**ON THE TRACK FOR AN EFFICIENT DETECTION OF *ESCHERICHIA COLI* IN
WATER: A REVIEW ON PCR-BASED METHODS**

Diana Mendes, Lucília Domingues ♦

CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal.

Diana Mendes (diana.mls@gmail.com)

Lucília Domingues (luciliad@deb.uminho.pt)

♦ **corresponding author:**

Lucília Domingues, Centre of Biological Engineering (CEB), University of Minho, 4710-057 Braga, Portugal

Email: luciliad@deb.uminho.pt

Phone: +351 253 604402

Fax: +351 253 678986

22 **ABSTRACT**

23 Ensuring water safety is an ongoing challenge to public health providers. Assessing the
24 presence of faecal contamination indicators in water is essential to protect public
25 health from diseases caused by waterborne pathogens. For this purpose, the bacteria
26 *Escherichia coli* has been used as the most reliable indicator of faecal contamination in
27 water. The methods currently in use for monitoring the microbiological safety of water
28 are based on culturing the microorganisms. However, these methods are not the
29 desirable solution to prevent outbreaks as they provide the results with a considerable
30 delay, lacking on specificity and sensitivity. Moreover, viable but non-culturable
31 microorganisms, which may be present as a result of environmental stress or water
32 treatment processes, are not detected by culture-based methods and, thus, may result
33 in false-negative assessments of *E. coli* in water samples. These limitations may place
34 public health at significant risk, leading to substantial monetary losses in health care
35 and, additionally, in costs related with a reduced productivity in the area affected by
36 the outbreak, and in costs supported by the water quality control departments
37 involved. Molecular methods, particularly polymerase chain reaction-based methods,
38 have been studied as an alternative technology to overcome the current limitations, as
39 they offer the possibility to reduce the assay time, to improve the detection sensitivity
40 and specificity, and to identify multiple targets and pathogens, including new or
41 emerging strains. The variety of techniques and applications available for PCR-based
42 methods has increased considerably and the costs involved have been substantially
43 reduced, which together have contributed to the potential standardization of these

44 techniques. However, they still require further refinement in order to be standardized
45 and applied to the variety of environmental waters and their specific characteristics.
46 The PCR-based methods under development for monitoring the presence of *E. coli* in
47 water are here discussed. Special emphasis is given to methodologies that avoid pre-
48 enrichment during the water sample preparation process so that the assay time is
49 reduced and the required legislated sensitivity is achieved. The advantages and
50 limitations of these methods are also reviewed, contributing to a more comprehensive
51 overview toward a more conscious research in identifying *E. coli* in water.

52

53 **Keywords:** *Escherichia coli*, water quality, PCR, rtPCR

54

1. INTRODUCTION

Water is frequently contaminated with human and/or animal feces (Ahmed et al., 2005; Davis et al., 2005; Gibson, 2014; Higgins et al., 2005; Scott et al., 2002; Walters et al., 2007), and the presence of faecal-derived pathogenic microorganisms (bacteria, virus, protozoa) in water is responsible for several infectious diseases (Straub and Chandler, 2003; WHO, 2005). Outbreaks of waterborne diseases remain a major challenge to public health providers worldwide, claiming millions of lives annually (Breathnach et al., 2012; Cabral, 2010; Cheun et al., 2013; Gelting et al., 2011; Jones et al., 2009; Pitkanen, 2013; Walser et al., 2012; Yang et al., 2011). The World Health Organization (WHO) estimates that 2.5 million people died with diarrheal disease in 2008 and that the number of cholera cases increased 85% in 2011, compared to 2010 (WHO, 2013). Although developing countries are particularly susceptible to these outbreaks due to their poor sanitation and water quality control, the developed countries are also affected by emerging pathogens resistant to water treatment, technological failure and/or inappropriate detection measures (Brettar and Höfle, 2008; de Man et al., 2014; Vale et al., 2009). Therefore, it is imperative to provide microbiologically safe water in order to protect Public Health.

It is not feasible to identify each one of the pathogens that can be found in water due to the excessive costs and labour involved. Instead, one or more microorganisms are chosen to indicate the possible presence of pathogens in water. Historically, many countries have been using faecal indicator bacteria, comprising total coliforms, faecal coliforms, *E. coli* and enterococci, as a monitoring tool to predict the presence of bacterial, viral and protozoan pathogens originated in faecal

78 contamination (Savichtcheva and Okabe, 2006). The European Commission (EC) (EC,
79 1998; EC, 2006; EC, 2009) the United States Environmental Protection Agency (USEPA)
80 (Agency, 2002; Boehm et al., 2009; Wade et al., 2003) and the WHO (WHO, 2011)
81 recommend the use of *E. coli* as the most effective available faecal indicator bacteria
82 for predicting the presence of pathogens in water intended for human consumption
83 and bathing water, and *Enterococcus sp.* as the most appropriate for marine waters
84 and as a useful indicator in bathing water as well (Bridle, 2013). *E. coli* is widely
85 accepted as an indicator bacteria (Khan et al., 2007) since it is a species of faecal
86 coliform bacteria that is specific to the intestines of humans and other warm-blooded
87 animals but not normally pathogenic, it is easy to detect and culture, and it is found at
88 higher concentrations than other pathogens in waters (Agency, 2002; WHO, 2011).
89 Moreover, its persistence in water and the effects of water treatment in cells viability
90 is similar to that of waterborne pathogens (Ahmed et al., 2005; Lemarchand et al.,
91 2004; McLellan et al., 2001). However, some studies have shown that *E. coli* is not a
92 reliable indicator of some pathogens since illness has been found in the absence of the
93 indicator microorganism. It has been shown that some pathogens are more resistant
94 to water treatment processes than *E. coli* and, consequently, faecal contamination may
95 not be detected, resulting in possible human exposure through drinking and
96 recreational waters (Cashdollar et al., 2013; Gregory et al., 2011; Sinclair et al., 2009).
97 Hence, the relationship between faecal indicator bacteria and the occurrence of
98 diseases may not be accurate in all the locations of sampling (Boehm et al., 2009;
99 Colford Jr. et al., 2012). Nevertheless, *E. coli* is still the microorganism that best
100 satisfies the faecal indicator microorganism criteria (Bridle, 2013).

101 Extensive research has been conducted to achieve flawless methods to detect
102 indicator microorganism(s), i. e., methods capable of detecting up to a single cell of the
103 indicator microorganism(s), providing fast and reliable results. Since the Pasteur era,
104 the microbiological quality of water has been monitored worldwide mainly through
105 officially approved culture-based methods. In the European Union, the detection of *E.*
106 *coli* in drinking water is described by ISO 9308-1 and is based in a membrane filtration
107 procedure, with an incubation period of 21 ± 3 h in TTC medium. This culture medium
108 is relatively non-selective, producing large numbers of false-positive results and is only
109 recommended for use with high quality waters (Fricker et al., 2008a). The detection of
110 *E. coli* in bathing water is described by ISO 9308-3 (or ISO 9308-1) and includes a 36 h
111 incubation time using a β -D-glucuronidase (enzyme encoded by the *uidA* gene) culture-
112 based method (EC, 2006). Despite the several advantages of enzymatic detection, lack
113 of ubiquity (ability to detect all strains of the targeted species) may still occur since the
114 enzymes are not found in all the strains of a target indicator (Boissinot and Bergeron,
115 2002; Clark et al., 2011). For example, the *uidA* gene is found in the majority of *E. coli*
116 strains, but some of them (e.g. the pathogenic *E. coli* O157:H7) do not exhibit the
117 corresponding enzymatic activity (Fricker et al., 2008b; Vale et al., 2009), which can
118 lead to false-negative results (Chao et al., 2004; Maheux et al., 2008; Martins et al.,
119 1993). Moreover, indicators may be severely underrepresented when injured by
120 disinfectants used in water treatment processes; these are known as viable but non-
121 culturable cells (VBNC) since they have lost the ability to form colonies (Juhna et al.,
122 2007; Keer and Birch, 2003; Rockabrand et al., 1999). Low specificity is also a problem,
123 as interference from other microorganisms can occur, resulting in false-positive
124 outcomes (Bej et al., 1991b; Clark et al., 2011; Fricker et al., 2008a; Omar et al., 2010;

125 Pisciotta et al., 2002; Sousa et al., 2010; Tryland and Fiksdal, 1998). For example, the
126 enzyme β -D-glucuronidase is found in several microorganisms, including some
127 *Salmonella* and *Shigella* strains, and some *Yersinia*, *Edwardia*, *Citrobacter* and *Hafnia*
128 strains. *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp. and *Clostridium* are
129 also capable of producing β -D-glucuronidase. To minimize interference from non-
130 target microorganisms, antibiotics and antifungals were included in the formulation of
131 several culture media, while other culture media (e.g. mFC agar) attribute its high
132 specificity to the high incubation temperature (44.5 °C) which limits the growth of non-
133 targeted microorganisms (Hakalehto et al., 2013; Maheux et al., 2014). Nevertheless,
134 several drawbacks (e.g. inability to detect VBNC microorganisms, lack of ubiquity, the
135 viability of the indicator microorganisms is not assured in the time between the sample
136 collection and the analysis, requirement of 18-24 h for detection and sometimes an
137 additional 24-72 h to confirm the positive results) have induced an increasing interest
138 in molecular techniques as a method to achieve a more efficient monitoring of the
139 microbiological water quality (Maheux et al., 2014; Maheux et al., 2012; Maheux et al.,
140 2008; Oliver et al., 2014).

141 This review aims to provide an outlook of the accomplishments achieved in the
142 adaptation of PCR-based methods to the detection of *E. coli* as part of the
143 microbiological water quality monitoring, and their capability to fulfil the legislated
144 requirements. Emphasis is given to water intended for human consumption and to
145 bathing water.

146

2. PCR-BASED METHODS

Several of the molecular-based techniques rely on protocols that amplify and detect nucleic acids. These methods, specifically those based on the PCR technique, amplify *in vitro* specific segments of the genome from the indicators or pathogens (DNA or RNA) (Girones et al., 2010). The advantages of these methods over culture-based techniques are due to the possibility of achieving a higher level of specificity and sensitivity on a faster period of time, without the need for complex cultivation or confirmation steps, although they are not yet established enough in the EU for regulatory monitoring (Girones et al., 2010; Oliver et al., 2014). Moreover, both culturable and non-culturable microorganisms can be detected with this technology within hours (Rompré et al., 2002).

The microorganisms are often disperse in a large volume of water: the water sample volume regulated by the European Commission Directives for assessing the microbiological water quality is of 100 mL (water intended for human consumption and bathing water) and of 250 mL (bottled water) (EC, 1998; EC, 2006; EC, 2009). Therefore, it becomes necessary to include in the assay a water sample preparation step to concentrate the microorganisms (and, in some cases, to reduce contaminants) before performing the molecular analysis, ideally to the template volume used in the molecular method (in PCR-based methods, it is usually used a volume of 1 or 2 μ L) (Girones et al., 2010; Hahn and Lapaire, 2013; Pitkanen, 2013; Ram et al., 2011). A pre-enrichment step may be performed before the molecular analysis to increase the amount of the target microorganisms in the water sample. Despite the outstanding

169 advantages of PCR-based techniques over classical methods, in what concerns water
170 quality monitoring, it is essential to reduce the time to provide the results and, thus,
171 reduce health care costs and protect public health. Therefore, it is essential to remove
172 the pre-enrichment step from the water sample preparation to reduce the assay time.
173 This comprises the major challenge in the adaptation of PCR-based techniques to
174 water quality analysis, since it directly influences the sensitivity of the method. Given
175 the high importance of the water sample preparation procedure to the successful
176 adaptation of PCR-based methods in the detection of microorganisms in water, some
177 of the techniques developed with this purpose are described in the next section.

178 **2.1 – WATER SAMPLE PREPARATION**

179 In what concerns water quality monitoring, the sensitivity of detection, time of
180 analysis, volume assayed and the presence of inhibitory substances in the water are
181 critical factors for implementing molecular methods (Higgins et al., 2001; Noble and
182 Weisberg, 2005). Depending on the final purpose of the analysis, different paths can
183 be chosen when designing a new water sample preparation protocol, as including or
184 not a pre-enrichment step, extracting the nucleic acids or performing a direct
185 detection by PCR, among others. An overview of the different paths that can be
186 adopted for water sample preparation is exemplified in Figure 1. The main challenge is
187 to avoid culturing the microorganisms and, thus, reduce the assay time in order to
188 increase the competitive potential of PCR-based methods over the culture-based
189 methods currently available.

190 After collecting the water samples, the water sample preparation procedures
191 for PCR-based analysis usually comprise three demanding steps: concentration of the
192 target organism (e.g. filtration, centrifugation), extraction of the nucleic acids (e. g.
193 heat-shock; chemical, mechanical and/or enzymatic lysis) and purification (e. g.
194 chemical precipitation, solvent extraction, magnetic separation) to eliminate possible
195 inhibitors (Jofre and Blanch, 2010). Therefore, an ideal water sample preparation
196 protocol must efficiently release the nucleic acids from the indicator, protect them
197 from degradation, eliminate or neutralize the inhibitors of the amplification reaction
198 and provide the necessary analytical sensitivity (Boissinot and Bergeron, 2002).

199 Environmental water samples are naturally diluted (Quilliam et al., 2011), which
200 implies a small concentration of the target microorganisms in a large volume of water.
201 The legislation states that volumes of 100 mL (water intended for human consumption
202 and bathing water) and 250 mL of water (bottled water) are to be analyzed; however,
203 PCR-based technologies typically use small volumes of template (usually, 1 or 2 μ L),
204 which inevitably arises the problem of insufficient sensitivity due to the difficulty to
205 concentrate all the microorganisms in the water sample 100.000-fold into the PCR
206 reaction tube without loss of microorganisms (EC, 1998; EC, 2006; EC, 2009; Noble and
207 Weisberg, 2005). This problem is usually minimized by including a concentration step
208 that increases by several times the number of target microorganisms in a gradually
209 smaller volume of water sample (Noble and Weisberg, 2005; Quilliam et al., 2011).

210 In what concerns nucleic acid extraction and purification procedures, an
211 impressive variety of commercial solutions are available (e.g. GenElute™ Bacterial
212 Genomic DNA, Sigma-Aldrich; QIAamp DNA Mini Kit, Qiagen; PowerWater® DNA

213 Isolation Kit, Mo Bio Laboratories), but these are only efficient in the presence of a
214 considerable amount of the target microorganisms due to a poor extraction recovery
215 efficiency at lower cell concentrations; moreover, they are frequently inhibited by
216 contaminants present in the water samples and are usually developed for low sample
217 volumes, which challenges the requirement to identify up to only 1 cell of the indicator
218 in the water sample (Clark et al., 2011; Jiang et al., 2005).

219 The complexity and composition of environmental water samples can challenge
220 the accurate detection of the indicator microorganisms by PCR-based techniques
221 (Green and Field, 2012). Suspended contaminants such as sediments, humic acids,
222 organic and inorganic compounds, cellular debris and heavy metals can interfere with
223 DNA extraction, reduce the recovery of target microorganisms and nucleic acids, and
224 inhibit polymerase activity which may lead to an underestimation of the indicator's
225 concentration (Green and Field, 2012; Noble and Weisberg, 2005; Rompré et al., 2002;
226 Schriewer et al., 2011). Moreover, many of these inhibitors have solubility properties
227 similar to those of the nucleic acids (Schriewer et al., 2011) and, as a consequence,
228 they are concentrated along with the nucleic acids (Noble and Weisberg, 2005), not
229 being removed from the water sample during the preparation steps (Lemarchand et
230 al., 2004). In these cases, a purification step could be included in order to reduce
231 inhibition of the polymerase activity. However, some of the reagents used in DNA
232 purification protocols (e.g. ethanol, isopropanol) may be difficult to eliminate from the
233 water sample and may cause inhibition of the amplification as well (Green and Field,
234 2012). Other approaches to reduce interference from the contaminants in the
235 detection include water sample dilutions when the target gene is present in high copy
236 numbers, the addition of facilitating molecules (e.g. bovine serum albumin - BSA) and

237 the use of designed polymerases (Baar et al., 2011; Schriewer et al., 2011).
238 Nevertheless, dilutions can increase variation in measured targets, and lead to false-
239 negatives when contaminants cannot be diluted to levels below interference or when
240 the targets are diluted to below the detection limit (Schriewer et al., 2011). Although it
241 was observed a reduction in the interference when BSA is used in the assay, it was
242 observed that the target's amplification efficiency can be reduced, cycle threshold (CT)
243 values can be increased and melting temperature values can be altered (Jiang et al.,
244 2005). Despite the developments achieved, target detection interference still occurs
245 and other methods were developed to identify interference, such as adding an internal
246 amplification control (non-target DNA sequence) used to identify false-negative
247 results (i. e., a negative result even though the water sample is contaminated) or
248 reduced amplification efficiency (i. e., a positive result with an error in quantifying the
249 concentration of the microorganisms in the water sample) due to polymerase
250 inhibition (Maheux et al., 2013; Schriewer et al., 2011; USEPA, 2013).

251 Numerous molecular procedures (Higgins et al., 2001; Ibekwe et al., 2002; Liu
252 et al., 2008; Sen et al., 2011; Takahashi et al., 2009) still include a culture step before
253 extracting the genetic material of *E. coli* to increase the number of cells in the water
254 sample. In recent years, some authors (Bertrand and Roig, 2007; Clark et al., 2011;
255 Heijnen and Medema, 2009; Horáková et al., 2008a; Horáková et al., 2008b; Khan et
256 al., 2007; Maheux et al., 2013; Maheux et al., 2011a; Maheux et al., 2011b; Mull and
257 Hill, 2009; Omiccioli et al., 2009; Patel et al., 2011; Ram et al., 2011; Takahashi et al.,
258 2009; Wolffs et al., 2006; Zhang et al., 2012) started pursuing the highest sensitivity
259 without a pre-enrichment step by using different strategies. Some strategies resulted

260 in interesting results (concerning the sensitivity, time, environmental sampling) and
261 are described as follows.

262 • Clark et al. (2011) tested ten different methods for recovering
263 waterborne bacterial pathogens, combining membrane filtration and
264 centrifugation to concentrate the cells, with mechanical, chemical and enzymatic
265 lysis techniques for nucleic acid extraction, providing results in 12 h with 73.3%
266 of recovery. Purification was performed using a commercial kit, but inhibitors
267 were not completely removed.

268 • An interesting and innovative procedure was developed by Maheux et
269 al. (2011), designated as CRENAME (concentration and recovery of microbial
270 particles, extraction of nucleic acids, and molecular enrichment) (Maheux et al.,
271 2011a; Maheux et al., 2011b). This method employed filtration and
272 centrifugation to concentrate cells, and the membrane was dissolved to recover
273 cells that could be trapped among the cellulose fibers. DNA was extracted by
274 bead beating and was amplified using a whole-genome amplification (WGA) kit,
275 increasing the total DNA amount available for analysis and, thus, increasing the
276 sensitivity. The whole procedure allowed a detection of 4.5 enterococcal and 1.8
277 *E. coli/Shigella* CFU per 100 mL of potable water in less than 5 hours.

278 • A simple protocol based on a repeated centrifugation of large volumes
279 of water samples was developed by Ram et al. (2011), where DNA was extracted
280 by boiling, and detection was carried out by rtPCR. A sensitivity of 100 genomic
281 equivalents (GE) per mL, which refers to the DNA amount needed to ensure that

282 one copy of the full genome of the organism is present, was achieved in less than
283 2 h.

284 • Heijnen and Medema (2009) accomplished a detection limit of 1 viable
285 CFU of *E. coli* in 100 mL of water through a complex procedure that included
286 filtration, heat-shock and several extraction reagents, as well as magnetic
287 extraction. Despite its complexity, the method was performed in 3-4 h.

288 • Mull et al. (2009) and Zhang et al. (2012) stated that the membrane
289 filtration of turbid surface waters can be limited due to filter clogging, since
290 organic matter and colloids are a common presence in water (Mull and Hill,
291 2009; Zhang et al., 2012). To avoid this problem, Mull et al. (2009) suggested
292 hollow-fiber ultrafiltration as a primary step for concentrating microorganisms
293 and approximately 50 CFU could be recovered from a 40-liter surface water
294 sample. However, there is no indication of the length of this protocol, and, since
295 it includes an incubation period of 20 h to 24 h, it has a disadvantage over other
296 faster methods (Mull and Hill, 2009). Zhang et al. (2012) developed a chemical
297 flocculation protocol. *E. coli* cells were captured and concentrated inside the
298 flocs by a lanthanum-based flocculation, which were dissolved with EDTA,
299 filtered and, after extraction, DNA was detected by rtPCR. This method was able
300 to detect 15 cells of *E. coli*/mL in raw and finished water (Khan et al., 2007).

301

302 Difficulties still prevail, particularly when these techniques are applied to
303 environmental water samples, since it is not yet possible to completely eliminate the
304 losses of target cells and/or genetic material, nor to remove the inhibitors during the

305 sample preparation procedures. The most significant technical problems associated
306 with water sample preparation are listed in Table 1. Nevertheless, remarkable
307 improvements, as those mentioned above, have been accomplished in the recent
308 years, providing attractive results concerning sensitivity and time.

309

310 **2.2 – AMPLIFICATION TECHNIQUES**

311 Polymerase chain reaction is a technique used to exponentially amplify (copy) a
312 segment of DNA or RNA, generating thousands to millions of copies of this particular
313 section (Caetano-Anollés, 2013; Hoy, 2013). This method is based on thermal cycling, a
314 method in which a solution containing the genetic material is repeatedly heated and
315 cooled, causing the enzymatic replication of the specific segment of the DNA or RNA
316 (Caetano-Anollés, 2013). A typical PCR reaction essentially requires the presence of the
317 DNA sequence to be amplified, a set of primers, nucleotides and a DNA synthesis
318 enzyme (e.g. Taq DNA polymerase). Each reaction typically involves 20 to 40 cycles,
319 divided in three steps of different temperatures for DNA denaturation, primer
320 annealing and DNA synthesis.

321 The selectivity of the PCR technique is originated in the use of a primer set that
322 is complementary to the DNA region targeted for amplification under specific thermal
323 cycling conditions. The exponential amplification of the target sequence significantly
324 increases the probability of detecting a rare sequence or relatively low numbers of
325 target microorganisms in a sample (Bej et al., 1990; Rompré et al., 2002). For most of
326 the published PCR assays, the target DNA sequences for the detection of *E. coli* and
327 total coliforms are the genes coding the same enzymes identified in the culture-based

328 methods: β -D-glucuronidase (*uidA* gene) and β -D-galactosidase (*lacZ* gene),
329 respectively (Bej et al., 1991a; Bej et al., 1991b; Bej et al., 1990; Farnleitner et al.,
330 2001; Rompré et al., 2002). The distinction here is that PCR-based methods can detect
331 and identify *E. coli* strains that carry the *uidA* gene but do not exhibit β -D-
332 glucuronidase activity, whereas culture-based methods cannot. This is particularly
333 important considering that one of the strains that remains undetected by culture-
334 based methods is the pathogenic *E. coli* O157:H7, which was recently the cause of
335 outbreaks in Europe (Frank et al., 2011; Friesema et al., 2008). On the other hand, *uidA*
336 and *lacZ* genes are not exclusive to *E. coli* spp., and can be found in other closely
337 related bacteria (e.g. *Salmonella* sp.) (Feng et al., 1991; Rice et al., 1990; Rompré et al.,
338 2002). Therefore, primers targeting other genes were studied for these indicators to
339 increase specificity (ability to target only the desired species) and ubiquity (ability to
340 detect all strains of targeted species) (Boissinot and Bergeron, 2002; Maheux et al.,
341 2008). Maheux et al. (2009) compared nine PCR primer sets targeting other genes
342 (including the *uidA* gene) that were designed to detect *E. coli* and *Shigella* in water
343 (Maheux et al., 2009). Traditionally, *E. coli* and *Shigella* have been considered as two
344 different genera, but more recently some authors introduced the idea that *Shigella* is,
345 in fact, “*E. coli* in disguise” (Lan and Reeves, 2002) and that genetically they belong to
346 the same species (Lan and Reeves, 2002; Maheux et al., 2009; Paradis et al., 2005; Zuo
347 et al., 2013). Therefore, Maheux and colleagues (Maheux et al., 2009) tested the ability
348 of the primers to identify both *E. coli* and *Shigella*, and concluded that only the primers
349 targeting the *tuf* gene were able to detect all the strains of both microorganisms.
350 However, *tuf* gene was also identified in *Escherichia fergusonii* and, therefore, none of

351 the primers was totally ubiquitous and should be selected according to the purpose of
352 the analysis.

353 The PCR-based method has often been described for the detection and
354 identification of microorganisms in foods, soils, sediments and waters (Ghosh and
355 Bodhankar, 2012; Green and Field, 2012; Khan et al., 2007; Pitkanen, 2013; Staley et
356 al., 2013). Several adaptations of the standard PCR protocol have been developed for
357 the detection of the indicator microorganisms in water samples. In spite of the
358 reported success in the studies presented in Table 2, common limitations were
359 identified as challenges to be overcome. Pathogens are naturally at low number in
360 environmental samples due to the dilution effect (Quilliam et al., 2011), which leads to
361 one of the most significant difficulties: the inefficient recovery of all the
362 microorganisms present in the water sample, which is necessary to achieve the
363 required sensitivity of a single cell. Moreover, the template volumes used in PCR-based
364 assays are of only a few microliters, being mandatory to apply a concentration step
365 before the nucleic acids extraction. A purification procedure is also advised to reduce
366 or, preferably, eliminate the inhibitors naturally present in environmental samples
367 (Girones et al., 2010). These three main steps (concentration, extraction and
368 purification) bring, as a consequence, the loss of a considerable amount of the target
369 cells and/or nucleic acids, due to the complexity of the procedures and the excessive
370 manipulation of the water samples. In Table 3, the main advantages and difficulties
371 observed in the several research works were summarized for a more comprehensive
372 perspective.

373 The different methods that have been explored to detect *E. coli* and
374 diarrheagenic *E. coli* in water samples were Multiplex-PCR (Gómez-Duarte et al., 2009;
375 Horáková et al., 2008a; Lang et al., 1994) RT-PCR (Liu et al., 2008), rtPCR and qrtPCR
376 (Chern et al., 2011; Heijnen and Medema, 2009; Khan et al., 2007; Patel et al., 2011;
377 Ram et al., 2011; Ram et al., 2008; Sen et al., 2011; Zhang et al., 2012), NASBA rtPCR
378 (Heijnen and Medema, 2009), and Multiplex rtPCR (Ibekwe et al., 2002; Maheux et al.,
379 2011a; Mull and Hill, 2009). Some of these adaptations of the standard PCR protocol
380 are here described with further detail:

381 • **Real-time PCR (rtPCR)** is based on the cycling principle of standard PCR with
382 the difference that successful amplification at each cycle is monitored by the
383 release of a fluorescent signal, which results in the possibility to follow the
384 detection in real time (Hahn and Lapaire, 2013). Moreover, measuring the
385 intensity of the fluorescent signal at the end of each cycle allows a quantitative
386 assessment of the initial concentration of the target in the reaction tube, which
387 can be achieved by employing a standard curve, and is designated by
388 quantitative real-time PCR (qrtPCR) (Hahn and Lapaire, 2013). rtPCR-based
389 technologies have emerged in recent years as a leading technology for rapid
390 detection of microorganisms due to their high degree of sensitivity and
391 specificity, introducing the possibility of a much faster detection of the target
392 microorganism in real time, with no need for additional time to detect the
393 rtPCR products by electrophoresis (Mackay, 2007; Quilliam et al., 2011).
394 Furthermore, quantification of the target has become possible by qrtPCR (Hahn
395 and Lapaire, 2013). Khan et al. (2007) used rtPCR in the detection of *E. coli* from
396 agriculture watersheds by employing newly designed species-specific

397 oligonucleotide primers derived from conserved flanking regions of the 16S
398 rRNA gene, the internal transcribed spacer region (ITS) and the 23S rRNA gene
399 (Pérez-Luz et al., 2004). This analysis led to the development of the first rtPCR
400 assay in the ITS region for detecting and enumerating *E. coli*, as an attempt to
401 solve the limitations of other primers normally used (for *lacZ*, *lamB*, *uidA*, *malB*
402 genes), which are not totally specific for *E. coli*, or are of insufficient ubiquity
403 (Boissinot and Bergeron, 2002; Khan et al., 2007). However, no specificity
404 assessment was performed in this research to confirm the advantage of these
405 primers over others.

406 An appealing sensitivity was achieved with the *Enterococcus* CRENAME-rtPCR
407 and *E. coli/Shigella* CRENAME-rtPCR methods (Maheux et al., 2013; Maheux et
408 al., 2011a; Maheux et al., 2011b). These authors used molecular enrichment
409 (WGA) as an alternative to culture enrichment. Performing an unspecific
410 amplification of nucleic acids enabled improvements in the sensitivity within a
411 significantly reduced period of time (3 h), when compared to culturing and
412 other PCR-based methods. Nevertheless, this unspecific amplification of the
413 genetic material makes it impossible to quantify the amount of the target
414 originally present in the sample. A more comprehensive evaluation of the
415 adaptability of this method to other water samples, such as polluted river water
416 and seawater, as well as its behavior in the presence of higher levels of
417 inhibitors, should be carried out in order to assess its adaptability to water
418 quality monitoring.

419 The USEPA is implementing two qrtPCR methods for recreational beach water
420 monitoring, capable of detecting as little as 31 cells per 100 mL, using Enterococci
421 as the indicator microorganism: Method 1611 and Method 1609 (Gonzalez and
422 Noble, 2014; USEPA, 2012; USEPA, 2013). A specific region of the large subunit
423 ribosomal RNA gene (lsrRNA, 23S rRNA) of these microorganisms (Haugland et
424 al., 2014; Sivaganensan et al., 2014; USEPA, 2012) is the amplification target.
425 These methods are initiated by filtering the water samples through a
426 membrane filter, the membrane is placed in a microcentrifuge tube containing
427 glass beads and buffer, and then shaken at high speed to extract the nucleic
428 acids into solution. The supernatant (5 µL) is used for qrtPCR amplification
429 where the Enterococci target DNA sequences are detected using either the
430 TaqMan[®] Universal master mix PCR reagent and TaqMan probe system
431 (Method 1611), or the TaqMan[®] Environmental master mix PCR reagent and
432 probe system (Method 1609) (USEPA, 2012; USEPA, 2013). Method 1609
433 includes an internal amplification control (Salmon DNA as the non-target DNA
434 sequence) that is added to each qrtPCR analysis and is co-amplified
435 simultaneously with the Enterococcus target sequence to identify the
436 occurrence of polymerase inhibition during amplification (USEPA, 2013). The
437 advantage of both methods over currently accepted culture-based methods is
438 to provide results within 3-4 hours of elevated levels of bacteria, allowing
439 same-day notification of recreational water quality. However, these methods
440 still have a limited experience with the performance in a broad range of
441 environmental conditions, and have been tested in a limited number of sites to
442 date (Sivaganensan et al., 2014). Therefore, the USEPA advises to perform site-

specific analysis of the method's performance before being used for beach notification programs (Sivaganensan et al., 2014). In addition, it was observed a considerable interlaboratory variation in the results, as well as significant variation when different lots or sources of commercial preparations or laboratory-grown cultures, or when inhibitors are present in the water samples (Haugland et al., 2014). To sum up, these methods provide fast results of a contamination with a considerable amount of microorganisms and can enable a quick response to protect public health but need further validation. In another embodiment, the USEPA developed a PCR-based method for detecting the pathogenic *E. coli* O157:H7 in water samples by culturing the microorganisms in either selective or non-selective media, followed by biochemical characterization and serological confirmation (USEPA, 2010). Broth cultures may be subjected to rtPCR confirmation in place of biochemical and serological confirmation. Although this procedure requires several days to provide the results, the rtPCR technique is efficiently employed to confirm the presence of *E. coli* O157:H7.

Multiplex-PCR/rtPCR is rather useful as it allows the simultaneous detection of different gene sequences and/or different microorganisms through the introduction of different primer-pairs in the same reaction tube (Velusamy et al., 2010). Horakova et al. (2008) developed a multiplex-PCR method for reliable detection of *E. coli* isolated from water samples, which enables the differentiation from biochemically and phylogenetically related bacteria (Horáková et al., 2008a). To improve the specificity of the PCR-based method for *E. coli* identification, four target genes were used: *uidA*, *lacZ*, *lacY* (coding for lactose permease) and *cyd* (coding for cytochrome bd complex) genes,

467 whose products could be considered as biochemical hallmarks of *E. coli* spp.
468 (Horáková et al., 2008a). The four fragments were observed only on *E. coli*
469 strains, including those that did not exhibit β -D-glucuronidase, and not on other
470 close relatives. In addition to the identification of several genes of one specific
471 target, multiplex enables the possibility of detecting different microorganisms
472 in the same analysis. Garrido and coworkers (2013) developed a multiplex
473 rtPCR for the simultaneous identification of *Salmonella* spp., *Escherichia coli*
474 O157 and *Listeria monocytogenes* in food and environmental, natural and
475 spiked samples. Since this procedure involves culturing the microorganisms, it
476 was obtained a very low limit of detection (5 CFU/25 g) for a simultaneous
477 detection of these three pathogens (Garrido et al., 2013). This work brings
478 evidence of the potential of a multiplex-rtPCR in the simultaneous detection of
479 the indicator and other microorganisms (e.g. pathogens and/or other
480 indicators).

481 • **Nested PCR** comprises two consecutive rounds of PCR amplification, being
482 the second round to increase the PCR product to detectable levels (Tallon et al.,
483 2005). The first primer set is used to amplify a sequence which will serve as a
484 template for the second amplification performed with a second primer set (Shi
485 et al., 2010). Juck et al. (1996) used this method targeting the *uidA* gene of *E.*
486 *coli* by designing two sets of primers: the first pair produced an amplicon of 486
487 bp that served as template for the second primer pair, which resulted in a
488 fragment of 186 bp (Juck et al., 1996).

489 PCR-based technologies are used to determine whether the target DNA is
490 present in the sample or not, being unable to provide information about the viability of
491 the microorganisms, a key factor for their pathogenicity. When released in water, the
492 microorganisms often enter in a VBNC state due to starvation or inefficient water
493 treatment procedures (Girones et al., 2010). Even when the water treatment is
494 successful and is able to completely eradicate the microorganisms, DNA remains stable
495 after cell-death, which may result in the detection of both viable and dead cells,
496 becoming difficult to conclude about the safety of the water tested. It is also frequent
497 to encounter the PCR reagents contaminated with trace amounts of DNA from the
498 target microorganism, as the recombinant polymerases used are frequently produced
499 in *E. coli*. Therefore, assessing the viability of the indicators is an absolute prerequisite
500 for the application to water quality monitoring and to fulfill the requirements of the
501 legislation (Gensberger et al., 2013). Some adaptations of the PCR-based technology
502 have been introduced as a way to distinguish viable from dead cells:

503 • **Reverse Transcriptase PCR (RT-PCR)** is a two-stage process: a target
504 messenger RNA (mRNA) sequence is first transcribed into a complementary
505 DNA (cDNA) sequence, which subsequently can be directly amplified by PCR
506 (Keer and Birch, 2003). Liu et al. (2008) applied this method to river water (Liu
507 et al., 2008). Samples were filtered with a low-protein-binding membrane, RNA
508 was extracted and purified directly. Target *E. coli* O157:H7 was detected using a
509 combination of RT-PCR and electronic microarray, with a sensitivity of 50 VBNC
510 cells in 1 liter of river water (the duration of the assay is not referred). It is
511 known that mRNA has a very short half-life and is able to provide a much better
512 association with the viability of the cell. Even though mRNA has a potential

513 advantage for distinguishing viable from non-viable cells, the complexity of the
514 method and practical difficulties in extracting detectable levels of intact mRNA
515 from only a few cells can be a serious drawback (Keer and Birch, 2003;
516 Lemarchand et al., 2004).

517 Ribosomal RNA (rRNA) has also been studied as a possible target of detection
518 (Chern et al., 2011; Keer and Birch, 2003; Lemarchand et al., 2004), since it is
519 more abundant and easier to detect than mRNA. However, its longer half-life
520 and instability makes it a weaker option.

521 • **Nucleic Acid Based Sequence Amplification (NASBA)** is an improvement of
522 RT-PCR, as it enables selective amplification of a RNA fragment, without
523 interference of background DNA (Keer and Birch, 2003). Heijnen and Medema
524 (2009) developed a real-time NASBA (NASBA-rtPCR) for the detection of *E. coli*
525 in water samples using a molecular beacon probe for the amplification of a
526 fragment of mRNA coding for the *clpB* heat shock protein (Heijnen and
527 Medema, 2009; Min and Baeumner, 2002). Several inactivation procedures
528 (starvation, chlorine treatment, UV-irradiation and chlorine) were used to test
529 the correlation between culturability and the ability to detect *E. coli* with
530 NASBA (Whitman et al., 2004). A 100% specificity of the NASBA assay was
531 demonstrated in the tested strains and a sensitivity of 1 viable *E. coli*/100 mL
532 was determined using serially diluted spiked tap water samples. Moreover, a
533 good correlation was observed between the number of colonies on the culture
534 plates and the results obtained with NASBA. It should be taken into
535 consideration that the VBNC cells do not form colonies on the culture plates

536 but can interfere in the NASBA detection and, thus, alter that correlation. With
537 an assay time of 3-4 h, this study has demonstrated that the NASBA method
538 has potential as a rapid test for microbiological water quality monitoring.

539 • **Propidium monoazide rtPCR (PMA-rtPCR)** has been introduced recently as
540 an alternative method to distinguish viable from dead cells. Propidium
541 monoazide (PMA) is a DNA intercalating dye that, when photo-activated, forms
542 a stable covalent bond with DNA, thus resulting in permanent DNA
543 modification (Xing-Long et al., 2013). This dye is cell membrane-impermeable,
544 which implies that live cells with intact membranes can exclude PMA, and free
545 DNA or the DNA from cells with the membrane integrity compromised are
546 irreversibly modified (preceding the PCR amplification) (Gensberger et al.,
547 2013). As a result, a selective PCR amplification of the DNA from only live cells
548 occurs. This method has proven to be effective in evaluating the sterilization
549 rate of water (Xing-Long et al., 2013) and in significantly reducing the false-
550 positive signal from the DNA amplification of dead cells, even in a background
551 of a highly abundant and complex microflora (Gensberger et al., 2013).
552 However, matrices with high solid content can hamper the detection (Bae and
553 Wuertz, 2009).

554 Table 2 assembles some of the PCR-based techniques reported in the literature
555 as methods to assess the microbiological quality of water, selected either due to the
556 interesting approaches used in the development of a new protocol, or for the
557 promising results obtained. The technology applied, microorganism and type of

558 sample, whether a pre-enrichment step is included or not, and the length of the whole
559 procedure is indicated.

560 On the whole, many improvements and adaptations of PCR-based methods
561 have been published, reinforcing the high potential of these techniques in the
562 detection of microorganisms, particularly when it is possible to include a pre-
563 enrichment step. In addition to the increased sensitivity and specificity, PCR-based
564 methods offer the possibility to test multiple targets in the same analysis (not only the
565 indicators), to detect the pathogenic *E. coli* O157:H7 and VBNC cells, which are not
566 identified by culture-based methods, and to reduce the assay time. However, several
567 assays were performed using samples of clean water spiked with cultured strains of
568 bacteria, or using simple dilutions of DNA. Even though this procedure is the first step
569 in developing a valid protocol for the detection of microorganisms, a follow-up step
570 must be taken in environmental water samples, so that the developed methods can be
571 completely validated and its applicability range determined.

572 Another key issue is the observed variability in molecular detection. It was
573 shown that the extremely low concentrations of target microorganisms in water
574 samples can cause high unpredictable fluctuations in the PCR efficiency, which leads to
575 tube-to-tube variability and, thus, to false-negatives (Jofre and Blanch, 2010). Another
576 cause of variability concerns the water composition differences between samples,
577 place of sampling, atmospheric conditions (e.g. rain drags faeces from the streets to
578 the wells, rivers, beaches) and temperature variations throughout the day (Heaney et
579 al., 2014; Kozuskanich et al., 2011). Additionally, extracellular DNA that persists in the
580 environment can affect the detection, increasing the risk of false-positives (Lavender

581 and Kinzelman, 2009). However, Maheux et al. (2011b) noticed that free DNA in
582 drinking water could flow through the filter during the filtration step without
583 interfering with the detection protocol. Thus, the detection of microorganisms in
584 environmental water samples requires the adjustment and optimization of the
585 protocol to each type of water and place of sampling, since the contaminants
586 (inhibitors and background DNA), the concentrations and types of indicators, the
587 weather and other variables differ from location to location (Boehm et al., 2009;
588 Lavender and Kinzelman, 2009; Shanks et al., 2011; Whitman et al., 2010).
589 Standardization of these methods is, therefore, a challenge for a routine use, since
590 accuracy and reproducibility are still hindered by these problems. Some studies
591 concerning other indicators, with the purpose to achieve standardization and
592 optimization for specific locations, are already exhibiting some promising results
593 (Ashbolt et al., 2010; Benedict and Neumann, 2004; Haugland et al., 2005; Mulugeta et
594 al., 2012; Shanks et al., 2011; Whitman et al., 2010).

595 PCR-based methods have been proving their utility not only in detecting water
596 contamination, but also in identifying the source of contamination and in predicting *E.*
597 *coli* in water. The current methods for microbiological water quality monitoring offer
598 no information about the originating sources of faecal pollution, which is a valuable
599 knowledge needed to characterize pollution and make decisions (Shanks et al., 2014).
600 Microbial source tracking (MST) describes methods and research strategies to identify
601 faecal pollution sources in ambient waters based in the association of distinct faecal
602 microorganisms with a particular host (Harwood et al., 2014). For example, a qrtPCR
603 method targeting *Bacteroides dorei*, a human-associated genetic marker, was
604 developed with the purpose to expand the use of PCR-based methods to identify

605 human faecal pollution in ambient water samples (Shanks et al., 2014). In another
606 embodiment, predictive empirical regression models (based on wind speed/direction,
607 rainfall, etc.) have been studied as a method to estimate the faecal indicator density
608 for a particular day (Ashbolt et al., 2010). Therefore, a quantitative microbial risk
609 assessment (QMRA) modeling of faecal indicator microorganisms is suggested as an
610 approach to simulate and explore the diversity of scenarios for hydrological events and
611 faecal contamination (Ashbolt et al., 2010).

612 In conclusion, PCR-based methods are promising tools to provide sensitive,
613 rapid and quantitative analysis for the detection of the indicators currently used in
614 microbiological water quality monitoring, VBNC cells, and new emerging pathogens
615 and indicators. Currently, USEPA has qrtPCR-based methods (Method 1611 and
616 Method 1609) with adequate sensitivity to be implemented for microbiological water
617 quality monitoring in bathing water. Nevertheless, the currently available or under
618 development techniques still require further improvement before being standardized
619 and adapted to the different characteristics of environmental samples of water, each
620 comprising its own particularities.

621

3. FUTURE PERSPECTIVES

Numerous approaches in the detection of indicator microorganisms and pathogens are being deeply explored with the purpose of efficiently identifying harmful contaminations in water.

PCR-based methods have shown immense potential in the characterization and recognition of targets, identifying non-culturable microorganisms and providing information concerning the presence of indicators and/or pathogens in water. This is particularly accurate when the pre-enrichment step is performed. However, as faster results are pursued, the pre-enrichment step is avoided and thus, the detection limit of only 1 CFU per sample becomes a challenge. Therefore, the complexity of these methods, the partial loss of the target cells and/or genetic material during these processes, and the presence of inhibitors and contaminants in environmental samples are barriers not yet fully overcome. Achieving the required levels of reliability, precision and robustness is essential for water quality monitoring and, so far, analysis in replicate and an adaptation of the protocol to each location of sampling are still a necessity. Nevertheless, detection using rtPCR techniques is growing quickly. In particular, USEPA has published revised standards based on the voluntary use of molecular methods and is moving toward the implementation of qrtPCR for the detection of *Enterococci* and *Bacteroidales* for ambient water quality monitoring (Haugland et al., 2014; Oliver et al., 2014; Sivaganansan et al., 2014). In the United Kingdom (UK), it was established a Working Group (WG), under the auspices of the “Delivery Healthy Water” project, with the purpose to debate the utility of qrtPCR methods versus culture-based methods for microbiological water quality analysis

646 linked to regulatory monitoring, interrogate the existing evidence-base and provide an
647 evaluation of the related uncertainties, advantages and limitations of this approach
648 concerning the implementation in the UK and the European Union for bathing water
649 monitoring and regulation (Oliver et al., 2014).

650 The new methods to be developed for the detection of microorganisms should
651 overcome the current methods on what concerns speed, specificity and sensitivity
652 (Connelly and Baeumner, 2012). Novel molecular methodologies such as loop-
653 mediated isothermal amplification (LAMP) of DNA techniques have emerged as an
654 alternative to the use of PCR-based methods (Daher et al., 2014; Niessen et al., 2013;
655 Pitkanen, 2013). This new technique is faster than other PCR-based techniques as the
656 cycling equipment is unnecessary, thus being a simpler and cheaper method for the
657 identification of target microorganisms, capable of providing equivalent sensitivity and
658 specificity (Daher et al., 2014; Niessen et al., 2013). Additionally, the next-generation
659 sequencing is a promising tool to sequence massive amounts of DNA per run, providing
660 the opportunity to screen large proportions of nucleic acids in a reduced time and cost
661 (Buermans and den Dunnen, 2014; Gobernado et al., 2014).

662 A recent interest is growing in coupling the molecular-based techniques with
663 electronic devices (biosensors), as they promise to combine the advantages of a higher
664 sensitivity and specificity of the molecular methods with the portability and easy-to-
665 use technology of sensors and microchips (Connelly and Baeumner, 2012; Sanvicens et
666 al., 2009). It is expected that in the near future these technologies will bring the
667 possibility of *in situ* real-time monitoring using low cost technology (Lopez-Roldan et
668 al., 2013; Quilliam et al., 2011; Skottrup et al., 2008; Tosar et al., 2010).

669 Research must continue its efforts on detecting indicators in their natural
670 environment and on its subsequent sample preparation steps. Miniaturization
671 strategies, by confining a reaction within a micro or nanoscale fluidic channel, can
672 benefit sensitivity, since the sample losses are reduced by the possibility to comprise
673 multiple operations together in one device (Connelly and Baeumner, 2012). Coupled
674 with new materials and multiplexing, these efforts are envisaged to bring new
675 methods capable of detecting several relevant pathogens at once, with the desired
676 sensitivity, specificity and speed.

677

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1068 **Table 1** – Technical challenges associated with the preparation of the water samples
1069 for the detection of microorganisms by molecular methods, when pre-enrichment is avoided
1070 (adapted) (Gensberger et al., 2013; Girones et al., 2010; Green and Field, 2012; Mulugeta et
1071 al., 2012; Rodríguez et al., 2012; Schriewer et al., 2011)

LIMITATIONS OF WATER SAMPLES PREPARATION FOR MOLECULAR DETECTION

- Need to concentrate the microorganisms from variable volumes of water due to the dilution effect (low concentration of microorganisms per volume), or to perform a previous culture step in order to increase the number of microorganisms in the sample
- Contaminants are concentrated along with the target microorganisms
- Membrane-clogging during filtration of environmental water samples
- Loss of target cells and/or nucleic acids during the several steps of the water samples preparation (concentration, extraction, purification), which causes an inefficient concentration of the target microorganisms to be detected and, as a consequence, false-negatives may occur
- Lengthy and complex procedures
- Inhibitors are difficult to remove and are not totally eliminated (some have the same solubility properties as nucleic acids)

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Table 2 – Selected PCR-based methods for microbiological water quality monitoring described by method used, microorganism selected, sample preparation procedures, type of sample tested, detection target, sensitivity, time of analysis and the presence of a pre-enrichment step in the procedure

METHOD	MICROORGANISM	SAMPLE PREPARATION	TYPE OF SAMPLE	TARGET	PRE-ENRICHMENT	SENSITIVITY	TIME OF ANALYSIS	REFERENCE
PCR	<i>E. coli</i> O157:H7	Filtration, membrane and filtrates re-suspended, boiled, extraction (phenol-chloroform-isoamyl alcohol), precipitation	Wastewater	<i>rfbE</i>	No	200 CFU/L in pure water Inhibition on wastewater samples	Not reported	(Bertrand and Poig, 2007)
RT-PCR and electronic microarray	<i>E. coli</i> O157:H7	Filtration, RNA extraction (ethanol, phenol-chloroform, TRIzol reagent)	Drinking water River water	<i>rfbE</i> and <i>fliC</i>	No	3-4 CFU/L in drinking water 7 CFU/L in river water 50 VBNC/L in river water	Not reported	(Liu et al., 2008)
NASBA rPCR (molecular beacon probe)	<i>E. coli</i>	Filtration, lysis buffer, heat-shock, magnetic nucleic acids extraction	Drinking water Treated sewage Surface water	<i>cjxB</i> -mRNA	No	1 viable/100 mL	3-4 h	(Heijnen and Medema, 2009)
qPCR (SYBR Green)	<i>E. coli</i>	Centrifugation, cells lysis and purification	Agriculture watersheds	Internal transcribed spacer (ITS) region between 16S-23S rRNA subunit genes	No	10 cells/mL	□ 3 h	(Khan et al., 2007)
Multiplex rPCR (SYBR Green)	<i>E. coli</i> O157:H7	1 colony suspended in water, heat-shock, centrifugation, supernatant used for amplification	Laboratorial	<i>stx1</i> and/or <i>stx2</i>	Yes (overnight)	8.4x10 ³ CFU/mL	Not reported	(Jothikumar and Griffiths, 2002)
Multiplex rPCR (SYBR Green)	Enterohemorrhagic <i>E. coli</i>	Boiling, sonication, centrifugation	Laboratorial	<i>stx1</i> , <i>stx2</i> and <i>eae</i>	Yes (overnight)	10 ³ to 10 ⁴ CFU/mL	Not reported	(Chassagnon et al., 2005)

METHOD	MICROORGANISM	SAMPLE PREPARATION	TYPE OF SAMPLE	TARGET	PRE-ENRICHMENT	SENSITIVITY	TIME OF ANALYSIS	REFERENCE
qPCR (molecular beacon probe)	Shiga toxin-producing <i>E. coli</i> (STEC)	Repeated centrifugation, boiling, precipitation	River water Drinking water	stx2	No	10 GE/PCR 100 GE/100 mL	□ 2 h	(Ram et al., 2011)
qPCR (molecular beacon probe)	Enterotoxigenic <i>E. coli</i> (ETEC)	Repeated centrifugation, boiling, precipitation	Surface water	L71	No	2 CFU/mL in spiked water 1.2×10 ³ to 1.4×10 ⁶ CFU/100 mL in polluted river water	Not reported	(Ram et al., 2008)
rtPCR (TaqMan probe)	<i>E. coli</i> O157:H7	Ultra-filtration, incubation, immunomagnetic-separation, incubation, colony DNA extraction	Surface water	stx1, stx2 and <i>rfbE</i>	Yes (> 24 h)	50 cells / 40 L	Not reported	(Mull and Hill, 2009)
rtPCR (TaqMan probe)	<i>Enterococcus</i> sp. <i>Enterococcus faecalis/faecium</i> <i>E. coli</i> and <i>Shigella</i> spp.	Filtration, dissolution of the filtration membrane, concentration by repeated centrifugation, lysis with glass beads, whole genome amplification	Drinking water	23S rRNA, <i>mif</i> , <i>ddl</i> , <i>atpD</i>	No (molecular enrichment: 3 h)	4.5 enterococcal CFU/100 mL 1.8 <i>E. coli</i> / <i>Shigella</i> CFU/100 mL	□ 5 h	(Maheux et al., 2013; Maheux et al., 2011a; Maheux et al., 2011b)
Multiplex qPCR (Minor groove binding probes)	Stressed <i>E. coli</i> O157:H7	Nucleic acids extraction from 1 colony	Drinking water	eae, stx1 and stx2	Yes (≈24 h)	3-4 cells/L	24 h	(Sen et al., 2011)
qPCR (TaqMan probe)	<i>E. coli</i> <i>Helicobacter pylori</i>	Lanthanum-based flocculation	Raw and finished water	<i>lacZ</i>	No	15 <i>E. coli</i> cells/mL	Not reported	(Zhang et al., 2012)
qPCR (SYBR Green)	ETEC	Boiling and precipitation	Potable waters derived from civic water supply	L71 and ST1	No	1 CFU/PCR	Not reported	(Patel et al., 2011)

1078 **Table 3** - Summary of the main advantages and limitations observed in PCR-based
 1079 methods for microbiological water quality monitoring (adapted) (Gensberger et al., 2013;
 1080 Girones et al., 2010; Green and Field, 2012; Maheux et al., 2013; Mulugeta et al., 2012;
 1081 Pitkanen, 2013; Ram et al., 2011; Schriewer et al., 2011)

METHOD	ADVANTAGES	LIMITATIONS
Standard PCR	<ul style="list-style-type: none"> – Higher sensitivity and specificity when compared to culture-based methods – Possibility of Multiplex-PCR for multiple pathogen detection – Detects VBNC cells – Simultaneous detection of different targets within the same species is possible (multiplex-PCR) 	<ul style="list-style-type: none"> – Post-PCR confirmation step needed (electrophoresis) – Non-quantitative – No distinction between viable and dead cells (detects both) – Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants (e.g. organic, inorganic and biomass content) – Low nucleic acid concentration causes frequent variability on the results, which leads to tube-to-tube variability
RT-PCR	<ul style="list-style-type: none"> – Distinguishes viable from dead cells 	<ul style="list-style-type: none"> – Complexity of the procedures – Short half-life of RNA – Technical expertise is necessary – Environmental samples can inhibit the detection
rtPCR	<ul style="list-style-type: none"> – Faster than conventional PCR – High level of sensitivity and specificity – Real-time detection – Quantification of the target in the sample is possible (qrtPCR) 	<ul style="list-style-type: none"> – Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants – No distinction between viable and dead cells (detects both)
NASBA-rtPCR	<ul style="list-style-type: none"> – Distinguishes viable from dead cells – No interference from background DNA 	<ul style="list-style-type: none"> – The same as in RT-PCR
PMA-rtPCR	<ul style="list-style-type: none"> – Distinguishes live from dead cells and from free DNA – Simple to perform 	<ul style="list-style-type: none"> – Possible inhibition from high solid content samples (further research is required) – Use of an extremely toxic compound (e.g. propidium monoazide)

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Figure 1
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