the similarity between them (Holland, 2008). The Bray-Curtis similarity measure is often used in ecology to quantify the similarity in species composition between two sites or comparison of DGGE-profiles. The method takes both the presence, or absence and abundance data into account. Results from comparison of diversity indices and band richness presented in this report are not statistically evaluated due to the low amount of obtained data.

2.7.3 FISH

Sample preparation

Preparation of the media samples prior to FISH analysis included fixation and permeabilization of the bacterial cells and detachment of biofilm from biofilter media. The protocol for sample preparation was optimized to fit this study. The final FISH protocol can be found in Appendix A.

Fixation and permeabilization

Collected media samples from the biofilter were fixed within an hour by adding 3% w/v paraformaldehyde (PFA), and incubated for 3-12 h at 4 °C. This inactivates the microbial cells by cross-linking nucleic acids and associated protein complexes, which prevents growth or decay after sample collection and enzymatic activity by e.g. endogenous RNAses, which can degrade RNA. The fixative agent is also crucial for cell wall permeabilization, probe penetration, and preserves the morphological details of the cells. PFA is the most effective agent for fixing gram-negative bacteria, and quickly penetrates cells (Nielsen et al., 2009).

Sonication procedures for detachment of biofilm from media

After fixation, the fixative was removed and the media was resuspended in 1x phosphate buffered saline (PBS). To analyze abundance of media attached bacteria with FISH the bacterial cells had to be detached from the media and disaggregated after fixation. The literature presents several techniques for disaggregating and detaching cells from substrata, including chemical treatment (i.e. using surfactants such as tetrasodium pyrophosphate (NaPP$_i$) or Tween), or physical treatment (i.e. manual shaking, vortexing, sonication, stomaching and density gradient centrifugation), or a combination of both. Sonication in combination with a surfactant appears to be the most efficient biomass removal technique from sand (Epstein and Rossel, 1995; Mermillod-Blondin et al., 2001).

For evaluation of the optimal detachment of biofilm from sand, methods from Mermillod-Blondin et al. (2001), Luef et al. (2009) and Epstein and Rossel (1995) were adopted and applied in this study. Sonication bath and sonication probe in combination with NaPP$_i$ was tested and evaluated to establish an optimal protocol. NaPP$_i$ was added to the samples (10 mM) already suspended in 3% PFA, and incubated for 30-60 min on a shaker. For each ultrasound exposure time, each analysis was performed on three sand samples.

To evaluate ultrasonic bath (Elmasonic S 15/(H), Elma Hans Schmidbauer GmbH & Co. KG, type D-78224, power 95 W, 37 kHz), nine different ultrasound exposure times were tested: 1, 5, 10, 15, 30, 45, 60, 75 and 90 min. 1 g (wet weight) of fixed sand samples were placed in 50 mL sterile Falcon tubes, and placed into the bath filled with approximately 1.5 L cold water. The sonication was stopped every 10 min to replace the water in the bath by cold water, to prevent overheating of the samples and denaturation of cells (Mermillod-Blondin et al., 2001).

To evaluate ultrasonic probe, a Branson Sonifier® 250 cell disruptor equipped with a 3 mm tapered microtip was used. Six different ultrasonic probe exposure times were investigated: 30, 60, 90, 120, 150 and 180 sec. 1 g (wet weight) of fixed sand sample was placed in a 20 mL sterile glass centrifuge tube. The samples were placed on ice to prevent overheating. The settings applied were: 50% duty cycle, output control 2 (= 40 W)) and interrupted for 30 sec every minute (Mermillod-Blondin et al., 2001) (Luef et al., 2009). The tip was located approximately 2 cm from the bottom of the glass tube to prevent the tip from touching the sand, which may destroy the tip.

The sonicated liquid phase was transferred to a centrifuge tube and centrifuged at 11000 RPM for 3 min. The supernatant was removed and the pellet resuspended in 1x PBS. This was repeated once. Finally the pellet was suspended in 1:1 1x PBS and cold ethanol (-20 °C), and stored at -20 °C until further FISH preparation.

The quality of the samples were evaluated by manually investigating the appearance of the biofilm seen in the microscope (i.e. bacterial abundance and cell disaggregation) and fixed bacteria were visualized by adding SYTO 9 bacterial nucleic acid stain from LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, Molecular Probes, Europe BV) for a rapid evaluation of the detachment methods. Lebaron et al. (1998) demonstrated that SYTO 9 decreases in fluorescence when cells are fixed prior to staining relative to unfixed bacteria. However, the dye was only applied to detect presence of the bacteria and to visualize if they were disaggregated

or remaining in big clusters. Disaggregating the cells from the EPS is desired since EPS can show autofluorescence (Marzorati et al., 2014). The EPS was not stained. The samples were analyzed with the Zeiss LSM Axio Imager.Z2 CLSM and the software ZEN 2010 by Carl Zeiss. A detailed LIVE/DEAD protocol is presented in Appendix H.

Oligonucleotide probes and stringency conditions

The fluorescently labeled probes chosen for this study are listed in Table 2.4. They were selected based on information from Ricardo et al. (2012), Moita and Lemos (2012) and probeBase (Loy et al., 2007). The oligonucleotide probes EUB338, EUB338-II and EUB338-III are used together for detection of all bacteria (general probes), and ALF969, BET42a and GAM42a were used for the detection of the specific α-, β- and γ-proteobacterial populations, respectively.

Table 2.4: Fluorescently labeled probes selected for this study, presented with their target population, sequence and reference.

Probe name	Target population	Sequence 5' to 3'	Reference
EUB338	Most bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
EUB338-II	Planctomycetales	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
EUB338-III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
ALF969	α-proteobacteria	TGG TAA GGT TCT GCG CGT	Oehmen et al. (2006)
BET42a	β-proteobacteria	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
GAM42a	γ-proteobacteria	GCC TTC CCA CAT CGT TT	Manz et al. (1992)

Non-fluorescent competitor probes were used in equal amounts with probes ALF969, BET42a and GAM42a to obtain optimal stringency conditions (Loy et al., 2007; Manz et al., 1992). cALF969a and cALF969b served as competitors for the ALF969 probe. cBET42a and c1033 served as competitors for the BET42a probe, and

cGAM42a and c1033 as competitors for the GAM42a probe. More details of the probes are presented in Appendix C.

6-carboxyfluorescein (6-FAM) and cyanine dyes (Cy3 and Cy5) were applied in this study. Details of the fluorophores' wavelength and color are given in Table 2.5. As a positive control to assess hybridization quality, freshly collected activated sludge was used.

Table 2.5: Excitation and emission wavelength and color of the fluorophores Cy3, Cy5 and 6-FAM.

| Fluorophore | Wavelength [1] | | Color [2] |
	Excitation (nm)	Emission (nm)	
Cy3	550	570	Orange-red
Cy5	651	674	Dark red
6-FAM	494	515	Green

[1] Information provided by Sigma-Aldrich
[2] Information provided by Thurnheer et al. (2004)

In situ hybridization

The FISH protocol applied in this study is based on information provided by Amann (1995), Daims et al. (2005), Daims et al. (2006) and Daims (2009). A detailed FISH protocol and preparation of reagents can be found in Appendix A and B, respectively. Detached biofilm samples suspended in 1:1 1x PBS and 96% ethanol were applied onto glass slides (3 x 10 µL) and dried at room temperature. The samples were dehydrated as described in Appendix A, and the oligonucleotide probes were hybridized at a formamide concentration of 35%.

Microscopy and image acquisition

The prepared samples were observed with a Zeiss LSM Axio Imager.Z2 confocal laser scanning microscopy (CLSM) through a 40x oil objective. The software ZEN 2010 by Carl Zeiss acquired the images. To obtain reliable quantification results,

several important issues were addressed when acquiring the images, as recommended by Daims et al. (2006). Settings applied for microscopy and image acquisition are listed in Appendix D.

Image analysis

The micrograph images acquired by the CLSM were analyzed using the software *daime* ("digital image analysis in microbial ecology") (Daims et al., 2006). *daime* was chosen for quantification of the bacteria since the software was developed having applications in microbiology in mind, especially for analyzing microbial cells as obtained by FISH or other fluorescence labeling techniques. Per sample analyzed, ≥ 34 images were acquired. ImageJ was used for adjusting the brightness of the CLSM micrographs presented in this report. More information about settings applied for obtaining the images are listed in Appendix D.

Statistical analysis

The standard deviation of the mean (SDOM) was calculated for each sample according to Equation 2.6. N is the number of images acquired per sample, and SD is the standard deviation calculated automatically by *daime*.

$$\text{SDOM} = \frac{SD}{\sqrt{N}} \qquad\qquad \text{(Equation 2.6)}$$

One-way ANOVA in conjunction with Tukey's pairwise was used to estimate significant differences between the means of the three proteobacterial populations. The null hypotheses (H_0) stated that there were no significant differences between the mean of the populations, and H_1 hypotheses stated that there were significant differences between the mean of the populations. The H_0 hypotheses were rejected if the p-value was < 0.05. The univariate statistical analyses applied assumes normal distribution. The analyses were performed using PAST (Hammer et al., 2001).

3. Results

3.1 Performance of batch and continuous flow biofilters

This section presents the results demonstrating the performance of the batch biofilter and bench-scale biofilter with continuous flow prior to and after exposure to PFOA.

Batch biofilter

The pilot-scale biofilters for treatment of drinking water was designed to analyze biodegradation of micropollutant and response of the microbial population. The installation of the pilot was included in this study. The system was not completed on time to spike with micropollutant due to leakage. A batch system was therefore evaluated for analysis of microbial response to PFOA (Figure 2.3).

Water samples collected from the closed batch showed an increase in DOC and conductivity of 95% and 36%, respectively, while the pH declined from 8.2 to 7.5 after 7 days of operation. To evaluate the possibility that equipment leaked carbonaceous compounds, the biologically active sand was removed, and the system was operated with Milli-Q water while samples for TOC and DOC analyses were collected from the recirculating water. Results demonstrated that TOC and DOC increased by 12.6 and 12.4 mg/L, respectively, after 12 days of operation, indicating that carbonaceous compounds leaked from the equipment.

After re-seeding the column with biologically active media, replacing tubes and valves for elimination of possible carbon-sources, a new batch experiment using fresh enrichment water without PFOA was performed to monitor carbon mass flow. After 36 hours, the easily biodegradable carbon, i.e. sodium acetate and –formate, was consumed, as seen by DOC stabilizing at approximately 1.2 mg/L (Figure 3.1),

resulting in nutrient poor conditions. The remaining DOC mostly derived from humic substances, which are hardly biodegradable.
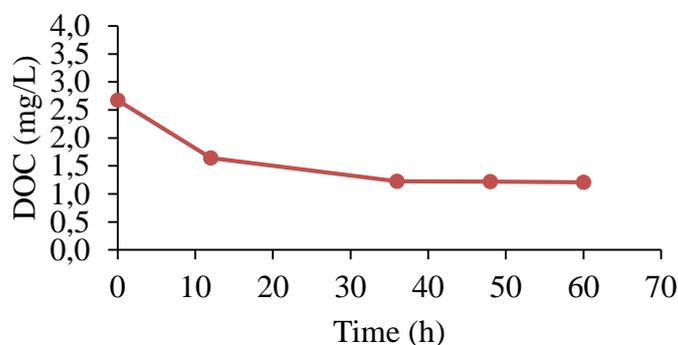
Figure 3.1: Batch system feed DOC concentration as a function of time.

During the latter experiment, the color of the recirculating water turned yellow after a few days of operation. Spectrophotometric analysis of the water revealed that the color, measured at 410 nm, increased from 14.2 to 33.4 mg Pt/L during 7 days, which demonstrates accumulation of compounds within the system. Moreover, a decline in pH from 8.2 to 7.5 during 7 days, most likely due to biochemical reactions, was observed. This demonstrates the difficulties of maintaining a stable pH in a closed batch system.

Bench-scale biofilter with continuous flow

It proved difficult maintaining a stable environment in the batch system due to accumulation of compounds originating from equipment or biochemical reactions, as shown by an increase in DOC and conductivity and decrease in pH. Moreover, the biofilter microbes are exposed to starvation conditions after 36 hours, which is not desired in this study. Due to parameters causing instability in the batch, as mentioned above, a bench-scale biofilter system with continuous flow was designed for the PFOA exposure test (Figure 2.4). A continuous flow system was more representative for the actual purpose of this study, which included bacterial community dynamics in

response to a micropollutant in a lab-scale DWT system. Prior to spiking the synthetic surface water with PFOA, the performance of the biofilter was monitored and evaluated to ensure that steady-state conditions were present.

Stabilization of feed water pH and temperature and consequences for biofilter performance

Biofilter performance is impacted by factors such as water quality (e.g. pH, turbidity), temperature and backwash procedure (Moll et al., 1999). Water samples from the feed water (influent) and biofiltered water (effluent) were collected prior to and after pH stabilization, and during PFOA exposure (see operational timeline, Figure 2.5). Physicochemical parameters, including DOC, conductivity, UV absorbance and color were analyzed for evaluation of biofilter performance and steady-state conditions.

The initial feed water pH was approximately 9.2 directly after preparation, due to the high pH of the humic concentrate added to the feed. A significant decline in the pH of the influent feed water was observed. After approximately 48 hours, the pH stabilized to 8.3, which is more representative of pH for surface waters. However, due to risk of microbial growth and consumption of nutrients in the feed tank during 48 hours, it was necessary to manually stabilize the pH immediately after the feed was prepared. The temperature of the tap water was very low, i.e. about 8.4 °C. To obtain a stable influent temperature, the tap was stored in a barrel one day prior to feed preparation to reach room temperature.

Alkalinity is a measure of the capacity of the water to resists a change in pH that would tend to make the water more acidic. The alkalinity of the synthetic feed water was measured by two-step titration, and found to be 1.26 mmol/L. From the alkalinity, the acid concentration for stabilizing the pH of the feed was calculated. The feed water pH stabilized at 8.2 by adding 0.12 mmol HCl/L. Table 3.1 presents the pH and temperature values prior to and after stabilization.

Table 3.1: pH and temperature in influent before and after pH and temperature stabilization, shown with p-values obtained by unpaired two-sample t-tests

Parameter	Influent values during		p-value
	unstable conditions	stable conditions	
pH	8.8 ± 0.4	8.2 ± 0.0	0.0056
Temperature (°C)	15.0 ± 5.2	19.9 ± 0.3	0.0378

Physicochemical parameters measured to monitor biofilter performance prior to and after stabilization of pH and temperature, are given in Table 3.2 and Table 3.3. The biofilter operated at stable conditions at pH of 8.2 and temperature of 19.9 °C, achieved significantly higher removal of DOC ($p<0.05$), compared to when it was operated under unstable conditions at a pH of 8.8 and temperature of 15.0 °C (Table 3.4). The removal of conductivity, color and compounds measured at UV 254 nm did not change significantly ($p>0.05$), and was generally low both prior to and after stabilization (Table 3.4).

Table 3.2: Biofilter performance prior to pH and temperature stabilization.

Parameter	Influent	Effluent	p-value
DOC (mg/L)	4.69 ± 0.50	4.54 ± 0.54	0.0029
Color (mg/L Pt)	17.7 ± 0.2	17.4 ± 0.2	0.1136
UV absorbance	0.1736 ± 0.0032	0.1708 ± 0.0014	0.0250
Conductivity (µS/cm)	276 ± 5	276 ± 4	0.5029

Table 3.3: Biofilter performance after pH and temperature stabilization.

Parameter	Influent	Effluent	p-value
DOC (mg/L)	5.05 ± 0.39	4.09 ± 0.21	0.0415
Color (mg/L Pt)	17.4 ± 0.5	17.2 ± 0.3	0.5118
UV absorbance	0.1699 ± 0.0047	0.1674 ± 0.0046	0.0414
Conductivity (µS/cm)	277 ± 3	276 ± 4	0.1390

Table 3.4: Biofilter removal efficiency before and after pH and temperature stabilization.

| Parameter | Removal (%) during | | p-value |
	unstable conditions	stable conditions	
DOC (mg/L)	3.26 ± 2.26	18.85 ± 5.75	3.10E-05
Color (mg/L Pt)	1.56 ± 2.25	0.70 ± 1.97	0.5408
UV absorbance	1.57 ± 1.40	1.47 ± 0.86	0.9031
Conductivity (μS/cm)	0.22 ± 0.88	0.41 ± 0.64	0.6344

The biofilter performance data were collected in parallel to sand media sampling for microbial analysis, to ensure that stable conditions were present throughout the PFOA exposure. Table 3.5 shows the overall biofilter performance after PFOA exposure. DOC influent concentration decreased on average by 12.7 ± 6.8 % within 24 hours after feed preparation (results not shown). As a result, the removal of DOC through the biofilter therefore slightly decreased (13.6 ± 7.7 %) relative to the DOC removal directly after new feed was prepared (19.7 ± 3.5 %).

Table 3.5: Biofilter performance after PFOA exposure.

Parameter	Influent	Effluent	p-value[2]	Removal (%)
DOC[1] (mg/L)	4.99 ± 0.22	4.00 ± 0.12	0,0004	19.7 ± 3.5
DO (mg/L)	9.0 ± 0.4	8.2 ± 0.4	4.78E-11	8.7 ± 1.4
pH	8.2 ± 0.0	8.1 ± 0.1	N.a.	N.a.
Color (mg/L Pt)	17.3 ± 0.2	17.1 ± 0.1	0.0391	1.2 ± 1.3
UV absorbance	0.1660 ± 0.0008	0.1648 ± 0.0016	0.0546	0.8 ± 1.0
Conductivity (μS/cm)	275 ± 6	274 ± 6	0.4418	0.5 ± 2.2

[1] DOC influent and effluent concentrations shortly after feed preparation
[2] p-values correspond to comparative analysis of influent and effluent concentrations of the different parameters

3.2 Optimization of FISH protocol

Establishment of protocol for detaching sand-associated biofilm

For FISH quantification of biofilm bacteria, the biomass had to be detached from the biofilter sand media. In combination with NaPP$_i$, ultrasonic bath and ultrasonic probe were tested and evaluated as techniques for optimal removal of sand-attached biomass and disaggregation of bacterial cells.

For both techniques (ultrasonic bath and ultrasonic probe), the amount of biofilm removed from the surface of the media was proportional to the exposure time. Using ultrasound bath for 30-45 min achieved satisfactory detachment; however the cells did not disaggregate well. The cells remained in thick biofilm aggregates (Figure 3.2A). Using ultrasound probe into the sample for a total of 3 min achieved satisfactory detachment and better disaggregation of cells relative to the ultrasonic bath (Figure 3.2B). The optimized protocol for detaching and disaggregating sand-attached biofilm is listed in Appendix A.
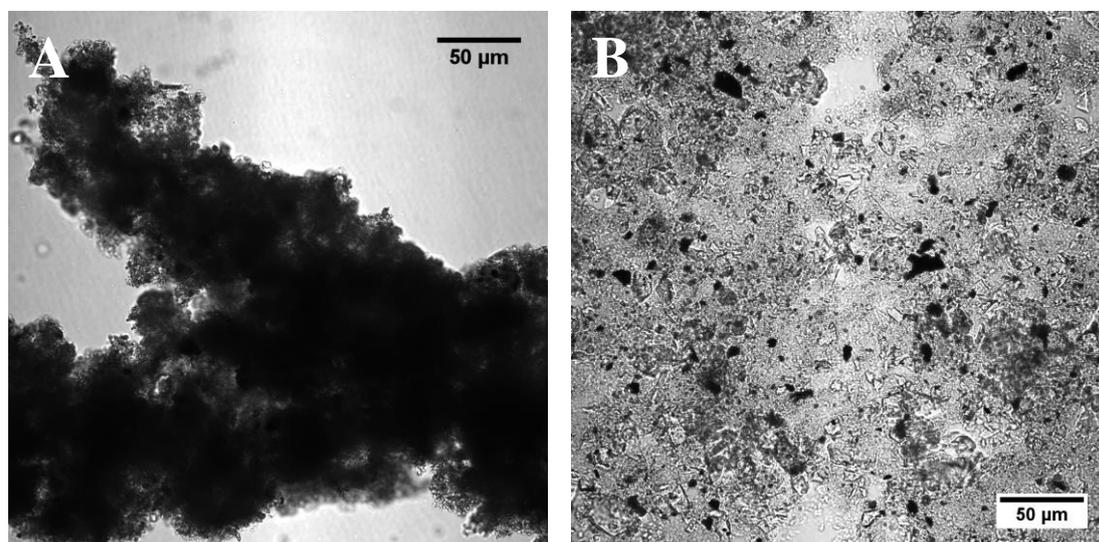
Figure 3.2: CLSM phase contrast micrographs of detached biofilms using two ultrasonic methods; sonication bath (A) and sonication probe (B).

Evaluation of 6-FAM as an alternative to Cy5 for probe labeling

Figure 3.3 demonstrates an example of autofluorescent artifacts in a biofilm sample collected from the bench-scale continuous flow biofilter. The sample was not subject to contamination of fluorescent staining. The artifacts emit fluorescent light at the same wavelength as the chosen emission detection wavelength of the Cy5 fluorophore (640 to 700 nm).

A long pass filter in the range of 630 to 700 nm was used for detection of Cy5 by CLSM. There was no possibility to change the settings due to software issues. A shorter band pass from e.g. 670 nm to 680 nm would be preferred to eliminate signals from autofluorescent particles that potentially may have emitted fluorescent light in the range of e.g. 640 to 669 and 681 to 700 nm.
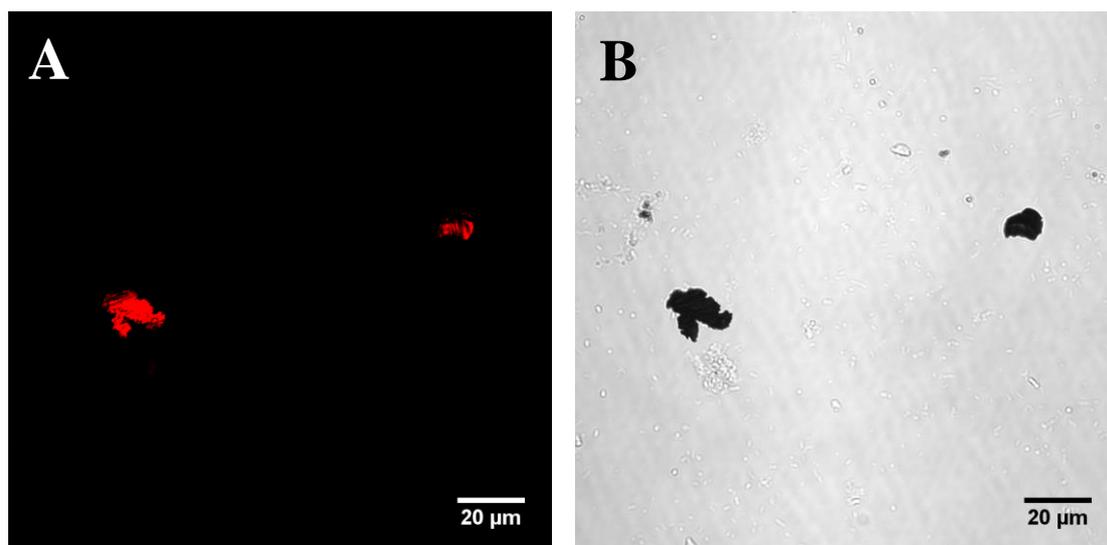
Figure 3.3: CLSM micrographs of autofluorescent artifacts shown for identical microscopic fields.
(A) Autofluorescent artifacts aquired at filter detector emission wavelength of 640 – 700 nm.
(B) Phase contrast image of the same particles as in (A).

The fluorophore 6-FAM, with a different excitation and emission wavelength than Cy5, was tested and evaluated as an alternative to the Cy5 fluorophore for labeling the oligonucleotide probes. Figure 3.4A demonstrates presence of what may look like autofluorescent bacteria or particles present in a biofilm sample from the bench-scale continous flow biofilter. The sample was not hybridized with any fluorescently labeled probe. 6-FAM was detected by a short pass filter (spilt 525 nm). The abundance of these autofluorescent particles was much higher than the abundance of the autofluorescent particles emitting light between 640 to 700 nm (Figure 3.3A).

The CLSM micrographs of beta-proteobacteria hybridized with 6-FAM labeled probes showed high intensity of backgrounds autofluorescence from materials surrounding the bacteria (figure 3.4B). Due to the higher abundance of autofluorescent signals in samples using 6-FAM relative to Cy5, it was decided to apply Cy5 for labeling of probes used for quantification of bacteria in biofilter samples.
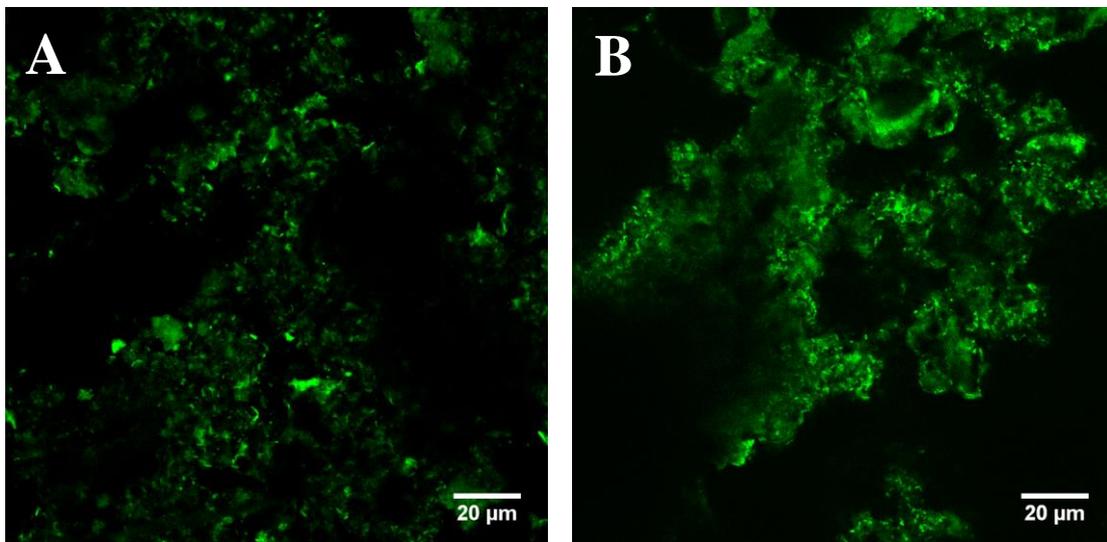
Figure 3.4: CLSM micrographs of biofilm from sand biofilter.
(A) Autofluorescence
(B) Fluorescence *in situ* hybridization, hybridization with 6-FAM labeled BET42a probe.

3.3   Bacterial community dynamics

Bacterial community dynamic analysis was performed on samples collected prior to pH and temperature stabilization, and before and after PFOA exposure. Samples representing top and bottom layers of filter bed were collected prior to pH and temperature stabilization. Samples collected after pH and temperature stabilization were taken at the bottom layer of the filter bed. The resulting DGGE-gel, including samples collected prior to and after PFOA exposure, is shown in Figure 3.5. For DGGE profiles of all samples, the band richness, Shannon's diversity index and evenness index were determined (Figure 3.6).
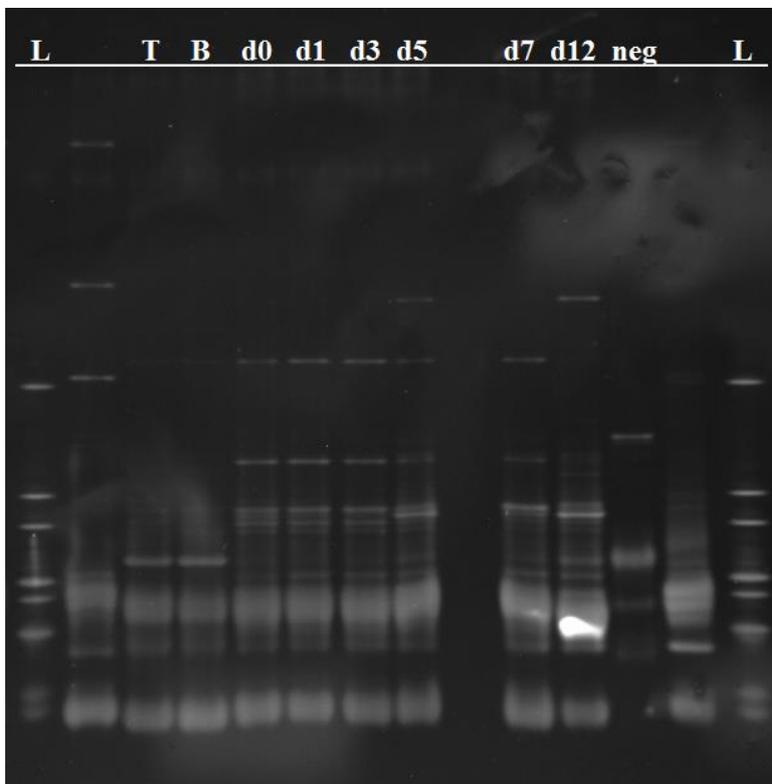
Figure 3.5: DGGE-gel image of PCR-amplified 16S rDNA representing the bacterial communities in the bench-scale continuous flow biofilter, for two samples taken before pH and temperature stabilization; B (bottom layer) and T (top layer) of the biofilter, and for samples collected in the bottom of filter bed after stabilization, both before (d0) and after (d1-d12) PFOA exposure. L: ladder; neg: non-template control; d=day.
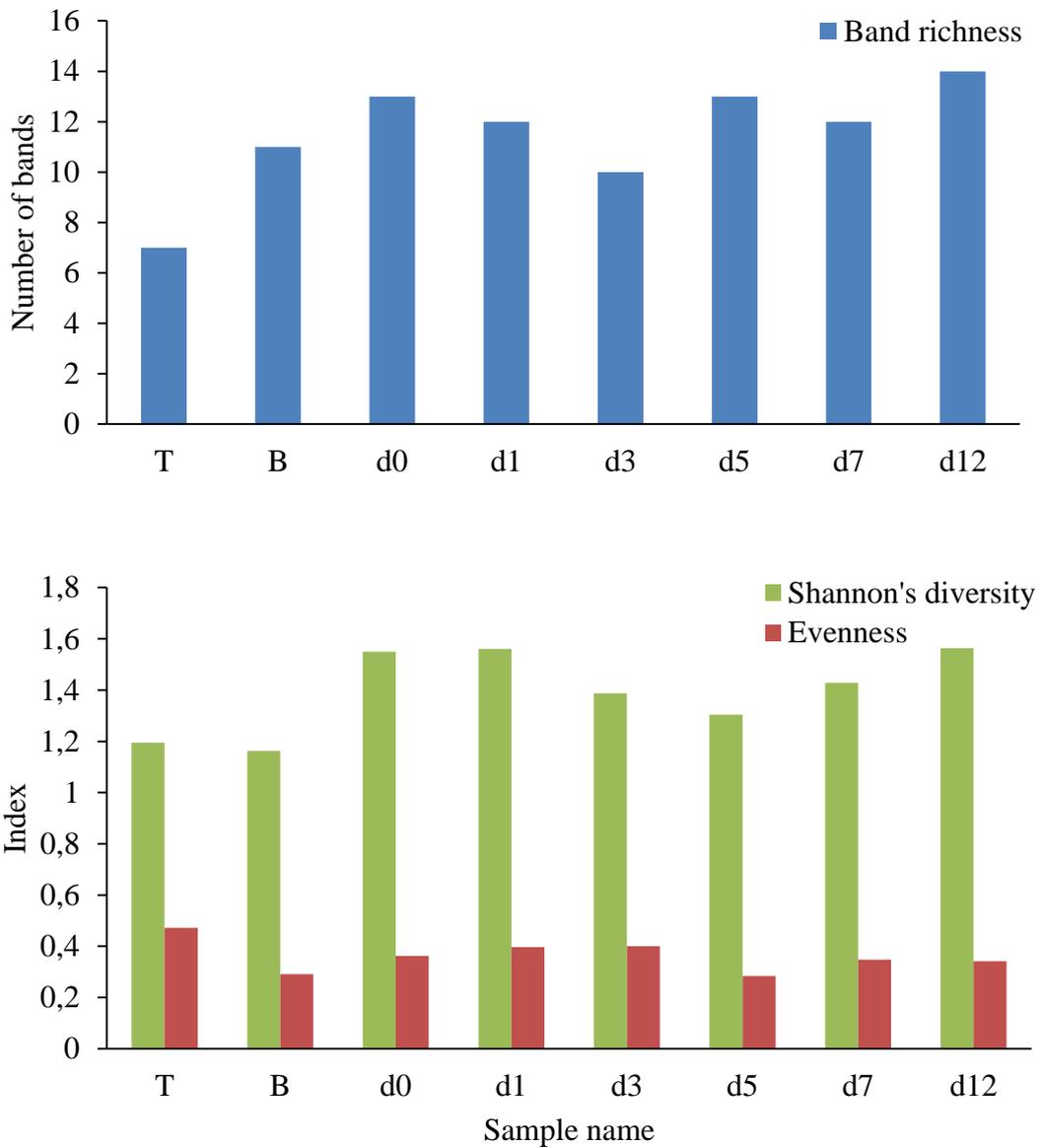
Figure 3.6: Band richness (A) and Shannon's diversity index and evenness (B) for 16S rRNA DGGE profiles representing the bacterial communities in a biofilter used in DWT, for two samples taken before pH and temperature stabilization; B (bottom layer) and T (top layer) of the biofilter, and for samples collected in the bottom of filter bed after stabilization, both before (d0) and after (d1-d12) PFOA exposure; d=day.
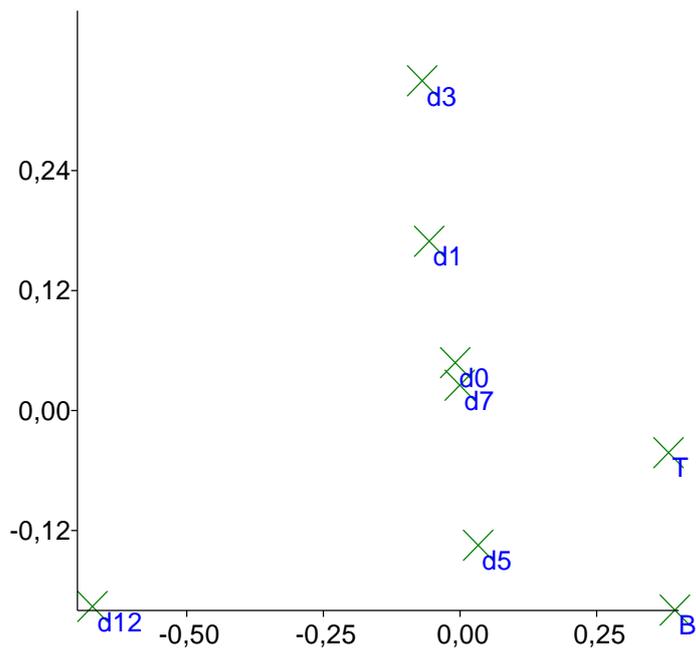
Figure 3.7: NM-MDS 2D plot based on Bray-Curtis distances measure for comparison of DGGE community profiles representing the bacterial communities in a biofilter used in DWT, for two samples taken before pH and temperature stabilization; B (bottom layer) and T (top layer) of the biofilter, and for samples collected in the bottom of filter bed after stabilization, both before (d0) and after (d1-d12) PFOA exposure; d=day.

3.4 Community response to differences in pH, temperature and filter bed depth

Biofilter bacterial communities were differentiated as a function of filter depth. Figure 3.6A shows that the band richness of DGGE profiles representing samples collected prior to pH and temperature stabilization was higher in the bottom of the filter bed relative to the top. Shannon's diversity (Figure 3.6B) was virtually the same for communities in the top relative to the bottom of the filter bed, while evenness was slightly higher in the top. However, only two samples were compared. To conclude

that this is a general trend for communities thriving at different filter bed depths, more samples are required, preferably collected over a longer time period.

The band richness (Figure 3.6A), Shannon's diversity and evenness (Figure 3.6B) appeared to increase after the feed water pH and temperature had stabilized and reached favorable microbial growth conditions. However, only two samples were compared. To further conclude that the biofilter bacterial communities treating water sources with different pH and temperatures, more samples are required, preferably collected over a longer time period.

The NM-MDS plot indicate that the bacterial community composition in samples collected prior to pH and temperature stabilization differs from that of after (Figure 3.7). DGGE profiles show presence of a particular band which is characteristic for samples collected prior to pH and temperature stabilization, but which seems to disappear after stabilization (Figure 3.5).

3.5   Response in biofilter communities to PFOA

DGGE profiling

There were no clear tendencies in evolution of the bacterial communities in response to PFOA exposure with respect to richness and diversity indices (Figure 3.6). Band richness (Figure 3.6A) and diversity (Figure 3.6B) appeared to be highest for samples collected prior to and after 12 days of continuous PFOA exposure, with a temporary reduction in between.

The NM-MDS plot shows no consistent temporal development of the bacterial community structure from day 0 to day 7. However, the community structure had changed 12 days after continuous exposure (Figure 3.7). This is also apparent in the DGGE gel (Figure 3.5), in which DGGE profile representing day 12 clearly differs from the other sample profiles. An interesting observation is the disappearance of two

particular bands in the DGGE profile representing day 12, which is seems to be present in samples from day 0 to day 7 (Figure 3.5).

Phylogenetic analysis by FISH

More than 600 micrographs were acquired by the CLSM, and used in the analysis of biofilm community dynamics in response to PFOA. Results obtained through FISH analysis were compared to biofilter performance data, collected in a parallel study. FISH with probes for three major phylogenetic groups, α-, β-, and γ-proteobacteria, quantified the abundance of the biofilm bacterial populations in the bottom of the biofilter bed throughout the 12-days PFOA exposure (Figure 3.8).
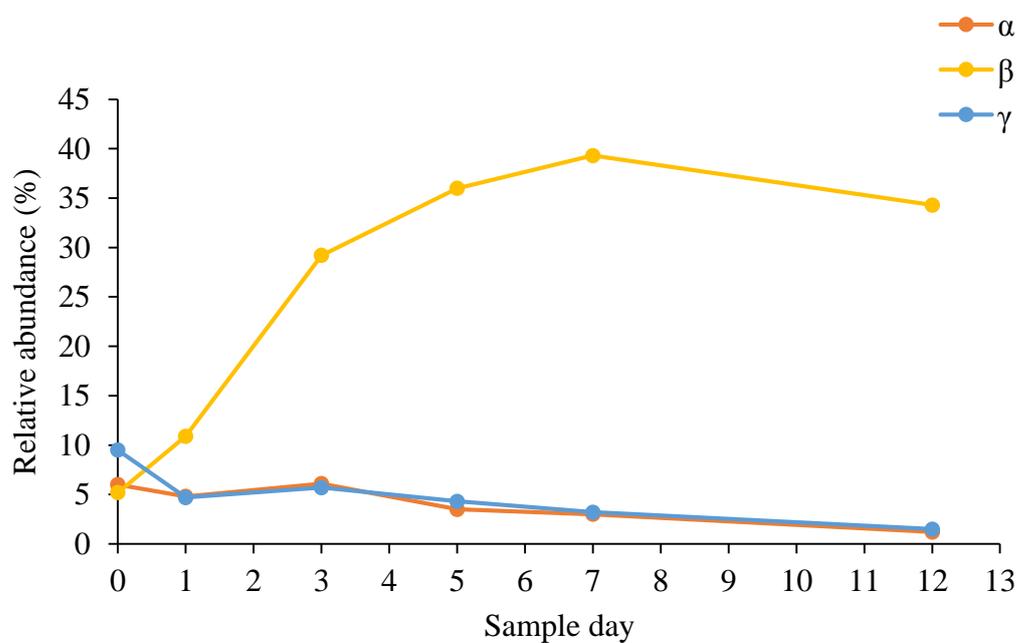
Figure 3.8: Relative α-, β- and γ-proteobacterial abundance as determined by FISH analysis of sand-attached biofilm samples from the biofilter treating synthetic surface water before (day 0) and after exposure to PFOA (day 1-12).

FISH revealed that the biofilter bacterial community was significantly dominated by members of the γ-proteobacteria prior to PFOA exposure ($p < 0.05$). Nearly 10% of the total hybridized cells from the biofilm samples were affiliated with the γ-proteobacteria prior to PFOA exposure, followed by α- and β-proteobacteria, respectively.

After one day of continuous PFOA exposure, the dominant phylogenetic group had significantly shifted to members belonging to the β-proteobacterial population, while the γ-, and α-subclass significantly decreased by approximately 50 and 20%, respectively ($p < 0.05$). Figure 3.8 illustrates that the abundance of the β-proteobacterial population overall increased after PFOA exposure, while those of α- and γ-proteobacteria showed an overall decline ($p < 0.05$). A slight decrease in abundance of the β-proteobacteria was observed after day 7 of PFOA exposure ($p < 0.05$).

Figure 3.9 illustrates micrograph images of the three proteobacterial populations at day 0 (A-C) and day 3 (D-F). From the images, it is evident that the β-proteobacteria are changing over time (Figure 3.9 B and E).
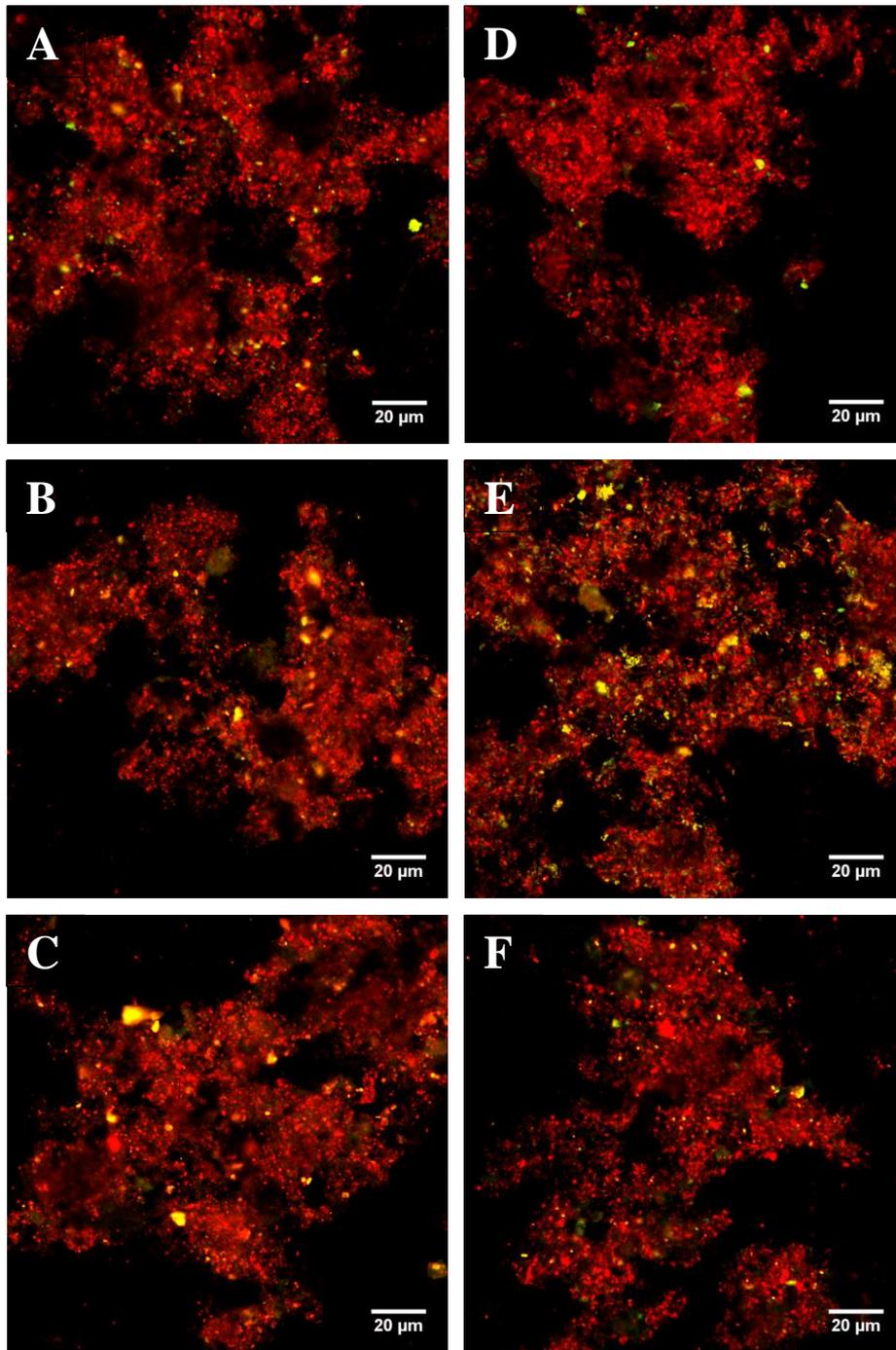
Figure 3.9: FISH micrographs of bacteria from sand-attached biofilm from biofilter treating synthetic surface water prior to PFOA exposure (A to C), and 3 days after continuous PFOA exposure (D to F).

(A, D) Total bacteria shown in red and α-proteobacteria in yellow.
(B, E) Total bacteria shown in red and β-proteobacteria in yellow.
(C, F) Total bacteria shown in red and γ-proteobacteria in yellow.

4. Discussion

4.1 Evaluation of methods and materials

4.1.1 Batch biofilter

The main objective of this project was to investigate biofilter bacterial community dynamics in response to PFOA. The pilot-scale biofilters for DWT were not ready for spiking with a micropollutant early in the project period, therefore a closed batch biofilter was tested as a temporarily solution for investigation of bacterial community dynamics in response to PFOA.

Results from the initial batch setup demonstrated a significant increase in DOC, which is surprising since DOC is expected to decrease due to microbial consumption. Due to suspicion of cell lysis and release of cellular carbonaceous substances, the biologically active sand and recirculating water was removed from the column. The batch system was rinsed with Milli-Q, and new measurements revealed DOC increase. The valve and tubes used in the batch system were replaced due to suspected carbon leakage. Biologically active sand was added the column. New measurements showed a decrease in DOC. Materials applied in the former batch setup may have leaked carbonaceous substances accumulating within the system, hence interfering with DOC measurements. These results demonstrate the importance of assessing materials prior to use.

However, the color of the recirculating water turned yellow after a few days of operation during the latter batch test. Apparent color was measured at a wavelength of 410 nm using a spectrophotometer, and the results demonstrated a significant increase, which was consistent with the observed color change in recirculating water. Hydrolysis of the biofilm due to anaerobic conditions, causing release and accumulation of cellular compounds, i.e. biopolymers and cations (Novak et al.,

2003), was a highly unlikely cause of the observed phenomenon. The average DO in the recirculating water was 8.8 mg/L throughout the experiment, which is adequate for aerobic microbial growth. The yellow color appearing may have been caused by production and build-up of compounds from biochemical reactions happening within the biofilm (Yu et al., 2001).

In order to conclude that the micropollutant is the direct cause of shifts in bacterial community dynamics, steady-state conditions should be present. A stable mass flow is difficult to maintain in a batch, due to risk of compounds accumulating and rapid consumption of easily BDOC, as demonstrated in Figure 3.2. The microbes are then exposed to starvation. The microbial community dynamics will naturally change when the nutrient availability is altered (Degerman et al., 2013).

Batch biofilters are applicable for micropollutant removal analyses (Buchanan et al., 1993; Zwiener et al., 2002); however proved to be inappropriate for studying bacterial community dynamics in response to PFOA exposure in this study.

4.1.2 Steady-state conditions in bench-scale biofilter with continuous flow

Due to obstacles with the batch system, as described above, a smaller replica of the pilot scale columns for continuous flow system was implemented for the PFOA exposure test. A continuous flow system was more representative for the actual purpose of this study, which included bacterial community dynamics in response to PFOA in a lab-scale drinking water treatment system. Moreover, a continuous flow system provides the advantage of a stable mass flow relative to a closed batch system. The performance of the biofilter was monitored and stabilized to ensure that steady-state conditions were present in order to render the system suitable for studying community dynamics in response to PFOA.

The high pH of the humic concentrate used for preparation of synthetic feed water was due to the high content of NaOH, which is used in the process of isolating humic substances from surface waters. A significant decrease in the pH of the feed was observed after a few days. A well-known phenomena that can explain the observed decrease in pH is the carbon dioxide (CO_2) and carbonic acid (H_2CO_3)-base equilibrium (Harvey, 2008). The humic concentrate contained excess of positively charged ions, i.e. Na^+, which shifted the balance of carbonate species towards negative ions (e.g. CO_3^{2-}) to compensate. The free $CO_{2\,(aq)}$ and $H_2CO_{3\,(aq)}$ concentration decreased, which in turn lead CO_2 absorption from the atmosphere to restore the equilibrium. $CO_{2\,(aq)}$ reacted with H_2O and formed $H_2CO_{3\,(aq)}$. H_2CO_3 loses protons to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), which will decrease the pH of the water.

As mentioned in section 3.1, the influent DOC decreased in between preparation of new feed. This may have been due to microbial growth in the feed tank or influent tubes. When nutrient availability is altered, the bacterial community structure may change (Degerman et al., 2013). However, the biofilter was exposed to overall good growth conditions throughout the PFOA exposure (Table 3.4), which leads to believe that the influent DOC increase after one day did not have an impact on the biofilter community.

4.1.3 PCR-DGGE

Based on past laboratory experiences, a 35 to 55% denaturing gradient was expected to give satisfactory separation of the DGGE-band profiles (Figure 3.5). It seemed as if the bands in the bottom of the gel were poorly separated. However, Gel2K data obtained from the gel could be interpreted. To obtain a higher resolution profile and improved separation of bands, a narrower gradient could have been tried out. Short

run or errors connected to the electrophoresis (i.e. buffer or electricity) may also have contributed to the poor separation.

As with any PCR based technique, the DGGE method suffers from DNA extraction and amplification step biases. Presence of bacterial DNA in the Taq DNA polymerase and master mixture is a known problem, causing amplification of contaminating DNA, which gives rise to false positives (Tseng et al., 2003). Contamination was observed in the non-template control sample (Figure 3.5). However, as the agarose-gel showed (result not included), a much higher amplification of DNA isolated from biofilter samples was observed relative to the non-template control. Moreover, the DGGE-profiles were different for PCR-products from the samples as compared to the non-template control. This indicates that the amplified PCR products from the biofilter samples are not associated with Taq DNA polymerase.

Another problem associated with DGGE is that it does not reveal any taxonomic information, i.e. which bacterial species the DGGE-gel bands correspond to. It is possible to associate taxonomy to DGGE-gel bands by excising gel-material, re-amplifying and sequencing it. However, this is not always successful and taxonomic information gained from DGGE-profiles are restricted. That is why DGGE is a suitable method to study community dynamics, but not for describing the composition of a microbial community. A more potent method for describing the microbial community composition is sequencing of 16S rDNA amplicons (Rastogi and Sani, 2011). Some of the PCR-products obtained from biofilter samples were delivered for Illumina-sequencing, however the results were not available in time to be included in this report.

4.1.4 FISH

Establishment of protocol for detaching sand-associated biofilm

A comparative analysis for the detachment of biofilm from sand and disaggregation of biofilm flocs was performed prior to FISH. Two different detachment techniques were evaluated; ultrasonic bath and ultrasonic probe. NaPP$_i$ was included in both procedures. According to Joaquin et al. (2009) sonication bath achieves sufficient removal of biofilm and disaggregation after a few minutes of sonication exposure. In this study, it proved to be insufficient for complete disaggregation of biofilm clumps although longer exposure times were tested. The probe sonicator proved to be the most successful method for detachment of sand attached biofilms and disaggregation of cells (Figure 3.2), as also demonstrated by Mermillod-Blondin et al. (2001).

Evaluation of 6-FAM as an alternative to Cy5 for probe labeling

FISH has limitations regarding autofluorescent particles. Figure 3.3 demonstrates presence of nonspecific fluorescent signal from autofluorescent material in a biofilm sample from the bench-scale biofilter for DWT. The material emitted fluorescence at the same detection wavelength as the Cy5 fluorophore. As previously described, filtering the autofluorescence out during image acquisition was not possible due to the fixed emission spectrum (from 640 to 700 nm). These signals may bias the quantification results. The fluorophore 6-FAM was therefore tested for replacement of Cy5 for labeling of probes.

CLSM micrographs of β-proteobacteria hybridized with 6-FAM labeled probe, showed background autofluorescence (Figure 3.4B), indicating that the probe may bind to EPS surrounding the bacteria. CLSM micrograph from a biofilm sample that was not hybridized with any fluorescently labeled probes show high presence of autofluorescent microbes or material emitting fluorescent light at the same detection wavelength as 6-FAM (Figure 3.4A). Autofluorescence has been reported for many microorganisms, including fungi (Baschien et al., 2001) and cyanobacteria (Schönhuber et al., 1999), which complicates FISH analysis of environmental microbiota. Particles in e.g. drinking water samples can also show autofluorescence,

and is caused by natural fluorescent inorganic or biological debris (Vesey et al., 1997). Attempts adjusting CLSM detector settings for 6-FAM to reduce signals from the autofluorescent artifacts was not successful, causing a reduction in the fluorescent light from the stained bacteria. Due to the higher abundance of autofluorescent signals in samples using 6-FAM, Cy5 was selected for labeling of probes.

daime

To manually exclude individual artifacts detected in each image, *daime* provide a built-in *artifact rejection tool.* This was not performed during this study due to the large quantity of images. It is therefore important to have in mind that autofluorescent particles may have been present in some of the biofilm samples analyzed by FISH, causing bias in the results. However, this applies for all samples.

daime indicates the average congruence of the FISH image pair. Values above 90% should be considered acceptable (Daims, 2009). The results obtained by *daime* demonstrated that the congruence between the image pair for α-proteobacteria was generally lower than the image pairs for β- and γ-proteobacteria (results not shown). For β- and γ-proteobacteria, the congruence was above 90% for all samples except one sample for γ-proteobacteria at day 7 of the PFOA exposure test, where the congruence was 89% (Table D.3, Appendix D).

The lower congruence for the α-proteobacterial images may reflect nonspecific binding of the α-proteobacterial probe to materials in the biofilm (Moter and Göbel, 2000) or to microbes other than fixed gram-negative bacteria although stringency conditions were optimized by the use of non-labeled competitor probes. This elucidates the importance of using a negative control for detection of nonspecific binding, however, no reports have been found demonstrating nonspecific binding of the ALF969 probe. A negative control was not included in this study.

Steric hindering by ALF969 of the EUB338 probe(s) to target may also have been a cause for the lower observed congruence (Moter and Göbel, 2000). The *artifact rejection tool* in *daime* was applied for all α-proteobacterial images to automatically exclude objects in the images for the population specific probe and to obtain a congruency above 90%. The final bacterial counts did not significantly decline after the *artifact rejection tool* was applied. Manual approximate quantification control counts were performed to verify the quantified bacterial abundances.

4.2 Bacterial community dynamics in continuous flow biofilter

The study of naturally occurring fluctuations in steady-state (established) bacterial communities, i.e. run a control biofilter fed tap or synthetic surface water without PFOA should have been included in this project. Established microbial communities can naturally evolve in engineered systems although operational and physicochemical parameters are left unchanged (Falk et al., 2009; Falkentoft et al., 2002). However, the response usually takes significantly longer than one day as compared to a young biofilm (during acclimation) where significant changes usually requires more than one day (Poulsen et al., 1993). A repeated independent experiment should also have been included before concluding that the observed shift in bacterial community composition is due to PFOA exposure.

4.3.1 Response to differences in pH, temperature and filter bed depth

Biofilter bacterial communities were differentiated as a function of filter bed depth. Comparing DGGE profiles of samples collected from the bench-scale continuous flow biofilter indicate a higher richness in the bottom of the filter bed relative to the top (Figure 3.6). Shannon's diversity was virtually the same for top and bottom, while evenness was slightly higher in the top filter bed. Fast growing and less specialized bacterial communities, such as r-strategists, are usually adapted in the top of the filter bed for efficient utilization of the easily biodegradable fraction of DOC. In the deeper

layers of the filter bed, more specialized bacterial communities (K-strategists) are expected to thrive, feeding on less-biodegradable and complex organic substances, which usually requires more diverse microbial communities (Moll et al., 1998) (Andrews and Harris, 1986). An r-selected community is expected to be less even in the top relative to the deeper parts of the biofilter. The DGGE-gel shows no striking differences between samples representing top and bottom of the filter (Figure 3.5).

Comparing DGGE profiles representing bottom of filter bed prior to and after pH and temperature stabilization demonstrate a higher band richness and Shannon diversity index (Figure 3.6) after stabilization. Moreover, changes in the community composition prior to and after pH and temperature stabilization is apparent in the DGGE-profiles of the plot (Figure 3.5) and the NM-MDS plot (Figure 3.7). Altered pH causes altered selection pressures on the microbes, favoring growth of bacteria capable of tolerating the specific pH.

However, due to the low amount of samples, too much emphasis of the differences cannot be placed. The differences would be more reliable if additional samples isolated at different pH, temperature and filter bed depth were included. Certain variation are always present, since the method is not 100% reproducible, causing minor fluctuations in the bacterial community composition. Additional samples and statistics are necessary to conclude with the observed results.

4.3.2 Response to PFOA exposure

DGGE profiling

The band richness, Shannon diversity and evenness indices for DGGE profiles of samples collected throughout the PFOA exposure test is varying, however there is no indication of any specific trend in the results observed. The NM-MDS plot (Figure 3.7) does not demonstrate any specific evolution in community structure composition after PFOA exposure. The temporary reduction in band richness and Shannon's

diversity (Figure 3.6) may have been a response in the community, and due to adaption the community raised again towards the end of the PFOA exposure experiment.

Another interesting observation is that the bacterial community composition at day 12 was slightly different from all the other samples (Figure 3.7). This may indicate a shift in the bacterial community composition after 12 days of continuous PFOA exposure. The change at day 12 may be interpreted as a community succession, in which the bacterial community undergoes a succession in response to PFOA perturbation. Biological processes can be adapted in a very dynamic manner to changes in their environment due to the complex nature of the microbial ecosystem (Rittmann and McCarty, 2001). Compounds which are biodegraded via complex biochemical degradation pathways may require enzymes that are not necessarily constitutively produced by the bacteria, as demonstrated by Stratton et al. (1983). Microbial communities may therefore require some time to adapt to the new conditions.

Phylogenetic analysis by FISH

The FISH results demonstrate a statistically significant shift from γ- to β-proteobacterial dominance in the initial stage of continuous PFOA exposure. The β-proteobacteria continued to increase up until day 7, where a slight decline was apparent (Figure 3.8).

It would be interesting to examine if PFOA is biodegradable by drinking water biofilters, and if so, correlating these results with the observed increase in β-proteobacterial abundance, which may indicate that members of this proteobacterial population are responsible for biodegradation of PFOA. β-proteobacteria are known to be highly versatile in their degradation capacities (Parales, 2010). If PFOA is biodegraded by certain species of the β-proteobacterial population in the biofilm bacterial community in the biofilter, the shift may correspond to PFOA acting as a

primary substrate for growth. However, this is highly unlikely due to the trace concentration of the compound introduced in the feed water (250 ng/L). If biodegradable, micropollutants are typically utilized as secondary substrates or act as cometabolites, which do not benefit growth of the bacteria (Stratton et al., 1983). PFOA has been reported to be highly recalcitrant towards biodegradation via DWT biofilters (Rahman et al., 2014). If PFOA is biodegradable, longer EBCT (Eschauzier et al., 2011) and exposure time, i.e. months to years (Providenti et al., 1993) is expected.

The decline in β-proteobacterial abundance between day 7 and 12 and the overall decrease in both γ- and α-proteobacterial populations throughout the 12 days PFOA exposure may reflect changes due to toxicity of PFOA. PFOA has protein-binding capacities, as reported by Han et al. (2003). PFOA may have attached to proteins in the EPS, hence accumulated inside the biofilm. This may have caused high local concentrations of PFOA due to the constant mass flow of PFOA through the filter. At high doses, the compound may have a toxic effect on species of the α- and γ-proteobacterial populations shifting the community composition towards bacterial populations that are resistant to this compound. The reported acute toxicity of PFOA on bacteria is low, even at concentrations in the mg/L-range (Pasquini et al., 2013; Rosal et al., 2010).

The results from FISH and DGGE are not consistent. Results from DGGE profiling indicated that evolution in community structure required 12 days of continuous PFOA exposure, while FISH results demonstrated a significant change in bacterial community dynamics after only one day of PFOA exposure. However, there may be numerous explanations to these differences.

5. Conclusion and future prospects

5.1 Conclusion

A closed batch experimental system is not suitable for analysis of bacterial community dynamics in response to micropollutants. Biofilter performance of the bench-scale continuous flow system was influenced by differences in pH and temperature of the synthetic surface water.

DGGE analysis revealed minor differences in community structure in top and bottom layers of the bench-scale continuous flow biofilter. Decreased pH and increased temperature resulted in an apparent change in the community structure.

The main purpose of this study included characterizing changes in community structure of a biofilter bacterial community exposed to environmental concentrations of the micropollutant PFOA. Bacterial community structure changed during continuous PFOA exposure, as demonstrated by both FISH and PCR-DGGE. FISH analysis of α-, β- and γ-proteobacteria revealed a significant shift from γ- to β-proteobacterial dominance in the biofilter communities in response to the polluted synthetic surface water.

5.2 Future prospects

It would be interesting to study an equivalent system over a longer time period, while including PFOA biodegradation results and relate it to changes in bacterial community structure. Exposing the biofilm to PFOA over a longer time-range than 12 days, i.e. weeks or months, is recommended since microbial systems include interactions among microorganisms and between microbial ecosystems, and their environment, and may therefore require extended adaptation times (Muyzer et al., 1993). Due to time constraints, the aim of this study did not include achieving biodegradation of PFOA, but to investigate biofilter bacterial community dynamics in response to PFOA exposure. Moreover, an interesting study would be to expose microbes to PFOA under nutrient poor conditions, forcing them to utilize carbon from PFOA, and identify potential PFOA-degrading strains.

To obtain reliable conclusions on the issues that were addressed in this report, replicating the PFOA exposure test, extending the biofilter exposure time of PFOA, and collecting additional samples over a longer time period prior to PFOA exposure for investigation of natural fluctuations, would be of interest.

Moreover, it would be interesting to study microbial community dynamics in a PFOA-exposed biofilter by modern sequencing technology, e.g. Illumina sequencing of barcoded 16S rDNA amplicons, which provides both detailed information regarding taxonomy and changes in community structure.

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Appendices

Appendix A: FISH protocol

Appendix B: Equipment and reagents for preparation of FISH

Appendix C: Preparation of FISH probes

Appendix D: CLSM and image analysis

Appendix E: DNA isolation

Appendix F: DGGE protocol

Appendix G: Preparation of reagents for DGGE

Appendix H: LIVE/DEAD *Bac*Light™ protocol

Appendix A – FISH protocol

The following protocol describes in detail how FISH was performed in this study for obtaining optimal and reproducible results. Additional protocols are provided explaining equipment and preparation of reagents (Appendix B) and probes (Appendix C) applied for FISH analysis. The following protocol was based on information provided by different sources, and optimized hereafter to fit this study. Step 1 and 2 were based on recommendations from Epstein and Rossel (1995); Luef et al. (2009); Mermillod-Blondin et al. (2001). Step 3 through 6 were based on recommendations from Amann et al. (1995), Daims (2009) and Osborn and Smith (2005).

Step 1 – Sampling

In this study, approximately 4 g (wet weight) of sand media was carefully collected into sterile 50 mL Falcon tubes with a sterile spatula, and fixed within an hour after sampling to inactivate cellular biochemical reactions and avoid growth.

Step 2 – Sample fixation and biofilm detachment from media

1. Add 3 volumes frozen aliquots of 4% paraformaldehyde (PFA) fixative in the fume hood to 1 volume of 1x phosphate buffered saline (PBS) to the media samples, and mix.
2. Incubate sample at 4 °C for 3 - 12 hours. Longer fixation times or higher temperature may render the cell envelopes of gram-negative cells less permeable to oligonucleotide probes.
3. Remove fixative.
4. Resuspend the media in 1x PBS.

5. Add tetrasodium pyrophosphate (NaPP$_i$) to the samples to a final concentration of 10 mM, and incubate the sample on a shaker at 100 RPM for 30 – 60 min.

6. Transfer the sample to e.g. a 30 mL centrifuge glass tube with an inner diameter of approximately 2.5 – 3 cm.

7. Use a 3 – 4 mm microtip ultrasonicator for detachment of the biofilm from sand media.

8. Place the glass tube on ice to prevent overheating of the samples and denaturation of cells.

9. Place the tip approximately 2 cm from the top of the media to prevent the media from coming into contact with the tip. This may destroy the tip.

10. Sonicate the sample for 3 min at 50% duty cycle and 40W, and interrupt for 30 sec every minute.

11. Transfer the sonicated liquid phase into a centrifuge tube, and centrifuge the sample at 11 000 RPM for 3 min.

12. Remove the supernatant and resuspend the cell pellet in 1x PBS. Dissolve the cell pellet by vortexing.

13. Repeat previous step once.

14. Remove the supernatant, and resuspend the cell pellet in 1:1 1x PBS and 96% cold ethanol (-20 ^{o}C).

15. Store the sample at -20 °C until further FISH preparation. Fixed cells can be stored at -20 °C for several months.

Step 3 – Sample application and dehydration

1. Prepare 50%, 80% and 96% ethanol solutions in 50 mL Falcon tubes. The solutions may be used multiple times.

2. 10-well 6.7 mm Teflon coated slides (Thermo Scientific) can be used for sample application (Figure A.1). Identify the slides with a pencil. Prepare one slide for each specific probe.

Figure A.1: Teflon coated slide with 10 hybridization wells.

3. Apply three layers of 10 μL cell suspension in each hybridization well. Let the sample air dry between each layer.

4. Dehydrate the samples in the three different concentrations of ethanol starting with 50%, then 80% and finally 96%. Soak the sample for 3 min in each solution. Two slides can be dehydrated per tube, back to back.

5. Air dry.

The slides can temporarily be stored (days to weeks) in a dry and dark location at -20 °C.

Step 4 – Probe hybridization

1. Prepare a "bed" of tissue paper inside a 50 mL dark Falcon tube. If a dark tube is not available, cover the tube to prevent light from weakening fluorescence. Use one tube for each slide.

2. In a fume hood, prepare hybridization buffer in a 2 mL Eppendorf tube at the time of use. Since each probe require different concentrations of formamide (Table A.2), prepare one Eppendorf tube for each slide with volumes of:
   a. 360 μL NaCl
   b. 40 μL Tris-HCl
   c. x μL high quality formamide (see Table A.1 for volume)
   d. y μL Milli-Q water (see Table A.1 for volume)

    e. 2 µL SDS 10 % on the lid (SDS can interact with NaCl and precipitate. Applying the SDS on the lid ensures that the SDS is the last component to be mixed with the solution).

3. Apply 8 µL of hybridization buffer in each hybridization well. NB! Do not touch the slide with the tip of the pipette!

4. Apply the remainder of the buffer to the tissue in the Falcon tube to keep the inside of the tube at a constant hybridization atmosphere.

5. Add 1 µL of the mix containing the population specific probe and 1 µL of the mix containing the general probes. Keep one of the wells with sample and hybridization buffer without any probe as a control to check for autofluorescence.
Mix the probes with the hybridization buffer without touching the slide.

6. Place the slide into the prepared Falcon tube in a horizontal position and seal the tube with the cap and parafilm.

7. Place the Falcon tube in the oven at 46 °C for 90 min.
To ensure that the tubes stay in a horizontal position, a rack tipped on the side can be used.

Table A.1: Volume of formamide and Milli-Q to prepare hybridization buffer

Formamide (µL)	Formamide (%)	Milli-Q (µL)
0	0	1598
100	5	1498
200	10	1398
300	15	1298
400	20	1198
500	25	1098
600	30	998
700	35	898
800	40	798
900	45	698
1000	50	598

Table A.2: Concentration of formamide for preparation of the hybridization buffer

Probe	Formamide (%)*	Reference
EUB338	0-50	Amann et al. (1990)
EUB338-II	0-50	Daims et al. (1999)
EUB338-III	0-50	Daims et al. (1999)
ALF969	35	Oehmen et al. (2006)
cALF969a		
cALF969b		
BET42a	35	Manz et al. (1992)
cBET42a		
c1033		
GAM42a	35	Manz et al. (1992)
cGAM42a		
c1033		

*Use high quality formamide. Lower grade formamide may be contaminated with cations reducing hybridization stringency.

Step 5 – Washing

1. Prepare wash buffer in a 50 mL Falcon tube (one tube for each slide) with the following concentrations:

    a. x μL NaCl (see Table A.3 for volume)

    b. 1 mL TRIS-HCl 1M

    c. y μL EDTA (see Table A.3 for volume)

    d. Dilute with Milli-Q up to 50 mL

    e. 50 μL SDS 10% (added at the end to avoid precipitation).

2. Place the buffer in a water bath at 48°C before washing the slides.

3. Wash each slide with a Pasteur pipette and let the excess go in a beaker. Wash the slides from the well without probe (control) and downwards to prevent cross contamination.

4. Place the slide in the Falcon tube with the washing buffer and place the tube in a water bath at 48 °C for 10-15 min.

5. Remove the slides from the tube and wash front and back with Milli-Q water at 4 °C.

6. To prevent probe dissociation, dry the slides quickly to remove every single droplet from the slide. Use compressed air if available.

Table A.3: Volume of NaCl and EDTA to prepare wash buffer

% Formamide	NaCl (x M)	EDTA (y mM)
0	0.900	-
5	0.636	-
10	0.450	-
15	0.318	-
20	0.225	5
25	0.159	5
30	0.112	5
35	0.080	5
40	0.056	5
45	0.040	5
50	0.028	5

Step 6 – Mounting the samples

1. Apply a few drops of anti-fadent VECTASHIELD® Mounting Media (Vector Laboratories) to the dried slides.
2. Place a cover slip over the wells and gently press it to force the Vectashield to cover all wells.
3. Remove excess Vectashield with a paper tissue.
4. Apply nail polish to the edges of the cover slip to seal the slip and prevent the immersion oil from combining with the Vectashield.
5. Wait 5-10 min until the anti-fadent has spread and penetrated the biomass.

The slides can be stored at -20°C in the dark.

Appendix B – Equipment and preparation of reagents for FISH

Equipment

- Nitrile gloves
- Shaker
- Vortexer
- Micro centrifuge (up to 11 000 RPM)
- Hybridization oven
- Water bath
- Dark Falcon tubes 50 mL
- Eppendorf tubes 2 mL
- Micropipettes and -tips from 0.5 μL to 1000 μL
- 10-well 6.7 mm Teflon coated slides (Thermo Scientific)
- Cover slips
- Nail polish
- VECTASHIELD® Mounting Medium (Vector Laboratories)
- Autoclave
- Ultrasonic probe or ultrasonic bath for detaching biofilm from media

Reagents

Phosphate Buffered Saline (PBS), pH 7.2

30x PBS: add 38.7 g Na_2HPO_4-$12H_2O$, 6.6 g NaH_2PO_4-$2H_2O$ and 113.1 g NaCl. Dilute with Milli-Q water up to 500 mL, adjust pH and autoclave. Store as stock in room temperature. Dilute 1:10 for 3x PBS and autoclave. Dilute 1:30 for 1x PBS and autoclave.

4% Paraformaldehyde (PFA), pH 7.2

Set up the balance and a heated stirrer in the fume hood. Heat 65 mL Milli-Q water to 60 °C. Add 4 g PFA powder (DO NOT INHALE) to the heated water to obtain a cloudy solution. Add 2 drops of 2M NaOH. PFA should be dissolved in 1-2 minutes. Cool down to room temperature. Add 33 mL 3x PBS. Solution can now be removed

from the fume hood. Adjust pH to 7.2 with 1M HCl. Adjust with Milli-Q water to obtain final volume of about 100 mL. Filter through 0.2μm filter to remove any undissolved crystals. Aliquot to applicable volumes and freeze at -20 °C.

5 M NaCl

Add 58 g NaCl to 200 mL of Milli-Q water, dissolve and autoclave. Can be stored at room temperature.

1 M TRIS-HCl, pH 7.2

Add 31.5 g Tris-HCl to 150 mL of Milli-Q water, dissolve, adjust pH to 7.2 with 2 M NaOH and autoclave. Make up to 200 mL with Milli-Q water and autoclave.

0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 7.2

Add 18.6 g EDTA and dilute with 75 mL Milli-Q water, dissolve and adjust pH to 7.2 with NaOH pellets. Make up to 100 mL with Milli-Q water and autoclave. NB! It may take a while for the EDTA to dissolve. Adding NaOH pellets will help this process. Can be stored at room temperature.

10% Sodium Dodecyl Sulfate (SDS)

Dissolve 10 g of SDS in 100 mL Milli-Q water in the fume hood. NB! Do not autoclave. Can be stored in room temperature.

0.05 M Tetrasodium pyrophosphate (NaPP$_i$)

Add 1.33 g of Tetrasodium pyrophosphate (Na$_4$P$_2$O$_7$) in 100 mL Milli-Q water. Autoclave.

Appendix C – Preparation of FISH probes

DNA oligonucleotide probes were purchased online from Sigma-Aldrich (http://www.sigmaaldrich.com/norway.html). Probes were selected based on information from Moita and Lemos (2012); Ricardo et al. (2012) and probeBase (Loy et al., 2007). Details of the oligonucleotide probes applied in this study are provided in Table C.2.

Preparation of FISH probes directly after arrival from the provider:

Since the probes arrived lyophilized they had to be diluted before being frozen into aliquots. Preparing aliquots of the probes is recommended to prevent thawing and freezing of the probe solution multiple times. This will increase the chance of contamination and decrease the lifetime of the probes. Dilution was carried out according to information from the technical data sheet provided by Sigma-Aldrich.

1. It is highly recommended to work under sterile conditions to prevent contamination of the probes.
2. Add autoclaved Milli-Q water to each probe to obtain a concentration of approximately 500 ng/μL.
3. Divide the probes into aliquots (in this experiment; 5 uL) needed for the experiment.
4. Store the probes protected from light at -20°C.

Preparation of FISH probes directly prior to use:

Directly prior to use, the probes have to be diluted 1:10 with Milli-Q water to reach a final concentration of approximately 50 ng/μL. In this study the probes EUB338-I, EUB338-II, EUB338-III were chosen for fluorescent labeling of the total biomass, while Alf969, BET42a and GAM42a were chosen as probes for the specific target

populations. Alf969, BET42a and GAM42a have competitor probes as listed in Table C.1, which are not labelled with fluorophores. These probes were included in the 1:10 dilutions.

Table C.1: Details of probe dilution.

Mix no.	Name of probes	Amount of probe (μL)	Amount of Milli-Q water (μL)	Final conc (ng/μL)
1	EUB338	5	35	50
	EUB338-II	5		
	EUB338-III	5		
2	ALF969	5	35	50
	cALF969a	5		
	cALF969b	5		
3	BET42a	5	35	50
	cBET42a	5		
	c1033	5		
4	GAM42a	5	35	50
	cGAM42a	5		
	c1033	5		

Table C.2: Details of the oligonucleotide probes applied in this study for quantification of bacterial subpopulations.

Probe name	Specificity	rRNA target site	Fluorophore	Sequence 5' to 3'	Reference
EUB338	Most bacteria	16S, 338-355	Cy5	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
EUB338-II	Planctomycetales	16S, 338-355	Cy5	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
EUB338-III	Verrucomicrobiales	16S, 338-355	Cy5	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
ALF969	α-Proteobacteria	16S, 969-986	Cy3	TGG TAA GGT TCT GCG CGT	Oehmen et al. (2006)
cALF969a	Competitor probe		None	AGG TAA GGT TCT GCG CGT	Oehmen et al. (2006)
cALF969b	Competitor probe		None	GGG TAA GGT TCT GCG CGT	Oehmen et al. (2006)
BET42a	β-Proteobacteria	23S, 1027-1043	Cy3	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
cBET42a	Competitor probe		None	GCC TTC CCA CAT CGT TT	Manz et al. (1992)
c1033	Competitor probe		None	GCC TTC CCA CCT CGT TT	Yeates et al. (2003)
GAM42a	γ-Proteobacteria	23S, 1027-1043	Cy3	GCC TTC CCA CAT CGT TT	Manz et al. (1992)
cGAM42a	Competitor probe		None	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
c1033	Competitor probe		None	GCC TTC CCA CCT CGT TT	Yeates et al. (2003)

Appendix D – CLSM and image analysis

CLSM

CLSM and image analysis were based on recommendations from Daims et al. (2006) and Daims (2009). Zeiss LSM 700, Axio Imager.Z2 confocal laser scanning microscopy (CLSM) and the software ZEN 2010 by Carl Zeiss were used to observe the samples and acquire the images. Specific filters for the Cy3, Cy5 and 6-FAM fluorophores were used. General and specific settings applied for CLSM detection of the fluorophores in this study are presented in Table D.1 and D.2, respectively.

Correcting for background autofluorescence

First, optimal settings for detection of the fluorophores were obtained by visualizing biomass hybridized with the probe of interest. Then, the biomass without probe was examined using the same settings as obtained in the previous step. If a signal was observed, this sample was autofluorescent. This was corrected to obtain reliable quantification results. The detector settings were adjusted so that the image was dark with little background autofluorescence. Then the wells with probe were used to acquire the images needed for the analysis.

Table D.1: General image acquisition settings for CLSM detection of fluorophores.

Settings	Value
Objective	40x oil
Scan speed	6
Data depth	8 bits
Mode	Line (Linear)
Pinhole	1 Arbitrary Unit (AU)
Scan average	4
Pixel resolution	1024x1024
Number of pictures taken per well	≥34

Table D.2: Specific image acquisition settings for CLSM detection of fluorophores.

Settings	Cy5	Cy3	6-FAM
Laser diode (nm)	639	555	488
Filter	Long pass 640 nm	Short pass 640 nm, Split 575	Short pass 555, Split 525
Laser intensity	15.0	6.0	6.0
Master Gain	Adjusted for each sample	Adjusted for each sample	Adjusted for each sample
Digital Offset	-15	-15	-5
Digital Gain	1.0	1.0	1.0

Image acquisition

The image pairs were recorded at randomly chosen positions within the sample. The sample was viewed through a filter that blocked the fluorescence emitted by the specifically labeled target population. The target population cells were labeled by the specific probe and the general probe. These cells had to be congruent (i.e. overlapping) in the images of each image pair. In order to meet this condition, the detectors of the CLSM were adjusted.

The same detector settings for recording of the total biomass is recommended. If detector adjustments were needed to meet the congruency requirement, only the detector settings for the target population-specific probe signals are recommended to be adjusted to ensure that results obtained for the different target populations remain comparable. However, due to major variations in fluorescent signal, the Master Gain for the detection of general bacterial population was adjusted to obtain optimal image quality. The threshold for quantification using *daime* was adjusted according to the Master Gain settings applied for each sample.

20-30 image pairs for each quantified population are sufficient to get reliable results. However, this depends on the type of environmental sample and on the quantified target population. A minimum of 34 image pairs for each quantified population were acquired in this study.

Image analysis

Quantification of the bacterial subpopulations was done using the software *daime* (Daims et al., 2006). The software was downloaded from http://microbial-ecology.net/daime/. The *daime* settings applied for quantification are listed in Table D.3. ImageJ (Abràmoff et al., 2004) was used to adjust the brightness and contrast of the images presented in this report.

Prior to image analysis, the images were *segmented* using the *daime* software. This tool provides features to distinguish objects from background and fluorescence-labeled cells from autofluorescent material. It can be difficult to distinguish between weak positive signals and background noise. If necessary, a threshold can be adjusted manually to eliminate noise while visualizing all cells. To distinguish between background noise and fluorescence-labeled cells, the upper and lower threshold for the signal intensity for image segmentation was set manually. The *segmenting* tool also has options for ignoring artifacts or noise that are smaller than the biomass objects. It was chosen to ignore all objects with a size up to 10 pixels in all the images.

Moreover, using the object editor before image analysis tested the success and quality of the segmentation. The *artefact rejection* tool within the *object editor* can detect putative artifacts in the images of the target population and rejects them automatically. The tool compares each object in the target population image with an object at the same position in the total biomass image, and how much these two object overlap (i.e. how congruent they are). The threshold for the artefact rejection tool was specified manually, and set to 65% for the alpha-proteobacteria, due to generally low congruency, i.e. less than 90%.

Table D.3: Details of *daime* settings for quantification, results of abundance and calculated SDOM.

Day	Population	Threshold total	Threshold population	Artifact rejection (%)	Congruency (%)	Mean biovolume (%)	SD	Number of pictures	SDOM
0	Alpha	39	29	65	96	6,0	1,8	34	0,309
0	Beta	39	29		99	5,2	1,7	38	0,276
0	Gamma	39	29		97	9,5	2,7	34	0,463
1	Alpha	29	29	65	90	4,8	2,6	35	0,439
1	Beta	39	29		96	10,9	4,4	34	0,755
1	Gamma	39	29		97	4,7	1,5	34	0,257
3	Alpha	29	29	65	94	6,1	2,0	34	0,343
3	Beta	29	29		97	29,2	5,2	34	0,892
3	Gamma	29	29		94	5,7	1,3	34	0,223
5	Alpha	29	29	65	92	3,5	0,8	34	0,137
5	Beta	29	29		94	36,0	5,5	34	0,943
5	Gamma	29	29		94	4,3	1,0	34	0,171
7	Alpha	29	29	65	92	3,0	1,0	34	0,171
7	Beta	29	29		93	39,3	7,3	34	1,252
7	Gamma	29	29		89	3,2	1,0	34	0,171
12	Alpha	19	19	65	93	1,2	0,5	34	0,086
12	Beta	19	19		92	34,3	4,5	34	0,772
12	Gamma	19	19		97	1,5	0,2	34	0,034

Appendix E – PowerSoil® DNA Isolation Kit by MO BIO Laboratories, Inc.

Experienced User Protocol
Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 μl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 μl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 μl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 μl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 μl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 μl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 μl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 μl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 μl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
   Note: A total of three loads for each sample processed are required.
16. Add 500 μl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 μl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Appendix F – DGGE protocol for Ingeny

Step 1 – Mounting of glass plates

1. Wash the two glass plates, the spacer, and comb, using Deconex soap and hot tap water. Finally rinse well with water to remove any traces of soap. Polish one side of each glass plate using 96% ethanol and Kimwipe paper.

2. Assemble the glass plates and spacer, and place it all in the gel box. Assure that the spacer is aligned to the lower edge of the glass plates. Tighten the screws.

3. Loosen the two uppermost screws, mount the comb, and then tighten the screws again.

Step 2 – Preparation of DGGE solutions

1. Determine the acrylamide percent and the denaturating gradient of the gel (for recipes of solutions, -see below).
2. Make acrylamide solutions with the desired denaturating percentages in two 50 mL tubes (total volume in each tube will be 24 ml; see table below for volumes of 0% and 80% denaturating solutions).
3. The 0% denat. acrylamide solution can be added to the 50 mL tubes without sterile filtration. The 80% denat. acrylamide solution needs to be filtered upon addition.
4. Prepare a 50 mL tube with 8 mL 0% denat. acrylamide solution ("stacking gel" for the top part of the gel).
5. When ready to pour the gel, add 16µl TEMED to the 24 mL gel solutions, and 10µl TEMED til the 8 mL "stacking gel" solution.

6. Directly prior to pouring the gel, add 87µl APS (10% Ammonium persulphate) in both 24 mL gel solutions (for the stacking gel, add 40µl APS, but not until the stacking gel is ready for pouring).

Step 3 – Casting the gel

1. Rinse the gradient mixer and the tubes by pumping Milli-Q water through the system.
2. Turn off the pump, close the valve between the chambers of the gradient mixer, and put the gradient mixer on magnetic stirring.
3. Pour the gel solution with low denat. percentage in the "left" chamber. Quickly open and close the valve to remove any air bubbles in the channel between the chambers. Use a pipette to remove the small amounts of gel solution from the "right" chamber.
4. Pour the gel solution with high denat. percentage in the "right" chamber.
5. Start the pump, wait a few seconds until the gel solution from the "right" chamber has migrated ~7-8 cm out in the tube. Then open the valve between the chambers. Assure stirring in both chambers.
6. Place the syringe between the glass plates (assure that no water from the washing step is left in the tube).
7. When the gel reaches approximately 1 cm below the comb, stop the pump, remove the syringe, and empty leftovers from the mixing chambers and the flexible tubes. Rinse the system with a small amount of Milli-Q water.
8. When the mixing chambers are empty from water, close the valve and stop the pump. Add APS to the "stacking gel" solution, mix, and pour into the "right" chamber.
9. Start the pump again. When the glass plates are completely filled with the stacking gel, turn off the pump, and press the comb down to the correct position. Tighten the screws.
10. Leave the gel for polymerization for at least two hours.

11. Pump Milli-Q water through the system to avoid gel polymerization in the tubes.

Step 4 – Preparations and addition of samples

1. Make 20 l of 0.5 x TAE (200mL 50 x TAE + 20 l Milli-Q) and add appr. 17 l to the buffer tank (the buffer may be used for 3 runs). Turn on the instrument to heat the buffer to 60^0C.
2. Carefully remove the comb from the gel. Loosen all screws, and carefully push down the spacer. Tighten the screws at the sides of the glass plates (the screws at the bottom should be loose throughout the electrophoresis).
3. Place the gel system in the buffer tank. Avoid air bubbles beneath the gel.
4. Attach cables and tube, turn on the recirculation. Use a syringe with buffer to rinse the wells. Turn on the power (100 Volts; should result in approximately 27-35 mA) and let run while preparing the samples.
5. Add 2-4μl loading dye to 5-15 μl PCR sample. When all samples are ready for loading, turn of the recirculation and the push the "low voltage" button. Apply the samples to the wells. Avoid using the 2-3 outermost wells on each side due to "smiling effects".

Step 5 – Running the gel
1. Turn on the "high voltage button", set the voltage to 100. Run 5-10 min without recirculation.
2. Turn on the recirculation and run for 17-18 hours.

Step 6 – Staining and visualization
1. Turn off the instrument; lift the gel system over to the blue box.
2. Loosen the screws, and lift out the gel. Carefully separate the glass plates (use the small red plastic equipment).
3. Transfer the gel to a plastic foil sheet, and place it in the dark blue box.

4. Prepare the staining solution: 30mL Milli-Q + 3μl SYBR Gold + 600μl 50 x TAE in a 50 mL tube.

5. Distribute the staining solution on the gel, put the lid on the box, and leave for 1-2 hours.

6. Carefully take out the gel, rinse with Milli-Q water. Carefully let the water run of the gel, use a paper towel at the edges of the gel to remove excess water.

7. Wash the UV plate of the "gel doc" with distilled water and ethanol. Use Kimwipe paper, and take care to avoid dust and particles on the UV plate (easier to avoid dust if the plate is not allowed to dry). Finally distribute Milli-Q water on the plate (this makes it possible to move the gel on the UV plate).

8. Carefully transfer the gel from the plastic foil to the UV plate (by turning the plastic foil "upside down"). Before removing foil, position the gel at the plate.

9. Photograph the gel at different exposures, and save the pictures in original file format, and e.g. pdf or other formats.

Step 7 – Eluation of bands for sequencing

1. Print out a picture of the gel, and number the bands that are to be sequenced.

2. Add 20μl sterile Milli-Q water to eppendorf tubes, and number the tubes according to the numbering of bands.

3. Pull out the UV plate, and pull on the UV screen. Cover the wrists to protect from UV radiation. Use the blue 1 mL pipette tips to stick out material from the bands. Take care to avoid touching other bands. Use a pipette to blow out the material in the eppendorf tube with water (it should be possible to see whether there is material in the pipette tip when transferring it to the water).

4. Place the tubes in the fridge over night.

5. Use 1 μl of the eluate as template in a 25μl PCR reaction.

Appendix G – Preparation of reagents for DGGE

For all solutions, add distilled water to obtain the final volume.

50 x TAE-buffer

Tris base	242 g/L
Glacial acetic acid	57.1 mL/L
0.5 M EDTA (pH 8,0)	100 mL/L

Autoclave the buffer.

Deionized formamide

Deionize 200 mL formamide by adding 7,5g DOWEX RESIN AG 501X8, and stir for 1 hour at room temperature.

Acrylamide solution (0% denaturing)

8% acrylamide in 0.5 x TAE (per 250 mL):

40% acrylamide solution (BioRadLab Inc., Ca., USA)	50 mL
50 x TAE	2.5 mL

Store the solution at 4ºC, protect from light.

Denaturing acrylamide solution (80% denaturing)

8% acrylamide, 5,6M urea, 32% formamide in 0,5 x TAE (per 250 mL):

40% acrylamide solution (BioRadLab Inc., Ca., USA)	50 mL
50 x TAE	2,5 ml
Urea	84 g
Deionized formamide	80 mL

Store the solution at 4°C, protect from light. This solution **must** be sterile filtered before pouring the gel.

Table G.1: Composition of low and high denaturing solutions

Denaturing %	0%	80%	TEMED + 10% APS	Total volume
15	19,5ml	4,5ml	16μl + 87μl	24ml
25	16,5ml	7,5ml	16μl + 87μl	24ml
30	15 ml	9 ml	16μl + 87μl	24ml
40	12 ml	12 ml	16μl + 87μl	24ml
45	10,5ml	13,5ml	16μl + 87μl	24ml
50	9ml	15ml	16μl + 87μl	24ml
55	7,5ml	16,5ml	16μl + 87μl	24ml
60	6 ml	18 ml	16μl + 87μl	24ml
75	1,5ml	22,5ml	16μl + 87μl	24ml

0% "Stacking gel":

8 mL 0% acrylamide solution, 40μl 10% APS and 10μl TEMED.

10% APS (ammonium persulfate):

10g ammonium persulfate dissolved in 100mL dH$_2$O
Sterile filter the solution, distribute in eppendorf tubes (250μl in each), and keep frozen.

Appendix H – LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit protocol for fluorescence microscopy

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012, developed by Molecular Probes Inc., was applied for enumeration of live and dead bacteria in the biofilm. The protocol was optimized to fit this study. The stained samples were analyzed with Zeiss LSM 700, Axio Imager.Z2 CLSM. The software ImageJ was used for quantification of viable and dead bacteria in the biofilm.

Preparation of bacterial suspension prior to staining

1. Collect appropriate volume of bacterial sample into a centrifuge tube
2. Centrifuge sample at 11000 RPM for 3 minutes.
3. Remove supernatant and resuspend the bacterial cell pellet in autoclaved 0.85 % NaCl or appropriate buffer. Phosphate wash buffers are not recommended because they appear to decrease staining efficiency.
4. Repeat step 2 and 3 once. A single wash step is usually sufficient to remove significant traces of interfering media components from the bacterial suspension.

Staining bacteria in suspension with kit L7012

1. Combine equal volumes of SYTO 9 and propidium iodide dyes in a microfuge tube, mix thoroughly.
2. Add 3uL of the dye mixture for each mL of the bacterial suspension. When used at recommended dilutions, the reagent mixture will contribute 0.3 % DMSO (dimethyl sulfoxide) to the staining solution. Higher DMSO concentrations may adversely affect staining.
3. Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

4. Trap 5uL of the stained bacterial suspension between a slide and a 18 mm square coverslip.
5. Observe in a fluorescence microscope equipped with a suitable filter useful for simultaneous viewing of the SYTO 9 and PI dyes.

Microscopy and selection of optical filters

The excitation maxima for SYTO 9 and propidium iodide (PI) is 480 and 490 nm, respectively. The 488 nm steady state laser was therefore chosen for excitation of the dyes. The emission maxima for the SYTO 9 and PI are 500 and 635 nm, respectively. Any standard fluorescein long pass and dual emission filter set can visualize the fluorescence from both live and dead bacteria simultaneously. Viable cells were fluorescent green, while non-viable cells were fluorescent red.