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Review

On the track for an efficient detection of *Escherichia coli* in water: A review on PCR-based methods

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ABSTRACT

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

The PCR-based methods under development for monitoring the presence of *E. coli* in water are here discussed. Special emphasis is given to methodologies that avoid pre-enrichment during the water sample preparation process so that the assay time is reduced and the required legislated sensitivity is achieved. The advantages and limitations of these methods are also reviewed, contributing to a more comprehensive overview toward a more conscious research in identifying *E. coli* in water.

Contents

1. Introduction

Water is frequently contaminated with human and/or animal feces (Ahmed et al., 2005; Davis et al., 2005; Gibson, 2014; Higgins

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et al., 2005; Scott et al., 2002; Walters et al., 2007), and the presence of fecal-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 labor involved. Instead, one or more microorganisms are chosen to indicate the possible presence of pathogens in water. Historically, many countries have been using fecal indicator bacteria, comprising total coliforms, fecal coliforms, *Escherichia coli* and enterococci, as a monitoring tool to predict the presence of bacterial, viral and protozoan pathogens originated in fecal contamination (Savichtcheva and Okabe, 2006). The European Commission (EC) (EC, 1998, 2006, 2009), the United States Environmental Protection Agency (USEPA) (USEPA, 2002; Boehm et al., 2009; Wade et al., 2003) and the WHO (WHO, 2011) recommend the use of *E. coli* as the most effective available fecal indicator bacteria for predicting the presence of pathogens in water intended for human consumption and bathing water, and *Enterococcus sp.* as the most appropriate for marine waters and as a useful indicator in bathing water as well (Bridle, 2014). *E. coli* is widely accepted as an indicator bacteria (Khan et al., 2007) since it is a species of fecal coliform bacteria that is specific to the intestines of humans and other warm-blooded animals but not normally pathogenic, it is easy to detect and culture, and it is found at higher concentrations than other pathogens in waters (USEPA, 2002; WHO, 2011). Moreover, its persistence in water and the effects of water treatment in cells viability is similar to that of waterborne pathogens (Ahmed et al., 2005; Lemarchand et al., 2004; McLellan et al., 2001). However, some studies have shown that *E. coli* is not a reliable indicator of some pathogens since illness has been found in the absence of the indicator microorganism. It has been shown that some pathogens are more resistant to water treatment processes than *E. coli* and, consequently, fecal contamination may not be detected, resulting in possible human exposure through drinking and recreational waters (Cashdollar et al., 2013; Gregory et al., 2011; Sinclair et al., 2009). Hence, the relationship between fecal indicator bacteria and the occurrence of diseases may not be accurate in all the locations of sampling (Boehm et al., 2009; Colford Jr. et al., 2012). Nevertheless, *E. coli* is still the microorganism that best satisfies the fecal indicator microorganism criteria (Bridle, 2014).

Extensive research has been conducted to achieve flawless methods to detect indicator microorganism(s), i. e., methods capable of detecting up to a single cell of the indicator microorganism(s), providing fast and reliable results. Since the Pasteur era, the microbiological quality of water has been monitored worldwide mainly through officially approved culture-based methods. In the European Union, the detection of *E. coli* in drinking water is described by ISO 9308-1 and is based in a membrane filtration procedure, with an incubation period of 21 ± 3 h in TTC medium. This culture medium is relatively non-selective, producing large numbers of false-positive results and is only recommended for use with high quality waters (Fricker et al., 2008a). The detection of *E. coli* in bathing water is described by ISO 9308-3 (or ISO 9308-1) and includes a 36 h incubation time using a β-D-glucuronidase (enzyme encoded by the *uidA* gene) culture-based method (EC, 2006). Despite the several advantages of enzymatic detection, lack of ubiquity (ability to detect all strains of the targeted species) may still occur since the enzymes are not found in all the strains of a target indicator (Boissinot and Bergeron, 2002; Clark et al., 2011). For example, the *uidA* gene is found in the majority of *E. coli* strains, but some of them (e.g. the pathogenic *E. coli* O157:H7) do not exhibit the corresponding enzymatic activity (Fricker et al., 2008b; Vale et al., 2009), which can lead to false-negative results (Chao et al., 2004; Maheux et al., 2008; Martins et al., 1993). Moreover, indicators may be severely underrepresented when injured by disinfectants used in water treatment processes; these are known as viable but non-culturable cells (VBNC) since they have lost the ability to form colonies (Juhna et al., 2007; Keer and Birch, 2003; Rockabrand et al., 1999). Low specificity is also a problem, as interference from other microorganisms can occur, resulting in false-positive outcomes (Bej et al., 1991b; Clark et al., 2011; Fricker et al., 2008a; Omar et al., 2010; Pisciotta et al., 2002; Sousa et al., 2010; Tryland and Fiksdal, 1998). For example, the enzyme β-D-glucuronidase is found in several microorganisms, including some *Salmonella* and *Shigella* strains, and some *Yersinia, Edwardia, Citrobacter* and *Hafnia* strains. *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp. and *Clostridium* are also capable of producing β-D-glucuronidase. To minimize interference from non-target microorganisms, antibiotics and antifungals were included in the formulation of several culture media, while other culture media (e.g. mFC agar) attribute its high specificity to the high incubation temperature (44.5 °C) which limits the growth of non-targeted microorganisms (Hakalehto et al., 2013; Maheux et al., 2014). Nevertheless, several drawbacks (e.g. inability to detect VBNC microorganisms, lack of ubiquity, the viability of the indicator microorganisms is not assured in the time between the sample collection and the analysis, requirement of 18–24 h for detection and sometimes an additional 24-72 h to confirm the positive results) have induced an increasing interest in molecular techniques as a method to achieve a more efficient monitoring of the microbiological water quality (Maheux et al., 2014, 2012, 2008; Oliver et al., 2014).

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

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, 2006, 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 μL) (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 advantages of PCR-based techniques over classical methods, in what concerns water quality monitoring, it is essential to reduce the time to provide the results and, thus, reduce health care costs and protect public health. Therefore, it is essential to remove the pre-enrichment step from the water sample preparation to reduce the assay time. This comprises the major challenge in the adaptation of PCR-based techniques to water quality analysis, since it directly influences the sensitivity of the method. Given the high importance of the water sample preparation procedure to the successful adaptation of PCR-based methods in the detection of microorganisms in water, some of the techniques developed with this purpose are described in the next section.

2.1. Water sample preparation

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

After collecting the water samples, the water sample preparation procedures for PCR-based analysis usually comprise three demanding steps: concentration of the target organism (e.g. filtration, centrifugation), extraction of the nucleic acids (e.g. heat-shock; chemical, mechanical and/or enzymatic lysis) and

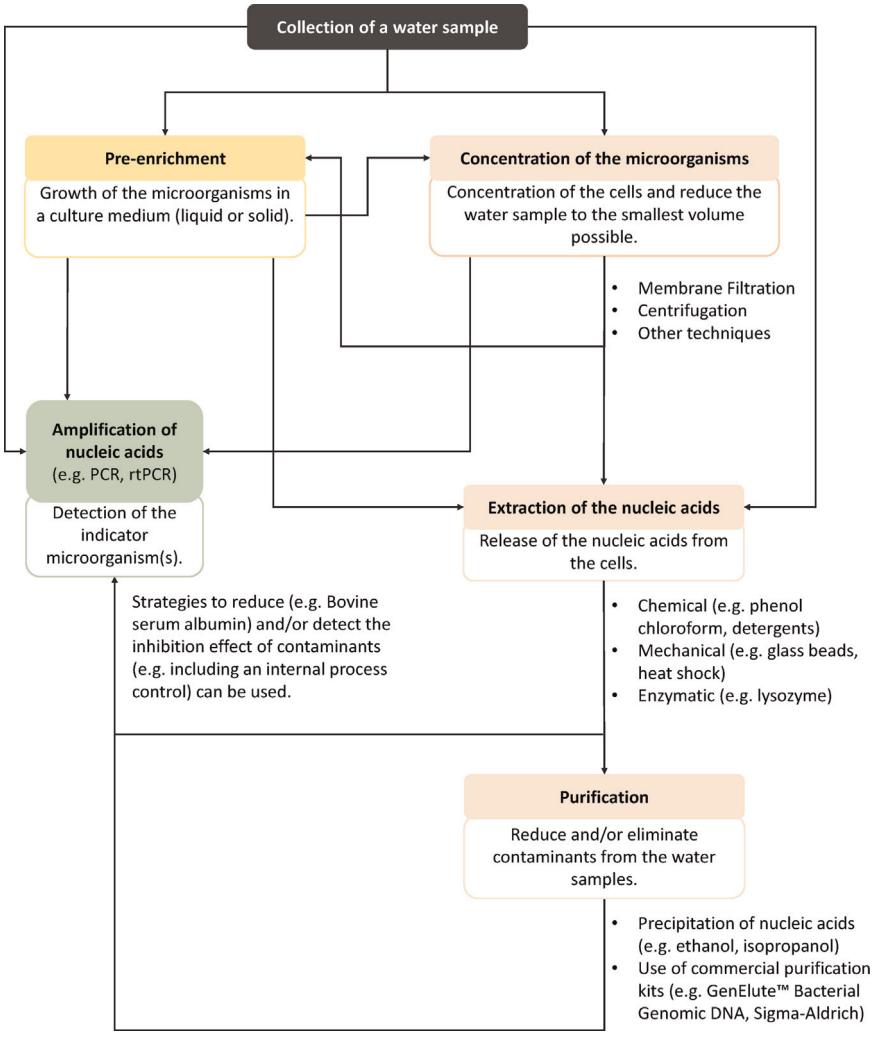

Fig. 1. Perspective of the different procedures that can be used in a protocol for the preparation of the water sample to be analyzed by amplification of the nucleic acids from the target microorganism (adapted) (Brettar and Höfle, 2008; Girones et al., 2010; Rompré et al., 2002)."

purification (e.g. chemical precipitation, solvent extraction, magnetic separation) to eliminate possible inhibitors (Jofre and Blanch, 2010). Therefore, an ideal water sample preparation protocol must efficiently release the nucleic acids from the indicator, protect them from degradation, eliminate or neutralize the inhibitors of the amplification reaction and provide the necessary analytical sensitivity (Boissinot and Bergeron, 2002).

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

In what concerns nucleic acid extraction and purification procedures, an impressive variety of commercial solutions are available (e.g. GenElute™ Bacterial Genomic DNA, Sigma-Aldrich; QIAamp DNA Mini Kit, Qiagen; PowerWater® DNA Isolation Kit, Mo Bio Laboratories), but these are only efficient in the presence of a considerable amount of the target microorganisms due to a poor extraction recovery efficiency at lower cell concentrations; moreover, they are frequently inhibited by contaminants present in the water samples and are usually developed for low sample volumes, which challenges the requirement to identify up to only 1 cell of the indicator in the water sample (Clark et al., 2011; Jiang et al., 2005).

The complexity and composition of environmental water samples can challenge the accurate detection of the indicator microorganisms by PCR-based techniques (Green and Field, 2012). Suspended contaminants such as sediments, humic acids, organic and inorganic compounds, cellular debris and heavy metals can interfere with DNA extraction, reduce the recovery of target microorganisms and nucleic acids, and inhibit polymerase activity which may lead to an underestimation of the indicator's concentration (Green and Field, 2012; Noble and Weisberg, 2005; Rompré et al., 2002; Schriewer et al., 2011). Moreover, many of these inhibitors have solubility properties similar to those of the nucleic acids (Schriewer et al., 2011) and, as a consequence, they are concentrated along with the nucleic acids (Noble and Weisberg, 2005), not being removed from the water sample during the preparation steps (Lemarchand et al., 2004). In these cases, a purification step could be included in order to reduce inhibition of the polymerase activity. However, some of the reagents used in DNA purification protocols (e.g. ethanol, isopropanol) may be difficult to eliminate from the water sample and may cause inhibition of the amplification as well (Green and Field, 2012). Other approaches to reduce interference from the contaminants in the detection include water sample dilutions when the target gene is present in high copy numbers, the addition of facilitating molecules (e.g. bovine serum albumin-BSA) and the use of designed polymerases (Baar et al., 2011; Schriewer et al., 2011). Nevertheless, dilutions can increase variation in measured targets, and lead to false-negatives when contaminants cannot be diluted to levels below interference or when the targets are diluted to below the detection limit (Schriewer et al., 2011). Although it was observed a reduction in the interference when BSA is used in the assay, it was observed that the target's amplification efficiency can be reduced, cycle threshold (CT) values can be increased and melting temperature values can be altered (Jiang et al., 2005). Despite the developments achieved, target detection interference still occurs and other methods were developed to identify interference, such as adding an internal amplification control (non-target DNA sequence) used to identify false-negative results (i.e., a negative result even though the water sample is contaminated) or reduced amplification efficiency (i.e., a positive result with an error in quantifying the concentration of the microorganisms in the water sample) due to polymerase inhibition (Maheux et al., 2013; Schriewer et al., 2011; USEPA, 2013).

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

- Clark et al. (2011) tested 10 different methods for recovering waterborne bacterial pathogens, combining membrane filtration and centrifugation to concentrate the cells, with mechanical, chemical and enzymatic lysis techniques for nucleic acid extraction, providing results in 12 h with 73.3% of recovery. Purification was performed using a commercial kit, but inhibitors were not completely removed.
- An interesting and innovative procedure was developed by Maheux et al. (2011a, 2011b), designated as CRENAME (concentration and recovery of microbial particles, extraction of nucleic acids, and molecular enrichment). This method employed filtration and centrifugation to concentrate cells, and the membrane was dissolved to recover cells that could be trapped among the cellulose fibers. DNA was extracted by bead beating and was amplified using a whole-genome amplification (WGA) kit, increasing the total DNA amount available for analysis and, thus, increasing the sensitivity. The whole procedure allowed a detection of 4.5 enterococcal and 1.8 *E. coli*/*Shigella* CFU per 100 mL of potable water in less than 5 h.
- A simple protocol based on a repeated centrifugation of large volumes of water samples was developed by Ram et al. (2011), where DNA was extracted by boiling, and detection was carried out by rtPCR. A sensitivity of 100 genomic equivalents (GE) per mL, which refers to the DNA amount needed to ensure that one copy of the full genome of the organism is present, was achieved in less than 2 h.
- Heijnen and Medema (2009) accomplished a detection limit of 1 viable CFU of *E. coli* in 100 mL of water through a complex procedure that included filtration, heat-shock and several extraction reagents, as well as magnetic extraction. Despite its complexity, the method was performed in 3–4 h.
- Mull and Hill (2009) and Zhang et al. (2012) stated that the membrane filtration of turbid surface waters can be limited due to filter clogging, since organic matter and colloids are a common presence in water. To avoid this problem, Mull and Hill (2009) suggested hollow-fiber ultrafiltration as a primary step for concentrating microorganisms and approximately 50 CFU could be recovered from a 40-l surface water sample. However, there is no indication of the length of this protocol, and, since it includes an incubation period of 20–24 h, it has a disadvantage over other faster methods (Mull and Hill, 2009).

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

Zhang et al. (2012) developed a chemical flocculation protocol. *E. coli* cells were captured and concentrated inside the flocs by a lanthanum-based flocculation, which were dissolved with EDTA, filtered and, after extraction, DNA was detected by rtPCR. This method was able to detect 15 cells of *E. coli*/mL in raw and finished water (Khan et al., 2007).

Difficulties still prevail, particularly when these techniques are applied to environmental water samples, since it is not yet possible to completely eliminate the losses of target cells and/or genetic material, nor to remove the inhibitors during the sample preparation procedures. The most significant technical problems associated with water sample preparation are listed in Table 1. Nevertheless, remarkable improvements, as those mentioned above, have been accomplished in the recent years, providing attractive results concerning sensitivity and time.

2.2. Amplification techniques

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

The selectivity of the PCR technique is originated in the use of a primer set that is complementary to the DNA region targeted for amplification under specific thermal cycling conditions. The exponential amplification of the target sequence significantly increases the probability of detecting a rare sequence or relatively low numbers of target microorganisms in a sample (Bej et al., 1990; Rompré et al., 2002). For most of the published PCR assays, the target DNA sequences for the detection of *E. coli* and total coliforms are the genes coding the same enzymes identified in the culture-based methods: β-D-glucuronidase (*uidA* gene) and β-D-galactosidase (*lacZ* gene), respectively (Bej et al., 1991a, 1991b, 1990; Farnleitner et al., 2001; Rompré et al., 2002). The distinction here is that PCR-based methods can detect and identify *E. coli* strains that carry the *uidA* gene but do not exhibit β-D-glucuronidase activity, whereas culture-based methods cannot. This is particularly important considering that one of the strains that remains undetected by culture-based methods is the pathogenic *E. coli* O157:H7, which was recently the cause of outbreaks in Europe (Frank et al., 2011; Friesema et al., 2008). On the other hand, *uidA* and *lacZ* genes are not exclusive to *E. coli* spp., and can be found in other closely related bacteria (e.g. *Salmonella* sp.) (Feng et al., 1991; Rice et al., 1990; Rompré et al., 2002). Therefore, primers targeting other genes were studied for these indicators to increase specificity (ability to target only the desired species) and ubiquity (ability to detect all strains of targeted species) (Boissinot and Bergeron, 2002; Maheux et al., 2008). Maheux et al. (2009) compared nine PCR primer sets targeting other genes (including the *uidA* gene) that were designed to detect *E. coli* and *Shigella* in water (Maheux et al., 2009). Traditionally, *E. coli* and *Shigella* have been considered as two different genera, but more recently some authors introduced the idea that *Shigella* is, in fact, "*E. coli* in disguise" (Lan and Reeves, 2002) and that genetically they belong to the same species (Lan and Reeves, 2002; Maheux et al., 2009; Paradis et al., 2005; Zuo et al., 2013). Therefore, Maheux et al. (2009) tested the ability of the primers to identify both *E. coli* and *Shigella*, and concluded that only the primers targeting the *tuf* gene were able to detect all the strains of both microorganisms. However, *tuf* gene was also identified in *Escherichia fergusonii* and, therefore, none of the primers was totally ubiquitous and should be selected according to the purpose of the analysis.

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

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

- *Real-time PCR (rtPCR)* is based on the cycling principle of standard PCR with the difference that successful amplification at

Table 2
Selected PCR-based methods for microbiological water quality monitoring described by method used, microorganism selected, water 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	Water sample preparation	Type of sample	Target	Pre-enrichment	Sensitivity	Time of analysis	Reference
PCR	*E. coli* O157:H7	Filtration, membrane and filtrates re-suspended, boiled, extraction (phenol-chloroform-isoamyl alcohol), precipitation	Wastewater	*rfbE*	No	200 CFU/L in pure water, inhibition in wastewater samples	Not reported	(Bertrand and Roig, 2007)
RT-PCR and electronic microarray	*E. coli* O157:H7	Filtration, RNA extraction (ethanol, phenol-chloroform, TRIzol reagent)	Drinking water, river water	*rfbE* and *fliC*	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-rtPCR (molecular beacon probe)	*E. coli*	Filtration, lysis buffer, heat-shock, magnetic nucleic acids extraction	Drinking water, treated sewage, surface water	*clpB*-mRNA	No	1 viable/100 mL	3–4 h	(Heijnen and Medema, 2009)
qrtPCR (SYBR Green)	*E. coli*	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-rtPCR (SYBR Green)	*E. coli* O157:H7	1 colony suspended in water, heat-shock, centrifugation, supernatant used for amplification	Laboratorial	*stx1* and/or *stx2*	Yes (overnight)	8.4×10^3 CFU/mL	Not reported	(Jothikumar and Griffiths, 2002)
Multiplex-rtPCR (SYBR Green)	Enterohemorrhagic *E. coli*	Boiling, sonication, centrifugation	Laboratorial	*stx1, stx2* and *eae*	Yes (overnight)	10^3–10^4 CFU/mL	Not reported	(Chassagne et al., 2009)
qrtPCR (molecular beacon probe)	Shiga toxin-producing *E. coli* (STEC)	Repeated centrifugation, boiling, precipitation	River water, drinking water	*stx2*	No	10 GE/PCR, 100 GE/100 mL	<2 h	(Ram et al., 2011)
qrtPCR (molecular beacon probe)	Enterotoxigenic *E. coli* (ETEC)	Repeated centrifugation, boiling, precipitation	Surface water	*LT1*	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)	*E. coli* O157:H7	Ultra-filtration, incubation, immunomagnetic-separation, incubation, colony DNA extraction	Surface water	*stx1, stx2* and *rfbE*	Yes (>24 h)	50 cells/40 L	Not reported	(Mull and Hill, 2009)
rtPCR (TaqMan probe)	*Enterococcus* sp., E*nterococcus faecalis/faecium, E. coli and Shigella spp.*	Filtration, dissolution of the filtration membrane, concentration by repeated centrifugation, lysis with glass beads, whole genome amplification	Drinking water	23S rRNA, *mtlf*, *ddl*, *atpD*	No (molecular enrichment: 3 h)	4.5 enterococcal CFU/100 mL, 1.8 *E. coli/Shigella* CFU/100 mL	<5 h	(Maheux et al., 2013, 2011a, 2011b)
Multiplex-qrtPCR (Minor groove binding probes)	Stressed *E. coli* 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)
qrtPCR (TaqMan probe)	*E. coli Helicobacter pylori*	Lanthanum-based flocculation	Raw and finished water	*lacZ*	No	15 *E. coli* cells/mL	Not reported	(Zhang et al., 2012)
qrtPCR (SYBR Green)	ETEC	Boiling and precipitation	Potable waters derived from civic water supply	*LT1 and ST1*	No	1 CFU/PCR	Not reported	(Patel et al., 2011)

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

Method	Advantages	Limitations
Standard PCR	– 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)	– 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	– Distinguishes viable from dead cells	– Complexity of the procedures – Short half-life of RNA – Technical expertize is necessary – Environmental samples can inhibit the detection
rtPCR	– Faster than conventional PCR – High level of sensitivity and specificity – Real-time detection – Quantification of the target in the sample is possible (qrtPCR)	– 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	– Distinguishes viable from dead cells – No interference from background DNA	– The same as in RT-PCR
PMA-rtPCR	– Distinguishes live from dead cells and from free DNA – Simple to perform	– Possible inhibition from high solid content samples (further research is required) – Use of an extremely toxic compound (e.g. propidium monoazide)

each cycle is monitored by the release of a fluorescent signal, which results in the possibility to follow the detection in real time (Hahn and Lapaire, 2013). Moreover, measuring the intensity of the fluorescent signal at the end of each cycle allows a quantitative assessment of the initial concentration of the target in the reaction tube, which can be achieved by employing a standard curve, and is designated by quantitative real-time PCR (qrtPCR) (Hahn and Lapaire, 2013). rtPCR-based technologies have emerged in recent years as a leading technology for rapid detection of microorganisms due to their high degree of sensitivity and specificity, introducing the possibility of a much faster detection of the target microorganism in real time, with no need for additional time to detect the rtPCR products by electrophoresis (Mackay, 2007; Quilliam et al., 2011). Furthermore, quantification of the target has become possible by qrtPCR (Hahn and Lapaire, 2013). Khan et al. (2007) used rtPCR in the detection of *E. coli* from agriculture watersheds by employing newly designed species-specific oligonucleotide primers derived from conserved flanking regions of the 16S rRNA gene, the internal transcribed spacer region (ITS) and the 23S rRNA gene (Pérez–Luz et al., 2004). This analysis led to the development of the first rtPCR assay in the ITS region for detecting and enumerating *E. coli*, as an attempt to solve the limitations of other primers normally used (for *lacZ, lamB, uidA, malB* genes), which are not totally specific for *E. coli*, or are of insufficient ubiquity (Boissinot and Bergeron, 2002; Khan et al., 2007). However, no specificity assessment was performed in this research to confirm the advantage of these primers over others.

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

The USEPA is implementing two qrtPCR methods for recreational beach water monitoring, capable of detecting as little as 31 cells per 100 mL, using Enterococci as the indicator microorganism: Method 1611 and Method 1609 (Gonzalez and Noble, 2014; USEPA, 2012, 2013). A specific region of the large subunit ribosomal RNA gene (lsrRNA, 23S rRNA) of these microorganisms (Haugland et al., 2014; Sivaganensan et al., 2014; USEPA, 2012) is the amplification target. These methods are initiated by filtering the water samples through a membrane filter, the membrane is placed in a microcentrifuge tube containing glass beads and buffer, and then shaken at high speed to extract the nucleic acids into solution. The supernatant (5 μL) is used for qrtPCR amplification where the Enterococci target DNA sequences are detected using either the TaqMan® Universal master mix PCR reagent and TaqMan probe system (Method 1611), or the TaqMan® Environmental master mix PCR reagent and probe system (Method 1609) (USEPA, 2012, 2013). Method 1609 includes an internal amplification control (Salmon DNA as the non-target DNA sequence) that is added to each qrtPCR analysis and is co-amplified

simultaneously with the Enterococcus target sequence to identify the occurrence of polymerase inhibition during amplification (USEPA, 2013). The advantage of both methods over currently accepted culture-based methods is to provide results within 3–4 h of high levels of bacteria, allowing same-day notification of recreational water quality. However, these methods still have a limited experience with the performance in a broad range of environmental conditions, and have been tested in a limited number of sites to 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). Horáková et al. (2008a) 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. 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, whose products could be considered as biochemical hallmarks of *E. coli* spp. (Horáková et al., 2008a). The four fragments were observed only on *E. coli* strains, including those that did not exhibit β-D-glucuronidase, and not on other close relatives. In addition to the identification of several genes of one specific target, multiplex enables the possibility of detecting different microorganisms in the same analysis. Garrido et al. (2013) developed a multiplex-rtPCR for the simultaneous identification of *Salmonella* spp., *E. coli* O157 and *Listeria monocytogenes* in food and environmental, natural and spiked samples. Since this procedure involves culturing the microorganisms, it was obtained a very low limit of detection (5 CFU/25 g) for a simultaneous detection of these three pathogens (Garrido et al., 2013). This work brings evidence of the potential of a multiplex-rtPCR in the simultaneous detection of the indicator and other microorganisms (e.g. pathogens and/or other indicators).
- *Nested PCR* comprises two consecutive rounds of PCR amplification, being the second round to increase the PCR product to detectable levels (Tallon et al., 2005). The first primer set is used to amplify a sequence which will serve as a template for the second amplification performed with a second primer set (Shi et al., 2010). Juck et al. (1996) used this method targeting the *uidA* gene of *E. coli* by designing two sets of primers: the first pair produced an amplicon of 486 bp that served as template for the second primer pair, which resulted in a fragment of 186 bp.

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

- *Reverse Transcriptase PCR (RT-PCR)* is a two-stage process: a target messenger RNA (mRNA) sequence is first transcribed into a complementary DNA (cDNA) sequence, which subsequently can be directly amplified by PCR (Keer and Birch, 2003). Liu et al. (2008) applied this method to river water. Samples were filtered with a low-protein-binding membrane, RNA was extracted and purified directly. Target *E. coli* O157:H7 was detected using a combination of RT-PCR and electronic microarray, with a sensitivity of 50 VBNC cells in 1 l of river water (the duration of the assay is not referred). It is known that mRNA has a very short half-life and is able to provide a much better association with the viability of the cell. Even though mRNA has a potential advantage for distinguishing viable from non-viable cells, the complexity of the method and practical difficulties in extracting detectable levels of intact mRNA from only a few cells can be a serious drawback (Keer and Birch, 2003; Lemarchand et al., 2004).
  Ribosomal RNA (rRNA) has also been studied as a possible target of detection (Chern et al., 2011; Keer and Birch, 2003; Lemarchand et al., 2004), since it is more abundant and easier to detect than mRNA. However, its longer half-life and instability makes it a weaker option.
- *Nucleic Acid Based Sequence Amplification (NASBA)* is an improvement of RT-PCR, as it enables selective amplification of a RNA fragment, without interference of background DNA (Keer and Birch, 2003). Heijnen and Medema (2009) developed a real-time NASBA (NASBA-rtPCR) for the detection of *E. coli* in water samples using a molecular beacon probe for the amplification of a fragment of mRNA coding for the *clpB* heat shock protein (Min and Baeumner, 2002). Several inactivation procedures (starvation, chlorine treatment, UV-irradiation and chlorine) were used to test the correlation between culturability and the ability to detect *E. coli* with NASBA (Whitman et al., 2004). A 100% specificity of the NASBA assay was demonstrated in the tested strains and a sensitivity of 1 viable *E. coli*/100 mL was determined using serially diluted spiked tap water samples. Moreover, a good correlation was observed between the number of colonies on the culture plates and the results obtained with NASBA. It should be taken into consideration that the VBNC cells do not form colonies on the culture plates but can interfere in the NASBA detection and, thus, alter that correlation. With an assay time of 3–4 h, this study has demonstrated that the NASBA method has potential as a rapid test for microbiological water quality monitoring.
- *Propidium monoazide rtPCR (PMA-rtPCR)* has been introduced

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

Table 2 assembles some of the PCR-based techniques reported in the literature as methods to assess the microbiological quality of water, selected either due to the interesting approaches used in the development of a new protocol, or for the promising results obtained. The technology applied, microorganism and type of sample, whether a pre-enrichment step is included or not, and the length of the whole procedure is indicated.

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

Another key issue is the observed variability in molecular detection. It was shown that the extremely low concentrations of target microorganisms in water samples can cause high unpredictable fluctuations in the PCR efficiency, which leads to tube-to-tube variability and, thus, to false-negatives (Jofre and Blanch, 2010). Another cause of variability concerns the water composition differences between samples, place of sampling, atmospheric conditions (e.g. rain drags feces from the streets to the wells, rivers, beaches) and temperature variations throughout the day (Heaney et al., 2014; Kozuskanich et al., 2011). Additionally, extracellular DNA that persists in the environment can affect the detection, increasing the risk of false-positives (Lavender and Kinzelman, 2009). However, Maheux et al. (2011b) noticed that free DNA in drinking water could flow through the filter during the filtration step without interfering with the detection protocol. Thus, the detection of microorganisms in environmental water samples requires the adjustment and optimization of the protocol to each type of water and place of sampling, since the contaminants (inhibitors and background DNA), the concentrations and types of indicators, the weather and other variables differ from location to location (Boehm et al., 2009; Lavender and Kinzelman, 2009; Shanks et al., 2011; Whitman et al., 2010). Standardization of these methods is, therefore, a challenge for a routine use, since accuracy and reproducibility are still hindered by these problems. Some studies concerning other indicators, with the purpose to achieve standardization and optimization for specific locations, are already exhibiting some promising results (Ashbolt et al., 2010; Benedict and Neumann, 2004; Haugland et al., 2005; Mulugeta et al., 2012; Shanks et al., 2011; Whitman et al., 2010).

PCR-based methods have been proving their utility not only in detecting water contamination, but also in identifying the source of contamination and in predicting *E. coli* in water. The current methods for microbiological water quality monitoring offer no information about the originating sources of fecal pollution, which is a valuable knowledge needed to characterize pollution and make decisions (Shanks et al., 2014). Microbial source tracking (MST) describes methods and research strategies to identify fecal pollution sources in ambient waters based in the association of distinct fecal microorganisms with a particular host (Harwood et al., 2014). For example, a qrtPCR method targeting *Bacteroides dorei*, a human-associated genetic marker, was developed with the purpose to expand the use of PCR-based methods to identify human fecal pollution in ambient water samples (Shanks et al., 2014). In another embodiment, predictive empirical regression models (based on wind speed/direction, rainfall, etc.) have been studied as a method to estimate the fecal indicator density for a particular day (Ashbolt et al., 2010). Therefore, a quantitative microbial risk assessment (QMRA) modeling of fecal indicator microorganisms is suggested as an approach to simulate and explore the diversity of scenarios for hydrological events and fecal contamination (Ashbolt et al., 2010).

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

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;

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

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

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

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

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