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Colorimetric and electrochemical genosensors for the detection of *Escherichia coli* DNA without amplification in seawater

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ABSTRACT

Monitoring seawater, particularly recreational water, for indicator bacteria presence is required to protect the public from exposure to fecal pollution and to guarantee the safety of the swimming areas. Two methods for the detection and quantification of *Escherichia coli* DNA were developed: a colorimetric assay in a microplate and an electrochemical biosensor. These assays were based on the double hybridization recognition of a single-strand DNA capture probe immobilized onto the microplate or the screen-printed carbon electrode to its complementary ssDNA, which is hybridized with an ssDNA signal probe labeled with horseradish peroxidase enzyme. The hybridization recognition step used the colorimetric monitoring of the oxidation state of the 3,3′,5,5′-tetramethylbenzidine. The electrochemical monitoring of the oxidation state of 5 methyl-phenazinium methyl sulfate was allowed when the horseradish-peroxidase was in the presence of the mediator (5 methyl-phenazinium methyl sulfate and hydrogen peroxide). These approaches allow for the detection and quantification of 10^2 to 10^3 cells of *E. coli* in 5 l of seawater samples in less than 5 h. Detection was achieved without a nucleic acid amplification step. The specificity of the two methods against *E. coli* was demonstrated by testing a panel of bacteria. The two methods can be used for on-site monitoring of seawater quality.

1. Introduction

Bacterial pathogens are distributed in soil, marine and estuarine waters, the intestinal tract of animals and water contaminated with fecal matter. The majority of these pathogens are enteric in origin; that is, they are excreted in fecal matter that contaminates the environment and then gains access to new hosts through ingestion (*i.e.*, the fecal–oral route). The presence of pathogenic organisms such as bacteria, viruses and other microorganisms in water, bathing water and, more particularly, seawater can have deleterious consequences to human health and the economy because of waterborne illnesses and the closing of recreational and seafood harvesting waters [1–4]. Among the fecal coliform bacteria used routinely to monitor aquatic systems for sewage contamination, *Escherichia coli* is often selected because it is specific for water pollution [5] and reflects the presence of fecal contamination and thus the risk of contamination by other fecal pathogens (*e.g.*, *Salmonella*, *Shigella*, enteric viruses) [6–9]. *E. coli* is present in the intestinal tracts of humans and warm-blooded animals, and some strains are associated with human infection. *E. coli* can cause diarrhea, urinary tract infections, inflammation and peritonitis in immunosuppressed patients as well as children and the elderly [10]. Therefore, a sensitive, rapid and specific detection and quantification of *E. coli* in water to help monitor the water quality is imperative for safety and hygiene reasons. Normalized microbiological methods for the isolation of the common bacterial pathogens and indicator bacteria such as *E. coli* from water and wastewater are based on cultures grown on differential agar media followed by counting the number of target organisms in the samples [8,11]. The basic steps for the detection of bacteria are preenrichment, selective enrichment, biochemical screening and serological confirmation. For *E. coli*, a rapid enzymatic test based on the activity of the β-D glucuronidase was used for the detection and quantification of the strain [7]. The interpretations of these tests are often difficult. The process is laborious and time consuming in the case of *E. coli*, and at least 24–48 h are necessary to obtain a result [12,13]. This method also requires well-trained personnel. Another drawback of the cultural method is its low sensitivity, risk of microbial contamination resulting in the inhibition of growth of bacteria of interest and the presence of viable but nonculturable bacteria (VBNC). The consequences of the presence of VBNC bacteria are the underestimation of viable cells pathogen numbers or a failure to isolate the pathogen from water [5,14,15]. The VBNC state is current with environmental samples, the bacteria in the environment are often starved or stressed, making them not readily cultivable. Difficulties

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encountered with normalized methods increase the motivation for the development of more sensitive, rapid and specific techniques for *E. coli* detection and quantification. New methods have been developed for the detection of pathogenic bacteria and the fecal indicator *E. coli*, including the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reactions (PCR). With the ELISA method, the results were obtained in 24–48 h, and the sensitivity was close to 10^5 CFU/ml. This sensitivity was insufficient to detect low levels of pathogens [16]. PCR is a more sensitive method, but it often results in false positives [17]. The PCR reaction may be inhibited by the presence of compounds in the sample matrix, making pretreatment by purification of the samples necessary [18]. Recently, biosensors have become attractive alternatives for bacteria detection due to their ability to work in very dirty environments with minimal sample preparation and their simplicity, versatility, portability, low cost and potential for accurate, real-time detection [12,19–31]. Several methods of biosensors have been proposed [32–36], including optical [37], magnetic [38], field effect transistors (FET) [39], quartz crystal microbalance [40] and electrochemical biosensor techniques [41]. Biosensors have many advantages, such as high-throughput analysis, high sensitivity and specificity [25]. Various electrochemical sensors for pathogenic bacteria detection have been reported. Electrochemical immunosensors using an antibody as the molecular recognition element can directly and rapidly detect pathogenic bacteria with high affinity and specificity [42–44]. However, the drawbacks associated with antibodies, such as the difficulty in producing them, their instability and their modification post-production limit their application [45]. Electrochemical DNA sensors can detect specific genes of specific bacteria [46]. This sensitivity is possible because these sensors have greater specificity regarding the hybridization between the probe and the complementary target sequence than the immunologically based detection systems [47]. Biosensors based on the direct electrochemical detection of target nucleic acid molecules have successfully been demonstrated in a variety of approaches by linking DNA or RNA hybridization events onto an oligonucleotide-modified electrode surface [19,21,22,48–51]. The detection strategy with these sensors is an electrochemical sandwich hybridization assay (SHA) in which target DNA or RNA are bound by both a capture and a signal probe [48,52–56]. The principle of sandwich hybridization is that a species capture probe is immobilized over the electrodes. If a target sequence binds to the immobilized capture probe on the working electrode, its detection occurs via a hybridization reaction with a second signal probe that is coupled to digoxigenin, which is later coupled to an antibody with horseradish-peroxidase (HRP) enzyme. Target and signal probes bind to the capture-modified chips by incubating all components in a hybridization mixture. The electrochemical detection takes place after this process. HRP catalyzes the reduction of hydrogen peroxide to water. Reduced peroxidase is regenerated by a mediator, which is reduced back at the working electrode by applying a fix potential. Once the potential is applied, a potentiostat measures the resulting electrochemical current, which can only be measured if the target nucleic acid sequence binds to both the capture and signal probes [24,57]. In field samples, total DNA would be extracted, and this would represent the target nucleic acid for the probes. For each target species, the DNA concentration per cell must be determined. A calibration curve must be developed for each probe set to determine the signal intensity at different DNA concentrations. Using the information on the curve, the electrical measurement of the potentiostat can be related to cell numbers in any field sample [24]. As a consequence, the DNA biosensors are able to directly detect genetic target of bacteria without cultivation or gene amplification and only after the extraction and purification of nucleic acids from natural environmental samples. This process is followed by hybridization with specific probes.

The ability of electrochemical sensors to directly identify nucleic acids in complex samples is a valuable advantage over other approaches, such as polymerase chain reactions (PCR), which require target purification and amplification [41,48]. Biosensors are a very versatile technology. For bacteria, these sensors should reduce the labor time required to detect genetic targets. Hence, automation must be an inherent attribute of the biosensor.

The aim of this research is to detect and quantify *E. coli* in seawater samples using colorimetric and electrochemical DNA biosensors. In this paper, we describe the development of the colorimetric assay in microplate and electrochemical DNA biosensors in screen-printed electrodes for the specific, sensitive and rapid detection of *E. coli* DNA in seawater. Highly specific DNA probes hybridize with an *E. coli* DNA sequence without PCR amplification. The colorimetric assay and the biosensor assay have been optimized including DNA probe concentrations, hybridization condition, conjugate concentration and binding reaction time. The colorimetric assay's and electrochemical biosensor's limits of detection and specificity have been determined.

2. Materials and methods

2.1. Materials and reagents

Anti-digoxigenin-horseradish peroxidase (Anti-DIG-HRP) was purchased from Roche (France). Liquid substrate 3,3′,5,5′-tetramethylbenzidine (TMB), 5-methyl-phenazinium methyl sulfate (MPMS), H_2O_2 (30%) and NeutrAvidin were purchased from Sigma (France), and the substrate solution was prepared daily in a 100 mM acetate buffer solution containing 300 mM NaCl pH 5. Hammersten-grade casein was purchased from MP Biomedicals (France).

Maxisorp™ polystyrene microtiter plates were purchased from NUNC™ (Denmark).

DropSens 110 screen-printed carbon electrodes (DropSens, S.L., Spain) were used. We used a three-electrode system with carbon working and counter electrodes and a silver reference electrode.

Synthetic oligonucleotide targets positive control (PC) and probes were designed by Liao [41], the negative control (NC) was a randomly selected sequence and were synthesized by Thermo Fisher Scientific (Germany) (Table 1). The capture probe was biotinylated in 5′, and the signal probe was labeled with a digoxigenin in 3′. The target and the probes were diluted in milliQ water to obtain 100 μM stock solutions. The oligonucleotide probe pair was designed to hybridize with species-specific region of the 16 S DNA gene of *E. coli*.

2.2. Apparatus

Colorimetric measurements were performed with a Victor 3 1420 Multilabel counter (PerkinElmer). Cyclic voltammetry and amperometry were carried out with an EmStat (Electrochemical Sensor Interface) using the PSTrace software from Palm Instruments BV (The Netherlands) and a DCS boxer connector from Dropsens (Spain).

Table 1
DNA probe sets.

Name	DNA sequences (5′→3′)	Modifications
EC434C	GTCAATGAGCAAAGGTATTAACTTTACTCCCTTCC	5′ Biotin
EC399S	TCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTT	3′ Digoxigenin
PC	AAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAG ĆGAAGGGAGTAAAGTTAATACCTTTGCTCATTGAC	
NC	ATATAGATTATCACCAGTTGTAAGAACCTTCCTTAA	

PC: Positive Control.
NC: Negative Control.

2.3. Bacterial strains and cultivation

Enterobacteriaceae strains *Escherichia coli* ATCC (American Type Culture Collection) 10536 and the negative controls *Salmonella enterica* subsp. *enterica serovar typhimurium* ATCC 23564 (*Salmonella typhimurium* ATCC 23564), *Enterobacter cloacae* CIP (Collection de l'Institut Pasteur) 79.28 and *Shigella boydii* CIP 82.50 were the strains used in this study. For each strain, the incubation was performed at 25 °C to approximate the seawater temperature as closely as possible in the bathing zone during summer.

To reach the stationary phase, bacterial strain cultures were grown overnight in Tryptic Soy Broth (TSB) medium (tryptic, 17 g l^{-1}; soy peptone, 3 g l^{-1}; NaCl, 5 g l^{-1}; K_2HPO_4, 2.5 g l^{-1}; glucose, 2.5 g l^{-1}; pH 7.3) (Sigma, France) at 25 °C with aeration by shaking at 200 rpm. To detect the density of *Escherichia coli, Enterobacter cloacae*, *Shigella boydii* and *Salmonella typhimurium*, the stationary phase of the strain cultures were serially diluted and plated on Trypticase Soy Agar (TSA) (tryptic, 15 g l^{-1}; soy peptone, 5 g l^{-1}; NaCl, 5 g l^{-1}; agar, 15 g l^{-1}; pH 7.3) (Sigma, France). After incubation at 25 °C for 24 h, *Escherichia coli, Enterobacter cloacae*, *Shigella boydii* and *Salmonella typhimurium* colonies on plates were counted to determine the number of colony-forming units per milliliter (CFU/ml).

2.4. Preparation of artificially contaminated seawater samples

Seawater was sampled in the Bay of Banyuls sur Mer, France.

One milliliter of freshly grown *Escherichia coli* culture in TSB at 10, 100, 1000 and 10,000 CFU/ml was centrifuged at 25 °C for 5 min at 5000 g in 1.5 ml microtubes. After removing the supernatant, the cell pellets were resuspended in 1 ml of seawater, and each sample (5 L) of seawater was spiked with the corresponding *Escherichia coli* cell concentration. The bacterial cell number in the TSB cultures was checked by spreading serial dilutions of the culture on sterile TSA and by counting the colonies of *Escherichia coli* that appeared on the TSA plate medium after 24 h of incubation at 25 °C. For the seawater control, *Escherichia coli* culture was replaced by sterile water, and this sample was centrifuged (5 min, 5000 g, 25 °C). The pellet was resuspended in 1 ml of seawater, and 5 L of seawater was spiked with this control. After a manual shaking (2 min at 25 °C), each 5 L spiked sample of seawater was pre-filtered over a 45 μm filter, and a final filtration was realized over a 0.2 μm filters (Millipore). Five batches of seawater samples were realized for each *Escherichia coli* concentration.

2.5. DNA extraction

Total DNA was extracted from the 0.2 μm filters. A treatment with RNase was realized prior to the steps of DNA extraction. One milliliter of extracting buffer (Tris–HCl, 100 mM, pH 8; EDTA, 100 mM, pH 8; NaCl, 100 mM; SDS, 2%) (Sigma, France) was added to the filter in a 2 ml sterile test tube containing 0.5 g of 106 μm diameter glass beads and two 2 mm diameter glass beads (Sigma, France). After five 20 s runs of high-speed mechanical disruption with a genie disruptor vortex (Scientific Industries, France), 20 μl of proteinase K (20 mg ml^{-1}) (Sigma, France) was added. The mixes were incubated at room temperature for 30 min and centrifuged (7000 g, 5 min, 4 °C), after which $NaClO_4$ (5 mol) (Sigma, France) was added (1:10, v/v) to the supernatants. After a 10 min incubation on ice, the extracts were centrifuged (14,000 g, 5 min, 4 °C). Total DNA was precipitated with cold isopropanol (v/v) for 30 min at −20 °C, washed with 70% ethanol and centrifuged (13,000 g, 5 min, 4 °C). Ultrapure sterile water was added to the pellet. Total DNA was purified using polyvinylpolypyrrolidone (PVPP) columns (Bio-Rad Laboratories, Marne la Coquette, France). The DNA was chilled in ice to obtain denatured, single-stranded DNA. The single-stranded DNA products were stored at −20 °C to prevent degradation.

2.6. Colorimetric test procedure

Colorimetric checkerboards were first performed on 96-well microtiter plates to optimize all concentrations of the involved reagents; they were subsequently performed on screen-printed graphite electrodes. Trials were performed at room temperature.

First, 96-well MaxiSorp plates were modified with 100 μL of 1 μg ml^{-1} NeutrAvidin solution (Sigma, France) in HEPES (Sigma, France) 0.1 M, pH 7.2, for 1 h at room temperature. The wells were thoroughly rinsed three times with 300 μL of phosphate-buffered saline (PBS) 1X (K_2PO_4, 0.1 M; KH_2PO_4, 0.1 M; KCl, 0.1 M). Before immobilization, the denaturation of the oligonucleotide functionalized probe was necessary. This process was achieved by heating the probe solution at 90 °C for 3 min with a thermomixer and allowing it to stand at 4 °C for 2 min and at room temperature for 5 min. The microtiter plate was then coated with 100 μL of 0.1 μM biotinylated DNA capture probe during 30 min of incubation. After blocking the unspecific sites with 1% Hammersten casein solution in binding buffer (BB) 1X (PBS 1X pH 7.4; $MgCl_2$ 1 mM; Tween 20 0.01%) for 45 min at room temperature, the hybridization step was performed for 30 min at 46 °C by adding 100 μL of the hybridization mix. The hybridization mix was prepared as follows: target and signal probes were assembled over the microtiter plate in a unique step by incubation in a hybridization mixture containing 25 μL 4X hybridization buffer (NaCl, 0.3 M; Tris 0.08 M; SDS 0.04%; pH 8), 7 μL DIG-labeled signal DNA probe (0.1 μM), 7 μL target sequence or non-complementary DNA sequence for the negative control and 61 μL milliQ water. For the calibration curve, the final concentrations in the mix of the targets' DNA sequences were 10^{-4}, 5×10^{-4}, 10^{-3}, 2.5×10^{-3}, 5×10^{-3}, 7.5×10^{-3}, 10^{-2}, 2.5×10^{-2}, 5×10^{-2} and 10^{-1} μM. A first negative control was included in which PBS 1X was used as the target instead of the target DNA sequence. The second negative control was realized with the negative control DNA sequence (Table 1). The hybridization mixture was incubated for 10 min at 65 °C. Subsequently, 100 μL anti-DIG-HRP conjugate at 75 mU/ml, corresponding to a 1: 2000 dilution of the stock solution in PBST-BSA, was added to the wells. Finally, 100 μL of HRP substrate solution (TMB liquid substrate) was added, and the absorbance was measured after 15 min at 620 nm. Each step was performed at room temperature with constant shaking and protection from light. The assays were performed three times for the optimization of the parameters and five times for the calibration curve.

2.7. Biosensor procedure

Cyclic voltammetry was used to investigate the redox mediator (MPMS) for HRP. Trials were performed at room temperature. First, the cyclic voltammogram corresponding to 0.001 M mediator in 100 mM acetate buffer, 30 mM NaCl, pH 5, was recovered at 0.010 V s^{-1} (10 mV s^{-1}). Afterward, HRP and H_2O_2 were added to the final 1: 2000 and 200 mM concentrations, respectively, and the cyclic voltammogram was recorded again.

The biosensor protocol was essentially the same as the colorimetric test but performed on screen-printed graphite electrodes. Briefly, the attachment of the biotinylated probe is performed through NeutrAvidin after incubation during 1 h at room temperature in 1 μg mL^{-1} NeutrAvidin solution. If a target sequence is bound to the immobilized capture probe on the working electrode, its detection takes place via a hybridization reaction to a second signal probe that is coupled to digoxigenin, which is later coupled to an antibody with the HRP. Target and signal probes are assembled over the capture-modified chips in a unique step by incubation in a hybridization mixture containing 2.5 μL 4X hybridization buffer, 0.7 μL DIG-labeled signal DNA probe (0.1 μM), 0.7 μL target sequence or non-complementary DNA sequence for

the negative control and 6.1 μL milliQ water. The same calibration curve as those carried out for the colorimetric assay, with a concentration range of target DNA between 10^{-4} and 10^{-1} μM, was performed. A negative control was included in which PBS 1X was used as the target instead of the target DNA sequence. The second negative control was realized with the negative control DNA sequence (Table 1). The hybridization mixture was incubated for 10 min at 65 °C. Chips were incubated in the hybridization mixture at 46 °C for 30 min after which the electrochemical detection was performed.

The major difference with the colorimetric assay was the measurement step. Amperometric measurements were carried out by placing a 70 μL drop of 100 mM acetate buffer, 300 mM NaCl, pH 5, and a 20 μL drop of 1 mM MPMS onto the carbon/SPE surface, covering the area of the three electrodes. When a steady-state current was obtained at an applied potential of −200 mV *vs.* Ag/AgCl of the carbon/SPE, a 10 μL drop of 200 mM H_2O_2 was deposited on the electrode surface and the change in the signal was recorded. Amperometric measurements were carried out at a set time of 100 s after H_2O_2 addition, which allowed for the

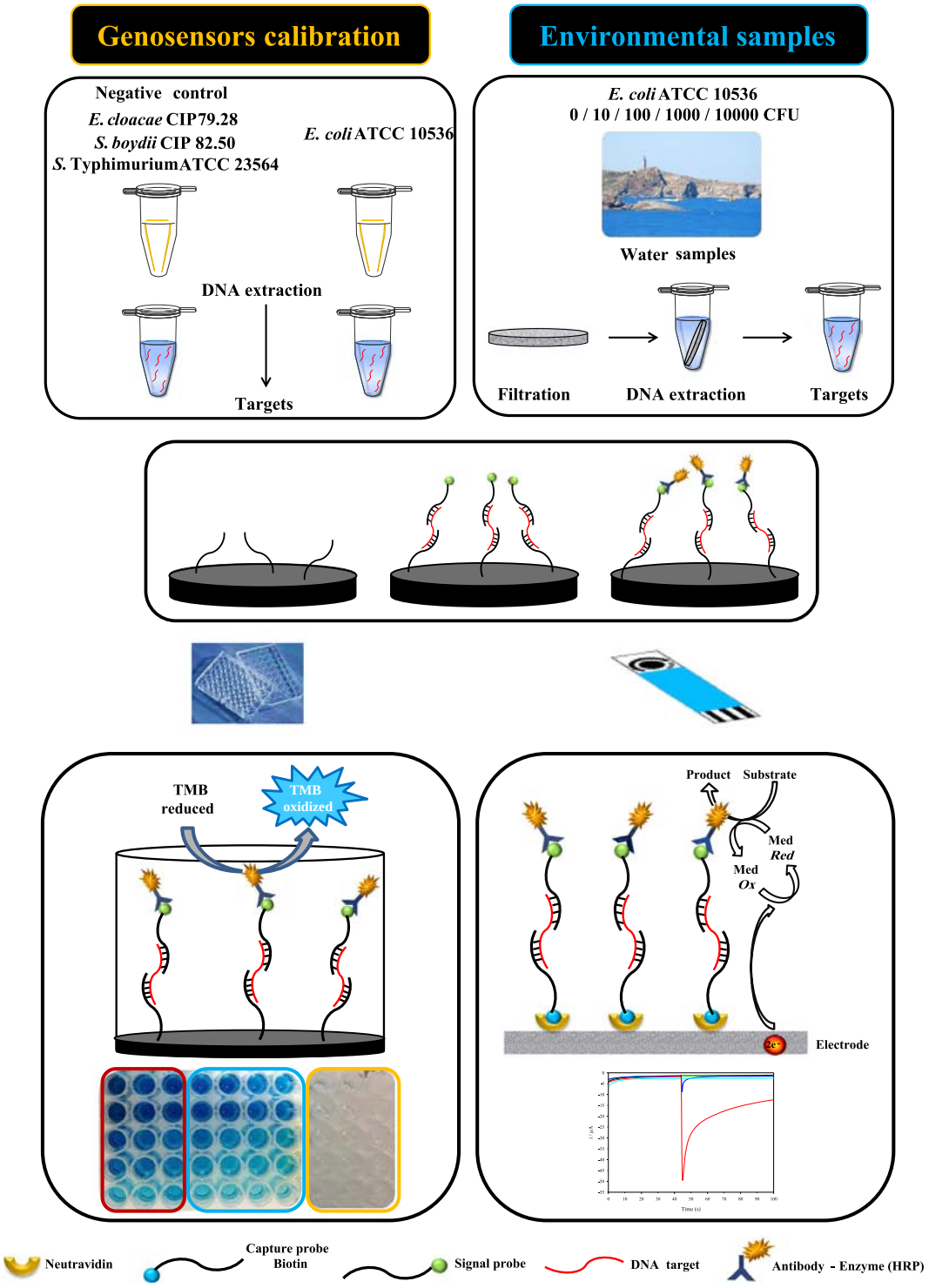

Fig. 1. Synoptic representation of the experimental approach.

stabilization of the amperometric signal and, therefore, reproducible responses. Controls without each of the components were always performed. Electrode assays were performed five times. All measurements were carried out at room temperature.

The HRP was oxidized by hydrogen peroxide (Eq. (1)) and regenerated upon donation of an electron to the MPMS (Eq. (2)). The sensor reader generated a charged electrode surface by applying millisecond pulses of −200 mV potential, and the MPMS was regenerated (Eq. (3)) upon transferring an electron to the carbon sensor surface, thus completing the catalytic cycle.

$$H_2O_2+2H^++HRP_{red}\rightarrow HRP_{ox}+2H_2O \quad (1)$$

$$2\ MPMS+HRP_{ox}\rightarrow 2\ MPMS^++HRP_{red} \quad (2)$$

$$2\ MPMS^++2e^-\rightarrow 2\ MPMS \quad (3)$$

2.8. Statistical tests

An ANOVA with $\alpha=0.05$ was used to test the equality of the average for the DNA concentration deduced using the colorimetric ($n=5$) and the electrochemical sensors ($n=5$) for each *Escherichia coli* concentration.

3. Results

In this study, we explored the feasibility of using colorimetric and electrochemical nucleic acid hybridization assays to detect and quantify *Escherichia coli* without PCR amplification of the target DNA. Capture probes were immobilized on the different solid surfaces of a microtiter plate and SPE. First, assays were carried out on the microtiter well to define all the parameters and to verify the performance of the protocol.

Fig. 1 schematizes the two assay configurations of colorimetric and electrochemical sensors developed in this work for the detection and quantification of *Escherichia coli* without target DNA amplification.

3.1. Calibration of the two genosensors

The aim of this assay was to improve certain parameters, such as the sensitivity and selectivity, by means of a double-recognition hybridization event. The double hybridization method was chosen because some authors demonstrated the improvement of certain parameters, such as the sensitivity and specificity, compared with the single hybridization format [58]. Pividori et al. (2001) demonstrated that the double-recognition hybridization process provides higher amperometric signals compared with the single hybridization format. Furthermore, this format improves the specificity of the genosensor because a wider sequence of the target is identified with two different complementary probes [58].

3.1.1. Colorimetric test for Escherichia coli synthetic DNA detection

In this experiment, the dilution ratios of the DIG-HRP conjugate, the binding reaction time and the capture and signal probes were investigated and optimized. The immobilization of the DNA capture probe onto the wells was optimized by testing a coating time ranging from 15 min to 120 min. No difference in the signal intensity was observed between 60 min to 120 min, whereas the signal intensity was lower between 15 min to 60 min. Therefore, a coating time of 1 h was chosen (data not shown). The blocking step was also studied because of its central point in colorimetric test and to avoid non-specific adsorption of the HRP. Different concentrations of Hammerstein casein were tested, and the best results were obtained from a concentration of 1% w/v (data not shown). The reaction time appears to be a crucial parameter because the absorbance value increased with increasing reaction time from 5 min to 20 min (Fig. 2). Longer incubation (40 min) resulted in a weak decrease of the response, indicating a possible dissociation of the complex DNA signal probe-conjugate. Therefore, a reaction time of 20 min was selected. The dilution of the conjugate was critical for the sensitivity of the analytical system. The absorbance value increased with an increasing conjugate (DIG–HRP) dilution ratio (Fig. 2). A plateau was reached for each time of incubation due to the low dilution ratio of the conjugate, which saturated the available binding sites (Fig. 2). To compromise between a measurable signal and a highly sensitive and cost-effective assay, the dilution of 1/2000 (75 mU/ml) was chosen as the optimum dilution of the conjugate to be used during the experiment, with a 100 μL DNA target.

After the optimization and the validation of all the parameters, the calibration curve with *Escherichia coli* synthetic DNA was performed. The working response and performance characteristics of the DNA-based sensor were evaluated by colorimetry (Fig. 3). Fig. 3A shows the response of the device when different concentrations of target probe, up to 10^{-1} μM, were set to react with the capture and signal probes. To validate the reproducibility of the sensor, five microplates prepared with the same volume and concentration of DNA probes were tested for each concentration of *Escherichia coli* synthetic DNA. The relative standard deviation average was 5% (Table 2). Such high precision reflects the reproducibility of the probe immobilization on the electrode surface, the hybridization and the colorimetric procedure. The stabilization of the signal from a concentration of *Escherichia coli* synthetic target DNA of 2.5×10^{-2} μM suggests that the limit of concentration in which the maximum quantity of capture, target and signal probe strands are linked was overcome (Fig. 3A). The colorimetric genosensor could detect the target DNA with a detection limit of 5×10^{-5} μM. A linear response in the colorimetric detection for the concentration of DNA was found between 5×10^{-4} μM and 10^{-2} μM, with a slope of 103.94 and a correlation coefficient of 0.9957 (Fig. 3B and Table 2).

The DNA extracts from the negative control strains (*Enterobacter cloacae* CIP 7928, *Shigella boydii* CIP 82.50 and *Salmonella typhimurium* ATCC 23564) and the negative control DNA sequence (Table 1) were tested. The colorimetric responses of the three strains and of the negative control DNA sequence corresponded to the background noise of the microplate with the solvent (data not shown). These results confirm the specific recognition of the colorimetric genosensor for *Escherichia coli*.

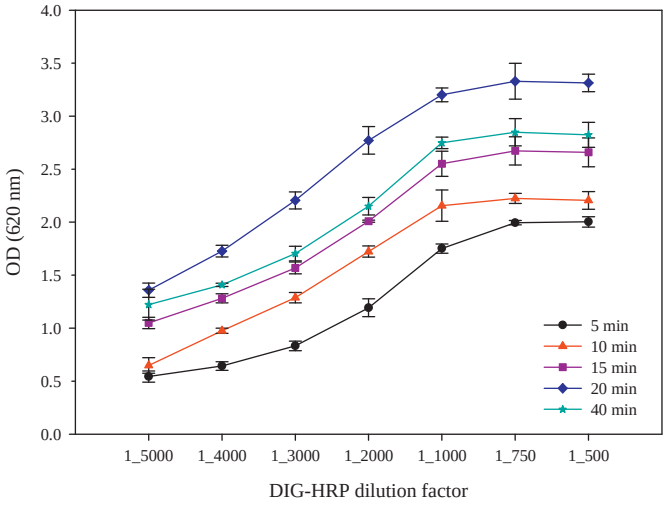

Fig. 2. Optimization of the experimental factors by colorimetric detection. Direct assay using several dilution ratios of DIG-HRP conjugate and five binding reaction times (5, 10, 15, 20 and 40 min). Error bars are standard deviations of the mean with $n=3$.

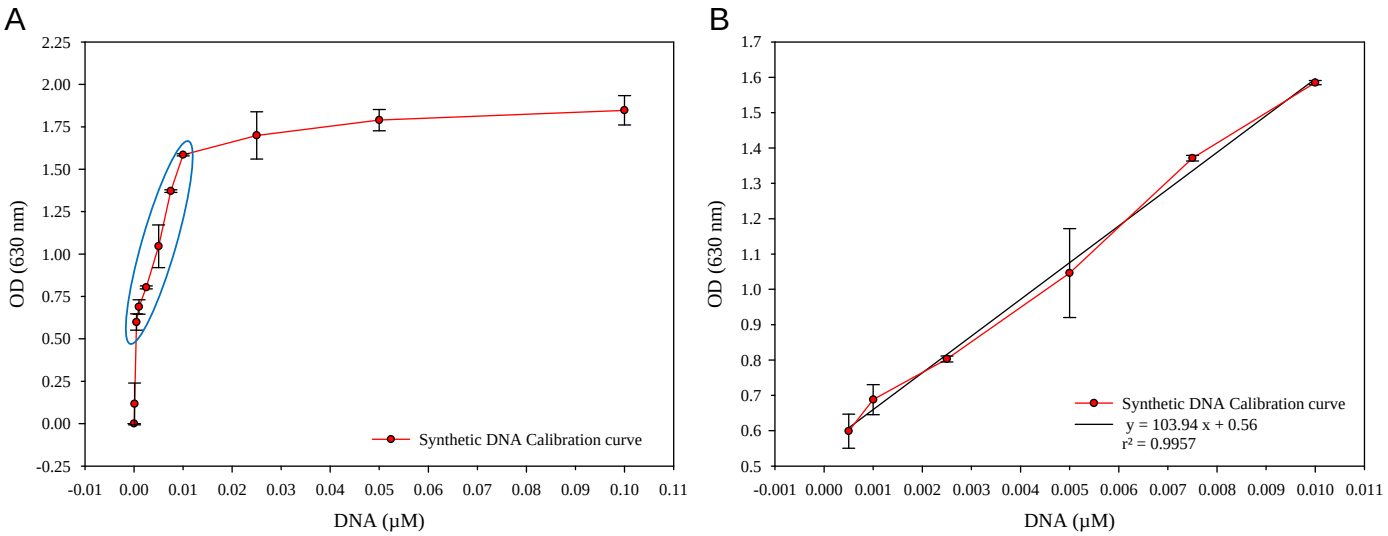

Fig. 3. Genosensor colorimetric responses. (A) Corresponding calibration curve. The blue circle corresponds to the linear portion. and (B) Linear portion of the calibration plot. Error bars are standard deviations of the mean with $n=5$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analytical parameters for the colorimetric and the electrochemical genosensors.

Calibration curves with synthetic DNA		
	Colorimetric assay	***Electrochemical assay***
Linear relationship	5×10^{-4} µM to 10^{-2} µM	5×10^{-4} µM to 25×10^{-3} µM
Regression equation	$Y=103.94\ X+0.56$	$Y=587.03\ X+13.24$
Correlation coefficient	0.9957	0.9945
Relative standard deviation (RSD)	5%	5%
Detection limit	5×10^{-5} µM	10^{-5} µM

The response of the colorimetric genosensor validates the parameters in terms of incubation time, concentration of the different constituents and specificity. These parameters were transferred for the development of the electrochemical genosensor.

3.1.2. *Electrochemical assay for Escherichia coli synthetic DNA detection*

The electrochemical detection was carried using SPEs to increase the sensitivity of the method. SPEs has the advantage of low cost and easy preparation. Additionally, no pre-treatment or cleaning step is required before the immobilization of the analyte. The immunosensor construction requires the immobilization of the probe on the electrodes. To this end, screen-printed graphite electrodes were used to guarantee the viability of the approach. Hybridization was monitored amperometrically at −0.2 V after the addition of 200 mM hydrogen peroxide. MPMS on the electrode surface mediated the catalytic reduction of H_2O_2 by HRP, and the electrochemical reduction of the generated $MPMS^+$ was measured at the above-mentioned potential.

First, the electrochemical behavior of both MPMS and $MPMS_{red}$ were investigated to optimize the conditions for the determination of HRP activity by amperometry. A cyclic voltammetric investigation of MPMS was carried out using a carbon electrode. The addition of HRP to a solution containing the two substrates (MPMS and H_2O_2) led to the consumption of MPMS and, consequently, to a decrease in the oxidation current and an increase in the reduction current. Cyclic voltammetry measurements demonstrated that the highest bioelectrocatalytic currents were achieved with MPMS. A working potential of −0.2 V (−200 mV) *vs.* Ag/AgCl for the measurement of HRP activity was chosen for this study (data not shown) [59]. At this potential, the current was near zero, and no substrate reduction occurred. These conditions were optimal for enzymatic activity determinations when a small amount of product ($MPMS_{red}$) was measured in the presence of a high concentration of substrate.

The detection takes place when the antibody of the signal probe (anti-DIG-probe) recognizes and binds to the conjugate (DIG-HRP) that is involved with the electrochemical signal amplification. HRP electrochemically converts an inactive substrate to an electroactive product that can be detected amperometrically. The current signal is proportional to the amount of the bound enzyme (and hence to the analyte concentration in the sample).

The biosensor performance is based on the catalytic reduction of the H_2O_2 to H_2O, which takes place with the concomitant oxidation of MPMS. These oxidized species can be reduced by polarizing the electrode.

Once the biosensor was characterized and the working potential and conditions were fixed, the analytical signal was studied by amperometry. This procedure was performed with optimized chemical conditions by applying a fix potential of −0.2 V.

In first the amperometric response, different buffers and reagents were studied (Fig. 4). No amperometric response was observed when the acetate buffer (Fig. 4, black curve (1)) or a solution of acetate buffer and MPMS (Fig. 4, green curve (2)) were added to the modified electrode before the hybridization process. The absence of signals in the buffer background voltammogram shows that no direct electron transfer between the heme group of the HRP structure and the electrode took place. A very weak amperometric response was recorded for the solution of acetate buffer, MPMS and H_2O_2 (Fig. 4, dark blue curve (3)). This response was taken as the background response of the hydrogen peroxide at the surface of the electrode.

The capture and signal probes' specificity for *Escherichia coli* DNA was also tested for the response to non-complementary DNA extract from a *Enterobacter cloacae* CIP 7928, *Shigella boydii* CIP 82.50, *Salmonella typhimurium* ATCC 23564 strain and the negative control DNA sequence (Table 1). Only the results obtained with *Salmonella typhimurium* ATCC 23564 were presented because the results were the same with the other strains and the negative

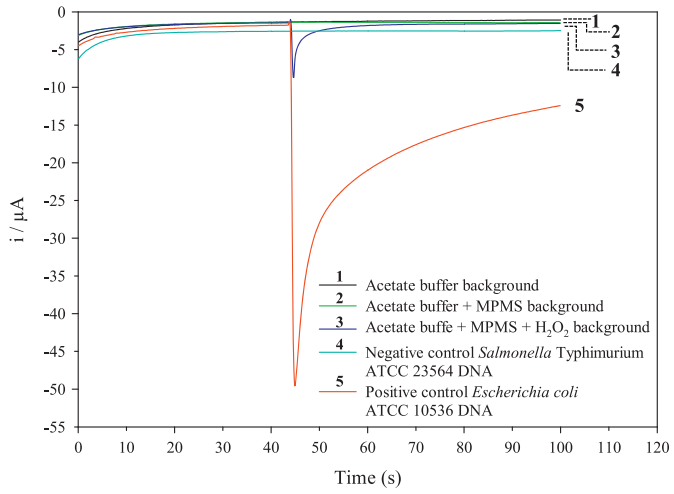

Fig. 4. Amperometric response of the biosensor. Analytical signal recorded at a fixed potential of −0.2 V. Background responses: in black (1) acetate buffer, in green (2) solution of acetate buffer and MPMS, in dark blue (3) solution of acetate buffer and MPMS and H_2O_2. Control responses: in light blue (4) negative control *Salmonella typhimurium* ATCC 23564 and in red (5) positive control *Escherichia coli* ATCC 10536. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

control DNA sequence. No change in the amperometric response was observed when *Salmonella. typhimurium* ATCC 23564 DNA was exposed to the capture and signal probes (Fig. 4, light blue curve (4)). In contrast, the response for *Escherichia coli* DNA was very high (Fig. 4, red curve (5)). This result demonstrated that these electrochemical genosensors were specific for *Escherichia coli* DNA detection.

The blocking step was very crucial because HRP could be easily absorbed on the working surface of the screen-printed electrode, leading to an unspecific response. The working response and performance characteristics of the DNA-based sensor were evaluated by amperometry. Fig. 5 shows the response of the device when different concentrations of target DNA, up to 10^{-1} µM, were set to react with the capture and signal probes. To validate the reproducibility of the sensor, five electrodes prepared with the same volume and concentration of DNA probes were tested for each concentration of *Escherichia coli* synthetic DNA. The relative standard deviation average was 5% (Table 2). Such high precision reflects the reproducibility of the probe immobilization on the electrode surface, the hybridization and the amperometric procedure. The stabilization of the signal from the concentration of 5×10^{-2} µM suggests that the limit of concentration in which the maximum quantity of capture, target and signal probe strands are linked was overcome (Fig. 5A). The electrochemical genosensor could detect the target DNA with a detection limit of 10^{-5} µM. A linear response in the colorimetric detection for the concentration of DNA was found between 5×10^{-4} and 25×10^{-3} µM, with a slope of 587.03 and a correlation coefficient of 0.9945 (Fig. 5B and Table 2).

The average RSD value for the colorimetric and the amperometric sensors ($n=5$) was 5%, indicating a good reproducibility of the protocol of hybridization in the microplates and on electrode surfaces. This value also indicates the appropriateness of the colorimetric and amperometric procedure (Table 2). The limit of detection was determined using the synthetic target sequence. The colorimetric and electrochemical sensors were used with 0.1 µM of the target and the signal probes. Excellent detection limits of only 5×10^{-5} µM and 10^{-5} µM for the colorimetric assay and the biosensor assay, respectively, were determined without PCR amplification of the target DNA (Table 2). The linear range of the standard curve of target sequence per assay was between 5×10^{-4} and 10^{-2} µM for the colorimetric assay and between 5×10^{-4} and 25×10^{-3} µM for the biosensor assay (Table 2). We observed a good correlation between the results obtained for the *Escherichia coli* colorimetric test and the electrochemical assay. These results are very interesting and are promising for the *in situ* detection of *Escherichia coli* from contaminated seawater. To test the efficiency of the two sensors, seawater was used as a matrix.

3.2. *Escherichia coli* detection and/or quantification from environmental samples

Colorimetric and electrochemical DNA biosensors were applied to detect the presence of *Escherichia coli* in environmental water samples. For methods evaluation, seawater samples were collected and spiked with different numbers of *Escherichia coli* cells to simulate real contamination as closely as possible. After sample prefiltration through a 45 µm pore size filter to remove the large planktonic particles, a filtration through a 0.2 µm filter was performed. DNA extractions from the 0.2 µm filters and the *Escherichia coli* DNA detection were carried out by colorimetry and amperometry. The *Escherichia coli* number from each cell suspension inoculated in seawater was checked by a cultural method, and cell enumeration values were similar to those expected (data not shown).

The results obtained by colorimetric and electrochemical detections from the different samples were reported in the calibration curves (Fig. 6A and B). The colorimetric and electrochemical values for the *Escherichia coli* culture concentrations of 100, 1000 and 10,000 CFU were in the linear range of the calibration curves (Fig. 6A and B). Only the optical density and the intensity values of the 10 CFU concentrations were below the initial lower values of the linear ranges of the calibration curves for the two biosensors (Fig. 6A and B).

Escherichia coli DNA concentrations for each sample were deduced from each plot equation of the corresponding calibration curves calculated for colorimetric and electrochemical sensors. A statistical ANOVA ($\alpha=0.05$) was applied to test the equality of the average for the DNA concentration deduced with the colorimetric sensors and the electrochemical sensors for each CFU. For 10 and 10,000 CFU of *Escherichia coli*, the ANOVA test concluded that we could reject the hypothesis of equality of the two averages at the level $\alpha=0.05$ (Fig. 7). Thus, for these two concentrations, we have only the detection of the *Escherichia coli* DNA target and no quantification because, for *Escherichia coli* at 10 CFU, the response of the two sensors was out of the linear part of the calibration curve (Fig. 7). For *Escherichia coli* at 10,000 CFU, the response of the two sensors was at the limit of the linear part of the calibration curve (Fig. 7). Thus, an imprecision in the result appears. In the case of *Escherichia coli* at 100 and 1000 CFU, the ANOVA test concluded that we could not reject the hypothesis of equality of the two averages at the level $\alpha=0.05$ because, for *Escherichia coli* at 100 and 1000 CFU, the detection values obtained by the two biosensors were included in the linear part of the calibrations curves (Fig. 7). Finally, for *Escherichia coli* at 100 and 1000 CFU, both detection and quantification of the DNA target were possible in spiked seawater samples.

With these results, we obtained the responses for the two sensors for a concentration range of *Escherichia coli* between 10 to 10,000 CFU. The *Escherichia coli* culture concentration in this study was for 5 L of seawater; thus, we tested 2 and 20 CFU/100 ml for the 100 and 1000 CFU cultures spiked in the seawater, respectively.

4. Discussion

The detection capability of *Escherichia coli* by colorimetric and electrochemical sensors is comparable or even better than several

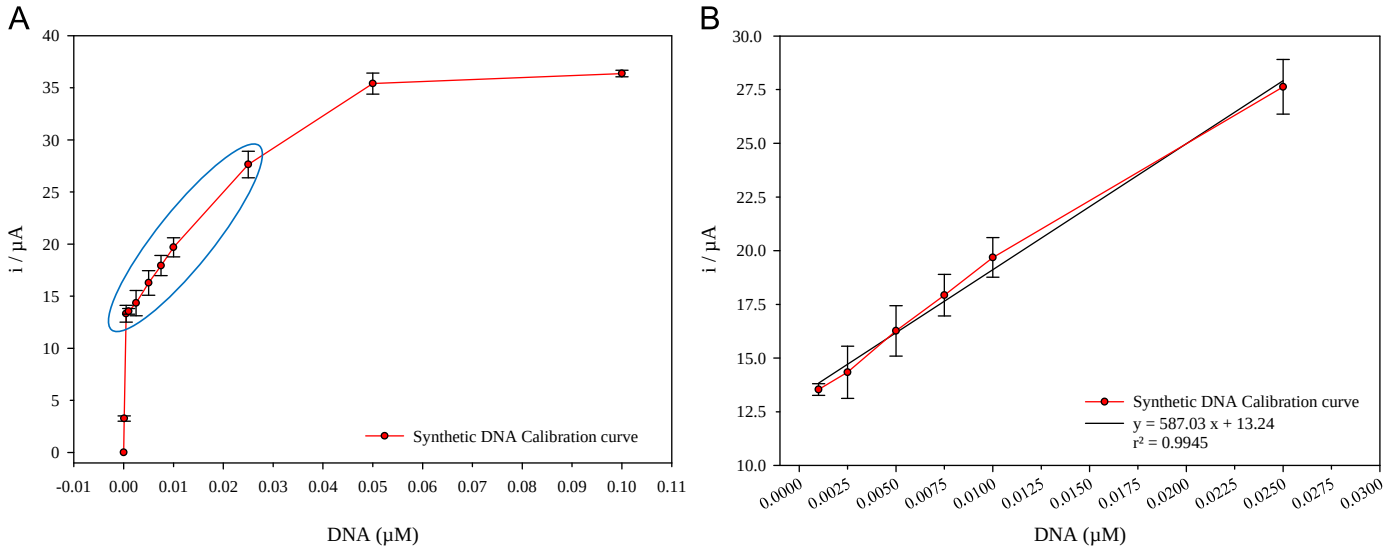

Fig. 5. Genosensor amperometric responses. (A) Corresponding calibration curve; the blue circle corresponds to the linear portion. (B) Linear portion of the calibration plot. Error bars are standard deviations of the mean with $n=5$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

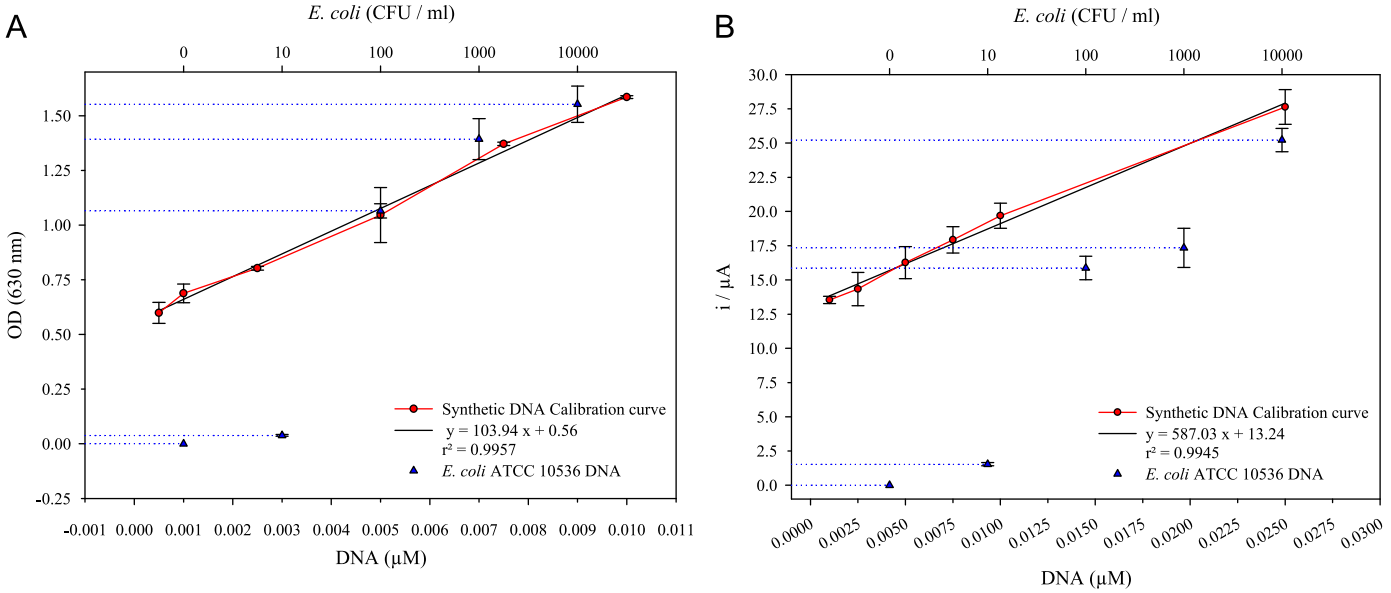

Fig. 6. Comparison of the performance of the colorimetric (A) and the electrochemical sensor (B) for the detection of the target DNA in seawater. Error bars are standard deviations of the mean with $n=5$.

methods developed by other researchers. For example, in the case of the direct detection of the entire bacteria *Escherichia coli* O157:H7, an amperometric enzyme-linked immunoassay method using 1-naphtyl phosphate as an enzymatic substrate with a minimum detectable level of 4.7×10^3 cells/ml was developed [60] as well as an immunomagnetic separation/flow injection analysis/mediated amperometric detection with a detection limit of 10^5 cells/ml [61]. Hasebe et al. [62] describe a tyrosinase-based chemically amplified biosensor for the detection of *Escherichia coli* and employed a tyrosinase-coupling electrode to detect polyphenolic compounds produced microbially from salicylic acid. The polyphenolic compounds are enzymatically oxidized to *o*-quinones by dissolved oxygen in a reaction catalyzed by tyrosinase. The *o*-quinones are then recycled to the original polyphenols by L-ascorbic acid and repeatedly reoxidized, which amplifies the signal. The sensor has shown promise in its capability to carry out sample analysis when presented with wastewater samples. However, if the samples contain any inherent tyrosinase-active compounds, these may have to be eliminated prior to analysis. The sensor is capable of detecting 10^3–10^4 cells/ml but requires a 3 h pre-enrichment period. Li et al. [63] describe an Fe_2O_3/Au core/shell nanoparticles-based electrochemical DNA biosensor that was developed for the amperometric detection of *Escherichia coli*. Magnetic Fe_2O_3/Au nanoparticles were prepared by reducing $HAuCl_4$ on the surface of the Fe_2O_3 nanoparticles. This DNA biosensor is based on the sandwich detection strategy, which involves the capture probe being immobilized on magnetic nanoparticles and the target and reporter probes labeled with horseradish peroxidase (HRP). Once the magnetic field was added, these sandwich complexes were magnetically separated, and the HRP confined at the surface of the magnetic nanoparticles could catalyze the enzyme substrate and generate electrochemical signals. The biosensor could detect concentrations higher than the

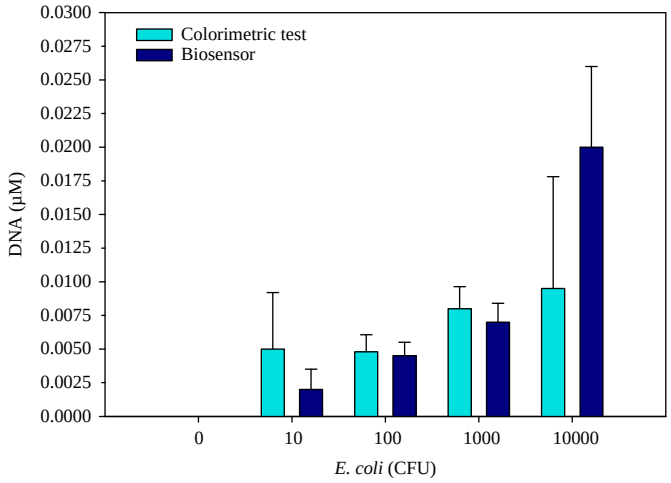

Fig. 7. Comparison of the performance between the two assays for *Escherichia coli* DNA detection and quantification. Error bars are standard deviations of the mean with $n=5$.

0.01 pM target DNA and higher than 500 CFU/ml of *Escherichia coli* without any nucleic acid amplification steps. The detection limit could be lowered to 5 CFU/ml of *Escherichia coli* after 4.0 h of incubation.

Our sensor can detect and quantify *Escherichia coli* for concentrations between 2 CFU and 20 CFU per 100 ml of the sample without amplification of the target DNA. In addition, we demonstrated the robustness, rapidity, selectivity and sensitivity of the double-recognition hybridization event by developing protocols for colorimetric and electrochemical detection of *Escherichia coli*. We designed two procedures that can ultimately be automated to continually monitor water quality.

5. Conclusions

The potential for DNA biosensors as a sensitive, rapid and portable tool for several applications in clinical, environmental and food analysis has been demonstrated. The DNA biosensor and the colorimetric assay described can detect specific sequences of oligonucleotides with high selectivity. They have also exhibited an elevated sensitivity to discriminate differences in concentrations of target DNA. In this study, the development of a highly specific and very sensitive colorimetric and electrochemical genosensor for the rapid detection of *Escherichia coli* in seawater was successfully demonstrated. The two sensors can detect and quantify 2 to 20 CFU/100 ml of *Escherichia coli* in seawater. The advantage of the two sensors is that they enable researchers to skip first step of DNA amplification by PCR and instead directly use *Escherichia coli* DNA extract. These sensors allow for the rapid detection (in 3–5 h) and quantification of the strain in environmental samples. No false-positive signals were obtained from other microorganisms. Thus, these biosensors may be an important tool for determining the presence of low concentrations of *Escherichia coli* in environmental samples. DNA biosensors act as simple tools than can give reliable, clear, rapid and inexpensive results directly at the site of interest by implementation of the sensors with a portable device.

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