Chapter 7

E. coli as an Indicator of Contamination and Health Risk in Environmental Waters

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Additional information is available at the end of the chapter

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Abstract

Good public health depends on regular monitoring of water quality as faecal contamination is a serious problem due to the potential for contracting disease. Bacterial contamination in water is measured using indicator organisms, notably *Escherichia coli and Enterococci* which are used as primary indicators of contamination in fresh and marine water quality, respectively, rather than the total coliforms present. Although most *E. coli and Enterococci* strains cause only mild infections, their presence is indicative of the potential presence of other more pathogenic organisms which are a danger to human health. The acceptable levels of indicator organisms are defined in legislation and are set for drinking, river, well and marine water. This chapter will consider current gold standard culture methods of analysis for *E. coli* and compare them with molecular DNA procedures. Established culture methods use β-D-glucuronidase to identify *E. coli* and β-D-galactosidase to detect coliforms. Emphasis will be placed on newer procedures that can be used onsite supported by laboratory procedures used for confirmation. Available rapid fluorimetric procedures which have been developed for use in the field, based on the assay of β-D-glucuronidase, will be discussed. The rapid advances in procedures using a molecular approach will be considered and compared with the more established methods for determining *E. coli* in water. It is essential that all these methods should be quantitative in order to comply with legal norms, and in this regard, the potential involvement of biosensor technology will be of great value in successfully transferring laboratory procedures to the field.

Keywords: water quality monitoring, *E. coli*, β-D-glucuronidase, faecal bacteria, rapid detection of contamination, biosensors

1. Introduction

Pathogen contamination of environmental waters is a major health risk and a threat to future supplies of water for living and recreational activities. Acceptable bacterial limits have been

defined in legislation by among others WHO [1], US EPA [2] and the European Union [3]. Health risks can be assessed and monitored using a series of tests for specific indicators which are defined in regional legislation. Water-borne contamination and related diseases are discussed in detail elsewhere [4, 5]. A recent comprehensive review [6] considered recent reports on recreational water and infection comparing epidemiological studies and quantitative microbial risk assessment (QMRAs). While potential sources of contamination are considered in a review [7] which concentrates on the transport of pathogens in the agricultural setting and their health implications. In this chapter, we assess the methodology currently available for monitoring the presence of *Escherichia coli* in environmental waters. Although laboratory analyses will remain the reference procedures, the current trend is to develop onsite methodology which will yield more rapid results allowing more immediate action to be taken if contamination is found. This development also benefits, for example, developing countries where good laboratory procedures may not be easily accessible and accurate and reliable onsite technology will be the key to progress and improved public health.

E. coli are present in the intestine of men and animals and are released into the environment in faecal material. As faecal matter is the main source for disease-causing agents in water, faecal bacteria are widely used as indicators of contamination which can affect rivers, sea beaches, lakes, ground water, surface water, recreational water and the many and diverse activities associated with these [5]. Contamination can result from leakage of sewage, sewer overflow caused by storm events and accidental or deliberate release into receiving water bodies, as well as water draining from agricultural land or directly from livestock and birds [4, 8]. Sewage treatment plants can also be a source of pathogenic *E. coli* and these can spread in the river systems [9, 10]. Even low levels of contact with contaminated water in rivers [11] or beaches [12] are significant and can result in outbreak of gastroenteritis. However, the amount of water likely to be ingested is most important when determining the risk of certain activities (e.g. swimming, boating) [13]. Although coliforms do not usually cause serious illness they are used to indicate the presence of more pathogenic bacteria and viruses. The health risk to humans and animals can be assessed using a series of tests which are defined in regional legislation. Coliforms and in particular *E. coli* are the most valuable markers. *E. coli* is widely accepted as the better faecal indicator organism than total coliforms. Contamination of water supplies by pathogenic strains like *E. coli* O157:H7 is more serious but usually localised. A number of reports have shown that most *E. coli* (over 95%) express β-D-glucuronidase (GUD) activity [14] making this enzyme a sensitive and specific marker for *E. coli* detection and thus faecal pollution. The current acceptable upper limit for *E. coli* content in fresh surface water is 900 cfu/100 ml and 500 cfu/100 ml in marine water [3]. Although *E.coli* is the marker of choice a number of other markers are used in environmental monitoring. *Enterococci* for example are used as a maker for contamination particularly in marine waters [15]. Further details of other markers used in environmental monitoring are detailed in Price and Wildeboer [16].

The widespread use of antibiotics in agriculture and their release from sewage works has accelerated the development of antibiotic-resistant strains in environmental water bodies increasing the need for accurate and regular monitoring. A recent study [17] demonstrated the presence of bacteria resistant to a number of antibiotics, some of which were of faecal origin highlighting concerns about release and spread of antibiotic-resistant organisms in receiving

waters. Another important aspect of faecal contamination of rivers and lakes is its effect on fish. This has both public health and economic impacts and is of critical importance in water bodies used for aquaculture [18–20]. The release of microorganisms into a lake or river and the use of antibiotics in fish farming can combine to create a perfect storm of an environment that is highly selective for the development of multi-drug resistant strains. Effective monitoring of water quality and rapid detection of contamination as well as more sustainable approaches and a reduction of drug use in aquaculture will need to go hand-in-hand to improve food safety and environmental health.

In this chapter, the different procedures currently available for monitoring *E. coli* will be critically assessed and particular emphasis will be on comparing recent and older methods for the detection *E. coli* (**Table 1**).

Culture-based methods	Sensitive, 1–2 days to obtain a result. Detects primary indicator organism and others present, relies on biochemical or immunological methods of identification, underestimates bacterial load as only viable organisms detected.
Chromogenic agars	Detects non-viable VBNC as well as viable bacteria, based on the assay of β-D-glucuronidase and β-D-glucuronidase.
API®ID Strip Range	Based on miniaturised laboratory techniques used as confirmatory tests.
Membrane filtration method	Reference method used for ISO standards. Culture on chromogenic media after filtration.
Most probable number	Depends on growth of target organism in liquid medium, more time consuming and labour intensive than MFM, not suitable for marine organisms.
Direct methods	Direct measurement of indicator enzyme activity in water, usually using fluorescent substrates for β-glucuronidase and β-galactosidase. Suitable for onsite monitoring.
Semi-automated methods	Commercial procedures, e.g. Colilert analysers, use selective media and fluorescent substrates, suitable for online monitoring, can give results within 24 h.
Nucleic acid-based procedures	Sensitive and rapid but when low numbers of bacteria are present and enrichment step is needed. Invaluable technique to identify individual pathogens, e.g. *E. coli* O157:H7.
Quantitative PCR	qPCR determines the number of genomes per volume of water for a bacterial species, can be coupled to fluorescence *in situ* hybridisation (FISH) or plate counting. FISH is used to identify different mixtures of bacteria in a sample, usually requires enrichment step.
qPCR and qPRT-PCR	A rapid sensitive method used in study of emerging as well as established pathogens. Needs further standardisation.
Biosensor techniques	Able to directly detect target bacteria and provide real-time results. Portable and easy to use for onsite testing. Avoids cultivation step and can measure viable and non-viable cells. Sensitive optical biosensors can detect 7 cfu/ml *E. coli* in water samples. Biosensors based on electrochemical immunosensors are also used while biosensors based on physicochemical methods, e.g. Raman spectroscopy are currently under development but probably would not be suitable for routine analysis.

Table 1. Established and developing methods used to monitor *E. coli* in environmental water.

2. Established analytical methods to detect *E. coli* in environmental waters

Methods involving culturing procedures are essentially laboratory based and, although they are sensitive, usually involve one or two days before the result is known. They can be used to detect the presence of a range of potential contaminating organisms in addition to the primary target organism. Culture procedures rely on either biochemical, immunological or molecular methods to identify the bacteria present. However, culture methods may underestimate the bacterial load or fail to grow relevant organisms as they measure only the viable organisms present in the samples that can be cultured. In environmental samples, a significant number of cells may not be detected despite being viable. Viable but non-culturable cells (VBNC) result from stress encountered in the environment or the condition and content of the samples [21]. Therefore, alternative new technologies that do not rely on growing the bacteria in culture are required; many of these involve nucleic acid based methods [6]. Chromogenic agar can detect non-growing cells by measuring the presence of an enzyme e.g. β-D-galactosidase for coliforms [22] and β-D-glucuronidase for *E. coli* [23]. A wide range of media is available for the characterisation of environmental microorganisms [24]. Detailed descriptions of standard laboratory procedures which are used in environmental studies including microscopic as well as biochemical characterisation are given in Alexander and Strete [25]. The rapid identification of known bacteria can be achieved using the API® ID Strip Range (BioMerieux, France) which consists of a series of miniaturised techniques based on established laboratory procedures.

The reference methods for detection and isolation of *E. coli* and coliforms in water are the membrane filtration method (ISO 9308-1:2014) and the multiple tube fermentation (Most Probable Number, MPN, ISO 9308-2:2012). ISO 9308-1:2014 is based on membrane filtration and subsequent culture on a chromogenic coliform-agar medium [26]. Due to the low selectivity of the differential agar medium, background growth can interfere with the reliable enumeration of *E. coli* and coliform bacteria, for example, in surface waters or shallow well waters. This method is not suitable for these types of water. As the MPN method (ISO 9308-2:2012) is based on the growth of the target organisms in liquid medium it is suitable for most waters but should not be used for enumeration of bacteria in marine samples as dilution of the sample is required. A recent study compared membrane filtration (MF) and multiple tube fermentation (MTF) procedures to analyse water obtained from a dockside and a beach in California [27]. The MF method gave more reliable and precise data than the MTF method. The later method was more time consuming, labour intensive and less precise. The MF procedure also has the advantage of being able to examine large volumes of water but it has limitations when dealing with turbid water samples. The *E. coli* and coliform content in water samples from five Environmental Protection Agency regions (EPA) in the USA were compared using the Colilert™ automated test and MTF procedure [28]. Similar results were obtained with both methods; however, the Colilert™ procedure was easier to perform and interpret.

Enumeration and characterisation of bacteria in environmental samples requires a tiered approach. The samples collected from, e.g., rivers are diluted or centrifuged to remove

particulate matter followed by a filtration step; the bacteria retained on the membrane are incubated on a growth medium for up to 18 h. *E. coli* are selected on colony colour and identified using chromogenic agar, confirmation can be via API 20E strips or PCR. Some strains of *E. coli* which are β-D-glucuronidase negative, such as *E. coli* O157:H7, will not be detected as *E. coli* but, as they are β-D-galactosidase positive, they will appear as coliform bacteria on selected chromogenic agars [29]. A range of chromogenic agars are available for the detection of *E. coli* O157:H7 which have improved specificity when compared to cefixime-tellurite Sorbitol MacConkey (CT-SMAC) [30] when tested against eight environmental samples inoculated with *E. coli* [31].

Amirat *et al*. [32] used the membrane filtration procedure followed by culture on selective chromogenic media to monitor bacterial contamination of the river Thames. This procedure successfully identified *Salmonella, Enterococci, Klebsiella pneumoniae* and *E. coli*. Sixty percent of the samples were in excess of the EU standard for bathing water and the study demonstrated frequent sewage pollution of the Thames which was most noticeable after heavy rainfall. The relationship between sewage contamination of rivers and heavy rainfall has also been reported in other studies: Tryland *et al*. [33] used the Colifast early warning system while Kolarevic *et al*. [34] studied the river Tisa in India using the membrane filtration method. The MPN method was also used to demonstrate an increase in indicator organisms including *E. coli* 2 days after rainfall in the river Göta Älv in Sweden [35] and to measure faecal pollution and antibiotic resistance in the river Cauvery in India [36]. Faecal contamination of the river Danube was measured using the indicator organisms: coliforms and *Enterococci* [37]. A rapid onsite method for *E. coli*, ColiSense, is based on the direct fluorescent analysis of β-D-glucuronidase activity in recreational water samples [38]. Total time taken to complete the analysis was approximately 103 minutes of which 75 minutes were needed to complete the assay. The total time taken to obtain a result depends on the time to transport the sample from the test site to the laboratory and time for any pre-treatment steps. This method does offer greater rapidity and portability but there may be differences in the results obtained with this procedure than with standard culture procedures.

Monitoring of environmental water samples is usually carried out using culture-based faecal indicators of microbial contamination. However, these methods are expensive and time-consuming and recently efforts have been made to develop methods which give more rapid results at lower cost and greater specificity. Indirect detection of *E. coli* and total coliforms in water samples from Canadian fresh water beaches using a portable detection system has been described [39]. The detection procedure was based on the fluorescent detection of β-D-glucuronidase and β-D-glucuronidase using novel anthracene-based enzyme substrate. The method is able to detect single cells of either *E. coli* or total coliforms within 18 h and turbidity and colour and turbidity of the water samples does not affect the result. False-positive coliform results due to the presence of *Aeromonas spp.* could be eliminated by the inclusion of Cefsulodin in the growth medium.

A number of semi-automated systems are currently available which utilise selective growth media and fluorometric substrates. The Colifast Analyser system utilises 4-methylumbelliferone-β-D-glucuronide to detect *E. coli* and 4-nitrophenyl-β-D-galactoside to detect coliforms using defined

substrate technology which is used for online monitoring. The endpoints are yellow for total coliforms and fluorescent for *E. coli*. There is also a micro hand held version available. Results can be obtained using this procedure within 2–12 h. An alternative system, Colilert® 3000 (Seres, France) utilises fluorescent or chromogenic substrates and can deliver results within 24 h. These methods correlate well with standard laboratory methods although the results were two to three orders of magnitude higher than MTF and MPN methods probably due to the presence of *Aeromonas spp.* and *Vibrio spp.* (natural inhabitants of the surface water) known to interfere with the Colilert test [40]. A comprehensive study by Schang [41] compared four methods to analyse riverine, estuarine and marine environments near Melbourne, Australia. They compared the industry-standard IDEXX (Colilert®) culture-based method with three alternative approaches: the TECTA™ automated system uses fluorometric assays [42] and while still under development they found a good correlation between the IDEXX and TECTA™ procedures while the later had the advantage of a faster turnaround time. Good correlation was found between the IDEXX method and the US EPA Method 1611 for qPCR detection of *Enterococci*. Good correlation was found between next-generation-sequencing (NGS) and the culture-based procedures; however, the cost of NGS is too high at present, but future developments might make the use of this procedure suitable for routine screening.

The use of indicator organisms is well established and will probably continue as the gold standard of microbial contamination until reliable alternative procedures are developed. There are however several promising areas of development which are considered in the sections below which provide valuable supplementary information and have the potential to evolve in specific easy to use onsite procedures. Culture procedures take a minimum of 24 h to complete and the availability of more rapid techniques will allow earlier appropriate management decisions to be made.

3. Molecular techniques for bacterial identification in environmental waters

Molecular techniques for the specific detection and quantification of bacteria are highly sensitive, rapid and specific, they can be readily automated and standardised so have some advantages over the standard culture-based techniques. Detection does not rely on the target organisms being viable and multiply under culture conditions or on the expression and activity of enzymes or other biochemical markers. However, where low numbers of bacteria are present, an enrichment step is often required limiting the aforementioned advantages. Quantitative PCR methods provide accurate numbers of genomes present and multiplex approaches can simultaneously identify the target organism and test for genes associated with pathogenicity or antibiotic resistance [43, 44] and host-specific detection thus linking the contamination to a source [45–47]. Both sample clean-up and PCR protocols have recently been developed to be fast and simplified and requiring a limited set of laboratory resources thus making molecular analysis a more attractive option for routine monitoring and even field testing. The development of automated DNA extraction and PCR methods have been utilised to develop an autonomous

system for the *in situ* detection of faecal indicator bacteria [48] showing the future potential for bringing molecular analysis out of the laboratory and constructing robotic analysers.

Recent advances in sequencing technology and the decrease in costs for whole genome sequencing have made this technology the forefront of investigations into outbreaks of infectious diseases and food or water contamination [49–51]. Rapid identification can be achieved and the outbreak quickly be traced to its source allowing for more effective treatment and containment. This provides an entirely new and effective tool that allows tracing a faecal contamination of water to its source. Measures can then be put in place to contain the current release, prevent future events and if the cause is found to be a careless or deliberate release, legal proceeding can be initiated. However, for routine monitoring of water quality this technology is not a viable alternative as it is more expensive, requires specialist equipment and trained analysts and does not provide rapid or onsite results.

The coupling of microarray technology with PCR enhances detection and identification of bacterial contaminants in water samples. Several commercial kits are now available for the assay of *shiga* toxin producing *E. coli* O157:H7 in environmental samples. More recently, detection techniques using biosensors have shown potential for onsite monitoring. These combine a rapid biochemical reaction with a physicochemical signal that is proportional to the concentration of the target molecule and thus the number of bacteria present in a sample. The biomarkers targeted are most commonly the enzymes established in laboratory-based assays. We have shown that a direct assay of 1 ml river water sample for β-D-glucuronidase activity analysed with a portable fluorimeter can achieve detection limits of 7 cfu/ml within 30 min [52], the ColiSense system described by Heery [38] combines incubation and fluorescent detection in a portable device achieving below 100 cfu/ml in 75 min and a recent study by Hesari [53] describes a biosensor, sensitive enough for the detection of *E. coli* in drinking water with a significant fluorescent signal generated in under 2 h and no sample processing. Wutor [54] describes a biosensor targeting β-D-galactosidase that can detect 1 cfu/100 ml in 15 min using voltammetry to detect the enzyme activity. A system that combines concentration of *E. coli* with a colorimetric detection of enzyme activity and is easy to use, portable and not requiring any instrumentation was recently developed and commercialised [55]. Several immunosensors have also been developed, mostly in order to detect specific bacterial antigens correlated with virulence. A detection limit of 100 cfu/ml is achieved by a specific immunosensor for *E. coli* O157:H7 [56], and with a gold-nanoparticle sensor described more recently, *E. coli* O157:H7 were detected as low as 10 cfu/ml in 1 h [57]. An electrochemical biosensor capable of specifically detecting ESBL *E. coli* strains was developed and achieved a detection limit of 5000 cfu/ml [58]. A third type of biosensors targets nucleic acids and Paniel [59] has shown that both optical and electrochemical detection methods can achieve detection limits below 20 cfu/ml *E. coli* in seawater. Capacitors can be utilised to detect whole cells and a recent paper describes a biosensor that can specifically detect *E. coli* to a limit of 70 cfu/ml in river water by combining a capacitive biosensor with microcontact imprinting [60]. A number of different biosensor systems for the detection of bacteria in water and studies evaluating these are reviewed by Lopez-Roldan [61].

Proteomics methods have been developed and extensive databases created allowing the identification of microorganisms directly in complex samples. Several studies have shown how MALDI-TOF-MS (Matrix-Assisted Laser Desorption Ionisation Time-of-flight Mass Spectrometry) can be employed to identify organisms at species level, and detect virulence and resistance markers in environmental waters [62, 63]. A study by Loff [64] compares proteomics analysis with molecular and biochemical methods for the detection of microorganisms commonly associated with water safety. It can be expected that future developments of this technology will widen its application in many diagnostic and analytical applications.

It has to be noted that the identification of organisms and detection of virulence or resistance by both molecular and proteomics approaches relies on the comparison of results with existing databases. This limits to the identification of known strains and characterised genes and proteins and is thus unlikely to achieve detection of uncultivable microorganisms. However, a combination of recent advances in bioinformatics and novel methods like the one described by Kaeberlein [65] have increased our knowledge about the microbial world and extended our database resources. Molecular and proteomics methods have shown great potential in the identification in temporal and special distribution of microorganisms in the aquatic environment and to combine species identification with detection of virulence and drug resistance. Future developments are likely to combine the best of both worlds to achieve robust assessment of water quality by quantifying indicator organisms to detect contamination and identify virulence and resistance markers to assess public health risks and inform stakeholders on the need and nature of required interventions.

4. Disadvantages in relying solely on *E. coli* to monitor water contamination

Although historically total coliforms, faecal coliforms, *Enterococci* and *E. coli* have all been used as indicator organism for faecal water pollution and currently employed methods continue to largely rely on these, it is clear that alternative indicators need to be developed to address limitations in identifying other water contaminants of considerable public health concerns. Water-borne diseases including diarrhoea and gastrointestinal illness can be caused by bacteria, viruses and protozoa [4]. Approximately 3.4 million people, mainly children, die from water-borne diseases [66] and solely relying on *E. coli* can result in misleading information [67]. Major etiological agents including *Giardia*, *Cryptosporidium*, *Vibrio cholerae* and *Salmonella* would be missed by current testing procedures. Often outbreaks are due to local flood or storm events or releases of untreated sewage which result in significant contamination of environmental water. Worldwide morbidity and mortality caused by contaminated drinking water is of considerable magnitude. The WHO ranks diarrhoeal diseases sixths highest in the list of causes of environmental deaths with an estimate of 846,000 deaths annually [68]. This highlights the need for a concentrated effort to make both recreational and drinking water safe in both developing and developed countries [4]. The development of methods detecting a wide range of significant pathogens is most likely to be achieved by extraction and antibody based detection, as described for pathogenic protozoa [69] or molecular techniques such as PCR, shown for *Cryptosporidium parvum* and *Giardia lamblia* [70], and

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with further developments of NGS and MALDI. However, the advantage of the currently used *E. coli*–based procedures is their simplicity, low cost and functionality for rapid onsite detection. Additional more broad ranging tests would need to be rigorously assessed in a wide variety of environmental situations before they could be adapted as international standards. There is, therefore, a clear need to re-examine the precision and reproducibility of both culture and molecular-based methods in the assessment of environmental samples to take into account local variations and design new methods to be applicable for a wide range of scenarios in order to make a significant contribution to improving water safety globally.

5. Conclusions

Sensitive and frequent monitoring of environmental waters is essential to minimise adverse effect on human health. The current approach to monitoring for contamination in environmental waters is shown in **Figure 1**.

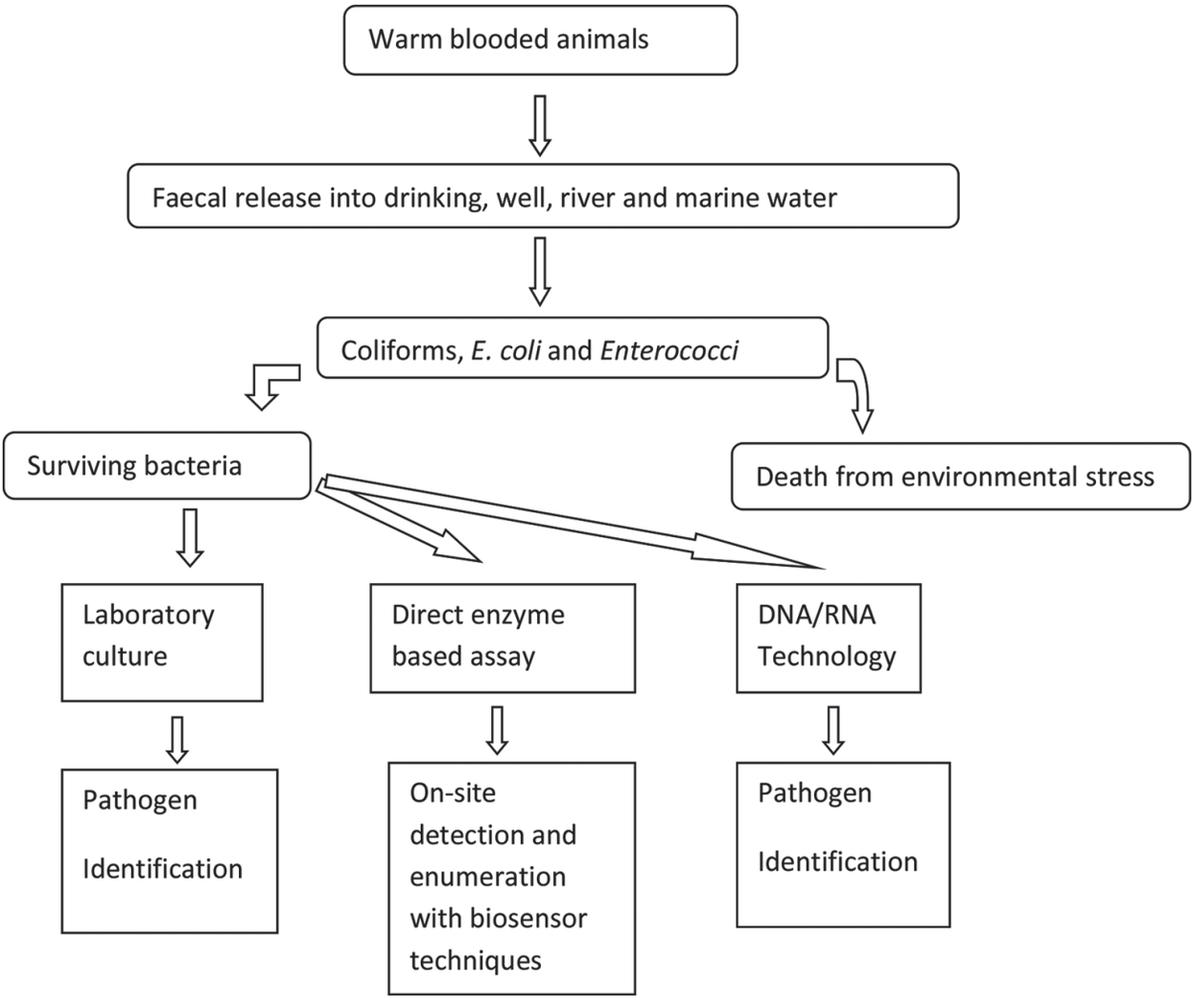

Figure 1. Current approach to monitoring and identifying bacteria in environmental water.

A wide range of techniques are now available for monitoring but culture-based techniques are used to define the legally accepted limits of environmental water contamination. The quantification of the indicator organisms *E. coli* and *Enterococci* are used to detect faecal pollution. Routine analysis is still largely based on the enumeration of these two intestinal organisms by culture coupled with the detection of β-D-galactosidase and β-D-glucuronidase activity. A secondary objective of environmental monitoring is the identification and quantification of bacteria present in water samples and this is best achieved by molecular methods. Whereas culture methods have the limitation of only providing information the day after collection of the sample, all the other methods currently available have some limitations as well when used for environmental samples. In the case of molecular methods this is the need to concentrate the sample or amplify the DNA, further the highly specific target sequences that are used could result in an underestimation of the actual level of indicator organism. The most promising area is the development of a wide range of biosensor systems which show promising simplicity for direct and *in situ* analysis.

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