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Methodological approaches for studying the microbial ecology of drinking water distribution systems

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ABSTRACT

The study of the microbial ecology of drinking water distribution systems (DWDS) has traditionally been based on culturing organisms from bulk water samples. The development and application of molecular methods has supplied new tools for examining the microbial diversity and activity of environmental samples, yielding new insights into the microbial community and its diversity within these engineered ecosystems. In this review, the currently available methods and emerging approaches for characterising microbial communities, including both planktonic and biofilm ways of life, are critically evaluated. The study of biofilms is considered particularly important as it plays a critical role in the processes and interactions occurring at the pipe wall and bulk water interface. The advantages, limitations and usefulness of methods that can be used to detect and assess microbial abundance, community composition and function are discussed in a DWDS context. This review will assist hydraulic engineers and microbial ecologists in choosing the most appropriate tools to assess drinking water microbiology and related aspects.

Contents

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1. Introduction

The safety of drinking water is assumed and taken for granted by consumers in most developed countries. Yet, our understanding of the microbial ecology of drinking water distribution systems (DWDS) is limited, partly as these environments are not easily accessible and because they have traditionally been considered as challenging environments for microbial life when compared with other aquatic ecosystems. However, available scientific literature fuelled by the application of recent advances in molecular-based methods to drinking water ecosystem indicates that DWDS are diverse microbial ecosystems, with high bacterial and fungal abundance, but where a variety of microbial life from viruses to protozoa can be found (Szewzyk et al., 2000).

Modern water treatment works can produce safe drinking water reliably, efficiently and effectively, starting from a variety of sources and initial qualities. While safe and of high quality, this water is far from sterile. Treated water is transported to end users through a diverse and complex water distribution infrastructure. Preventive measures are taken to control water quality, including microbial contamination, at treatment works and via the provision of disinfection residuals in the majority of DWDS. Nonetheless, some microorganisms can persist after treatment and enter and live within distribution systems (LeChevallier et al., 1987; Szewzyk et al., 2000). Additionally, treatment works have not always been operated to the current high standard, historically providing a range of nutrients to the communities developed within DWDS. Microorganisms can also enter distribution networks during installation, repair or replacement of infrastructure and by net ingress under dynamic or other depressurisation events (Besner et al., 2011). Once microorganisms are within a DWDS they will face a challenging environment, with limited nutrients and changing water flow and pressure fluctuations. As a consequence, microorganisms will often have a better chance of survival attached to the pipe surfaces within a biofilm (Henne et al., 2012), where they are protected from external adverse factors and benefit from the interaction with other microorganisms. More than 95% of the microbial biomass in a DWDS is attached to the pipe walls forming biofilms (Flemming, 1998).

The common questions arising when trying to study microorganisms in DWDS, irrespective of their life style are; (1) which type of microorganisms are present; (2) how abundant are they; (3) how their activities shape the environment or influence other organisms, including any possible effects on human health; and (4) how the environment influences the structure and function of the microorganisms present. Where function refers to those components of biodiversity that influence how an ecosystem works (Tilman, 2001).

Different methods have been used to study DWDS in an attempt to answer these questions, ranging from cultured-dependent methods to culture independent-techniques. In accordance with regulatory requirements, water companies routinely use culture-dependent methods to assess the quality of drinking water. Culture-dependent detection and enumeration of faecal coliforms are useful for monitoring drinking water for faecal contamination providing water utilities with data at a reasonable cost. However, they provide limited information about the total microbial community (encompassing < 1% of the diversity) and changes therein. The application of culture-independent techniques has overcome these limitations and has recently revealed a new and improved view of the microbial world in DWDS. The implementation of these techniques as the method of choice to investigate microbial communities by water utilities is slow, since they require more specialised equipment, trained personnel and are more expensive than the culture-dependent methods. However, it is expected that a number of culture-independent methods will be used routinely in the near future (as the prices for the analysis are dropping).

This review presents an overview of the available methods that can be used to detect microorganisms and assess their abundance, composition and function within DWDS. The methods discussed are critically assessed with respect to their advantages, limitations, relevance and applicability to drinking water research. A full understanding of the microbial ecology of DWDS is of fundamental importance to preserve and guarantee safe and good quality drinking water. Better insights into microbial ecology of drinking water can provide more reliable risk assessments and help to improve current control and management strategies.

2. Sampling water distribution systems

2.1. *Bulk water sampling*

Appropriate sampling procedures are essential for collecting representative water samples for microbiological parameters. Sampling programmes, guidelines for practices and procedures to monitor water quality within DWDS have been designed and developed by international organisations and water companies. The World Health Organisation (WHO) have published several editions of the *Guidelines for Drinking Water Quality* (2011), where information about standardised methods for microbial analysis of DWDS can be found (ISO5667-5:2006). At a national level, in the USA, the Safe Drinking Water Act authorises the Environmental Protection Agency (US EPA) to set standards for drinking water and has developed a guide to help collect water samples according to these standards (http://water.epa.gov/lawsregs/rulesregs/sdwa/index.cfm). In the European Union (EU). Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal L, 330 (05/12/1998), regulates the quality of water for human consumption and requires that the EU countries meet a number of health parameters and standards (Weinthal et al., 2005). In the UK, the Environmental Agency (EA) also provides guidance on methods of sampling and analysis for determining the quality of drinking water and the Drinking Water Inspectorate regulates water companies in England and Wales to ensure that drinking water quality is safe and acceptable to consumers.

Despite rigorous standards for regulatory purposes, there is often a lack of detail in the scientific literature about sampling methodologies, making the evaluation and comparison of data across systems and research difficult. Several basic considerations need to be taken into account when sampling, such as the use of appropriate sampling containers, transport, storage and avoidance of contamination during collection. However, if the research objective is to apply methods besides the standard analysis of drinking water, which are molecular-based (DNA/RNA) or based on proteomics or metabolomics approaches the current official regulations and guidelines described above do not provide any protocol guidance. For example, there are no standards regarding the minimal representative sampling volume needed to capture the complete microbiome present in DWDS. Different volumes of water ranging from 1 L to 100 L have been used in the literature to concentrate microbial biomass for downstream molecular analysis (Martiny et al., 2003; Lautenschlager et al., 2010; Revetta et al., 2010; Gomez-Alvarez et al., 2012). While sampling standards do exist for regulated parameters (e.g. random day time sampling in the UK, requiring tap sterilisation, flushing, etc.) the suitability of these for advanced microbial analysis should be reviewed, including consideration of how, where and when samples are taken. The lack of standards for molecular work makes comparison of results between laboratories extremely difficult. However, molecular techniques are more frequently used and it is expected that standards and guidance for these will be developed in the near future.

2.2. *Biofilm sampling*

Biofilm research is a key component in DWDS microbial studies, but as pipes are not readily accessible, collecting samples from real systems is a substantial challenge. Habitually, bench-top laboratory biofilm reactors such as the Rotating Disc Reactor (Murga et al., 2001; Mohle et al., 2007), the Biofilm Annular Reactor (Batte et al., 2003a,b), and the Propella Reactor (Appenzeller et al., 2001) have been used to study various abiotic factors that might influence biofilm formation. However, it is well known that they poorly replicate the conditions of real pipe networks (Deines et al., 2010).

Currently two different approaches exist for studying biofilms *in situ* in DWDS. One involves cut-outs of pipes; the other one relies on devices inserted into the pipe. Pipe cut-out sampling protocols are labour-intensive, expensive and classed as destructive sampling methods (LeChevallier et al., 1998; Wingender and Flemming, 2004). Furthermore, the excavation and cutting processes often lead to concerns with contamination and representative sampling. The use of devices, commonly coupons, that can be deployed repeatedly either within a pilot-scale test facility or in an operational DWDS, allows the study of biofilm dynamics over time in relation to changing abiotic and biotic factors *in situ*. Commonly, the main limitation of some of these devices is that they distort hydraulic conditions in pipes and, in most cases, shear stress and turbulence regimes are different from those expected in real pipes, artificially influencing the way biofilms develop. The Robbin device (Manz et al., 1993; Kalmbach et al., 1997) and the "Pipe Sliding Coupon" holder (Chang et al., 2003) present these types of hydraulic limitations. Some devices such as the "Biofilm Sampler" (Juhna et al., 2007) are directly connected to a DWDS avoiding the distortion of hydraulic conditions on biofilm processes but to study *in situ* biofilms, for example via microscopy techniques, biofilms need to be removed from the coupon. The Pennine Water Group coupon, 'PWG Coupon', takes the benefits of the "Biofilm Sampler" a step further, since the coupon is curved and therefore sits flush with the pipe wall reducing the distortion of hydraulic conditions (Deines et al., 2010). Another advantage is that the coupon comprises two parts; a removable 'insert', which allows the analysis of biofilms *in situ* and an outer part that can be used to extract nucleic acids for further characterisation of microbial communities (Deines et al., 2010).

The application of coupon techniques in both experimental and live DWDS makes it possible for us to advance our understanding of biofilms and the numerous abiotic factors that might play a role in their formation and properties.

3. Conventional and current microbiological techniques and methodological advancements to address the challenges of maintaining potable water quality

Fig. 1 shows the techniques most frequently used to detect, quantify, and characterise microbial communities in drinking water-related samples (i.e. bulk water and biofilm). Conventional microbial techniques have been traditionally applied to monitor changes in the microbial quality of water. Despite their usefulness, these techniques are certainly limited and they only show a relatively small proportion (<1%) of the total diversity of the water samples (Riesenfeld et al., 2004). Recently, molecular approaches have circumvented these limitations, allowing us to obtain a more detailed image of microbial communities. In this section, the applications, advantages and limitations of these techniques are discussed in detail.

3.1. *Microbial detection and enumeration*

3.1.1. *Culture-dependent techniques*

Despite the well-known limitations of culture-dependent methodologies (Amann et al., 1995; Theron and Cloete, 2000), they are the current regulatory requirement used by water companies and analytical laboratories to routinely monitor microbial quality of drinking water, including the detection of faecal contamination.

The reference method used for routine bacteriological monitoring in drinking water is **heterotrophic plate count (HPC) measurements**, which assess only heterotrophic bacteria able to form colonies on a solid medium at a specific temperature. Counting the number of colonies grown after a defined incubation time provides a general estimation of the bacteriological load in the water samples. There are several standardized HPC methods but not an approved standard operating procedure. These methods include incubation of plates using temperatures ranging from 20 °C to 37 °C and over periods from a few hours to several days (Allen et al., 2004). HPC yields only information about a limited fraction of the whole microbial community in a sample but the low cost, relative simplicity, wide acceptance and long history of the method makes HPC a convenient tool for water utilities to assess the efficiency of water treatment and to infer regrowth of microorganism in the network (WHO, 2003).

Culture-dependent tests are also used to detect indicator microorganisms such as coliform bacteria. Coliform bacteria (e.g. *Escherichia* spp., *Enterobacter* spp. and *Citrobacter* spp.) are habitual inhabitants of animal faeces and for this reason their presence above certain concentrations, established in specific legislations, is used to infer faecal contamination in the water (Boubetra et al., 2011). The **membrane filtration (MF)** technique and the **multiple tube fermentation (MTF)** method are often used to detect coliforms in drinking water. The MF technique consists of filtering a water sample to concentrate cells followed by incubation of the filter on a specific medium and after a given period of time the developed colonies are enumerated. In the MTF technique, the concentration of bacteria is estimated by inoculating a series of tubes containing liquid medium with ten-fold dilutions of the water sample. If the medium supports microbial growth it will become turbid and the results can be expressed using an estimation of the average number of bacteria in the sample known as the **most probable number (MPN)** technique (Sutton, 2010). However, further testing is generally required

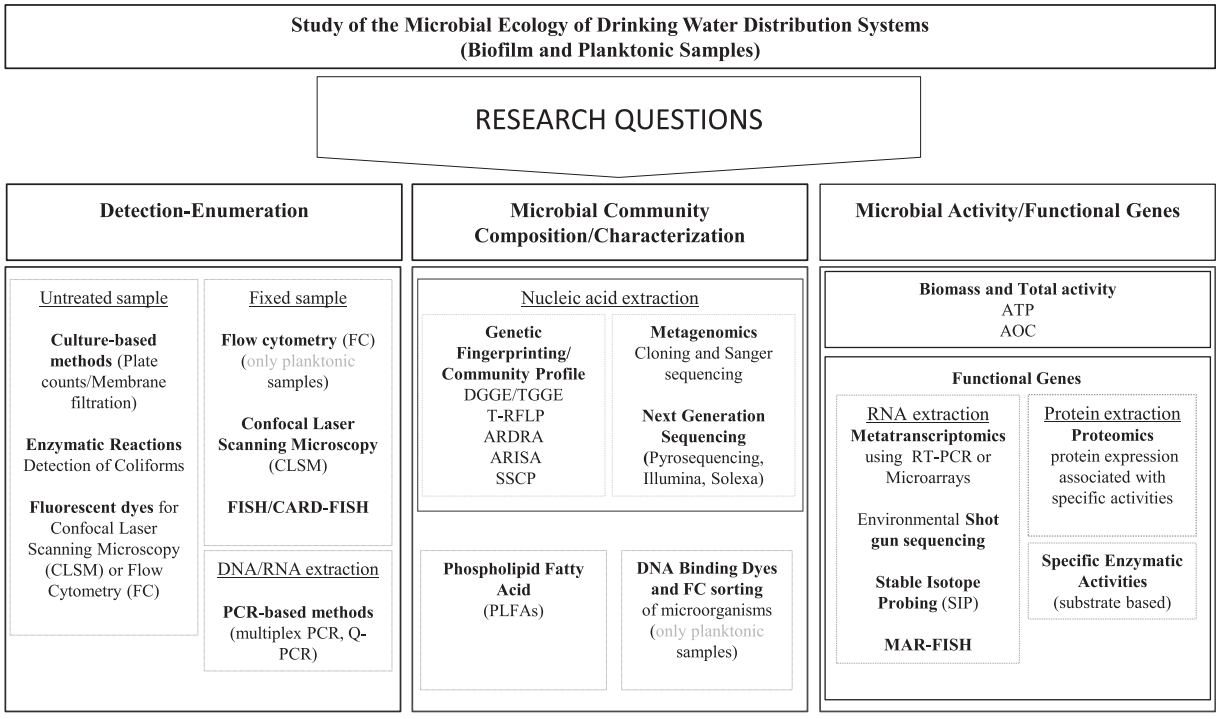

Fig. 1 – Scheme showing the different techniques available to characterise microbial communities in drinking water distribution systems.

to confirm the presence of specific coliform organisms (Ashbolt et al., 2001). The tests used to analyse these bacteria are relatively cheap, easy and safe to execute, providing water companies and analytical laboratories with a convenient tool to assess risk of faecal contamination. In *Standard Methods for the Examination of Water and Wastewater* (APHA, 2012), detailed methodological information can be found regarding the detection of total and faecal coliforms.

An alternative and more sensitive approach to detect coliforms is based on **enzymatic reactions**, using the enzymes β-D galactosidase and β-D glucoronidase. Briefly, the water sample is used to inoculate a medium containing specific enzyme substrates which in contact with a particular microorganism produce a quantifiable colour change (Rompre et al., 2002). The most widely used test based on enzymatic reactions to detect coliforms is ColilertR (IDEXX Laboratories) and a modified version, Quantity-Tray (QT), allows for their quantification. These methods are easy to use and they can detect non-culturable coliforms (George et al., 2000), but they are more expensive when compared with cultivation methods.

Alternative indicators of faecal pollution are sometimes monitored in addition to coliforms. The sulphite-reducing anaerobe bacterium *Clostridium perfringens* is considered a good indicator of faecal contamination (Ashbolt et al., 2001). Spores formed by this bacterium are mainly of faecal origin and can survive disinfection as they are more resistant than vegetative cells. Consequently, *Clostridium* spp. is a better indicator than *Escherichia coli* of the presence of more long-lasting organisms such as viruses and protozoa because they can survive under similar conditions (Ashbolt et al., 2001). There is an established ISO procedure to detect *C. perfringens* (ISO/TC 147/SC 4) using a selective medium for this microorganism.

In conclusion, culture-dependent methods are convenient diagnostic tools used by water companies given that they are simple to perform, relatively low-cost and fast ways of detecting general microbial failures in the system. However, they are only representative of a limited and specific fraction of microbial communities in water samples.

3.1.2. *Culture-independent techniques*

To circumvent the limitations of culture-dependent techniques in representing the actual microbial diversity, culture–independent methods have been developed to detect and quantify microorganisms. In Table 1, we comment on the main applications, advantages and disadvantages of the most commonly used techniques to study microorganisms in drinking water distribution systems.

3.1.2.1. Microscopy methods. **Epifluorescence microscopy** based methods offer a faster alternative for monitoring the quality of drinking water than traditional plate counts, which have long incubation times. Different fluorescent dyes can be used to directly stain cells in biofilms and/or water samples and to estimate total cell counts using an epifluorescence microscope. Some of the most useful dyes to quantify microorganisms in water and biofilm samples are acridine orange (AO) (Hobbie et al., 1977), 4,6-di-amino-2 phenylindole (DAPI) (Schaule et al., 1993) and 5-cyano-2,3 Dytolyl Tetrazolium Chloride (CTC) (Schaule and Flemming, 1996). To estimate viable cells a viability staining method might be used, such as the **LIVE/DEAD®** Bacterial Viability Kit (BacLight™) which contains two nucleic acid stains: SYTO 9™ (green-fluorescent) and propidium iodide (PI) (red-fluorescent). The SYTO 9™ dye penetrates all membranes while PI can only penetrate cells with damaged membranes. Therefore, cells with compromised membranes will stain red, whereas cells with undamaged membranes will stain green (Boulos et al., 1999).

Fluorescent *in situ* hybridization (FISH) effectively extends epifluorescence microscopy, allowing for the fast detection and enumeration of specific microorganisms (Wagner et al., 1993). This method uses fluorescent labelled oligonucleotides probes (usually 15-25 bp) which bind specifically to microbial DNA in the sample, allowing the visualization of the cells using an epifluorescence or confocal laser scanning microscope (CLSM) (Gilbride et al., 2006). FISH has been successfully used to characterise microorganisms within biofilms and to detect pathogens in drinking water samples (Batte et al., 2003b; Wilhartitz et al., 2007). An improvement of the FISH method is the **catalysed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)** (Pernthaler et al., 2002a). This method uses oligonucleotides probes labelled with a horse radish peroxidase (HRP) to amplify the intensity of the signal obtained from the microorganisms being studied (Schauer et al., 2012). CARD-FISH is useful when dealing with drinking water samples since it can enhance the fluorescent signal from cells in samples with low microbial concentration (Dorigo et al., 2005). The method has been successfully applied to investigate changes in microbial communities in DWDS (Deines et al., 2010), to detect pathogens such as *Legionella pneumophila* (Aurell et al., 2004) and faecal indicators (Baudart and Lebaron, 2010). In general, FISH is not used as a standalone technique and is mostly used in combination with other methods to characterise microbial communities. An example of these combined techniques is **high–affinity peptide nucleic acid (PNA)-FISH**, useful to study pathogens in biofilms due to the enhanced capability of the probe to penetrate through the Extracellular Polymeric Substance (EPS) matrix (Lehtola et al., 2007). Another example is **LIVE/DEAD–FISH** which combines the cell viability kit with FISH (Savichtcheva et al., 2005) and has been used to assess the efficiency of disinfection in DWDS (Hoefel et al., 2003). Despite its numerous advantages when compared with culture-dependent techniques, FISH also has several limitations. First of all, knowledge of the nucleotide sequence of the target organisms is needed and the design of new probes and the optimization of the hybridization conditions can be time consuming and complex (Sanz and Köchling, 2007). The efficiency of the hybridization might be influenced by the physiological state of the cells and, to conclude the signal emitted by auto-fluorescence cells can interfere with the signal emitted by the target microorganisms (Dorigo et al., 2005).

An alternative fast and reliable method to monitor bacterial abundance and viability of planktonic cells or cells in suspensions is **flow cytometry (FC)**. This technique uses fluorescent dyes to stain the water samples before analysing them with a flow cytometer. The cells in solution pass through a capillary that is intersected by a laser beam, when the laser

Table 1 – Current molecular techniques to study microbial consortia and communities of drinking water distribution systems (advantages and disadvantages).

Method	Description	Application	Advantages	Disadvantages
Fingerprinting techniques DGGE/TGGE SSCP,T-RFLPs Ribosomal Intergenic Space Analysis (RISA/ARISA) Length Heterogeneity PCR (LH-PCR)	PCR-based fingerprinting techniques provide community structure based on DNA sequence variation (length and nucleotide sequence)	• Monitoring of microbial communities over time and/or in response to changes in environmental conditions • Characterization of planktonic and biofilm communities in distribution pipes and corrosion scales in cast iron pipes	• Quick profiling of spatial-temporal variability • Simultaneous analysis of large number of samples • Bands on DGGE/TGGE and SSCP gels can be excised, amplified and sequenced	• Bias associated with PCR • Only predominant species are detected • No direct taxonomic identification • Time consuming, requires post-PCR analysis of samples • Analysis of short sequences(<500 bp) • DGGE, difficult comparison between gels • T-RFLPs and ARDRA; difficult resolution of microbial profiles
Fluorescent in situ hybridization (FISH) and catalyzed reporter deposition FISH (CARD–FISH)	Fluorescent rRNA oligonucleotide probes are used for *in situ* detection and enumeration of microorganisms	• Specific detection and abundance of microorganisms in drinking water and biofilms	• Phylogenetic identification • Visualization of non- cultivable microorganisms • Highly sensitive and quantitative • Detection of different microorganisms simultaneously by using multiple fluorescent dyes	• Sequence information is required for probe design and specific detection • Difficult to differentiate between live and dead cells • Difficult accessibility to target gene
Cloning and Sequencing	Extraction of nucleic acids, amplification and cloning the gene of interest in a vector, followed by sequencing and taxonomic assignments using bioinformatics	• Microbial community analysis of drinking water and biofilms	• Taxonomic and phylogenetic analysis	• Time consuming and laborious • Semi-quantitative • Sequencing of a limited number of clones describe only the dominant members of the microbial communities
High–throughput sequencing techniques (Roche 454 FLX, Illumina/Solexa Genome Analyzer, etc.)	DNA fragment libraries are amplified and sequenced using massively parallel platforms	• Microbial diversity and structure analysis in water, biofilms and water meters	• Faster and less expensive than traditional Sanger sequencing • Multiple samples can be combined in a run	• High cost and time-consuming data analysis
Quantitative PCR (Q–PCR) or Real Time (RT–PCR)	Uses intercalating fluorescent probes (TaqMan) or dyes (SYBR Green) to measure the accumulation of amplicons in real time during each cycle of the PCR	• Detection of pathogens and faecal indicators • Abundance and expression of taxonomic and functional genes (e.g. denitrifiers and sulphate reducers)	• Highly sensitive and quantitative • Fast and accurate gene quantification	• RT-PCR; difficult to obtain enough and good quality RNA
DNA–chip array/microarrays DNA/RNA	Fluorescent PCR amplicons are hybridized to known molecular probes attached on the microarrays	• Community functional analysis • Detection of pathogens and faecal indicators	• No bias associated with PCR • Rapid evaluation with replication • The intensity of the hybridization signal is proportional to the abundance of the target organisms	• Very costly and highly trained personal is needed for data analysis
Biosensors	Direct detection of microorganisms using immunoassays techniques, integrated optics and surface chemistry	• Detection of faecal indicators	• Fast detection	• Depends on cultivation of the microorganisms • No discrimination between live and dead microorganisms

interacts with the cells this causes the light to scatter and also excite the dye, the fluorescence intensity and the scattering generated can be quantified using different detectors (Hammes et al., 2008). Different fluorescent dyes can be used to estimate total bacterial counts (Hammes et al., 2008), virus-like particles (Rinta-Kanto et al., 2004) and *Cryptosporidium* and *Giardia* (Vesey et al., 1993, 1994; Ferrari et al., 2000) in water samples. FC provides much more realistic quantification of the total number of cells in water samples than traditional plate counts and recently has been established as a reference method in Switzerland by the Swiss Federal Institute of Aquatic Science and Technology (Eawag). However, when epifluorescence microscopy and flow cytometry are used to measure cell volume and/or estimate the viability or total cell counts of biofilms and sediments, both methods are susceptible to errors due to the formation of cell clusters and the attachment of cells to inorganic compounds (Van der Kooij et al., 2014).

3.1.2.2. *PCR based methods*. The **polymerase chain reaction (PCR)** is a method used to amplify (i.e. obtain multiple copies) fragments of DNA. **PCR** based methods require the extraction of nucleic acids (DNA/RNA), followed by the amplification of a target gene or genes via PCR and post-PCR analysis. It is important to notice that the amplicons obtained from PCR form the basis for all the community fingerprinting techniques and next generation sequencing methods explained in the following sections of this review. The most useful PCR-based techniques to detect microorganisms in drinking water are **multiplex–PCR** and quantitative **real time (q–PCR)**. Multiplex-PCR uses several oligonucleotide probes to simultaneously detect different microorganisms and has been used in drinking water-related research to detect faecal indicators and/or pathogens (Bej et al., 1991). q-PCR is a sensitive tool to detect and quantify microorganisms in environmental samples based on quantifying the number of target gene copies present in a sample. This technique can monitor the amount of PCR product obtained during the exponential phase of the PCR reaction by quantifying a fluorescent reporter. The amount of detected reporter is then correlated with the initial amount of target template allowing the quantification of the target organism (Kubista et al., 2006). Despite the general limitations of the PCR-based techniques discussed in detail in Table 1, several studies have shown the applicability of these methods to detect viral indicators of human faecal contamination (Albinana-Gimenez et al., 2009), pathogenic bacteria such as *Helicobacter pylori* (McDaniels et al., 2005; Sen et al., 2007), *Mycobacterium avium and Legionella* sp. (Dusserre et al., 2008) and to quantify *Giardia* and *Cryptosporidium* (Guy et al., 2003).

3.2. *Microbial community composition*

The techniques discussed in this section are useful to obtain information about the microbial members of drinking water-related samples. This information is essential in order to detect pathogens, microorganisms associated with corrosion or water discolouration, to monitor biofilm formation on pipes, to assess the influence of abiotic factors on microbial communities and to compare diversity between different samples.

3.2.1. *Phospholipid fatty acids*

Phospholipid fatty acids (PLFAs) are useful to overcome the limitations of culturing techniques when assessing the microbial community composition of environmental samples. The membranes of microorganisms have phospholipids which contain fatty acids (Zelles, 1999) and these can be used to obtain microbial communities fingerprints (Vestle and White, 1989). This technique has been applied in drinking water research, to study biofilms (Keinanen et al., 2004; Lehtola et al., 2004) and to detect pathogens (White et al., 2003). It should be noted that such techniques provide a fingerprint which describes a microbial community, and hence measures and compares overall diversity but does not provide identification of specific species actually present in the samples.

3.2.2. *Molecular techniques*

The advent of molecular techniques has enabled the characterisation of natural microbial communities without the need of culturing microorganisms and has introduced new insights into the microbial ecology of different ecosystems. Molecular analysis of samples includes the extraction and purification of DNA and/or RNA. DNA provides information of the total microbial community of the samples while RNA-based analysis represents only the active part (Kahlisch et al., 2012). The nucleic acid extraction is followed by PCR amplification of "marker genes" to obtain taxonomic information. The most commonly used marker gene in microbiological research is the ribosomal RNA (rRNA) gene, 16S rRNA for prokaryotes and 18S rRNA for eukaryotes. The rRNA gene has different regions, some are highly conserved across all phylogenetic domains (i.e. bacteria, eukarya and archaea), other regions are variable between related species (Woese, 1987) and this variability allows for inferring phylogenetic information from microorganisms inhabiting different ecosystems (Prosser, 2002).

During recent years, to aid identification of sequences recovered from environmental samples, databases of small (16S/18S) and large subunits (23S/28S) rRNA sequences for bacteria, archaea and eukarya have been developed and are constantly expanding. SILVA rRNA database project provides good quality, aligned ribosomal RNA sequence data which is regularly updated (www.arb-silva.de). Other good databases are accessible through the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) and the Greengenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

An overview of the choice of primers pairs available for bacteria and archaea can be found in Klindworth et al. (2012). The authors discuss the best available primers pairs for different amplicon sizes with respect to the SILVA 16S/18S rDNA non-redundant reference dataset (SSURef 108 NR). Once the adequate primers have been selected, the resulting PCR products (i.e. amplicons) can be separated and analysed using different techniques as will be discussed in the following sections.

3.2.2.1. *Fingerprinting techniques*. Among the different molecular tools available to assess the microbial community

Table 2 – Methods used to extract and analyse different components of biofilms and the EPS (extracellular polymeric substance) matrix.

Aim/process	Method	Advantages/Disadvantages	References
Extraction of EPS	Cation Exchange Resin (CER)	Used in drinking water samples; reported to increase extraction yield and quality from biofilms in different environments, although limited comparison with other methods	Jahn and Nielsen, 1995; Frolund et al., 1996; McSwain et al., 2005; Denkhaus et al., 2007; Michalowski et al., 2009
	Freeze-drying (ethanol precipitation)	Used to assess carbohydrates in estuarine sediments but has not been applied in a drinking water context	Hanlon et al., 2006; Haynes et al., 2007; Hofmann et al., 2009
	Ethylenediaminetetraacetic acid (EDTA)	Commonly used method but inhibits protein analysis; found to release nucleic acids in a study of *Rhodopseudomonas acidophila*	Zhang et al., 1999; Sheng et al., 2005; Eboigbodin and Biggs, 2008
	Formaldehyde	Stated as best method for subsequent carbohydrate analysis	Zhang et al., 1999
Cell Lysis	Nucleic Acid	Does not distinguish between free DNA already in EPS from intracellular DNA due to cell lysis	Wingender et al., 1999; Michalowski et al., 2009
	G6PDH Enzyme Assay[b]	G6PDH is an accurate indicator of cell lysis as it is not found naturally outside of cells	Lessie and van der Wijck, 1972; Frolund et al., 1995; McSwain et al., 2005
	DAPI[c]	Cannot differentiate between DNA present in cells or EPS	Jahn and Neilsen, 1995; Frolund et al., 1995;
Quantification	TOC[d]	Commonly used to assess biomass and EPS amount, relatively quick and reliable	Jahn and Neilsen, 1995; McSwain et al., 2005
	TS or TSS or VSS[e]	Used to indicate biofilm or cell mass	Zhang et al., 1999; Sheng et al., 2005
	Dry Weight (via Freeze-driying)	Samples are freeze-dried and weighed before being resuspsended in sterile water; provides a dry weight for quantification	Hofmann et al., 2009
Protein Assay	Bradford Assay	Recommended due to: speed, simplicity and insensitivity to other compounds (compared to Lowry).Variable sensitivity to different proteins	Bradford et al., 1976; Raunkjaer et al., 1994; Frolund et al., 1995
	Lowry	Subject to interference; laborious; slight variability in sensitivity to different proteins but distinguishes between molecules as small as dipeptides	Lowry, 1951; Raunkjaer et al., 1994; Jahn and Neilsen, 1995; Sheng et al., 2005;
		Modified Lowry has been used with drinking water, removes humic acids but is more complex and time consuming. The RC DC[a] assay is based on the Lowry method and is available as a kit.	Bradford et al., 1976; Frolund et al., 1995; Michalowski et al., 2009
Carbohydrate Assay	Phenol-Sulfuric Acid	Used with drinking water and commonly used in other biofilm studies. It is more comprehensive than the anthrone method, has high specificity for all carbohydrates, which undergo the colour change with the same intensity	Dubois et al., 1956; Raunkjaer et al., 1994; Hanlon et al., 2006; Haynes et al., 2007; Michalowski et al., 2009; Hofmann et al., 2009
	Anthrone	Commonly used; more complex than phenol-sulfuric method, not all carbohydrates produce colour of the same intensity – problem if protein composition is unknown	Raunkjaer et al., 1994; Jahn and Neilsen, 1995; Frolund et al., 1995

[a] Reducing Agent Compatible, Detergent Agent Compatible.
[b] Glucose-6-phosphate Dehydrogenase.
[c] 4′,6-diamidino-2-phenylindole.
[d] Total organic carbon.
[e] TS – total solids; TSS – total suspended solids; VSS – volatile suspended solid.

composition of drinking water ecosystems, fingerprinting techniques are the most commonly employed. Fingerprinting techniques are particularly useful to simultaneously analyse multiple samples and to compare different microbial community structures. **Denaturing gradient gel electrophoresis (DGGE)** (Muyzer et al., 1993) and **temperature gradient gel electrophoresis (TGGE)** (Po et al., 1987) are fingerprinting techniques where specific fragments of the rRNA gene are amplified and then separated based upon their sequence composition in a denaturing polyacrylamide gel (DGGE) or using a temperature gradient (TGGE). The final result is a gel with a pattern of bands which is a visual profile of the most abundant species in the studied microbial community. This approach allows for monitoring changes in microbial communities and it can be used, similarly to other fingerprinting techniques, as a semi-quantitative method to estimate species abundance and richness (Muyzer, 1999). In addition, specific bands on the gel can be excised and sequenced for subsequent taxonomic identification. DGGE is the most cited fingerprinting method used to characterise microbial communities in drinking water. DGGE has been used to assess opportunistic pathogens in urban drinking water biofilms (Pryor et al., 2004) to monitor biofilm formation and activity in distributions systems (Boe-Hansen et al., 2003), to study the effect of stagnation in taps (Lautenschlager et al., 2010), corrosion on cast iron pipes (Teng et al., 2008), nitrification in drinking water networks (Yapsakli et al., 2010), occurrence of fungi in biofilms (Pereira et al., 2010) and to assess bacterial water quality in real distribution systems (Sekar et al., 2012). Despite its broad application, this technique has several disadvantages; first of all handling polyacrylamide gels and obtaining the optimal denaturing conditions is highly laborious. In terms of the analysis of the gels, associating a single band with a particular species is complicated and cloning and sequencing of particular bands is ultimately needed for confirmation of results (Muyzer, 1999). Despite the use of markers on the gels, comparison of patterns across gels and the detection of rare members of the microbial community are challenging.

Although used to a much lesser extent than DGGE, there are other fingerprinting techniques useful to characterise microbial communities in DWDS. **Terminal restriction fragment length polymorphism (T–RFLP)** is a technique based on the amplification of short fragments of a marker gene using end-labelled primers (Liu et al., 1997). The amplicons are then digested with restriction enzymes (e.g. *Alu I, Cfo I, Hae III*) and the digested fragments are normally separated by capillary electrophoresis. Despite being less technically laborious than techniques such as DGGE, the application of T-RFLPs in drinking water is limited and has been used in only a few studies, for example to identify protozoa in unchlorinated drinking water (Valster et al., 2009) or to study changes in biofilm microbial communities over time in distribution systems (Douterelo et al., 2014).

Amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte et al., 1992) is another fingerprinting tool in which amplicons of rRNA genes are digested with a set of restriction enzymes, producing a pattern of fragments representative of a given microbial community (Heyndrickx et al., 1996). ARDRA has been used to characterise biofilms (Ludmany et al., 2006) and to identify non-tuberculous *Mycobacterium* (Tsitko et al., 2006). **Automated ribosomal intergenic spacer analysis (ARISA)** (Fisher and Triplett, 1999) is normally used to characterise fungal communities. In ARISA, the ITS regions of nuclear DNA located between the 18S (SSU) and 28S (LSU) genes are amplified using fluorescent labelled primers, then the amplicons are analysed in a sequencer to determine their size and to ultimately obtain a fingerprint of the studied microbial community. The method known as **single strand conformational polymorphism (SSCP)** (Orita et al., 1989) also separates amplicons as a result of variation in their sequence (Widjojoatmodjo et al., 1995). The amplicons are treated to obtain single DNA strands, which are separated via gel electrophoresis. SSCP use in drinking water is also limited but has been used for *in situ* genotyping of *L. pneumophila* (Kahlisch et al., 2010).

In general, fingerprinting techniques are frequently used in combination with cloning and sequencing, in order to obtain specific phylogenetic information from selected samples. Despite providing interesting results, the disadvantages of these techniques are discussed in Table 1 and certainly the main drawbacks are that they require specialist equipment and can be very labour intensive.

3.2.2.2. Sequencing-based approaches. **Cloning and sequencing** is the conventional and more widespread genomic approach used when detailed and accurate phylogenetic information from environmental samples is required. The method involves the extraction of nucleic acids, amplification of the rRNA gene with suitable primers and the construction of clone libraries using sequencing vectors (Rondon et al., 2000). Selected clones are then sequenced (Sanger-based) (Sanger et al., 1977) and the nucleotide sequence of the rRNA gene retrieved, allowing estimates of the microbial diversity in the samples by comparison with sequences available in databases (e.g. GenBank, EMBL and Silva). The generation of DNA clone libraries followed by sequencing has being extensively applied in drinking water microbiology, a selection of these applications are discussed in brief. This method has been used to study long term succession in biofilms (Martiny et al., 2003), disinfection efficiency (Hoefel et al., 2005), nitrifier and ammonia-oxidizing bacteria in biofilms (Lipponen et al., 2004), to characterise the microorganisms present in red water events (Wullings and van der Kooij, 2006) and to detect *Bacteroidetes* in unchlorinated water (Saunders et al., 2009).

The approach known as **metagenomics**, involves sampling the entire genome of an environmental sample in order to obtain sequence information from the microorganisms contained in it and to ultimately make taxonomic assignments to characterise them. A sequencing-based approach useful to sequence the entire genome and characterise microbial communities in environmental samples is known as **shot gun sequencing**. Genomic DNA is cut into smaller fragments; these fragments can be sequenced individually and then reassembled into their original order in the genome, based on sequence overlaps, to obtain the complete genome sequence. Environmental genome shotgun sequencing has been used in ocean water to assess the diversity and relative abundance of organisms (Venter

et al., 2004). To our knowledge this molecular approach has not been used to explore drinking water ecosystems but we consider that its application might bring new insights into the microbial ecology of DWDS.

Independently of the sequencing approach employed, taxonomic assignments of the sequences are typically identified using search algorithms such as the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), sequences are also aligned, clustered and phylogenetic trees are constructed using software such as MEGA (Tamura et al., 2011), PHYLIP (Felsenstein, 1989) and ARB (Ludwig et al., 2004).

Despite being enormously successful, cloning and sequencing approaches are very expensive and time consuming and since the introduction of **next generation sequencing (NGS)** techniques their use has declined substantially. In the last decade, the use of NGS has incredibly enhanced the understanding of the microbial ecology of different ecosystems. The NGS platforms have improved the depth of sequencing since they can produce thousands of short reads in a single run allowing for the detection of less abundant members of microbial communities (Metzker, 2010). In addition, the use of high-throughput sequencing techniques avoids the need of the laborious and time consuming steps in conventional cloning and sequencing. However, NGS techniques provide sequence information with a limited base pair length (max ~600 bp) and, despite increases in read lengths as these technologies advance, phylogenetic comparisons are based on shorter sequences when compared with conventional Sanger sequencing (max ~1500 bp). This restriction might result in less accurate gene annotation and overestimation of microbial richness in samples. In addition, it should be noticed that the resolution of NGS methods is currently too low to identify microorganisms to the species level. The most frequently used NGS platforms are Roche 454 and Illumina/Solexa. Nowadays, Illumina is replacing Roche 454 as the sequencing method of choice for most of microbial-related studies. While Illumina yields shorter reads than Roche 454, the sequencing error of both platforms is comparable and Illumina is much cheaper than 454 (Luo et al., 2012).

Several bioinformatics software and analysis tools are available to analyse the numerous sequences reads obtained from NGS runs, the most useful ones are MOTHUR (Schloss et al., 2009), QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010) and the pyrosequencing pipeline in the Ribosomal Database Project (RDP) (Cole et al., 2009). The use of these high throughput sequencing techniques in drinking water is constantly increasing, recent studies have used pyrosequencing to characterise bacterial communities from impellers retrieved from customer water meters (Hong et al., 2010), in membrane filtration systems from a drinking water treatment plant (Kwon et al., 2011), to assess the influence of hydraulic regimes on bacterial community composition in an experimental distribution system (Douterelo et al., 2013) and to assess the influence of different disinfectant regimes on microbial community dynamics (Gomez-Alvarez et al., 2012; Hwang et al., 2012). With sequencing costs decreasing, NGS is enabling an increasing number of laboratories to taxonomically (and functionally) classify a wide range of the organisms that are present in drinking water.

3.3. *Microbial activity and analysis of functional genes*

Studying the structure and composition of microorganisms in environmental samples is important; however, the understanding of their activity and function is vital to get a complete picture of the microbial ecosystems. Linking the presence of microorganisms to specific biochemical or physical processes is an ultimate goal in any environmental microbial research. The methods described in this section can help to assess the viability and stability of microbial communities in response to specific treatments or conditions or to study specific processes such as corrosion, discolouration or denitrification in distribution systems.

3.3.1. *Estimation of biomass*

Two methods widely used in drinking water research to estimate biomass and bacterial growth are the quantification of **adenosine triphosphate (ATP)** and of **assimilable organic carbon (AOC)** respectively. ATP quantification enables active microbial biomass to be measured (van der Wielen and van der Kooij, 2010). Briefly, cellular ATP reacts with a luciferin-luciferase complex, the luminescence produced in this reaction is proportional to the concentration of ATP, which is then correlated to the quantity of biomass in the sample (Hammes et al., 2010). Nowadays, ATP can be easily assessed using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega, UK), which allows quantification of several samples simultaneously using a microplate reader. This method is fast, low-cost and easy to perform, thus is an ideal tool for monitoring purposes. The use of ATP is well-established in drinking water-related research and is used as a relievable method to estimate microbial activity (Hammes et al., 2010). Numerous studies have successfully used this technique to assess microbial viability and the biological stability of water in DWDS (Lehtola et al., 2002; Berney et al., 2008; Hammes et al., 2008; Lautenschlager et al., 2013). **AOC** is widely used in drinking water research to assess growth of heterotrophic bacteria in water (van der Kooij, 1992). Hammes and Egli (2005) developed a new and faster AOC method using natural microbial communities as inoculum and flow cytometer to estimate cell counts in water samples. The method has been used to test the influence of disinfectant on microbial growth in distribution systems (Choi and Choi, 2010; Ohkouchi et al., 2013) and for assessing the potential growth of biofilms on different pipe material (Zacheus et al., 2000; Liu et al., 2002).

ATP and AOC methods have been tested in order to assess the biological stability of drinking water, which is defined as the inability of water or a material in contact with water to support microbial growth (van Delft, 2010; Liu et al., 2013) and implies that the concentration of cells and the microbial community composition should not change during water distribution (Lautenschlager et al., 2014). Most of the research related to biological stability is focused on estimating the potential microbial activity in water and/or in particle-associated bacteria (i.e. associated to suspended solids and loose deposits) (Liu et al., 2014). In general, the AOC method has several limitations; i) assumes that bacterial growth is limited by organic carbon, ii) quantifies available nutrients instead of bacteria and iii) depends on the type of bacteria used (Liu et al., 2013). Due to these limitations, it has been

considered that the potential contribution of nutrients and biomass contained by loose deposits is overlooked when using this method (Liu et al., 2014). ATP has been used to quantify the bacteria from different phases of an unchlorinated DWDS, including bulk water, pipe wall biofilm, suspended solids, and loose deposits (Liu et al., 2014). The study concludes that bacteria associated with loose deposits and pipe wall biofilm accumulated in the DWDS accounted for over 98% of the total bacteria. However, when using ATP to study particle–associated microorganisms it is necessary to perform some pre-treatment to the samples to detach the microorganisms into suspension for further analysis (Liu et al., 2013).

In general, ATP and AOC quantification methods can be used in combination with other techniques, such as flow cytometry, to enable better insights into the response of microbial communities to specific treatments or conditions (Vital et al., 2012) or to predict changes in their stability in response to different factors (Lautenschlager et al., 2013).

3.3.2. *Functional genes*

The study of functional genes involved in metabolic and catabolic pathways is essential when attempting to link microbial diversity with specific ecological functions. In drinking water research, better knowledge of the role of microorganisms in processes such as biofilm formation, disinfection efficiency, water discolouration and corrosion is without doubt required.

The molecular approach known as **metatranscriptomics** is based on the study of actively transcribed ribosomal and messenger RNA (rRNA and mRNA) and facilitates linking specific functions to certain members of a microbial community. Routinely, the first step is the extraction of RNA from a sample. This maybe a challenging process since RNA degrades easily outside the cells due to its short-half life and to the presence of RNAases. For an accurate estimation of gene expression, it is also important that the extracted RNA is free of contaminating DNA and inhibitors (Bustin et al., 2009). After RNA extraction, complementary DNA (cDNA) is synthesized from RNA by reverse transcription (RT) using random or specific primers (Sharkey et al., 2004) and the resulting cDNA can then be used to measure the expression of functional genes by for example **real time–PCR (RT–PCR)** or functional microarrays.

RT–PCR can be applied to study changes in expression of particular genes in response to different treatments (e.g. disinfection strategies) and/or changes in environmental conditions (e.g. pH, temperature and hydraulic regimes). RT-PCR is highly sensitive, accurate and allows the analysis of several samples and the use of different functional genes simultaneously on the same experiment. In drinking water research, RT-PCR has been mainly applied to quantify and to monitor the expression of genes involved in particular metabolic or catabolic pathways such as *amoA* genes to study ammonia oxidising bacteria and archaea in distribution systems (Hoefel et al., 2005; van der Wielen et al., 2009), *dsrB* genes to study sulphate reducing bacteria (Li et al., 2010) and the *nirS* gene to assess the distribution of denitrifiers in a water well field (Medihala et al., 2012).

The application of **functional microarrays** enables assessment of the overall gene expression of a microbial community. In microarrays, oligonucleotides probes targeting functional genes are immobilized on solid supports (chips) and arranged spatially in a known pattern, the subsequent hybridization between the target cDNA, labelled with a fluorescent dye, and the oligonucleotides on the chip indicates that the gene has been transcribed (Sharkey et al., 2004). With this technique patterns of hybridization are obtained and the intensity of the fluorescence is proportional to the gene expression (Gilbride et al., 2006). GeoChip is an example of a functional gene array (He et al., 2007), the last developed array of this type GeoChip4.0 contains 120,054 distinct probes, covering 200,393 coding sequences (CDS) for genes involved in different processes (e.g. biogeochemical cycles of carbon, nitrogen and phosphorus). The main advantage of using microarrays is that the expression of thousands of mRNAs can be assessed simultaneously. However, most of the arrays are normally developed using genes and metabolic pathways obtained from laboratory isolates and when microarrays are applied to environmental samples sequence divergence can affect hybridization leading to erroneous interpretations (Wilmes and Bond, 2006). Other limitations of microarrays to be aware of are low specificity in some cases and that mRNA expression and protein expression are not always directly correlated (Pradet-Balade et al., 2001). Despite the high potential of this technique for assessing functionality of microbial communities, the use of microarrays has not yet been explored for this purpose in drinking water research.

3.3.3. *Proteomics*

Proteomics is a discipline focused on the identification of proteins and **metaproteomics** can be defined as the characterisation of the entire protein complement of a microbial community (Wilmes and Bond, 2006). Protein expression can be directly associated with specific microbial activities. The fundamental steps in proteomics investigations are protein extraction, separation and/or fractioning, identification and quantification (Siggins et al., 2012). Traditionally, proteins are visualised and separated in a two dimensional polyacrylamide gel electrophoresis (2D-PAGE) then digested with enzymes and identified by mass spectrometric (MS) analysis (Schneider and Riedel, 2010). However, 2D-PAGE gels have several limitations, to name a few; they are highly laborious, proteins can co-migrate in the gel and some proteins such as membrane proteins, proteins with extreme molecular weights or isoelectric points are difficult to separate (Schneider and Riedel, 2010). Alternatively, proteins can be separated by **liquid chromatography** (LC). The combined approach using LC-MS has become widely used in environmental proteomics and the use of 2D-PAGE gels has currently decreased. Furthermore, the use of gel-free protein fractions has been recommended when possible since they provide higher levels of protein identification when compared with gel-based methods (Siggins et al., 2012). Ultimately, the mass spectrophotometer generates a peptide sequence or a peptide mass fingerprint (PMF) which can be compared with available databases. If sequencing data is not available, proteins can be identified from their corresponding *de novo* peptide sequences by means of a protein BLAST (BLASTp) (Wilmes and Bond, 2006; Pandhal et al., 2008).

Recently, protein identification has been facilitated by the development of NGS and new metagenomic sequences databases. Additionally, quantitative proteomics and the MS-based quantification method can be used to quantify microbial activities across different environmental or operating conditions. This approach is based on the use of stable isotopes as mass-tags to label proteins, the tags can then be identified and quantified by the MS (Bantscheff et al., 2007). Although *de novo* peptide sequences can be used for protein identification, the main limitation of metaproteomics is that it relies on genomic or metagenomics sequence data, which is used to identify proteins. As a consequence, it cannot be used as a 'stand-alone' method. To the best of our knowledge, metaproteomics has not yet been applied in microbial research in DWDS. However, it has been successfully used to investigate microbial community functions in other aquatic ecosystems such as marine environments (Morris et al., 2010), freshwater ecosystems (Lauro et al., 2011) and biofilms from an acid mine drainage (Denef et al., 2009, 2010; Mueller et al., 2010) which shows the potential for functional analysis using metaproteomics in DWDS in the future.

3.3.4. *Metabolomics*

Metabolomics studies the metabolome which includes cell metabolites that are produced or consumed as a result of biological activity (Beale et al., 2013). Within metabolomics, metabolic footprinting focuses on the analysis of extracellular metabolites which can provide information on functional genomics and on cell to cell communication mechanisms (Mapelli et al., 2008). This methodology can be used to monitor the presence and/or microbial mediated processes in DWDS since it allows associating specific metabolite profiles with different microorganisms (Beale et al., 2010). Profiles of intracellular and extracellular metabolites associated with microbial activity can be obtained using techniques such as **gas chromatography–mass spectrometry (GC–MS)**. GC–MS approaches have been used to study microbial influenced corrosion. Beale et al. (2012), used GC–MS to obtain specific metabolic markers in order to discriminate between water samples and to identify those exposed to bacteria involved in pipe corrosion. In another study, Beale et al. (2010) applied a metabolomics approach to also study pipe corrosion and were able to observe using 3D fluorescence spectroscopy the 'protein-like' fluorophore associated with presence of bacteria in water collected from corroded pipes and cross reference this with derivatised fatty acid metabolites using GC–MS analyses of the same water. Using samples from flushing a water main Beale et al. (2012), demonstrated the effectiveness of metabolomics to study biofilms in DWDS using also GC–MS, the chemometric analysis of the chromatograms in combination with mass spectrometer data allowed differentiating between biofilms from different pipe materials and planktonic bacteria. The same author, Beale et al. (2013) has also showed that a metabolomics approach can be used to rapidly (less than 24 h) detect and quantify viable and non-viable *Cryptosporidium* oocysts in water samples. In this research, the authors used a chemometric approach to analyse information obtained from chromatographic and mass spectral data to identify and quantify excreted metabolites from *Cryptosporidium* oocysts and found that a number of key metabolite features including aromatic and non-aromatic amino acids, carbohydrates, fatty acids and alcohol type compounds were able to explain the difference between the viable and non-viable oocysts in water samples.

3.3.5. *Other functional techniques and combined approaches*

Other molecular techniques which can be useful to investigate functionality in microbial ecosystems are environmental **shot gun sequencing, stable isotope probing (SIP)** and **RNA–FISH.** As explained in detail in a previous section, random environmental **shot gun sequencing** randomly samples sequencing data from fragmented DNA/RNA from an environmental sample (Eisen, 2007), allowing determination of the metabolic capability of a microbial community (Allen and Banfield, 2005). **SIP**, enables determination of the microbial diversity associated with specific metabolic pathways (Radajewski et al., 2000) and has been generally applied to study microorganisms involved in the utilization of carbon and nitrogen compounds. The substrate of interest is labelled with stable isotopes (^{13}C or ^{15}N) and added to the sample, only microorganisms able to metabolise the substrate will incorporate it into their cells. Subsequently, ^{13}C-DNA and ^{15}N-DNA can be isolated by density gradient centrifugation and used for metagenomic analysis. Manefield et al. (2002), suggest that RNA-based SIP could be a more responsive biomarker for use in SIP studies when compared to DNA, since RNA itself is a reflection of cellular activity (independent of replication) and because synthesis rates are higher for RNA than for DNA. To our knowledge SIP has not been applied to DWDS research however it has been used to assess hydrocarbons and oil contamination in aquifers (Busch-Harris et al., 2008; Winderl et al., 2010).

Methods such as **Bromodeoxyuridine (BrdU) incorporation, microautoradiography–FISH, Raman–FISH** and **isotope array** provide further insights into the metabolic activities of the microorganisms. **BrdU incorporation** is a non-radioactive approach that provides information of active and DNA synthesizing cells, when used in combination with FISH gives the identity and activity of targeted cells (Pernthaler et al., 2002b). The combination of BrdU magnetic bead immunocapturing and DGGE analysis have facilitated the exploration of the phylogenetic affiliations of DNA-synthesising and active bacteria (Hamasaki et al., 2007), such methods could be used to study the viability of bacteria in drinking water during treatment processes.

Microautoradiography (MAR) is a radioactive approach used in combination with FISH **(MAR–FISH)** to reveal the physiological properties of microorganisms with single-cell resolution (Wagner et al., 2006). MAR-FISH provides information on total cells, probe targeted cells and the percentage of cells that incorporate a given radiolabelled substance (Ouverney and Fuhrman, 1999). This method has been widely applied to study the structure and function of microbes in freshwater and marine ecosystems including biofilms (Lee et al., 1999; Nielsen et al., 2003; Kindaichi et al., 2004a). The method provides a picture of the *in situ* function of targeted microorganisms and is an effective approach to study the *in vivo* physiology of microorganisms in biofilms (Ginige et al., 2004). Lee et al. (1999) developed a microscopic method in combination with FISH and MAR for simultaneous

determination of identities, activities and substrate uptake by specific bacterial cells in complex microbial assemblages. Kindaichi et al. (2004a,b) used this approach to study the ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in biofilms. Such a study is relevant for drinking water as chlorination of water has been found to promote the growth of nitrifying bacteria (Eichler et al., 2006). Nielsen et al. (2003) developed a new technique for quantification of cell-specific substrate uptake in combination with MAR-FISH known as QMAR (quantitative MAR).

Most of the techniques explained above have not been widely used in drinking water microbiology and the lack of knowledge regarding the function of certain microorganisms in drinking water ecosystems could be addressed with these. Therefore, it is likely that in the near future the techniques discussed here will be explored and optimised to study in more detail the role of certain microorganisms in DWDS.

3.4. *Biofilms and species interaction*

Most of the microorganisms inhabiting DWDS are attached to the pipe surface forming biofilms (Flemming et al., 2002). The resistance of biofilms to disinfection and the difficulty of controlling their growth in distribution networks are common themes for research groups and water companies around the world. There are numerous studies regarding biofilm response to different chemicals and disinfectant strategies, most of them using bench top reactors and single species biofilms (Gagnon et al., 2005; Murphy et al., 2008; Simoes et al., 2010b). However, a better understanding of the physico-chemical structure of biofilms, and of the mechanisms regulating its formation such as adhesion and coaggregation, in DWDS is needed and fundamental to improve and/or develop control and management strategies.

3.4.1. *Cell adhesion*

The process of biofilm formation is initiated by attachment of planktonic cells to the pipe surfaces (Simoes et al., 2007). Bacterial adhesion is affected by environmental conditions in the network (e.g. hydraulic forces, disinfectant regime, pipe material) and by intrinsic characteristics of the cells such as hydrophobicity, surface charge, production of polysaccharides and cell motility (Li and Logan, 2004; Simoes et al., 2010b). Different parameters can be used to estimate the **potential of cell adhesion**, for example measurements of cell hydrophobicity, electrostatic potential and the thermodynamic potential of the cell (Van Loosdrecht et al., 1987; Simoes et al., 2007). Adhesion to surfaces is often mediated by hydrophobic interactions; often measured by **angle contact measurements** (Cerca et al., 2005). The method was initially described by Busscher et al. (1984) and has been used to study adhesion to surfaces mainly by clinically relevant microorganism such as *Pseudomonas aeruginosa* (Pasmore et al., 2002) and *M. avium* (Steed and Falkinham, 2006). This method has been also used to study the potential of bacteria isolated from drinking water to adhere to different materials (Simoes et al., 2007, 2010a). However, it has been suggested that to accurately predict bacterial adhesion the surface charge of the bacterial cell needs also to be incorporated in adhesion models (Van Loosdrecht et al., 1987). Habitually, to assess the cell surface charge the **zeta potential** is determined by measuring the electrical potential of the interface between the aqueous solution and the stationary layer of the fluid attached to the cell (Karunakaran et al., 2011). The zeta potential has been measured to assist in determining the potential for adhesion of drinking water-isolated bacteria to polystyrene (Simoes et al., 2010a). Soni et al. (2008) showed that the zeta potential among selected drinking water bacteria (i.e. *Pseudomonas* spp., *E. coli* and *Salmonella* spp.) varies depending on their physiological state.

Nowadays, spectroscopy techniques such as RAMAN, Fourier-transformed infrared (FTIR), and X-ray photoelectron are preferred options to study the chemical characteristics of bacterial cell surfaces in biofilm-related research (Karunakaran et al., 2011). These techniques are discussed in section 3.4.4 *Biochemical composition and visualization*.

3.4.2. *Coaggregation*

Once the substratum is colonised by microorganisms, cells will grow and produce EPS, microcolonies will develop and coadhesion and coaggregation of different bacterial cells will contribute towards the development of a multi species biofilm (Rickard et al., 2003). In the process known as coaggregation (cell to cell interactions), single species cells and multiple microbial species will interact and become attached to each other (Rickard et al., 2003).

The most commonly used method to study coaggregation in biofilms is the **visual coaggregation assay.** This technique involves mixing, generally in pairs, planktonic batch cultures of specific bacteria and assessing the degree of coaggregation visually in a semi-quantitative way (Cisar et al., 1979). If the mechanism of cell–cell recognition occurs, cells will form coaggregates that will tend to settle out, giving different levels of turbidity in the medium (Simoes et al., 2008). The visual aggregation assays use a subjective scoring criteria based on the method developed by Cisar et al. (1979) to assess the degree of coaggregation between species. Values ranging from 0 (no coaggregation) to 5 (large flocs of coaggregates settle down and leave a clear supernatant) are assigned to the biofilm cultures to subjectively classify the level of coaggregation. However, the subjectivity of this method can make accurate comparisons between studies difficult.

Bacterial coaggregates can also be visualised using **DAPI** (total cell counts) or **LIVE/DEAD** (viability) stains in combination with **fluorescence microscopy (**for details see 3.1.2.1 *Microscopy methods*). Growth rates of different combinations of bacteria isolated from drinking water can be monitored over time using **microtiter plates** with R2A as a medium and inoculating the plates with the cell suspensions to study (Stepanovic et al., 2000). If coaggregation occurs the biofilm will grow changing the initial optical density of the medium which can be detected using spectrophotometry. Biofilms can also be stained with **crystal violet** to quantify changes in biofilm mass during the process of coaggregation (Simoes et al., 2010a).

These methods have been useful to study coaggregation between species isolated from drinking water biofilms (Simoes et al., 2008, 2010a; Giao et al., 2011; Ramalingam et al., 2013). Most of these techniques have been applied to study biofilms under laboratory conditions at a bench top scale and

cannot be applied *in situ* to biofilms attached to drinking water networks. However, compared with molecular-based techniques, coaggregation assays are relatively cheap, easy to perform and results can be obtained in short periods of time.

Molecular-based techniques have also been applied to study coaggregation. Mutants defective in genes associated with cell–cell interactions have been used in biofilm research to discover their function in the process of coaggregation (Davey and O'toole, 2000).

3.4.3. *Quorum sensing*

The biochemical process of cell to cell communication known as **quorum sensing (QS)** plays an important role in initial cell attachment to surfaces and in the control of biofilm growth. QS systems are also involved in polysaccharide synthesis, microbial adherence, cell division and motility (Lazar, 2011). Molecular techniques such as **RT–PCR** have been used to study the expression of genes involved in QS (e.g. the QscR regulon, gene HapR), but mainly applied to pathogenic species such as *Pseudomonas aeruginosa* (Lequette et al., 2006) and *Vibrio cholerae* (Liu et al., 2007). Microscopy techniques such as **confocal laser scanning microscopy (CLSM)** are also useful to monitor morphological changes in biofilms and have been applied to study the formation of biofilms by pathogenic bacteria with mutations in QS genes (Purevdorj et al., 2002; Cole et al., 2004; Huang et al., 2009). QS processes are directly involved in inhibition or promotion of biofilm growth, consequently a better understanding of these mechanisms would contribute to control or prevent the negative consequences of biofilm growth. Due to the complexity of interactions between signalling molecules in multi-species biofilms, most QS research has been based on the study of a limited number of bacterial species, isolated from model drinking water systems and generally developed under laboratory conditions. As a consequence, the main drawback of these coaggregation and cell-to-cell techniques is that they ignore the actual diversity of real biofilms and the ecological complexity of DWDS. Future research should address this complexity using new -omics technologies, for example combining metagenomics and proteomics.

3.4.4. *Biochemical composition and visualization*

The **biochemical composition of biofilms,** particularly the EPS matrix components (i.e. carbohydrates, proteins, lipids and cells) may be quantified and evaluated via chemical assay techniques or microscopy based approaches. In order to apply **chemical assays** it is necessary to first isolate the EPS from the cellular fraction of the biofilm and ensure the isolate is free from intracellular contaminants due to cell lysis; a wide array of extraction and cell lysis detection methods are available, the most common of which are summarised in Table 2. Several authors have compared different extraction techniques (Jahn and Nielsen, 1995; D'Abzac, 2010) but no single method has been found to be consistently the most efficient, generally varying with the sample origin and methodology applied. Michalowski et al. (2009, 2010), evaluated several EPS extraction techniques using biofilms grown in a reactor fed with drinking water and showed that a cation exchange resin (CER) based protocol was the most efficient method.

Extraction processes facilitate the use of biochemical assays to quantify protein concentrations using, for example, the Bradford method (Bradford, 1976) or Lowry et al. (1951) based approaches (see Table 2). Carbohydrates may be quantified using a glucose assay kit (Karunakaran and Biggs, 2011), the phenol-sulfuric method described by DuBois et al. (1956) or the anthrone method proposed by Trevelyan et al. (1952), see Table 2 for more details. Extraction and chemical analysis is most successful when applied to well-developed biofilms with a maximum biomass. This is not an issue when biofilms are grown under idealised laboratory conditions, but when working with drinking water biofilms developed under more realistic conditions, which often have a lower biomass; these extractions methods may be less useful. For instance, Michalowski et al. (2009, 2010) successfully used these techniques with 14 day old drinking water biofilms from a reactor but their application to 28 day old biofilms from a full scale DWDS facility produced unreliable, inconsistent results (Fish, 2013). With extraction based processes more detail chemical-species analysis, via PAGE gels and protein sequencing, can be obtained using the same EPS isolates as used for evaluation of biochemical concentration. However, these techniques also require the sample to be physically disturbed; consequently the localisation of different biochemical components within the biofilm cannot be evaluated.

Non-invasive **microscopy techniques** offer a way to overcome some of the limitations of extraction techniques, providing the possibility of monitoring, quantifying and visualising cells and other biofilm components *in situ*, without perturbing their structure. **Confocal laser scanning microscopy (CLSM)**, in combination with different fluorescent dyes, is a common and useful approach for biofilm-related research. A range of fluorescent dyes can be used to detect and quantity different biofilm components. Some of the most commonly used ones are: DAPI for cells, Syto-60 and Syto-84 for nucleic acids, FITC and Sypro red for proteins, Nile red for lipids, and concanavalin A (ConA) labelled with Alexa fluor 488 for carbohydrates (Johnsen et al., 2000; Yang et al., 2006). The method based on the Green Fluorescent Protein (GFP) is widely used to detect specific bacterial cell types within biofilms (Wouters et al., 2010). To analyse confocal images different software is available such as DAIME (Digital Image Analysis in Microbial Ecology) (Daims et al., 2006), COMSTANT (Heydorn et al., 2000) and IMARIS (Bitplaine, St Paul, MN) which are particularly helpful to analyse 3D images and quantify Z-stacks (Hall-Stoodley et al., 2008).

Fluorescent staining and CLSM have been successfully used to assess the EPS carbohydrates and proteins of flocs (Schmid et al., 2003), granules (McSwain et al., 2005) and single-species cultured biofilms (Chen et al., 2007; Shumi et al., 2009). However, within a drinking water context, the scope of CLSM analysis is generally limited to the study of cells and carbohydrates, or targeting carbohydrates and proteins separately, using different samples (Ivleva et al., 2009). Wagner et al. (2009) analysed biofilms from a wastewater fed reactor using two dual combinations of fluorophores to target carbohydrates/cells of one sample, followed by the proteins/cells of another. Conversely, Fish et al. (2011) demonstrates the application of a triple stain combination, CLSM and digital image analysis to concurrently visualise and quantify the

carbohydrates, proteins and cells, of multi-species biofilms from a full scale DWDS. The use of fluorescent microscopy to study biofilms is restricted by the excitation wavelengths of the lasers available at the given imaging facility and the amalgamations of stains which can be separated using these laser combinations. While this approach does not enable the detailed analysis of chemical-species possible with the extraction processes, fluorescent staining and imaging techniques may be favoured as they enable visual investigations of the 3D arrangement of the biochemical compounds, as well as quantification.

Another useful technique to visualise biofilms is **scanning electron microscopy (SEM)**, this has been used to obtain 3D images of biofilms on surfaces of drinking water networks (Hammes et al., 2011). However, the samples for SEM need to be processed (i.e. fixed, dehydrated and coated with a conductive material) before they can be visualized which can create artefacts or the partial destruction of the biofilm structure (Bergmans et al., 2005). Alternatively, **environmental scanning electron microscopy (ESEM)** may be used, for which samples do not require processing, however, the maximum magnification obtained is less than with conventional SEM (Donald, 2003). It should be noted that both SEM and ESEM provide purely qualitative analysis, unlike CSLM or a technique termed **X–ray photoelectron spectroscopy,** which provides direct chemical analysis of the surfaces of microbial cells (Rouxhet et al., 1994) and can be used to investigate environmental samples under realistic conditions (Bluhm et al., 2006). Techniques, such as **transmission electron microscopy (TEM)** and **scanning transmission X–ray microscopy (STXM)** were successfully used by Lawrence et al. (2003) to map the distribution of lipids, polysaccharides, proteins, and nucleic acids within riverine biofilms. However, no references are available regarding the application of these approaches to drinking water samples. Several studies have also applied **fourier–transform infrared spectroscopy (FTIR spectroscopy)** to obtain *in situ* biochemical and physiological information on biofilms and to monitor and map changes during their development (Quiles et al., 2010; Naz et al., 2013). The main disadvantage of FTIR is that biofilm samples need to be dried before they can be analysed, which can have similar impacts upon sample integrity as the processing required for SEM.

The use of **raman microscopy (RM)** can overcome the problem of post-image processing since hydrated biofilm samples can be used (Karunakaran et al., 2011) and detailed chemical composition data can be obtained (Ivleva et al., 2009; Wagner et al., 2009). RM has been used in conjunction with **CLSM** to investigate the influence of hydrodynamic conditions on the chemical composition of wastewater biofilms (Ivleva et al., 2009; Wagner et al., 2010) and to identify bacterial species (Beier et al., 2010). Wagner et al. (2009) successfully applied the method using wastewater biofilms older than a month and it is unrealistic to expect that similar results can be obtained with drinking water biofilms given the low microbial content of drinking water in comparison to wastewater. Wagner et al. (2010) also concluded that RM is a slow, laborious method, which can promote photo bleaching of the samples, therefore it is suggested for RM to be used more widely in biofilm research, which should improve the technology.

Despite the array of technical advances in methods used to assess biofilm biochemical composition or distribution, the processes driving the expression or production of different cell biochemical components or how these are regulated at a genetic level, remain unknown. Further research to fill this knowledge gap will be needed to understand, for example the physiological differences of biofilm bacteria from that of their planktonic counterparts (Karunakaran et al., 2011) or the influence of environmental parameters upon their gene expression and activity.

4. Application and integration to better inform understanding and management of drinking water distribution systems

There is, arguably, a tendency to overlook the changes in water quality that can and does occur within DWDS. There is a reliance that the high quality water produced from modern water treatment works will not be deteriorated to an unacceptable level. However, we know that DWDS are not inert transport systems; complex physical, chemical and microbiological processes take place between the source and the consumer's tap.

Understanding and predicting bulk water changes within the DWDS can help secure potable water quality. Research and practice in this area is often led by the transfer and application of latest treatment work derived process science that is starting to utilise some of the tools and techniques presented here. However, it is known that the changes occurring within DWDS are influenced and often dominated by the interface between the pipe infrastructure and the bulk water (Sekar et al., 2012). It is increasingly accepted that microbial communities are ubiquitous at this interface and that biofilms are the dominant source of DWDS organic matter. Despite this, we lack applicable understanding of the microbial communities at the pipe wall interface, particularly with respect to their impact on water quality and, conversely, how the environmental conditions of the DWDS impact on the community.

The methods presented here facilitate research aiming to quantify the microbial community, evaluate microbial diversity and determine the potential function of those microorganisms present (including the potential to harbour pathogens). Ultimately, this research offers an assessment of the impact of the microbial ecology upon water quality and asset management It is however important that such microbial research is conducted in an integrated manner, for example there is a body of understanding concerning corrosion of cast iron pipes, driven by consideration of structural performance including bursts and leakage, that relates to the interface and includes consideration of microbial mediated corrosion. We need to strive to integrate physical, chemical and biological understanding. It is important to note that the techniques presented here are not always optimised for application to DWDS and that development work is needed to yield valid and informative data. These techniques are also often time and resource intensive, requiring careful consideration of the specific question(s) to be explored and how the resulting data can be utilised, such as to inform modelling. It

should also be noted that often a range and/or combination of techniques need to be applied in order to obtain the required knowledge. Careful consideration, planning and understanding of the implications of sampling method and regime is also critical, as discussed earlier.

Applied research is needed that can move asset management strategies away from the idea of a 'clean' pipe that can be maintained in perpetuity. It is generally accepted in most fields that the complete eradication of biofilms is impossible. To our knowledge a suitable surface entirely resistant to colonization of microorganisms does not exist. Even if such a material were found, it is unlikely it would be suitable for retrofitting to the vast, ageing, deteriorating infrastructure of DWDS. Hence, while a 'clean pipe' may be briefly achieved by highly invasive, aggressive cleaning approaches, the pipe will be compromised as soon as potable water is introduced, as a microbial community will establish, evolve and adapt over time. It is important that we learn how to understand, predict and manage this community such that we can estimate the risks to water quality that this community poses and can develop interventions to control and manage that risk. This need to understand predict and manage necessarily requires developing modelling tools to capture and extrapolate community composition, behaviour, function and impacts. Such modelling will be essential to enable extrapolation between bench top, pilot scale and real systems. It is impossible that we can sample and test for every combination of conditions and variables that exist in the real world, hence modelling is essential. Ultimately it would be desirable for such models to be driven by surrogate data rather than being reliant on the complex and expensive tools and techniques presented here. Hence there is a need to develop such surrogates and for the adoption of standard methods, these should not necessarily be driven by regulation, but by the need for best asset management and service delivery through a risk based management approaches.

With the latest generations of tools and techniques presented here we are now able to start generating data and understanding to inform and populate suitable modelling approaches and ultimately derive management and operational guidance. Ultimately such research will help ensure that the best sustainable use can be made of our existing infrastructure to safe guard water quality for future generations in the light of pressures such as climate change and increasing urban populations.

5. Conclusions and outlook

The advantages and limitations of approaches currently used in environmental microbiology have been discussed in relation to their applicability to DWDS. Ultimately, the choice of technique depends on the objective of the research, the required level of resolution, the availability of specialised equipment and the available funding.

Despite culture-dependent techniques still being used by water utilities to routinely monitor the microbial quality of drinking water, molecular methods are replacing these and some water companies are beginning to implement PCR-based approaches to detect pathogens. The application of NGS has exceptionally enlarged the existing knowledge about the diversity and structure of microbial communities in DWDS. The main sequencing platforms are constantly increasing quantity of sequences obtained and read length from samples while reducing the costs. These developments will make this technology more affordable and accessible and they will be soon considered a standard approach in environmental microbial research. The future automatisation of molecular methods might be indispensable for the development of online devices to for example detect pathogens in drinking networks.

The major knowledge gap in understanding the microbiology of DWDS is the lack of information required to link microbial diversity and function. Approaches that can fill this gap are microarrays, metabolomics and metaproteomics, unfortunately their use has not yet been explored in DWDS. Future research should use integrated approaches to improve our understanding of drinking water microbiology, combining a range of techniques, to explore and link the microbial diversity and activity to ultimately understand the relationship between microorganisms and system function. A system biology approach where environmental metagenomics is combined with other methodologies such as metatranscriptomics, metaproteomics and metabolomics should allow expansion of our understanding of DWDS.

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