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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.

Keywords: biofilms, drinking water distribution systems, methodological approaches, microbial diversity, microbial function.

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

48 within a DWDS they will face a challenging environment, with limited nutrients and
49 changing water flow and pressure fluctuations. As a consequence, microorganisms will often
50 have a better chance of survival attached to the pipe surfaces within a biofilm (Henne *et al.*,
51 2012), where they are protected from external adverse factors and benefit from the interaction
52 with other microorganisms. More than 95 % of the microbial biomass in a DWDS is attached
53 to the pipe walls forming biofilms (Flemming, 1998).

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

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

72 expected that a number of culture-independent methods will be used routinely in the near
73 future (as the prices for the analysis are dropping).

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

82 **2. Sampling water distribution systems**

83 **2.1 Bulk water sampling**

84 Appropriate sampling procedures are essential for collecting representative water samples for
85 microbiological parameters. Sampling programmes, guidelines for practices and procedures
86 to monitor water quality within DWDS have been designed and developed by international
87 organisations and water companies. The World Health Organisation (WHO) have published
88 several editions of the *Guidelines for Drinking Water Quality* (2011), where information
89 about standardised methods for microbial analysis of DWDS can be found (ISO5667-
90 5:2006). At a national level, in the USA, the Safe Drinking Water Act authorises the
91 Environmental Protection Agency (US EPA) to set standards for drinking water and has
92 developed a guide to help collect water samples according to these standards
93 (http://water.epa.gov/lawsregs/rulesregs/sdwa/index.cfm). In the European Union (EU), The
94 Drinking Water Directive (DWD) (98/83/EC), regulates the quality of water for human
95 consumption and requires that the EU countries meet a number of health parameters and
96 standards (Weinthal *et al.*, 2005). In the UK, the Environmental Agency (EA) also provides

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

119 **2.2 Biofilm sampling**

120 Biofilm research is a key component in DWDS microbial studies, but as pipes are not readily
121 accessible, collecting samples from real systems is a substantial challenge. Habitually, bench-

122 top laboratory biofilm reactors such as the Rotating Disc Reactor (Murga *et al.*, 2001; Mohle
123 *et al.*, 2007), the Biofilm Annular Reactor (Batte *et al.*, 2003a; Batte *et al.*, 2003b), and the
124 Propella Reactor (Appenzeller *et al.*, 2001) have been used to study various abiotic factors
125 that might influence biofilm formation. However, it is well known that they poorly replicate
126 the conditions of real pipe networks (Deines *et al.*, 2010).
127 Currently two different approaches exist for studying biofilms *in situ* in DWDS. One involves
128 cut-outs of pipes; the other one relies on devices inserted into the pipe. Pipe cut-out sampling
129 protocols are labour-intensive, expensive and classed as destructive sampling methods
130 (LeChevallier *et al.*, 1998; Wingender and Flemming, 2004). Furthermore, the excavation
131 and cutting processes often lead to concerns with contamination and representative sampling.
132 The use of devices, commonly coupons, that can be deployed repeatedly either within a pilot-
133 scale test facility or in an operational DWDS, allows the study of biofilm dynamics over time
134 in relation to changing abiotic and biotic factors *in situ*. Commonly, the main limitation of
135 some of these devices is that they distort hydraulic conditions in pipes and, in most cases,
136 shear stress and turbulence regimes are different from those expected in real pipes, artificially
137 influencing the way biofilms develop. The Robbin device (Manz *et al.*, 1993; Kalmbach *et*
138 *al.*, 1997) and the "Pipe Sliding Coupon" holder (Chang *et al.*, 2003) present these types of
139 hydraulic limitations. Some devices such as the "Biofilm Sampler" (Juhna *et al.*, 2007) are
140 directly connected to a DWDS avoiding the distortion of hydraulic conditions on biofilm
141 processes but to study *in situ* biofilms, for example via microscopy techniques, biofilms need
142 to be removed from the coupon. The Pennine Water Group coupon, 'PWG Coupon', takes
143 the benefits of the "Biofilm Sampler" a step further, since the coupon is curved and therefore
144 sits flush with the pipe wall reducing the distortion of hydraulic conditions (Deines *et al.*,
145 2010). Another advantage is that the coupon comprises two parts; a removable 'insert', which

146 allows the analysis of biofilms *in situ* and an outer part that can be used to extract nucleic
147 acids for further characterisation of microbial communities (Deines *et al*., 2010).
148 The application of coupon techniques in both experimental and live DWDS makes it possible
149 for us to advance our understanding of biofilms and the numerous abiotic factors that might
150 play a role in their formation and properties.

151 3. Conventional and current microbiological techniques and methodological 152 advancements to address the challenges of maintaining potable water quality

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

162 3.1 Microbial detection and enumeration

163 3.1.1 Culture-dependent techniques

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

168 The reference method used for routine bacteriological monitoring in drinking water is
169 **heterotrophic plate count (HPC) measurements**, which assess only heterotrophic bacteria
170 able to form colonies on a solid medium at a specific temperature. Counting the number of

171 colonies grown after a defined incubation time provides a general estimation of the
172 bacteriological load in the water samples. There are several standardized HPC methods but
173 not an approved standard operating procedure. These methods include incubation of plates
174 using temperatures ranging from 20°C to 37°C and over periods from a few hours to several
175 days (Allen *et al.*, 2004). HPC yields only information about a limited fraction of the whole
176 microbial community in a sample but the low cost, relative simplicity, wide acceptance and
177 long history of the method makes HPC a convenient tool for water utilities to assess the
178 efficiency of water treatment and to infer regrowth of microorganism in the network (WHO,
179 2003).

180 Culture-dependent tests are also used to detect indicator microorganisms such as coliform
181 bacteria. Coliform bacteria (e.g. *Escherichia* spp., *Enterobacter* spp. and *Citrobacter* spp.)
182 are habitual inhabitants of animal faeces and for this reason their presence above certain
183 concentrations, established in specific legislations, is used to infer faecal contamination in the
184 water (Boubetra *et al.*, 2011). The **membrane filtration (MF)** technique and the **multiple**
185 **tube fermentation (MTF)** method are often used to detect coliforms in drinking water. The
186 MF technique consists of filtering a water sample to concentrate cells followed by incubation
187 of the filter on a specific medium and after a given period of time the developed colonies are
188 enumerated. In the MTF technique, the concentration of bacteria is estimated by inoculating a
189 series of tubes containing liquid medium with ten-fold dilutions of the water sample. If the
190 medium supports microbial growth it will become turbid and the results can be expressed
191 using an estimation of the average number of bacteria in the sample known as the **most**
192 **probable number (MPN)** technique (Sutton, 2010). However, further testing is generally
193 required to confirm the presence of specific coliform organisms (Ashbolt *et al.*, 2001). The
194 tests used to analyse these bacteria are relatively cheap, easy and safe to execute, providing
195 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 *E. 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

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

225 **3.1.2.1 Microscopic methods**

226 **Epifluorescence microscopy** based methods offer a faster alternative for monitoring the
227 quality of drinking water than traditional plate counts, which have long incubation times.
228 Different fluorescent dyes can be used to directly stain cells in biofilms and/or water samples
229 and to estimate total cell counts using an epifluorescence microscope. Some of the most
230 useful dyes to quantify microorganisms in water and biofilm samples are acridine orange
231 (AO) (Hobbie *et al.*, 1977), 4,6-di-amino-2 phenylindole (DAPI) (Schaule *et al.*, 1993) and
232 5-cyano-2,3 Dytolyl Tetrazolium Chloride (CTC) (Schaule and Flemming, 1996). To
233 estimate viable cells a viability staining method might be used, such as the **LIVE/DEAD®**
234 Bacterial Viability Kit (BacLight™) which contains two nucleic acid stains: SYTO 9™
235 (green-fluorescent) and propidium iodide (PI) (red-fluorescent). The SYTO 9™ dye
236 penetrates all membranes while PI can only penetrate cells with damaged membranes.
237 Therefore, cells with compromised membranes will stain red, whereas cells with undamaged
238 membranes will stain green (Boulos *et al.*, 1999).

239 **Fluorescent *in situ* hybridization (FISH)** effectively extends epifluorescence microscopy,
240 allowing for the fast detection and enumeration of specific microorganisms (Wagner *et al.*,
241 1993). This method uses fluorescent labelled oligonucleotides probes (usually 15-25 bp)
242 which bind specifically to microbial DNA in the sample, allowing the visualization of the
243 cells using an epifluorescence or confocal laser scanning microscope (CLSM) (Gilbride *et al.*,

244 2006). FISH has been successfully used to characterise microorganisms within biofilms and
245 to detect pathogens in drinking water samples (Batte *et al.*, 2003b; Wilhartitz *et al.*, 2007).
246 An improvement of the FISH method is the **catalysed reporter deposition fluorescence *in***
247 ***situ* hybridization (CARD-FISH)** (Pernthaler *et al.*, 2002a). This method uses
248 oligonucleotides probes labelled with a horse radish peroxidase (HRP) to amplify the
249 intensity of the signal obtained from the microorganisms being studied (Schauer *et al.*, 2012).
250 CARD-FISH is useful when dealing with drinking water samples since it can enhance the
251 fluorescent signal from cells in samples with low microbial concentration (Dorigo *et al.*,
252 2005). The method has been successfully applied to investigate changes in microbial
253 communities in DWDS (Deines *et al.*, 2010), to detect pathogens such as *Legionella*
254 *pneumophila* (Aurell *et al.*, 2004) and faecal indicators (Baudart and Lebaron, 2010). In
255 general, FISH is not used as a stand-alone technique and is mostly used in combination with
256 other methods to characterise microbial communities. An example of these combined
257 techniques is **high-affinity peptide nucleic acid (PNA)-FISH**, useful to study pathogens in
258 biofilms due to the enhanced capability of the probe to penetrate through the Extracellular
259 Polymeric Substance (EPS) matrix (Lehtola *et al.*, 2007). Another example is **LIVE/DEAD-**
260 **FISH** which combines the cell viability kit with FISH (Savichtcheva *et al.*, 2005) and has
261 been used to assess the efficiency of disinfection in DWDS (Hoefel *et al.*, 2003). Despite its
262 numerous advantages when compared with culture-dependent techniques, FISH also has
263 several limitations. First of all, knowledge of the nucleotide sequence of the target organisms
264 is needed and the design of new probes and the optimization of the hybridization conditions
265 can be time consuming and complex (Sanz and Köchling, 2007). The efficiency of the
266 hybridization might be influenced by the physiological state of the cells and, to conclude the
267 signal emitted by auto-fluorescence cells can interfere with the signal emitted by the target
268 microorganisms (Dorigo *et al.*, 2005).

269 An alternative fast and reliable method to monitor bacterial abundance and viability of
270 planktonic cells or cells in suspensions is **flow cytometry (FC)**. This technique uses
271 fluorescent dyes to stain the water samples before analysing them with a flow cytometer. The
272 cells in solution pass through a capillary that is intersected by a laser beam, when the laser
273 interacts with the cells this causes the light to scatter and also excite the dye, the fluorescence
274 intensity and the scattering generated can be quantified using different detectors (Hammes *et*
275 *al.*, 2008). Different fluorescent dyes can be used to estimate total bacterial counts (Hammes
276 *et al.*, 2008), virus-like particles (Rinta-Kanto *et al.*, 2004) and *Cryptosporidium* spp. and
277 *Giardia* spp. (Vesey *et al.*, 1993; Vesey *et al.*, 1994; Ferrari *et al.*, 2000) in water samples.
278 FC provides much more realistic quantification of the total number of cells in water samples
279 than traditional plate counts and recently has been established as a reference method in
280 Switzerland by the Swiss Federal Institute of Aquatic Science and Technology (Eawag).
281 However, when epifluorescence microscopy and flow cytometry are used to measure cell
282 volume and/or estimate the viability or total cell counts of biofilms and sediments, both
283 methods are susceptible to errors due to the formation of cell clusters and the attachment of
284 cells to inorganic compounds (Van der Kooij *et al*., 2014).

285 **3.1.2.2 PCR based methods**

286 The **polymerase chain reaction (PCR**) is a method used to amplify (i.e. obtain multiple
287 copies) fragments of DNA. **PCR** based methods require the extraction of nucleic acids
288 (DNA/RNA), followed by the amplification of a target gene or genes via PCR and post-PCR
289 analysis. It is important to notice that the amplicons obtained from PCR form the basis for all
290 the community fingerprinting techniques and next generation sequencing methods explained
291 in the following sections of this review. The most useful PCR-based techniques to detect
292 microorganisms in drinking water are **multiplex-PCR** and quantitative **real time (q-PCR)**.
293 Multiplex-PCR uses several oligonucleotide probes to simultaneously detect different

294 microorganisms and has been used in drinking water-related research to detect faecal
295 indicators and/or pathogens (Bej *et al.*, 1991). q-PCR is a sensitive tool to detect and quantify
296 microorganisms in environmental samples based on quantifying the number of target gene
297 copies present in a sample. This technique can monitor the amount of PCR product obtained
298 during the exponential phase of the PCR reaction by quantifying a fluorescent reporter. The
299 amount of detected reporter is then correlated with the initial amount of target template
300 allowing the quantification of the target organism (Kubista *et al.*, 2006). Despite the general
301 limitations of the PCR-based techniques discussed in detail in Table 1, several studies have
302 shown the applicability of these methods to detect viral indicators of human faecal
303 contamination (Albinana-Gimenez *et al.*, 2009), pathogenic bacteria such as *Helicobacter*
304 *pylori* (McDaniels *et al.*, 2005; Sen *et al.*, 2007), *Mycobacterium avium* and *Legionella* sp.
305 (Dusserre *et al.*, 2008) and to quantify *Giardia* and *Cryptosporidium* (Guy *et al.*, 2003).

306 **3.2 Microbial community composition**

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

312 **3.2.1 Phospholipid fatty acids**

313 **Phospholipid fatty acids (PLFAs)** are useful to overcome the limitations of culturing
314 techniques when assessing the microbial community composition of environmental samples.
315 The membranes of microorganisms have phospholipids which contain fatty acids (Zelles,
316 1999) and these can be used to obtain microbial communities fingerprints (Vestle and White,
317 1989). This technique has been applied in drinking water research, to study biofilms

318 (Keinanen *et al.*, 2004; Lehtola *et al.*, 2004) and to detect pathogens (White *et al.*, 2003). It
319 should be noted that such techniques provide a fingerprint which describes a microbial
320 community, and hence measures and compares overall diversity but does not provide
321 identification of specific species actually present in the samples.

322 **3.2.2 Molecular Techniques**

323 The advent of molecular techniques has enabled the characterisation of natural microbial
324 communities without the need of culturing microorganisms and has introduced new insights
325 into the microbial ecology of different ecosystems. Molecular analysis of samples includes
326 the extraction and purification of DNA and/or RNA. DNA provides information of the total
327 microbial community of the samples while RNA-based analysis represents only the active
328 part (Kahlisch *et al.*, 2012). The nucleic acid extraction is followed by PCR amplification of
329 "marker genes" to obtain taxonomic information. The most commonly used marker gene in
330 microbiological research is the ribosomal RNA (rRNA) gene, 16S rRNA for prokaryotes and
331 18S rRNA for eukaryotes. The rRNA gene has different regions, some are highly conserved
332 across all phylogenetic domains (i.e. bacteria, eukarya and archaea), other regions are
333 variable between related species (Woese, 1987) and this variability allows for inferring
334 phylogenetic information from microorganisms inhabiting different ecosystems (Prosser,
335 2002).

336 During recent years, to aid identification of sequences recovered from environmental
337 samples, databases of small (16S/18S) and large subunits (23S/28S) rRNA sequences for
338 bacteria, archaea and eukarya have been developed and are constantly expanding. SILVA
339 rRNA database project provides good quality, aligned ribosomal RNA sequence data which
340 is regularly updated (www.arb-silva.de). Other good databases are accessible through the

341 Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) and the Greengenes database
342 (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

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

349 **3.2.2.1 Fingerprinting techniques**

350 Among the different molecular tools available to assess the microbial community
351 composition of drinking water ecosystems, fingerprinting techniques are the most commonly
352 employed. Fingerprinting techniques are particularly useful to simultaneously analyse
353 multiple samples and to compare different microbial community structures. **Denaturing**
354 **gradient gel electrophoresis (DGGE)** (Muyzer *et al.*, 1993) and **temperature gradient gel**
355 **electrophoresis (TGGE)** (Po *et al.*, 1987) are fingerprinting techniques where specific
356 fragments of the rRNA gene are amplified and then separated based upon their sequence
357 composition in a denaturing polyacrylamide gel (DGGE) or using a temperature gradient
358 (TGGE). The final result is a gel with a pattern of bands which is a visual profile of the most
359 abundant species in the studied microbial community. This approach allows for monitoring
360 changes in microbial communities and it can be used, similarly to other fingerprinting
361 techniques, as a semi-quantitative method to estimate species abundance and richness
362 (Muyzer, 1999). In addition, specific bands on the gel can be excised and sequenced for
363 subsequent taxonomic identification. DGGE is the most cited fingerprinting method used to
364 characterise microbial communities in drinking water. DGGE has been used to assess

365 opportunistic pathogens in urban drinking water biofilms (Pryor *et al.*, 2004) to monitor
366 biofilm formation and activity in distributions systems (Boe-Hansen *et al.*, 2003), to study the
367 effect of stagnation in taps (Lautenschlager *et al.*, 2010), corrosion on cast iron pipes (Teng *et*
368 *al.*, 2008), nitrification in drinking water networks (Yapsakli *et al.*, 2010), occurrence of
369 fungi in biofilms (Pereira *et al.*, 2010) and to assess bacterial water quality in real distribution
370 systems (Sekar *et al.*, 2012). Despite its broad application, this technique has several
371 disadvantages; first of all handling polyacrylamide gels and obtaining the optimal denaturing
372 conditions is highly laborious. In terms of the analysis of the gels, associating a single band
373 with a particular species is complicated and cloning and sequencing of particular bands is
374 ultimately needed for confirmation of results (Muyzer, 1999). Despite the use of markers on
375 the gels, comparison of patterns across gels and the detection of rare members of the
376 microbial community are challenging.

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

387 **Amplified ribosomal DNA restriction analysis (ARDRA)** (Vaneechoutte *et al.*, 1992) is
388 another fingerprinting tool in which amplicons of rRNA genes are digested with a set of
389 restriction enzymes, producing a pattern of fragments representative of a given microbial

390 community (Heyndrickx *et al.*, 1996). ARDRA has been used to characterise biofilms
391 (Ludmany *et al.*, 2006) and to identify non-tuberculous *Mycobacterium* (Tsitko *et al.*, 2006).
392 **Automated ribosomal intergenic spacer analysis (ARISA)** (Fisher and Triplett, 1999) is
393 normally used to characterise fungal communities. In ARISA, the ITS regions of nuclear
394 DNA located between the 18S (SSU) and 28S (LSU) genes are amplified using fluorescent
395 labelled primers, then the amplicons are analysed in a sequencer to determine their size and to
396 ultimately obtain a fingerprint of the studied microbial community. The method known as
397 s**ingle strand conformational polymorphism (SSCP)** (Orita *et al.*, 1989) also separates
398 amplicons as a result of variation in their sequence (Widjojoatmodjo *et al.*, 1995). The
399 amplicons are treated to obtain single DNA strands, which are separated via gel
400 electrophoresis. SSCP use in drinking water is also limited but has been used for *in situ*
401 genotyping of *Legionella pneumophila* (Kahlisch *et al.*, 2010).

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

407 **3.2.2.2. Sequencing-based approaches**

408 **Cloning and sequencing** is the conventional and more widespread genomic approach used
409 when detailed and accurate phylogenetic information from environmental samples is
410 required. The method involves the extraction of nucleic acids, amplification of the rRNA
411 gene with suitable primers and the construction of clone libraries using sequencing vectors
412 (Rondon *et al.*, 2000). Selected clones are then sequenced (Sanger-based) (Sanger *et al.*,
413 1977) and the nucleotide sequence of the rRNA gene retrieved, allowing estimates of the

414 microbial diversity in the samples by comparison with sequences available in databases (e.g. 415 GenBank, EMBL and Silva). The generation of DNA clone libraries followed by sequencing 416 has being extensively applied in drinking water microbiology, a selection of these 417 applications are discussed in brief. This method has been used to study long term succession 418 in biofilms (Martiny *et al.*, 2003), disinfection efficiency (Hoefel *et al.*, 2005), nitrifier and 419 ammonia-oxidizing bacteria in biofilms (Lipponen *et al.*, 2004), to characterise the 420 microorganisms present in red water events (Wullings and van der Kooij, 2006) and to detect 421 *Bacteroidetes* in unchlorinated water (Saunders *et al.*, 2009).

422 The approach known as **metagenomics,** involves sampling the entire genome of an 423 environmental sample in order to obtain sequence information from the microorganisms 424 contained in it and to ultimately make taxonomic assignments to characterise them. A 425 sequencing-based approach useful to sequence the entire genome and characterise microbial 426 communities in environmental samples is known as **shot gun sequencing**. Genomic DNA is 427 cut into smaller fragments; these fragments can be sequenced individually and then 428 reassembled into their original order in the genome, based on sequence overlaps, to obtain the 429 complete genome sequence. Environmental genome shotgun sequencing has been used in 430 ocean water to assess the diversity and relative abundance of organisms (Venter *et al.*, 2004). 431 To our knowledge this molecular approach has not been used to explore drinking water 432 ecosystems but we consider that its application might bring new insights into the microbial 433 ecology of DWDS.

434 Independently of the sequencing approach employed, taxonomic assignments of the 435 sequences are typically identified using search algorithms such as the Basic Local Alignment 436 Search Tool (BLAST) (Altschul *et al.*, 1990), sequences are also aligned, clustered and 437 phylogenetic trees are constructed using software such as MEGA (Tamura *et al.*, 2011), 438 PHYLIP (Felsenstein, 1989) and ARB (Ludwig *et al.*, 2004).

439 Despite being enormously successful, cloning and sequencing approaches are very expensive
440 and time consuming and since the introduction of **next generation sequencing (NGS)**
441 techniques their use has declined substantially. In the last decade, the use of NGS has
442 incredibly enhanced the understanding of the microbial ecology of different ecosystems. The
443 NGS platforms have improved the depth of sequencing since they can produce thousands of
444 short reads in a single run allowing for the detection of less abundant members of microbial
445 communities (Metzker, 2010). In addition, the use of high-throughput sequencing techniques
446 avoids the need of the laborious and time consuming steps in conventional cloning and
447 sequencing. However, NGS techniques provide sequence information with a limited base pair
448 length (max ~600 bp) and, despite increases in read lengths as these technologies advance,
449 phylogenetic comparisons are based on shorted sequences when compared with conventional
450 Sanger sequencing (max ~1500 bp). This restriction might result in less accurate gene
451 annotation and overestimation of microbial richness in samples. In addition, it should be
452 noticed that the resolution of NGS methods is currently too low to identify microorganisms to
453 the species level. The most frequently used NGS platforms are Roche 454 and
454 Illumina/Solexa. Nowadays, Illumina is replacing Roche 454 as the sequencing method of
455 choice for most of microbial-related studies. While Illumina yields shorter reads than Roche
456 454, the sequencing error of both platforms is comparable and Illumina is much cheaper than
457 454 (Luo *et al.*, 2012).

458 Several bioinformatics software and analysis tools are available to analyse the numerous
459 sequences reads obtained from NGS runs, the most useful ones are MOTHUR (Schloss *et al.*,
460 2009), QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.*, 2010) and the
461 pyrosequencing pipeline in the Ribosomal Database Project (RDP) (Cole *et al.*, 2009). The
462 use of these high throughput sequencing techniques in drinking water is constantly
463 increasing, recent studies have used pyrosequencing to characterise bacterial communities

464 from impellers retrieved from customer water meters (Hong *et al.*, 2010), in membrane
465 filtration systems from a drinking water treatment plant (Kwon *et al.*, 2011), to assess the
466 influence of hydraulic regimes on bacterial community composition in an experimental
467 distribution system (Douterelo *et al.*, 2013) and to assess the influence of different
468 disinfectant regimes on microbial community dynamics (Gomez-Alvarez *et al.*, 2012; Hwang
469 *et al.*, 2012). With sequencing costs decreasing, NGS is enabling an increasing number of
470 laboratories to taxonomically (and functionally) classify a wide range of the organisms that
471 are present in drinking water.

472 **3.3 Microbial activity and analysis of functional genes**

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

480 **3.3.1 Estimation of biomass**

481 Two methods widely used in drinking water research to estimate biomass and bacterial
482 growth are the quantification of **adenosine triphosphate (ATP)** and of **assimilable organic**
483 **carbon (AOC)** respectively. ATP quantification enables active microbial biomass to be
484 measured (van der Wielen and van der Kooij, 2010). Briefly, cellular ATP reacts with a
485 luciferin-luciferase complex, the luminescence produced in this reaction is proportional to the
486 concentration of ATP, which is then correlated to the quantity of biomass in the sample
487 (Hammes *et al.*, 2010). Nowadays, ATP can be easily assessed using the BacTiter-Glo^{TM}

488 Microbial Cell Viability Assay (Promega, UK), which allows quantification of several
489 samples simultaneously using a microplate reader. This method is fast, low-cost and easy to
490 perform, thus is an ideal tool for monitoring purposes. The use of ATP is well-established in
491 drinking water-related research and is used as a relievable method to estimate microbial
492 activity (Hammes *et al.*, 2010). Numerous studies have successfully used this technique to
493 assess microbial viability and the biological stability of water in DWDS (Lehtola *et al.*, 2002;
494 Berney *et al.*, 2008; Hammes *et al.*, 2008; Lautenschlager *et al.*, 2013). **AOC** is widely used
495 in drinking water research to assess growth of heterotrophic bacteria in water (van der Kooij,
496 1992). Hammes and Egli (2005) developed a new and faster AOC method using natural
497 microbial communities as inoculum and flow cytometer to estimate cell counts in water
498 samples. The method has been used to test the influence of disinfectant on microbial growth
499 in distribution systems (Choi and Choi, 2010; Ohkouchi *et al.*, 2013) and for assessing the
500 potential growth of biofilms on different pipe material (Zacheus *et al.*, 2000; Liu *et al.*, 2002).

501 ATP and AOC methods have been tested in order to assess the biological stability of drinking
502 water, which is defined as the inability of water or a material in contact with water to support
503 microbial growth (Delft *et al*., 2010; Liu *et al.,* 2013) and implies that the concentration of
504 cells and the microbial community composition should not change during water distribution
505 (Lautenschlager *et al.*, 2014). Most of the research related to biological stability is focused on
506 estimating the potential microbial activity in water and/or in particle-associated bacteria (i.e.
507 associated to suspended solids and loose deposits) (Liu *et al.*, 2014). In general, the AOC
508 method has several limitations; i) assumes that bacterial growth is limited by organic carbon,
509 ii) quantifies available nutrients instead of bacteria and iii) depends on the type of bacteria
510 used (Liu *et al.*, 2013). Due to these limitations, it has been considered that the potential
511 contribution of nutrients and biomass contained by loose deposits is overlooked when using
512 this method (Liu *et al.*, 2013). ATP has been used to quantify the bacteria from different

513 phases of an unchlorinated DWDS, including bulk water, pipe wall biofilm, suspended solids,
514 and loose deposits (Liu *et al.*, 2014). The study concludes that bacteria associated with loose
515 deposits and pipe wall biofilm accumulated in the DWDS accounted for over 98% of the total
516 bacteria. However, when using ATP to study particle–associated microorganisms it is
517 necessary to perform some pre-treatment to the samples to detach the microorganisms into
518 suspension for further analysis (Liu et al., 2013).

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

523 **3.3.2 Functional genes**

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

529 The molecular approach known as **metatranscriptomics** is based on the study of actively
530 transcribed ribosomal and messenger RNA (rRNA and mRNA) and facilitates linking
531 specific functions to certain members of a microbial community. Routinely, the first step is
532 the extraction of RNA from a sample. This maybe a challenging process since RNA degrades
533 easily outside the cells due to its short-half life and to the presence of RNAases. For an
534 accurate estimation of gene expression, it is also important that the extracted RNA is free of
535 contaminating DNA and inhibitors (Bustin *et al.*, 2009). After RNA extraction,
536 complementary DNA (cDNA) is synthesized from RNA by reverse transcription (RT) using

537 random or specific primers (Sharkey *et al.*, 2004) and the resulting cDNA can then be used to
538 measure the expression of functional genes by for example **real time-PCR (RT-PCR)** or
539 functional microarrays.

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

550 The application of **functional microarrays** enables assessment of the overall gene
551 expression of a microbial community. In microarrays, oligonucleotides probes targeting
552 functional genes are immobilized on solid supports (chips) and arranged spatially in a known
553 pattern, the subsequent hybridization between the target cDNA, labelled with a fluorescent
554 dye, and the oligonucleotides on the chip indicates that the gene has been transcribed
555 (Sharkey *et al.*, 2004). With this technique patterns of hybridization are obtained and the
556 intensity of the fluorescence is proportional to the gene expression (Gilbride *et al.*, 2006).
557 GeoChip is an example of a functional gene array (He *et al.*, 2007), the last developed array
558 of this type GeoChip4.0 contains 120,054 distinct probes, covering 200,393 coding sequences
559 (CDS) for genes involved in different processes (e.g. biogeochemical cycles of carbon,
560 nitrogen and phosphorus). The main advantage of using microarrays is that the expression of
561 thousands of mRNAs can be assessed simultaneously. However, most of the arrays are

562 normally developed using genes and metabolic pathways obtained from laboratory isolates
563 and when microarrays are applied to environmental samples sequence divergence can affect
564 hybridization leading to erroneous interpretations (Wilmes and Bond, 2006). Other
565 limitations of microarrays to be aware of are low specificity in some cases and that mRNA
566 expression and protein expression are not always directly correlated (Pradet-Balade *et al.*,
567 2001). Despite the high potential of this technique for assessing functionality of microbial
568 communities, the use of microarrays has not yet been explored for this purpose in drinking
569 water research.

570 **3.3.3 Proteomics**

571 **Proteomics** is a discipline focused on the identification of proteins and **metaproteomics** can
572 be defined as the characterisation of the entire protein complement of a microbial community
573 (Wilmes and Bond 2006). Protein expression can be directly associated with specific
574 microbial activities. The fundamental steps in proteomics investigations are protein
575 extraction, separation and/or fractioning, identification and quantification (Siggins *et al.*,
576 2012). Traditionally, proteins are visualised and separated in a two dimensional
577 polyacrylamide gel electrophoresis (2D-PAGE) then digested with enzymes and identified by
578 mass spectrometric (MS) analysis (Schneider and Riedel, 2010). However, 2D-PAGE gels
579 have several limitations, to name a few; they are highly laborious, proteins can co-migrate in
580 the gel and some proteins such as membrane proteins, proteins with extreme molecular
581 weights or isoelectric points are difficult to separate (Schneider and Riedel, 2010).
582 Alternatively, proteins can be separated by **liquid chromatography** (LC). The combined
583 approach using LC-MS has become widely used in environmental proteomics and the use of
584 2D-PAGE gels has currently decreased. Furthermore, the use of gel-free protein fractions has
585 been recommended when possible since they provide higher levels of protein identification
586 when compared with gel-based methods (Siggins *et al.*, 2012). Ultimately, the mass

587 spectrophotometer generates a peptide sequence or a peptide mass fingerprint (PMF) which
588 can be compared with available databases. If sequencing data is not available, proteins can be
589 identified from their corresponding *de novo* peptide sequences by means of a protein BLAST
590 (BLASTp) (Wilmes and Bond, 2006; Pandhal *et al.*, 2008).

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

606 **3.3.4 Metabolomics**

607 Metabolomics studies the metabolome which includes cell metabolites that are produced or
608 consumed as a result of biological activity (Beale *et al.*, 2013). Within metabolomics,
609 metabolic footprinting focuses on the analysis of extracellular metabolites which can provide
610 information on functional genomics and on cell to cell communication mechanisms (Mapelli

611 *et al.*, 2008). This methodology can be used to monitor the presence and/or microbial
612 mediated processes in DWDS since it allows associating specific metabolite profiles with
613 different microorganisms (Beale *et al.*, 2010). Profiles of intracellular and extracellular
614 metabolites associated with microbial activity can be obtained using techniques such as **gas**
615 **chromatography-mass spectrometry (GC-MS)**. GC–MS approaches have been used to
616 study microbial influenced corrosion. Beale et al., (2012), used GC-MS to obtain specific
617 metabolic markers in order to discriminate between water samples and to identify those
618 exposed to bacteria involved in pipe corrosion. In another study, Beale *et al*., (2010) applied a
619 metabolomics approach to also study pipe corrosion and were able to observe using 3D
620 fluorescence spectroscopy the 'protein-like' fluorophore associated with presence of bacteria
621 in water collected from corroded pipes and cross reference this with derivatised fatty acid
622 metabolites using GC–MS analyses of the same water. Using samples from flushing a water
623 main Beale *et al*., (2012), demonstrated the effectiveness of metabolomics to study biofilms
624 in DWDS using also GC-MS, the chemometric analysis of the chromatograms in
625 combination with mass spectrometer data allowed differentiating between biofilms from
626 different pipe materials and planktonic bacteria. The same author, Beale *et al*., (2013) has
627 also showed that a metabolomics approach can be used to rapidly (less than 24 h) detect and
628 quantify viable and non-viable *Cryptosporidium* oocysts in water samples. In this research,
629 the authors used a chemometric approach to analysing information obtained from
630 chromatographic and mass spectral data to identify and quantify excreted metabolites from
631 *Cryptosporidium* oocysts and found that a number of key metabolite features including
632 aromatic and non-aromatic amino acids, carbohydrates, fatty acids, and alcohol type
633 compounds were able to explain the difference between the viable and non-viable oocysts in
634 water samples.

635 **3.3.5 Other functional techniques and combined approaches**

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

653 Methods such as **Bromodeoxyuridine (BrdU) incorporation**, **microautoradiography-**
654 **FISH, Raman-FISH** and **isotope array** provide further insights into the metabolic activities
655 of the microorganisms. **BrdU incorporation** is a non-radioactive approach that provides
656 information of active and DNA synthesizing cells, when used in combination with FISH
657 gives the identity and activity of targeted cells (Pernthaler *et al.*, 2002b). The combination of
658 BrdU magnetic bead immunocapturing and DGGE analysis have facilitated the exploration of
659 the phylogenetic affiliations of DNA-synthesizing and active bacteria (Hamasaki *et al.*,

660 2007), such methods could be used to study the viability of bacteria in drinking water during
661 treatment processes.

662 **Microautoradiography (MAR)** is a radioactive approach used in combination with FISH
663 **(MAR-FISH)** to reveal the physiological properties of microorganisms with single-cell
664 resolution (Wagner *et al.*, 2006). MAR-FISH provides information on total cells, probe
665 targeted cells and the percentage of cells that incorporate a given radiolabelled substance
666 (Ouverney and Fuhrman, 1999). This method has been widely applied to study the structure
667 and function of microbes in freshwater and marine ecosystems including biofilms (Lee *et al.*,
668 1999; Nielsen *et al.*, 2003; Kindaichi *et al.*, 2004a). The method provides a picture of the *in*
669 *situ* function of targeted microorganisms and is an effective approach to study the *in vivo*
670 physiology of microorganisms in biofilms (Ginige *et al.*, 2004). Lee *et al.* (1999) developed a
671 microscopic method in combination with FISH and MAR for simultaneous determination of
672 identities, activities and substrate uptake by specific bacterial cells in complex microbial
673 assemblages. Kindaichi *et al.* (2004) used this approach to study the ecophysiological
674 interaction between nitrifying bacteria and heterotrophic bacteria in biofilms. Such a study is
675 relevant for drinking water as chlorination of water has been found to promote the growth of
676 nitrifying bacteria (Eichler *et al.*, 2006). Nielsen *et al.* (2003) developed a new technique for
677 quantification of cell-specific substrate uptake in combination with MAR-FISH known as
678 QMAR (quantitative MAR).

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

684 **3.4 Biofilms and species interaction**

685 Most of the microorganisms inhabiting DWDS are attached to the pipe surface forming
686 biofilms (Flemming *et al.*, 2002). The resistance of biofilms to disinfection and the difficulty
687 of controlling their growth in distribution networks are common themes for research groups
688 and water companies around the world. There are numerous studies regarding biofilm
689 response to different chemicals and disinfectant strategies, most of them using bench top
690 reactors and single species biofilms (Gagnon *et al.*, 2005; Murphy *et al.*, 2008; Simoes *et al.*,
691 2010b). However, a better understanding of the physico-chemical structure of biofilms, and
692 of the mechanisms regulating its formation such as adhesion and coaggregation, in DWDS is
693 needed and fundamental to improve and/or develop control and management strategies.

694 **3.4.1 Cell adhesion**

695 The process of biofilm formation is initiated by attachment of planktonic cells to the pipe
696 surfaces (Simoes *et al.*, 2007). Bacterial adhesion is affected by environmental conditions in
697 the network (e.g. hydraulic forces, disinfectant regime, pipe material) and by intrinsic
698 characteristics of the cells such as hydrophobicity, surface charge, production of
699 polysaccharides and cell motility (Li and Logan, 2004; Simoes *et al.*, 2010b). Different
700 parameters can be used to estimate the **potential of cell adhesion**, for example measurements
701 of cell hydrophobicity, electrostatic potential and the thermodynamic potential of the cell
702 (Van Loosdrecht *et al.*, 1987; Simoes *et al.*, 2007). Adhesion to surfaces is often mediated by
703 hydrophobic interactions; often measured by **angle contact measurements** (Cerca *et al.*,
704 2005). The method was initially described by Busscher *et al.* (1984) and has been used to
705 study adhesion to surfaces mainly by clinically relevant microorganism such as *Pseudomonas*
706 *aeroginosa* (Pasmore *et al.*, 2002) and *Mycobacterium avium* (Steed and Falkinham, 2006).
707 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; Simoes *et al.*, 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

732 will form coaggregates that will tend to settle out, giving different levels of turbidity in the
733 medium (Simoes *et al.*, 2008). The visual aggregation assays use a subjective scoring criteria
734 based on the method developed by Cisar *et al.* (1979) to assess the degree of coaggregation
735 between species. Values ranging from 0 (no coaggregation) to 5 (large flocs of coaggregates
736 settle down and leave a clear supernatant) are assigned to the biofilm cultures to subjectively
737 classify the level of coaggregation. However, the subjectivity of this method can make
738 accurate comparisons between studies difficult.

739 Bacterial coaggregates can also be visualised using **DAPI** (total cell counts) or **LIVE/DEAD**
740 (viability) stains in combination with **fluorescence microscopy (**for details see 3.1.2.1
741 *Microscopy methods*). Growth rates of different combinations of bacteria isolated from
742 drinking water can be monitored over time using **microtiter plates** with R2A as a medium
743 and inoculating the plates with the cell suspensions to study (Stepanovic *et al.*, 2000). If
744 coaggregation occurs the biofilm will grow changing the initial optical density of the medium
745 which can be detected using spectrophotometry. Biofilms can also be stained with **crystal**
746 **violet** to quantify changes in biofilm mass during the process of coaggregation (Simoes *et al.*,
747 2010a).

748 These methods have been useful to study coaggregation between species isolated from
749 drinking water biofilms (Simoes *et al.*, 2008, 2010a; Giao *et al.*, 2011; Ramalingam *et al.*,
750 2013). Most of these techniques have been applied to study biofilms under laboratory
751 conditions at a bench top scale and cannot be applied *in situ* to biofilms attached to drinking
752 water networks. However, compared with molecular-based techniques, coaggregation assays
753 are relatively cheap, easy to perform and results can be obtained in short periods of time.

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

757 **3.4.3 Quorum Sensing**

758 The biochemical process of cell to cell communication known as **quorum sensing (QS)**
759 plays an important role in initial cell attachment to surfaces and in the control of biofilm
760 growth. QS systems are also involved in polysaccharide synthesis, microbial adherence, cell
761 division and motility (Lazar, 2011). Molecular techniques such as **RT-PCR** have been used
762 to study the expression of genes involved in QS (e.g. the QscR regulon, gene HapR), but
763 mainly applied to pathogenic species such as *Pseudomonas aeruginosa* (Lequette *et al.*,
764 2006) and *Vibrio cholerae* (Liu *et al.*, 2007). Microscopy techniques such as **confocal laser**
765 **scanning microscopy (CLSM)** are also useful to monitor morphological changes in biofilms
766 and have been applied to study the formation of biofilms by pathogenic bacteria with
767 mutations in QS genes (Purevdorj *et al.*, 2002; Cole *et al.*, 2004; Huang *et al.*, 2009). QS
768 processes are directly involved in inhibition or promotion of biofilm growth, consequently a
769 better understanding of these mechanisms would contribute to control or prevent the negative
770 consequences of biofilm growth. Due to the complexity of interactions between signalling
771 molecules in multi-species biofilms, most QS research has been based on the study of a
772 limited number of bacterial species, isolated from model drinking water systems and
773 generally developed under laboratory conditions. As a consequence, the main drawback of
774 these coaggregation and cell-to-cell techniques is that they ignore the actual diversity of real
775 biofilms and the ecological complexity of DWDS. Future research should address this
776 complexity using new -omics technologies, for example combining metagenomics and
777 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 (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

803 (Fish, 2013). With extraction based processes more detail chemical-species analysis, via
804 PAGE gels and protein sequencing, can be obtained using the same EPS isolates as used for
805 evaluation of biochemical concentration. However, these techniques also require the sample
806 to be physically disturbed; consequently the localisation of different biochemical components
807 within the biofilm cannot be evaluated.

808 Non-invasive **microscopy techniques** offer a way to overcome some of the limitations of
809 extraction techniques, providing the possibility of monitoring, quantifying and visualising
810 cells and other biofilm components *in situ*, without perturbing their structure. **Confocal laser**
811 **scanning microscopy (CLSM)**, in combination with different fluorescent dyes, is a common
812 and useful approach for biofilm-related research. A range of fluorescent dyes can be used to
813 detect and quantity different biofilm components. Some of the most commonly used ones are:
814 DAPI for cells, Syto-60 and Syto-84 for nucleic acids, FITC and Sypro red for proteins, Nile
815 red for lipids, and concanavalin A (ConA) labelled with Alexa fluor 488 for carbohydrates
816 (Johnsen *et al.*, 2000; Yang *et al.*, 2006). The method based on the Green Fluorescent Protein
817 (GFP) is widely used to detect specific bacterial cell types within biofilms (Wouters *et al.*,
818 2010). To analyse confocal images different software is available such as DAIME (Digital
819 Image Analysis in Microbial Ecology) (Daims *et al.*, 2006), COMSTANT (Heydorn *et al.*,
820 2000) and IMARIS (Bitplaine, St Paul, MN) which are particularly helpful to analyse 3D
821 images and quantify Z-stacks (Hall-Stoodley *et al.*, 2008).

822 Fluorescent staining and CLSM have been successfully used to assess the EPS carbohydrates
823 and proteins of flocs (Schmid *et al.*, 2003), granules (McSwain *et al.*, 2005) and single-
824 species cultured biofilms (Chen *et al*., 2007; Shumi *et al.,* 2009). However, within a drinking
825 water context, the scope of CLSM analysis is generally limited to the study of cells and
826 carbohydrates, or targeting carbohydrates and proteins separately, using different samples
827 (Ivleva *et al.*, 2009). Wagner *et al.* (2009) analysed biofilms from a wastewater fed reactor

828 using two dual combinations of fluorophores to target carbohydrates/cells of one sample,
829 followed by the proteins/cells of another. Conversely, Fish *et al*., (2011) demonstrates the
830 application of a triple stain combination, CLSM and digital image analysis to concurrently
831 visualise and quantify the carbohydrates, proteins and cells, of multi-species biofilms from a
832 full scale DWDS. The use of fluorescent microscopy to study biofilms is restricted by the
833 excitation wavelengths of the lasers available at the given imaging facility and the
834 amalgamations of stains which can be separated using these laser combinations. While this
835 approach does not enable the detailed analysis of chemical-species possible with the
836 extraction processes, fluorescent staining and imaging techniques may be favoured as they
837 enable visual investigations of the 3D arrangement of the biochemical compounds, as well as
838 quantification.

839 Another useful technique to visualise biofilms is **scanning electron microscopy (SEM)**, this
840 has been used to obtain 3D images of biofilms on surfaces of drinking water networks
841 (Hammes *et al.*, 2011). However, the samples for SEM need to be processed (i.e. fixed,
842 dehydrated and coated with a conductive material) before they can be visualized which can
843 create artefacts or the partial destruction of the biofilm structure (Bergmans *et al.*, 2005).
844 Alternatively, **environmental scanning electron microscopy (ESEM)** may be used, for
845 which samples do not require processing, however, the maximum magnification obtained is
846 less than with conventional SEM (Donald, 2003). It should be noted that both SEM and
847 ESEM provide purely qualitative analysis, unlike CSLM or a technique termed **X-ray**
848 **photoelectron spectroscopy,** which provides direct chemical analysis of the surfaces of
849 microbial cells (Rouxhet *et al.*, 1994) and can be used to investigate environmental samples
850 under realistic conditions (Bluhm *et al.*, 2006). Techniques, such as **transmission electron**
851 **microscopy (TEM)** and **scanning transmission X-ray microscopy (STXM)** were
852 successfully used by Lawrence *et al.* (2003) to map the distribution of lipids, polysaccharides,

853 proteins, and nucleic acids within riverine biofilms. However, no references are available
854 regarding the application of these approaches to drinking water samples. Several studies have
855 also applied **fourier-transform infrared spectroscopy (FTIR spectroscopy)** to obtain *in*
856 *situ* biochemical and physiological information on biofilms and to monitor and map changes
857 during their development (Quiles *et al.*, 2010; Naz *et al.*, 2013). The main disadvantage of
858 FTIR is that biofilm samples need to be dried before they can be analysed, which can have
859 similar impacts upon sample integrity as the processing required for SEM.

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

872 Despite the array of technical advances in methods used to assess biofilm biochemical
873 composition or distribution, the processes driving the expression or production of different
874 cell biochemical components or how these are regulated at a genetic level, remain unknown.
875 Further research to fill this knowledge gap will be needed to understand, for example the
876 physiological differences of biofilm bacteria from that of their planktonic counterparts

877 (Karunakaran *et al.*, 2011) or the influence of environmental parameters upon their gene
878 expression and activity.

879 4. Application and integration to better inform understanding and management of 880 drinking water distribution systems

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

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

896 The methods presented here facilitate research aiming to quantify the microbial community,
897 evaluate microbial diversity and determine the potential function of those microorganisms
898 present (including the potential to harbour pathogens). Ultimately, this research offers an
899 assessment of the impact of the microbial ecology upon water quality and asset management
900 It is however important that such microbial research is conducted in an integrated manner, for

901 example there is a body of understanding concerning corrosion of cast iron pipes, driven by
902 consideration of structural performance including bursts and leakage, that relates to the
903 interface and includes consideration of microbial mediated corrosion. We need to strive to
904 integrate physical, chemical and biological understanding. It is important to note that the
905 techniques presented here are not always optimised for application to DWDS and that
906 development work is needed to yield valid and informative data. These techniques are also
907 often time and resource intensive, requiring careful consideration of the specific question(s)
908 to be explored and how the resulting data can be utilised, such as to inform modelling. It
909 should also be noted that often a range and/or combination of techniques need to be applied
910 in order to obtain the required knowledge. Careful consideration, planning and understanding
911 of the implications of sampling method and regime is also critical, as discussed earlier.

912 Applied research is needed that can move asset management strategies away from the idea of
913 a 'clean' pipe that can be maintained in perpetuity. It is generally accepted in most fields that
914 the complete eradication of biofilms is impossible. To our knowledge a suitable surface
915 entirely resistant to colonization of microorganisms does not exist. Even if such a material
916 were found, it is unlikely it would be suitable for retrofitting to the vast, ageing, deteriorating
917 infrastructure of DWDS. Hence, while a 'clean pipe' may be briefly achieved by highly
918 invasive, aggressive cleaning approaches, the pipe will be compromised as soon as potable
919 water is introduced, as a microbial community will establish, evolve and adapt over time. It is
920 important that we learn how to understand, predict and manage this community such that we
921 can estimate the risks to water quality that this community poses and can develop
922 interventions to control and manage that risk. This need to understand predict and manage
923 necessarily requires developing modelling tools to capture and extrapolate community
924 composition, behaviour, function and impacts. Such modelling will be essential to enable
925 extrapolation between bench top, pilot scale and real systems. It is impossible that we can

926 that we can sample and test for every combination of conditions and variables that exist in the
927 real world, hence modelling is essential. Ultimately it would be desirable for such models to
928 be driven by surrogate data rather than being reliant on the complex and expensive tools and
929 techniques presented here. Hence there is a need to develop such surrogates and for the
930 adoption of standard methods, these should not necessarily be driven by regulation, but by the
931 need for best asset management and service delivery through a risk based management
932 approaches.

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

939 **5. Conclusions and outlook**

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

944 Despite culture-dependent techniques still being used by water utilities to routinely monitor
945 the microbial quality of drinking water, molecular methods are replacing these and some
946 water companies are beginning to implement PCR-based approaches to detect pathogens. The
947 application of NGS has exceptionally enlarged the existing knowledge about the diversity and
948 structure of microbial communities in DWDS. The main sequencing platforms are constantly
949 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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1667 **Figure and tables**

1668 **Figure 1**: Scheme showing the different techniques available to characterise microbial communities in drinking water distribution systems

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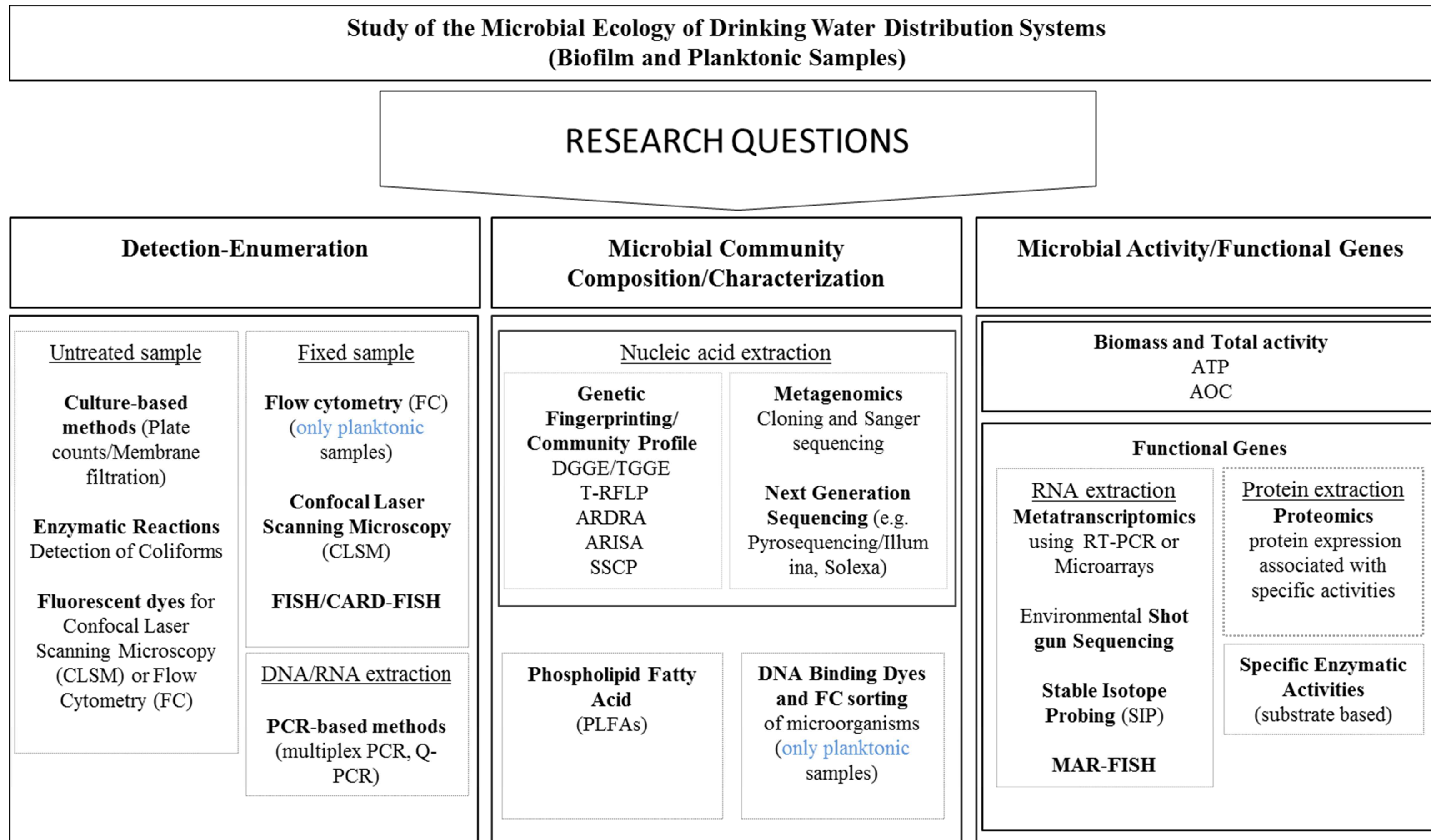

1670

1671 **Table 1**: Current molecular techniques to study microbial consortia and communities of

1672 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

1673

1674 **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 & Nielson, 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 & 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 cells	Lessie & van der Wijck, 1972; Frolund *et. al.*, 1995; McSwain *et. al.*, 2005
	DAPI [C]	Cannot differentiate between DNA present in cells or EPS	Jahn & Neilsen, 1995; Frolund *et. al.*, 1995
Quantification	TOC [D]	Commonly used to assess biomass and EPS amount, relatively quick and reliable	Jahn & 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-drying)	Samples are freeze-dried and weighed before being resuspsended in 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 & 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 & 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

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