Evaluation of seawater monitoring for the detection of *Escherichia coli* and *Enterococcus faecalis* on an integrated biosensor system

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Abstract

Purpose – This study aims to monitor seawater by determing two biological indicators, *Escherichia coli* and *Enterococcus faecalis*. The process of following standard procedures is mainly time-consuming. Thus, there is a demand for a biosensor, an appropriate device for rapid and accurate results that can give information about the microbiological quality of seawater in an effective and rapid way.

Design/methodology/approach – In the gold standard method for seawater monitoring, the filter method is applied as a condensation step. In this work, the authors evaluated six types of common syringe filters for bacteria concentration and then the best filter was used for seawater analysis for *E. coli* and *Enterococci* with loop-mediated isothermal amplification (LAMP) polymerase chain reaction (PCR).

Findings – Cellulose acetate filter had the highest efficiency (98%) for bacterial concentration. The limit of detection of the LAMP method was 104/1,000 mL for both *E. coli* and *E. faecalis*. The proposed method could be used for the development of seawater biosensors with advantages such as a simple heating element and the speed that the LAMP PCR presents.

Originality/value – The suggested protocol is proposed in an integrated in situ system, a biosensor, for seawater quality determination.

Keywords Seawater, Biosensor, *Escherichia coli*, *Enterococcus faecalis*, Biomarkers, LAMP

Paper type Research paper

Introduction

Coastal and swimming areas are globally considered to be dynamic zones in urban cities and affect their financial conditions (Tiwari *et al.*, 2021). For their protection, the European Union has taken care of establishing some legislation. The Bathing Water Directive includes the directives of other environmental policies such as the Water Framework Directive and the Marine Strategy Framework Directive (European Commission, 2008, 2006). Both of them lead to good water quality under the regime to which belongs the directive of bathing waters (European Commission, 2006). Fecal indicator bacteria (FIB) have been used as proxies for the presence and determination of sources associated with fecal contamination (Lee *et al.*, 2019a). The 2006 European Directive suggests 250 CFU of *E. coli* and 100 CFU of *E. faecalis* in 100 mL of water as "good quality." The number of indicators should not exceed 500 CFU for *E. coli* and 200 CFU for *E. faecalis* to be considered as "sufficient" (European Commission, 2006).

The main concern is the time required for water analysis. Despite the fact that the cultural methods are relatively economical, they require at least 24–48 h to export results (Foddai and Grant, 2020). However, molecular methods are faster, sensitive and more precise but they are expensive (Kotsiri *et al.*, 2022). Even though polymerase chain reaction (PCR) is superior in accuracy and reliability and can decrease the testing time to 24 h, it remains expensive to detect microorganisms in routine monitoring (Jasim *et al.*, 2019).

To overcome the needs of specialized equipment such as PCR equipment, the loop-mediated isothermal amplification (LAMP) method has attracted scientific interest in the past few years (Pang *et al.*, 2019). The simplicity, robustness and low cost of the method are what have attracted the most attention. The amplification of nucleic acid can be simply achieved under isothermal conditions ranging from 60°C to 65°C (Daddy Gaoh *et al.*, 2021; Houmansadr *et al.*, 2020). The amplification is performed by using the enzyme Bst DNA polymerase, which demonstrates a highly displacement activity (Bi *et al.*, 2020). Multiplex LAMP allows the detection of multiple genes at the same time. The comparative advantage of this assay is the time-saving and cost-effective analysis (Rainbow *et al.*, 2020).

In recent years, biosensors have emerged and been considered as a powerful tool for diagnosis (Andryukov *et al.*, 2020). Their applications have been applied to a variety of analyses, including not only health care but also environmental monitoring. A biosensor is a small instrument that delivers a signal through the integration of an active biological element with a suitable physicochemical transducer. The biological molecule could be DNA/RNA, an antibody, a protein or a

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microorganism (Nesakumar *et al.*, 2021). Biosensors are filling the gap and can export rapid results in a short period of time, are sensitive in the detection of biomolecules and can be miniaturized in portable devices where the conditions are required (Leonardo *et al.*, 2021). Finally, they need a small quantity of samples to test compared with conventional analytical methods (Choi *et al.*, 2018). This new technology could provide results in a few hours and inform the public and help stakeholders to improve health protection (Vidic *et al.*, 2019, 2017).

To perform water analysis, the European Commission's directives suggest a minimum volume of 100 mL of sample to deliver results. On the other side, sensors, until today, required small amounts of sample volumes, equal to or smaller than 1 mL in several cases. Therefore, a preconcentration step is necessary to diminish the initial volume (Ezenarro *et al.*, 2022). Preconcentration methods are usually based on magnetic preconcentration using aptamers (Kotsiri *et al.*, 2019), antibodies (Park and Choi, 2017) and centrifugation (Wu *et al.*, 2020). Filtration (Lee *et al.*, 2019b) has been widely used in large sample volumes (Zhang *et al.*, 2018).

An important part of FIB detection and limit of detection (LOD) is based on filter efficiency. Our study aims to evaluate and compare the market filters applied in bacterial seawater analysis. This efficient filtration could be integrated into a biosensor.

Materials and methods

Bacterial growth

Escherichia coli NCTC 9001 and *Enterococcus faecalis* NCTC 12697 were dissolved in Tryptic soy broth (TSB) (Oxoid, England). The solutions were spread on petri dishes containing Chromocult coliform agar (CCA) (Merck, USA) and Slanetz and Bartley (SB) agar (Oxoid, England), respectively. Following overnight incubation at 37°C for 24 h and at 37°C for 48 h, 30 mL of TSB was separately inoculated with a single colony of *E. coli* and *E. faecalis*. The liquid cultures were incubated at 37°C for 16–18 h while being shaken at 180 rpm. The following day, for bacterial enumeration, a scattered light turbid meter was used, with optical density measured at a wavelength of 600 nm. Then, serial dilutions were prepared, and bacterial concentrations were calculated by triplicate analysis using the plate count method. The stocks were stored at −20°C with glycerol at 70% in a ratio of 4:1.

Syringe filters

Five types of 0.45-μm membrane syringe filters were evaluated to determine the most appropriate filter for seawater analysis. The filters used are presented in Table 1. All filters were purchased from the same manufacturer (Whatman, Maidstone, UK), eliminating any analytical errors because of differences among manufacturers. All filter materials are hydrophilic.

Sample preparation and analysis

Seawater samples were collected from coastal sites in Patras, Greece (Figure 1). All samples were passed through a syringe filter with pore sizes of 5 μm (nitrocellulose membrane, Whatman) and then 0.2 μm [polyethersulfone (PES), Puradisc, Whatman] to capture sediment, bacteria and any other organism.

Seawater samples equal to 100 mL spiked with *E. coli* (10^4 CFU/mL) were filtered through different filters. A peristaltic pump (Cleaver Scientific, UK) was used to collect seawater through a filter from the sample tank. From a second reservoir, 10 mL of dH_2O was passed through a syringe filter to manage washing. In the backward flow process, 1 mL of dH_2O was backflushed, henceforth, with reversed rotational direction and the collected retentate flow, including bacteria, was transferred into a 1.5-mL tube for the next step of detection. The procedure is done in triplicate.

Cultures

For the evaluation of filters, the backflushed 1 ml was used by applying standard method of ISO 9308-1:2014 to determine the bacterial recovery. From the backflushed 1 mL, the surface plate technique was used for the detection of *E. coli* with CCA by placing 100 μL to evaluate the bacterial recovery. The dishes were incubated at 37°C for 16–18 h. The efficiency was assessed by calculating the number of bacterial colonies on plates after three replications on a colony counter (CC-J3; Bioevopeak).

Conventional polymerase chain reaction

For the extraction of DNA, 1 mL of backflushed sample was heated at 100°C for 10 min to achieve bacterial lysis. DNA quantity and purity were checked using the NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Conventional PCR was performed with yaiO primer (Table 2). All reactions were performed on a MJ Mini Personal Thermal Cycler (Bio-Rad, USA). The PCR reaction mixture had a total volume of 20 μL and consisted of 10× Dream Taq PCR buffer (Thermo Fisher Scientific, USA), 0.2 mM dNTPs, 0.6 mM betaine, 1.25 U of Dream Taq DNA polymerase, 0.25 μM forward and reverse primers, template DNA and nuclease-free water. All reactions were performed on tubes and flat cap strips of 8 (Thermo Fisher Scientific, USA), and the cycling conditions for PCR with target *E. coli* were carried out under the following program: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°

Table 1 Types of filter applied in this study

Filter type	Abbreviation	Pore size	Characteristics
Polyvinylidene difluoride	PVDF	0.45 μm	High solvent resistance, low protein binding
Regenerated cellulose	RC	0.45 μm	Low protein binding
Polyethersulfone	PES	0.45 μm	Stability in low pH, exhibits low protein binding
Glass microfiber/glass fiber	GD/X	0.45 μm	High-viscosity filters with a prefilter
Cellulose acetate	CA	0.45 μm	Low protein binding, suitable for protein recovery applications
Anotop		0.2 μm	Enables a high level of consistency in particle retention

Figure 1 Schematic illustration of water sampling for bacteria

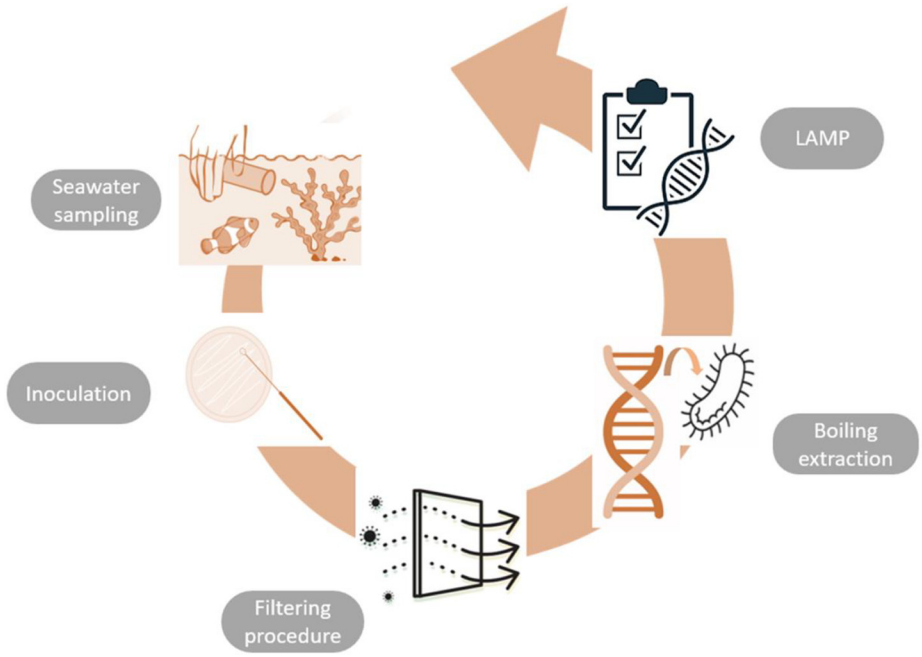


Table 2 Primers and probes used in this study

Target organism	Target gene	Type	Primer	Primer sequence (5′–3′)	Reference
E. coli	yaiO	PCR	F	TGATTTCCGTGCGT CTGAATG	Molina *et al.* (2015)
		PCR	R	ATGCTGCCGTAGCGTGTTTC	
E. coli	uidA	LAMP	FIP	TAACGCGCTTTCCCACCAACGGCCTGTGGGCATTCAGTC	Ahmad *et al.* (2017)
		LAMP	BIP	TAACGATCAGTTCGCCGATGCACTGCCCAACCTTTCGGTAT	
		LAMP	F3	CKGTAGAAACCCCAACCCG	
		LAMP	B3	AWACGCAGCACGATACGC	
		LAMP	LF	TCCACAGTTTTCGCGATCCA	
		LAMP	LB	ACGTCTGGTATCAGCGCGAAGT	
E. faecalis	23S rRNA	LAMP	FIP	GCAATCGTAACTCGCCGGTTCAAACCGTGTGCCTACAACAA	Lee *et al.* (2019)
		LAMP	BIP	TCGAAGAGACGGAGCCGCAG-TGGTTTCGGGTCTACGACT	
		LAMP	F3	GAAAAGCACCCCGGAAGG	
		LAMP	B3	ACCTGGACATGGGTAGATCA	
		LAMP	LF	CCATCACTCATTAACGAGCTTTGAC	
		LAMP	LB	CGAGTCTGAATAGGGCGAATGAGTA	

C for 30 s and finally 72°C for 10 min. The amplified products were analyzed by gel electrophoresis in 2% ultrapure agarose gels (AgaPure^{TM} Agarose LE, Canvax, Biotech) with the addition of TAE buffer at 100 V for 1 h. A UV light with a camera on it was used for the visualization of DNA bands and to determine the results. The loaded PCR products on a 2% agarose gel obtained fragments of about 115 bp for *E. coli*. The procedure was followed in triplicate.

Limit of detection analysis for the applied method

After the evaluation of filters, the LOD of the integrated procedure was performed for both FIB. A total of 1,000 mL of seawater sample was inoculated with serial dilutions ranging from 10^2–10^7 of *E. coli*, *E. faecalis* and mixed *E. coli* and *E. faecalis*. Then, samples were filtered through a cellulose acetate (CA)-S syringe filter. A peristaltic pump (Cleaver Scientific) leads the whole volume of seawater to the filter. Another tank containing dH_2O transfers 100 mL through a syringe filter. For the reversal procedure, 1mL of dH_2O was backflushed with and the supernatant transferred into a 1.5-mL tube. The tube was heated at 100°C for 10 min to achieve bacterial lysis.

Colorimetric loop-mediated isothermal amplification

The primers for the LAMP reactions of the *E. coli* and *E. faecalis* strains were designed to target the region of the b-D-glucuronidase (uidA) (Ahmad *et al.*, 2017) and Enterococcus 23S rRNA genes (Lee *et al.*, 2019b). A set of six primers, two outer primers, a forward outer primer (F3), a backward outer primer (B3), two inner primers, a forward inner primer (FIP), a backward inner primer (BIP) and two loop primers (LF and LB) were used (Eurofins, Germany).

The LAMP assay was performed in a total of 20 μL of a mixture containing WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs, Singapore), 10× LAMP primer mix (1.6 mM FIP and BIP, 0.2 mM F3 and B3, 0.4 mM LF and LB), 5.5 μL genomic DNA and sterile deionized water, according to the manufacturer's instructions. The LAMP reactions were carried out at 64°C for 60 min in triplicate. LAMP results were considered positive when the color of the reaction changed from pink to yellow-orange. LAMP products were supplementary examined on 2% agarose gel.

Results and discussion

The applied method combines simple steps such as filtering, extraction by boiling and LAMP to acquire accurate results.

The *E. coli* recovery for each filter with the ISO 9308-1:2014 method is illustrated in Table 3. CA-S filter displays the highest bacterial recovery (98.5%) when analyzed both by the surface plate and conventional PCR. CA is generally used in applications involving filtration for recovery or for the analysis of proteins, amino acids, etc. More investigation of the syringe filters was carried out in terms of robustness. Thus, CA filters showed optimal performance and they were chosen to be applied in seawater samples in accordance with other studies that indicated the main element of the membrane was cellulose acetate (Briciu-Burghina *et al.*, 2017). Equally good results arose from the use of the RC filter, with about 93.5% accuracy. Those filters are preferred for samples suspended in aqueous media. The low protein binding character suits applications where minimal binding and maximizing recovery of proteins and amino acids are important (Hinkley *et al.*, 2020). Moderate results regarding the detachment of the bacteria from the surface of the membrane were obtained for the glass microfiber/glass fiber (GD/X) and polyvinylidene difluoride (PVDF), at 67.5% and 48.33%, respectively. GD/X are constructed of a prefiltration stack of GMF 150 (graded density) and GF/F glass microfiber media that leads to the entrapment of contaminants, and thus makes backflushing difficult. PVDF is useful for filtering aqueous/organic suspensions, especially where minimal protein binding is required. Poor results were extracted from the analysis using PES (15%) and Anotop (7.5%) filters. PES filters present a hydrophilic membrane exhibiting low protein binding and low extractables, and in that way it is possible to trap bacteria. Anopore filters contain a membrane that has a well-defined pore structure and very narrow pore-size distribution, making it especially suitable for critical applications. In these types of filters, the solvents are clogged on the surface. The results of bacterial recovery, derived by the standard plate method, were confirmed after boiling a 1 mL backflushed sample by PCR, as it shows bands of moderate to high efficiency (Figure 2).

Volume reduction efforts of backflush at 100 μL, it was impossible to collect the eluted water and untrap bacteria. The syringe filter was filled with air and made the procedure impossible. That is why we increase the elution volume by about 1 mL. The results were precise, as there were no false positive samples in all revisions of the experiment. PCR is characterized by specificity, as the primers were unique for *E. coli* according to the BLAST database.

Table 3 *E. coli* recovery efficiency % from filters with standard deviation of three sets

Target	Type of filter	% recovery
E. coli	PVDF	48.3 ± 0.07
	RC	93.5 ± 0.07
	PES	15 ± 0.01
	GD/X	67.5 ± 0.07
	CA-S	98.5 ± 0.02
	ANOTOP	7.5 ± 0.9

Limit of detection of integrated procedure with seawater

Nanodrop was used to measure DNA concentration (Table 4). We further determined the LOD of our method using colorimetric LAMP for rapid results. Seawater samples containing against *E. coli*, *E. faecalis* and the inoculation with both bacteria. The detection results of four concentrations of bacteria, ranging from 10^4 to 10^7 CFU/mL are shown in Figure 3. Positive LAMP assay results were determined by the visible color change from pink to orange-yellow using the WarmStart Colorimetric. The color change indicates the presence of DNA. Each concentration was tested in triplicate in parallel experiments, and it was tested with the plate technique. LAMP assays are applied for rapid detection in food and water samples (Martzy *et al.*, 2017; Pang *et al.*, 2019). LAMP generates such vast amounts of DNA products, which is why assays that include dyes are preferred and the risk of carryover contamination is annihilated (González-González *et al.*, 2021).

The LOD reported was 10^4 for all inoculations. Both FIB are characterized by primer specificity according to the BLAST database. LAMP results had reproducibility in all experiments that were performed. Gel electrophoresis using the end-point of LAMP products confirmed DNA products in the assay (Supplementary Figures S1–S3).

There is a need for new, rapid and accurate methods for the determination of microbial indicators in seawater samples, and it is one of the main goals of research focused on the control and management of water quality. Those techniques that have a quick response could be used in platforms for accurate results in wastewater (Donia *et al.*, 2022).

Conclusions

Coastal water body quality is studied for bacteria to avoid diseases that are associated with swimming. The 2006/7/EC directives propose less than 250 CFU of *E. coli* and 100 CFU of *E. faecalis* in 100 mL of water as "good quality." The

Figure 2 Evaluation of bacterial recovery of used filters visualized on 2% agarose gel electrophoresis for amplified yaiO gene

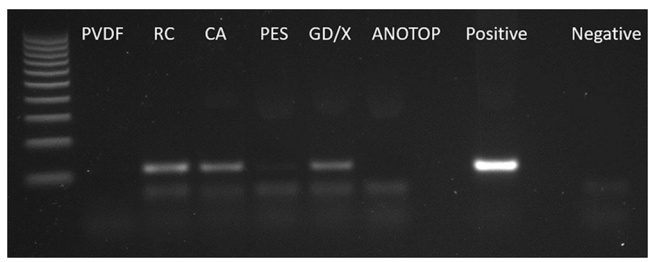
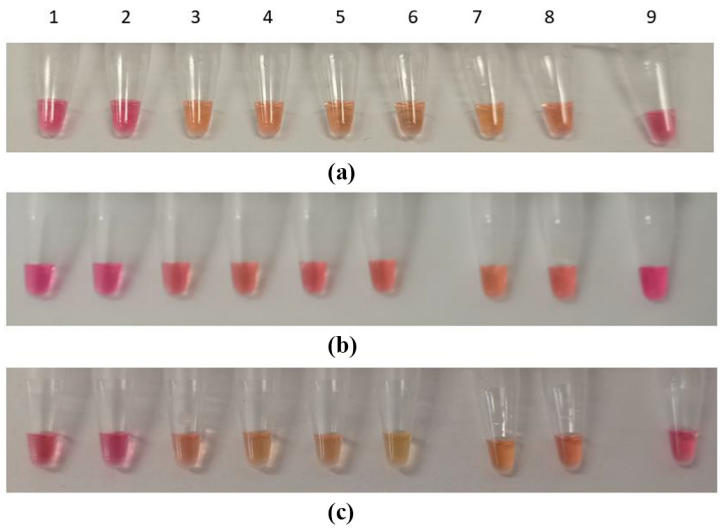


Table 4 Average of nanodrop measurements for FIB

CFU/mL	*E. coli*, ng/μL ± Std. dev.	*E. faecalis*, ng/μL ± Std. dev.	*E. coli* + *E. faecalis*, ng/μL ± Std. dev.
10^2	0.57 ± 0.03	0.47 ± 0.17	1.7 ± 0.14
10^3	1.12 ± 0.53	0.8 ± 0.14	2.2 ± 0.14
10^4	1.55 ± 0.35	1.37 ± 0.1	3.37 ± 0.17
10^5	1.97 ± 0.45	2 ± 0.14	4.15 ± 0.07
10^6	2.4 ± 0.35	2.45 ± 0.07	4.75 ± 0.14
10^7	2.87 ± 0.31	3.32 ± 0.74	5.85 ± 0.91

Figure 3 Sensitivities of LAMP assays for the detection of serially diluted bacterial DNA



Notes: A (*E. coli*): 1, 8.9×10^2 CFU/mL; 2, 8.9×10^3 CFU/mL; 3, 8.9×10^4 CFU/mL; 4, 8.9×10^5 CFU/mL; 5, 8.9×10^6 CFU/mL; 6, 8.9×10^7 CFU/mL; 7, (positive *E. coli*) 8.9×10^4 CFU/mL; 8, (positive *E. faecalis*) 2×10^4 CFU/mL; 9, negative. B (*E. faecalis*): 1, 2×10^2 CFU/mL; 2, 2×10^3 CFU/mL; 3, 2×10^4 CFU/mL; 4, 2×10^5 CFU/mL; 5, 2×10^6 CFU/mL; 6, 2×10^7 CFU/mL; 7, (positive *E. coli*) 8.9×10^4 CFU/mL; 8, (positive *E. faecalis*) 2×10^4 CFU/mL; 9, negative. C (*E. coli* + *E. faecalis*): 1, $(8.9 + 2) \times 10^2$ CFU/mL; 2, $(8.9 + 2) \times 10^3$ CFU/mL; 3, $(8.9 + 2) \times 10^4$ CFU/mL; 4, $(8.9 + 2) \times 10^5$ CFU/mL; 5, $(8.9 + 2) \times 10^6$ CFU/mL; 6, $(8.9 + 2) \times 10^7$ CFU/mL; 7, (positive *E. coli*) 8.9×10^4 CFU/mL; 8, (positive *E. faecalis*) 2×10^4 CFU/mL; 9, negative. Three replicates were tested (not shown)

number of biomarkers should not exceed 500 CFU for *E. coli* and 200 CFU for *E. faecalis* to be considered as "sufficient." Overall, six kinds of membranes were evaluated to choose the one that is more suitable for bacterial condensation. The results suggest CA filters for that purpose. The colorimetric LAMP assay combined with the syringe filter and boiling method gave rapid results for fecal biomarker detection. This assay gave accurate results when multiplex colorimetric LAMP was performed, and detected both bacteria. This flow could be used for sampling until detection in a portable system to test water quality with an unqualified staff in a single assay. It would be cost-effective and could give fast results. Another advantage of this method is the simple equipment that LAMP requires. Potentially, anyone from the municipalities could place the device, a biosensor, with those specific requirements and then share the results to the public.

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Supplementary materials

Supplementary materials of this article can be found online.

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