A Novel Handheld Fluorimeter for Rapid Detection of *Escherichia coli* in Drinking Water

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***Abstract*—The microbiological quality of drinking water is a concern to consumers, water suppliers, regulators and public health authorities alike. Monitoring the microbiological quality of drinking water relies largely on examination of indicator bacteria such as coliforms like *Escherichia coli*. *E. coli* is widely used as an indicator of fecal pollution when monitoring the microbial quality of drinking water because it is abundant in all mammal feces and therefore is found in sewage and in natural waters contaminated with fecal matter, from human origin, wild animals or derived from agricultural activity. This paper describes the development of a novel handheld fluorimeter for rapid detection of *E. coli* in drinking water based on a specific cellular biomarker. The measurement system is based on a photomultiplier tube that captures the fluorescence signal produced by the cellular biomarker when it is excited by an ultraviolet LED. The cellular biomarker is also developed and it consists of a chimeric protein with a Green fluorescent protein in the N-terminal domain (GFP) and a specific amino acid sequence in the C-terminal domain (Colicin S4) that targets specifically the structure of the microorganism to be detected. The instrument is simple to use, lightweight, and can be powered by either an AC/DC power adapter or a rechargeable battery, making it an excellent choice for rapid detection of *E. coli* in drinking water in field studies and laboratory measurements.**

***Index Terms*— Drinking Water, *Escherichia coli*, Green Fluorescent Protein, Photomultiplier, Microcontroller, Colony-forming Unit, Handheld Fluorimeter.**

I. Introduction

WATERBORNE health problems represent one of the most important problems of modern society, particularly so in developing countries and also in regions with limited economic resources to reinforce and complement quality controls and drinking water treatment. This is due to the disadvantages present in the source water treatment and the long distance between production stations and the consumer's tap. In this process, the water is exposed to an increasing number of pollutants that once housed in the environment, can be ingested by humans causing serious health problems.

Drinking water for consumption may therefore, in certain cases, be the cause of disease in humans. This is because drinking water can act as a vector of a number of environmental toxic compounds, both naturally occurring (toxins from microbial or mineral origin) and human (from industrial poisons). In addition, drinking water may also be the gateway to different pathogenic organisms, of parasitic, fungal, bacterial, or viral type, independently of the economic development stage of the country. In Norway or the USA, massive outbreaks of intestinal infection caused by intestinal parasites such as *Giardia* due to water supply network problems are regularly described [1]. Bacteria with high morbidity or mortality as *Legionella* and *Leptospira* are also transmitted by poorly maintained water distribution networks [2].

So, drinking water distributed by pipeline to the populations must meet quality levels that are highly regulated with respect to many parameters. At the European level, the applied law is the European Directive 98/83/EC [3], which since 1998 standardizes the quality of drinking water across the EU. This sets the values of quality drinking water, as well as the systems which must monitor and sample the sources from which it is obtained and distributed, and other measures to keep the public informed of the quality of their drinking water.

European legislation establishes a basis for treatment, control, storage and distribution of drinking water, allowing sanitary and control measurements to protect the health of consumers. Thus, it can be concluded that the analysis set up to control drinking water is necessary but not sufficient because it does not allow us to know in real time the quality of water that reaches the consumer's tap. Also, possible deviations in laboratory parameters observed between the analyzed water sample and water that reaches the consumer at a given time (due to heavy rain, for example), can create possible health problems. The reason is that current analyses follow classical microbiology methods which require 1-2 days of incubation in a laboratory [4]. Therefore, the current analytical system does not allow us to know, in real time, the state of the water quality parameters at the time of or just before it is consumed.

In the literature, several handheld fluorimeters are presented [5-7]. However, to our knowledge, there is no a specific handheld electronic instrument for *E. coli* detection. In [8] a

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This article has been accepted for publication in a future issue of this journal, but has not been fully edited. Content may change prior to final publication. Citation information: DOI 10.1109/JSEN.2016.2558649, IEEE Sensors Journal

procedure for rapid detection of *E. coli* in water is described. This procedure uses a standard handheld fluorescence detector and requires 30 min of incubation to acquire the detection limits imposed by legislation.

The instrument designed in this study will be key in detecting, with no waiting time, the presence of pathogen microorganisms in drinking water or water in direct contact with human beings, as for example, in recreational waters. It will also be of great importance for the control of industrial water quality. The device will allow early detection and quick decision-making by the people responsible for the verification of water quality, guarantying instantly the quality of the consumed water and the absence or presence of microbiological contaminants.

The main result is the development of a fast system for pathogen detection using an autonomous, portable and real-time device based on a specific cellular biomarker against *E. coli* and the optical measurement system. Moreover, the developed instrument is easy to manipulate so it can be used by anyone without specific knowledge.

The rest of the paper is organized as follows. Section II is a description of the cellular biomarker created to detect *E. coli*. Section III presents the electronic design of the measurement system. The experiment results and discussion are included in Section IV. Finally, conclusions are offered in Section V.

II. THE CELLULAR BIOMARKER

Diverse National laws provide that drinking water is considered to be potable if the value of bacterial species *E. coli*, *Enterococcus* sp. and *Clostridium perfringens*, obtained for a sample volume of 100 ml, shows a value of 0 colony-forming units (CFUs) (Council Directive 98/93/EC in EU [3], 74 FR 25651 and 21 CFR part 129 in USA [9], as examples). These three bacteria are used as indicators of fecal contamination.

In this work we have chosen *E. coli* as indicator bacteria because it is abundant in all mammal feces at concentrations of 10^7 per gram [10], and it is found in sewage and in natural waters recently contaminated with fecal matter, from human origin, wild animals or derived from agricultural activity. This bacterium is also used as an indicator of the effectiveness of water purification treatments. The majority of authors argue that *E. coli* provides adequate protection to public health if tests are done often enough [11-13].

Main methods for detection of *E. coli* in drinking water are based on DNA hybridization, PCR (and qRT-PCR for quantification), immunoassays, immunomagnetic separation, lateral flow tests, incubation micro-chambers as VITEK, labelled nanoparticles, NIRS (near infrared spectroscopy), DEP-FFF (dielectrophoretic field-flow fractionation), or β-D-glucuronidase assays [14-22]. But these current technologies are not inexpensive in equipment (as NIRS) or reactives (as labelled antibodies) and usually require an intense water sample manipulation and laboratory equipment (as DNA-hybridization), or may need several hours of incubation (β-D-glucuronidase assays) which impairs its portability as well as the economic feasibility of tests every few minutes.

Here we have chosen a method including filtration and the use of a fluorescence protein chimera, composed of a Green Fluorescent Protein (GFP) and the bacteriocin Colicin S4. These two proteins are linked in this chimera by a section of 100 amino acids with no secondary structure, in order to avoid steric problems.

Colicins are bacteriocins encoded in plasmids, produced by nearly 50% of *E coli* strains. Their function is to eliminate other competitor *E. coli* strains under stress conditions. Among the mechanisms of colicins toxicity is pore formation in the cytoplasmic membrane, which is accompanied by an alteration of the electrochemical gradients, killing other strains of the same species [23,24].

Colicin S4 is a protein of 499 amino acids comprising four domains: N-terminal translocation domain, two receptor-binding domains (R1 and R2), and the C-terminal domain for pore generation (Figure 2). This colicin binds to other *E. coli* strains by recognizing specifically the OmpW receptor at the outer membrane of *E. coli* cells, where this protein forms a hydrophobic channel of unknown function. OmpW is an outer membrane protein that belongs to the family of β-barrel proteins. This 230 amino acids long protein presents eight transmembrane β-strands and a single α-helix domain framed by two β-strands exposed to the extracellular environment [23]. Asp116, His117 y Glu120 in that α-helix form a charged patch showing a negative-positive-negative charge pattern that is totally complementary to the positive-negative-positive charge pattern of the α-helix present in the receptor-binding domain of the bacteriocin colicin S4. This receptor-binding domain is unique, because it doesn't have a homologous sequence in other colicins, so colicin S4 is the only colicin able to bind to OmpW [25]. This fact is responsible for the high specificity of the biosensor method described in this study (Figure 8).

GFP was first described by Shimomura, Johnson and Saiga in 1962 as a chaperone aequorin protein, in the jellyfish *Aequorea victoria* [26]. Green Fluorescent Protein has two excitation peaks, with a dominant maximum of approximately 395 nm and a minor one at 475 nm. GFP has also two emission peaks, at 508 nm and at 503 nm [27], as shown in Fig. 1.

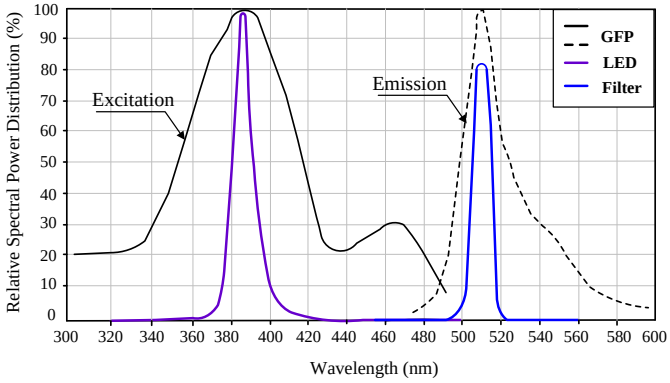

Fig. 1. Spectral response of GFP (excitation and emission peaks), LED (emission) and optical filter (detection).

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The chimeric synthetic gene, GFP-S4, has an optimized design to facilitate purification on Ni^{2+}-Sepharose FPLC columns and to avoid steric hindrance that may exist between the GFP and colicin S4. In order to facilitate purification, it contains six codons for histidine at 5´-end (polyhistidine-tag, His-Tag) that have affinity for the Ni^{2+} column. Also, to avoid steric hindrance, it contains a linker of 100 amino acids which is a flexible structure which does not have α-helix nor β-sheet structure. Its sole function is to bind the colicin S4 with GFP to thereby prevent the tertiary structure of GFP encompassing the chimera (Figure 8).

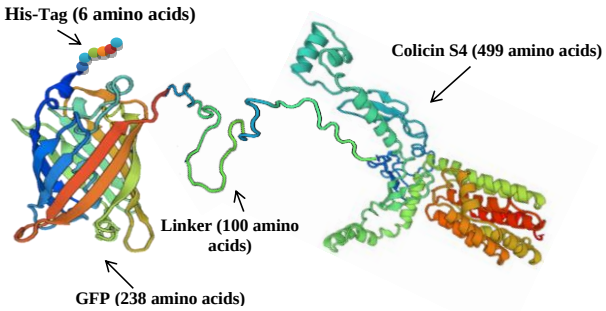

Fig. 2. Hypothetical spatial structure of the chimera GFP-colicin S4 generated in this work. This structure was generated from Swiss-model free software [28].

III. MEASUREMENT SYSTEM

The measurement system developed to analyze the presence of *E. coli* in water consists basically in detecting the fluorescent light emitted by the immobilized GFP in the *E. coli* cells. GFP protein is a common method for fluorescent labeling of eukaryotic cells, organelles and other biological structures. Here, we have used GFP to construct a chimeric biosensor protein which consists of GFP domain (at the C-terminus), a long linker, and a colicin S4 domain (at the N-terminus) [27]. The GFP C-terminal domain senses the 395 nm wavelength emitted by the LED light, and generates a fluorescence response at 509 nm which is detected and amplified by the PMT, generating at a further step an electrical signal, which is shown in the display of the handheld biosensor device (Figures 8 and 9). In the case of a water sample containing *E. coli* cells, the sample is excited following the previous description, measured, using a photodetector, and the corresponding fluorescence is emitted. Fig. 3 shows the functional blocks that make up the measurement system. The optical subsystem consists of a light source of a specific wavelength that excites the sample and a photodetector that permits the detection of the fluorescence emitted by the GFP protein linked to the *E. coli* bacteria. The presence of an optical filter is required to remove non-desired light that may affect the measurement.

The signal processing unit handles the activation of the optical system, measures the signal provided by the photodetector, calibrates the measurement system and provides a numerical indication of the level of *E. coli* present in the sample. To implement these tasks a microcontroller device is used. The user interface consists of a LCD touch-screen display that permits the display of the measured value and the entry of the system configuration parameters.

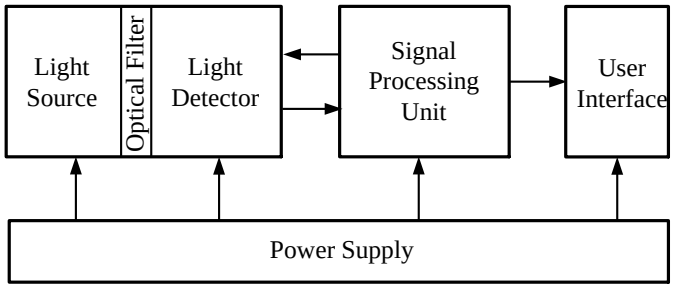

Fig. 3. Functional blocks of the measurement system.

A. Optical Subsystem

The light source must comply with the requirements established by the excitation spectrum of the sensing phase. As shown in Fig. 2 the fluorescent protein GFP has two excitation peaks, one at 475 nm and another at 395 nm. The excitation peak of 395 nm was used, since it is the furthest away from the emission peak (509 nm) and therefore there is less risk that the wavelengths overlap. We choose the Roither Lasertechnik UV HUUV-5102L LED, whose emission peak is at 395 nm. To avoid external interferences the light signal must be modulated [29]. Fig. 4 shows the block diagram of the light source. It consists of an oscillator that provides a 1-kHz sinusoidal signal followed by an offset circuit that enables the dc level to be shifted and to always have a positive excitation signal. Finally, a current amplifier provides the adequate excitation current level. The frequency of the oscillator must be neither very high—in order not to limit the dynamic response of components—nor so low as to be confused with other illumination sources.

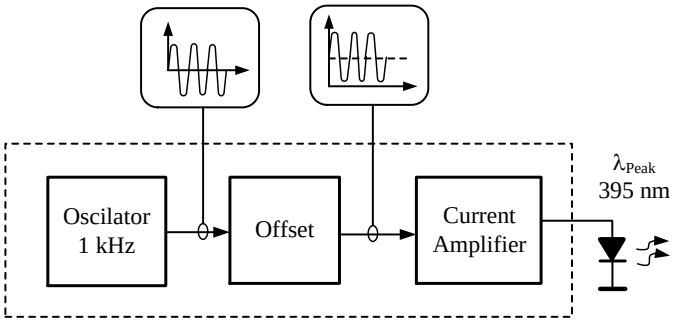

Fig. 4. Block diagram of the light source.

There are three basic technologies for the light detector: photomultiplier tubes (PMTs), avalanche photodiodes (APDs), and silicon photodiodes (PIN diodes). The question of which detector to use is not a simple one. In applications where there is ample light, a photodiode is suitable. A PMT is the best choice where there are very weak signals. In other applications, however, the choice is not so clear. A guide to choosing the right detector can be found in [30].

The ultimate test of a detector system is its signal-to-noise ratio (SNR) in a particular application. The main advantage of

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PMT is its excellent SNR at high gains. This feature has allowed this vacuum tube is still used today. Its high price, however, restricts its use to very demanding applications, in this case to measure very low levels of light. APDs have the advantage of being very fast and have high gain, but being a semiconductor their SNR is low and very dependent on temperature. We have evaluated the two options by measuring the SNR in the application, concluding that to use the APD in fluorimeters, you must cool it with a Peltier cell, bringing the final price becomes similar.

Because the fluorescent protein has its emission peak at 509 nm, the spectral response of the PMT must include this wavelength within the bandwidth. The PMTs are very sensitive in that range of the spectrum and have the ability to provide a great amplification retaining an excellent signal-to-noise ratio. They provide an output signal in the form of current, proportional to the intensity of incident light. Specifically the H7827-001 Hamamatsu PMT module was used. This module has the advantage of integrating the high voltage power supply that the PMT needs and the current-to-voltage converter.

Fig. 5 shows the variation of the PMT gain versus control voltage used to select the gain of the PMT. The control voltage is obtained from a reference voltage generated by the PMT itself. In order to have a good resolution the PMT gain must be chosen as large as possible without saturation. We have chosen a control voltage of 0.8 V to obtain a PMT gain of 105. The LED emission spectrum has a long tail that extends into the detection range for the PMT. To minimize as far as possible this kind of interference an optical filter has been placed in front of the window of the PMT. The characteristics of this filter are: Thorlabs FL508.5-10, ∅1", Laser Line Filter, CWL=508.5±2 nm, FWHM=10±2 nm.

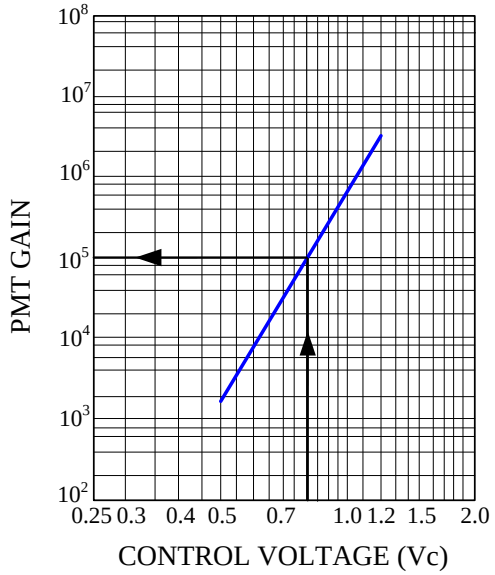

Fig. 5. Variation of the PMT gain with the control voltage.

Fig. 6 shows the block diagram of the light detector circuit. The PMT output signal is demodulated using a band-pass filter whose central frequency is 1 kHz, which is equal to the oscillator. Next, the signal is rectified by a precision rectifier and 1Hz low-pass filtered to eliminated the HF intrinsic noise due to the electronic components. Therefore the output voltage of detector module, VOUT, is proportional to the level of fluorescence radiation incident on its window.

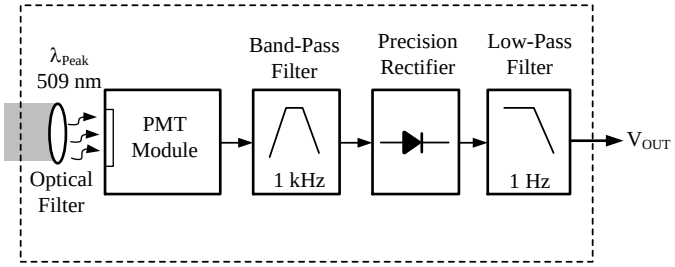

Fig. 6. Block diagram of the light detector.

B. *Signal Processing Unit*

Fig. 7 shows the simplified block diagram of the signal processing unit. The microcontroller (PIC24FJ128) reads the output of the PMT module by the AN0 input and generates an equivalent digital signal through the D0 output. Next, a digital-to-analog converter (DAC8552) obtains the equivalent analog signal. In these conditions the output of the instrumentation amplifier (IA INA118) should be zero volts. A 1 Hz RC low-pass filter is used to remove the 50/60-Hz noise.

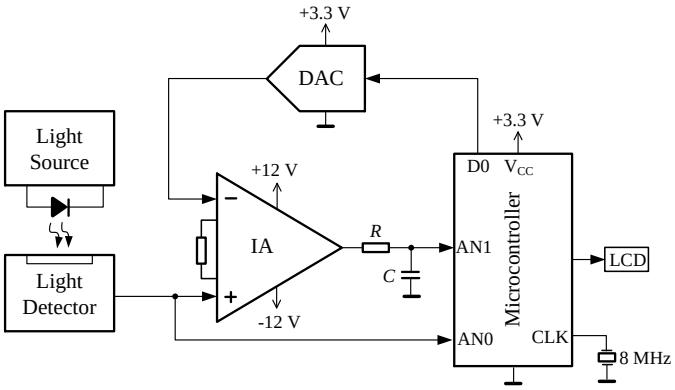

Fig. 7. Simplified block diagram of the signal processing unit

It was necessary to measure a blank sample because the prototype operates with very high gain and any small change in the measurement set-up (cuvette, LEDs, etc.) can be error sources on the output voltage. The procedure to minimize simultaneously these types of errors is to calibrate the instrument with each measure. In absence of fluorescence and even with the optical filter placed the PMT provides an output voltage that can be assimilated to an offset voltage. The origin of this voltage is the dark current of the PMT tube. It is assumed that the connection of the PMT to the measuring cell is good enough to assume that the radiation which reaches the PMT tube to come only from the measuring cell. The removal of the offset is performed during the calibration process using a sample of uncontaminated water. Under these conditions the output of the PMT module should be only the offset voltage.

The instrument can be powered from the mains through an AC/DC converter of +9 V, or from a pack of Lithium-ion

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batteries 9V/2000 mAh. The component of greatest consumption is the PMT module (max. 50 mA). The supply voltage of this module has been taken as +12 V. The remaining components are supplied at +3.3 V. To obtain these voltages two DC/DC switching converters, have been used. Tests have shown that the battery provides two hours of autonomy that can be enough to work in field environments.

The output variation of the PMT with operating time must be considered. Drift per unit time generally improves with longer operating time and this tendency continues even if the PMT is left unused for a short time after operation. Applying the power supply voltage to the PMT prior to use ensures more stable operation. Since these characteristics greatly depend on the magnitude of signal output current, keeping the average output current within a few microamperes is recommended [31,32]. On the other hand the gain and dark current of the PMT change slightly with the ambient temperature, approximately -0.2%/ºC, so that temperature control may not be required.

C. Microcontroller Firmware

Fig. 8 shows the flowchart of the microcontroller firmware. Programming the microcontroller has been developed in the C language, using the CCS compiler. First of all, device initialization is performed, booting the touch screen and preparing it for the correct detection of pulsations, then a splash screen appears with the corresponding logos. Next, initialization of the DAC from the values read from the EEPROM is performed. Then, the calibration screen is displayed, asking the user to place in the measuring cell the cuvette with a sample of uncontaminated water. It then reads the ADC. This reading is performed 10 times to minimize possible errors of manipulation or interference. An average of the measurements is performed and its value is saved. This value will be used later in the calculations. The next step is to show the user the measurement screen. A cuvette with the sample to be analyzed is put into the cell and the reading of the ADC is performed again. As in the previous case, 10 measurements will be taken. The average value is obtained and subtracted from the value corresponding to the pattern. This value is the actual measured value corresponding to the concentration of *E. coli*. This value will be transformed into CFUs and will be displayed to the user on the final screen. From this screen you can return to the calibration screen to perform the whole process again. Each measurement requires the calibration of the instrument, since the measurement conditions may have changed.

D. Confocal microscopy

Images were collected with a Leica TCS AOBS SP2 confocal microscopy using a 63x/1.40 Oil objective. GFP was excited using a 488 nm argon/krypton ion laser and fluorescence emission was detected at 502-556 nm. Data acquisition software: Leica confocal software (LCS) version 2.61 Build 1537. Copyright (C) 1997-2004. Leica Microsystems Heidelberg GmbH (Germany).

IV. EXPERIMENTAL RESULTS AND DISCUSSION

The GFP-S4 synthetic gene (accession number LT548289) was commercially synthesized and subcloned into pWHM3 bifunctional *E. coli/Streptomyces* plasmid vector [33], generating the final construction pLMF-BS2-NP, for its expression in the bacteria *Streptomyces albus*. From 4-day old cultures of this strain (six 2500 mL flasks, each one containing 250 mL R5A culture medium and 10^7 spores/mL), the chimeric protein was purified by FPLC using a Ni^{2+}-Sepharose column (affinity chromatography), and a Sephadex column (molecular exclusion chromatography). 3 ml of purified chimeric protein were measured by using capillary electrophoresis (Agilent Bioanalyzer 2100), giving rise to a concentration of 912 μg/ml in PBS buffer [34].

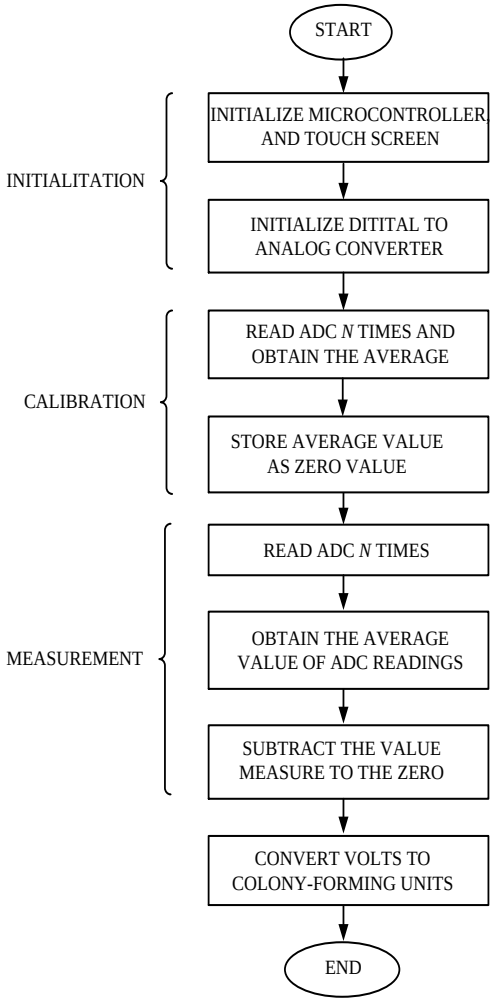

Fig. 8. Flowchart of the microcontroller firmware.

In order to generate a reference curve, for delimitating lower and upper detection limits (sensitivity and linear range), ten *E. coli* dilutions (1 ml each one) in LB medium were generated from overnight cultures in LB medium. These dilutions were incubated for 5 min at room temperature with the equivalent to 456 μg of GFP-S4 protein (three replicas). Then, each mixture was filtered using 0.2 μm pore size filters, in order to get rid of unbound GFP-S4 protein. After that, filters were washed with 2 ml of PBS. Then, the filter was

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inverted with respect to the flow axis, in order to recover all *E. coli* cells (with GFP-S4 labeling or without it) with 2 ml of PBS from filter membrane. These recovered 2 ml were placed into a spectrophotometer cuvette and measured by using the measurement system described in Section 3. Blank solution for these measurements was PBS with the corresponding filtered and recovered *E. coli* dilution, but without GFP-S4 protein labeling. These PBS blank samples were measured before each experimental sample, in order to eliminate putative background interferences. With the aim of measuring the *E. coli* CFUs by using a gold standard method, each dilution was also plated out on EMB [4] agar plates and incubated overnight at 37 ºC. CFUs were counted and average CFUs from plate dilutions were used as reference for X axis in Figure 9. Different dilutions of these *E. coli* cultures were analyzed, within a quantity range from 3 to $3x10^5$ CFUs/ml (Fig. 9).

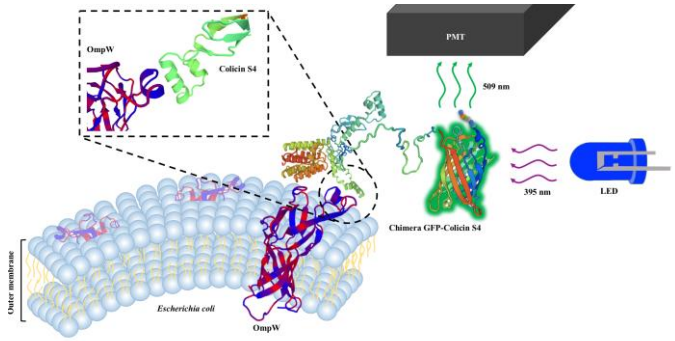

Fig. 9. Schema showing the biosensor process. A 395 nm LED light stimulates the GFP-Colicin S4 chimeric biosensor protein, causing the GFP portion to emit light at 509 nm that is amplified and detected by the PMT device. The GFP-Colicin S4 biosensor chimera recognizes and binds the OmpW protein at the outer membrane of *E. coli*, labeling those bacterial cells and letting them ready for fluorescence detector at the developed handheld device.

Under operation conditions, the new developed handheld device registers, for each binding assay, a mV value which corresponds to the equivalent fluorescence emitted by the GFP-S4 bound to the *E. coli* CFUs present in the experiment. With these data, the sensitivity curve of Fig. 10 was generated.

The curve from Fig. 10 shows that in binding experiments with more than $1x10^3$ *E. coli* CFUs, the systems gets saturated, and there is not a further increase in the mV signal generated. Also, with *E. coli* amounts below 20 CFUs, the signal is also no longer proportional to the number of CFUs. Therefore, the linear range of signal obtained with these experiments is between 20 and $1x10^3$ *E. coli* CFUs, and the minimum number of detectable CFUs, the sensitivity value, was 3. Fig. 11 shows a view of the handheld device. The enclosure is a Hammond 1455N2202 which dimensions are 220 mm x 10^3 mm x 53 mm.

Independently of the water volume to be analyzed (from 1 ml to a few liters with this filtration method), this method allows a final recovery of 2 ml of final volume (from the filter) in the final cuvette, for measurement of the putative fluorescence that may be present in case of any *E. coli* CFUs is present in the initial sample. The law establishes absence of *E. coli* CFUs in 100 ml of drinking water. Our developed method shows a lower linear detection limit of 20 CFUs. This implies that just filtering 2 liters of drinking water, it would conform to legislation.

In order to test this method with real drinking water samples, the assay was carried out (three replicas each) by using drinking water from a spring in a rural area in the Oviedo district (Spain). All other drinking water samples analyzed from different water pipeline distribution networks (in the cities of Oviedo, Gijón, Mieres and Avilés) were negative for *E. coli*, both by using the gold standard plating out method and this new biosensor method. 2 liters from this rural water spring were filtered (0.2 μm pore size) and all bacterial cells present were recovered by inverting the filter and washing with 2 ml PBS, and this was incubated for 5 min at room temperature with the equivalent to 456 μg of GFP-S4 protein in order to carry out the detection assay.

Simultaneously, the same spring water volume was treated equivalently, and recovered cells were used for counting CFUs by serial dilution plating on *E. coli* selective medium EMB [4]. The measurements obtained from these real drinking water 3 replicas gave rise to 363, 375 and 387 mV, which fall within the linear range of detection. Therefore, these values were positive for *E. coli* presence, as was confirmed from replica plating experiments (57, 92 and 103 CFUs per 2 liters spring water).

Similar experiments were carried out in order to establish specificity parameters, once the sensitivity level was established. In order to do this, triplicates of 50 mL of distilled water were spiked with 10^4 CFUs of different bacteria belonging to the family *Enterobacteriaceae*: *E. coli*, *Enterobacter cloacae*, *Serratia marcescens* and *Salmonella enterica*. These samples were processed in two ways. On one side, three replicas of 2 mL of each type of spiked water (with each one of the bacteria) were mixed with the chimeric biosensor protein (456 μg), filtered as described previously and processed towards measuring fluorescence in the developed biosensor device. In all cases but *E. coli* samples (384, 401 and 431 mV), recovered signal outputs were below 50-100 mV, which is lower than 250 mV for lower detection threshold, and therefore, the device was unable to detect any fluorescence signal, between linear range of detection, generated from bound colicin S4-GFP chimeric protein to these other three species.

When decimal serial dilutions of these spiked samples with five different types of bacteria were plated out on EMB medium, the corresponding CFUs were detected in all cases after overnight incubation at 37 ºC.

As a way to further determine the specificity binding of the chimeric colicin S4-GFP biosensor protein for different *Enterobacteriaceae* species, the filtered samples that were used for fluorescence detection (after mixing 2 mL of the corresponding spiked distilled water with the chimeric biosensor protein), were used for confocal microscopy (see section II, part D).

This article has been accepted for publication in a future issue of this journal, but has not been fully edited. Content may change prior to final publication. Citation information: DOI 10.1109/JSEN.2016.2558649, IEEE Sensors Journal

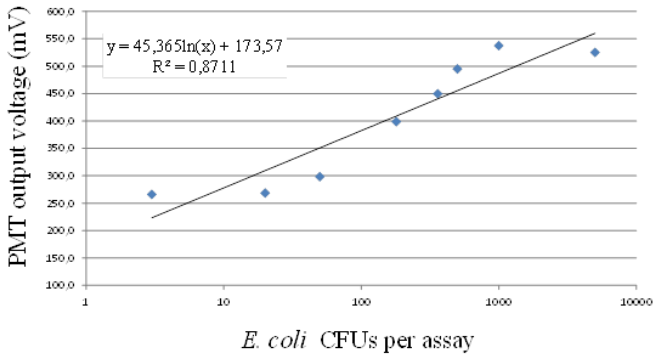

Fig. 10. Detection experiments with the GFP-S4 biosensor protein, using 3 to $3x10^5$ CFUs. The linear data range for this method is from 20 to $1x10^3$ CFUs. The regression line and R2 coefficient are also indicated. Diamonds represent the average values from 3 different measurements for each assay. The line connecting all measurement diamonds has been integrated by using Excel software. Saturation levels are observed at dilutions with more than 10^3 CFUs.

Fig. 11. Photographs of the handheld fluorimeter device.

Confocal microscopy images (Fig. 12) showed that only those water samples spiked with *E. coli* were showing the labeling with the fluorescent colicin S4-GFP chimera biosensor, with all cells emitting green fluorescence in the case of *E. coli*. In the cases of the other three bacterial spikings, no cell labeling was observed, just a homogeneous low level background fluorescence. This confocal microscopy experiment demonstrates that specificity of the chimeric biosensor protein is high, as only *E. coli* cells are highly labeled (Fig. 12).

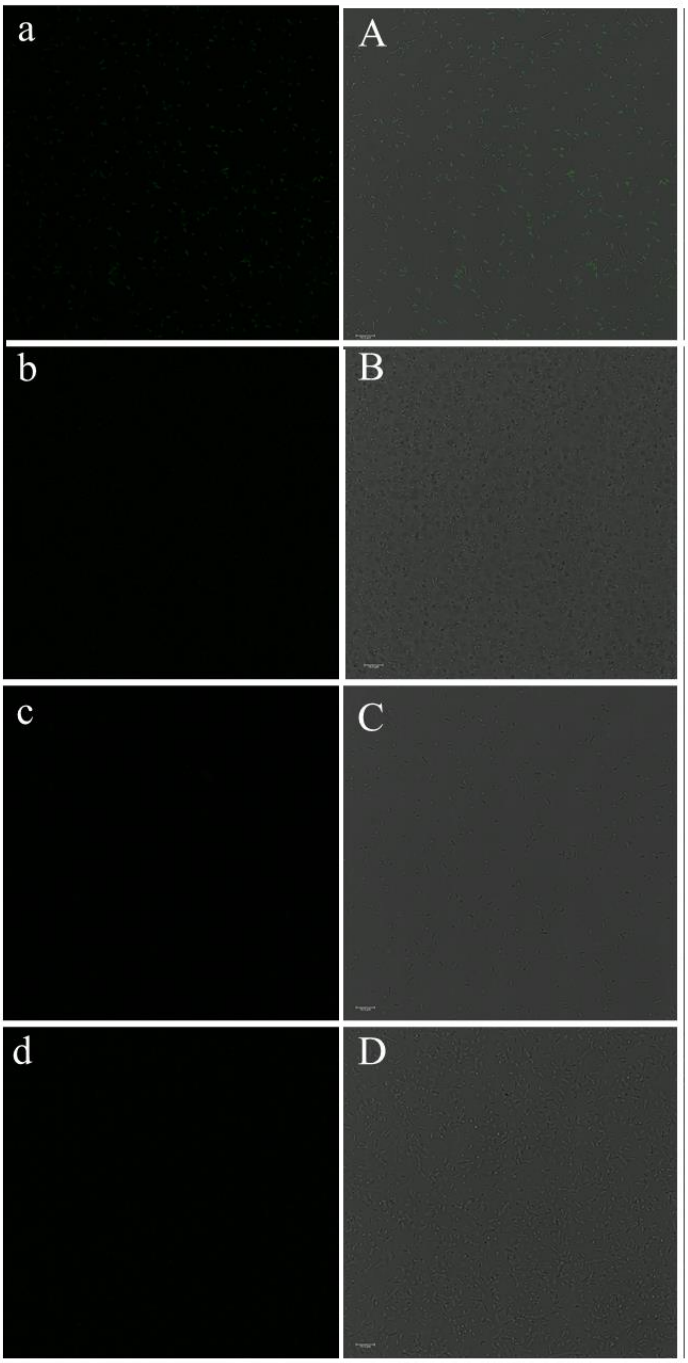

Figure 12. Confocal images of *E. coli* (A, a), *Serratia marcescens* (B, b), *Enterobacter cloacae* (C, c) and *Salmonella enterica* (D, d). Images for a, b, c and d sections correspond to fluorescence images. Only *E. coli* cells (pictures "a" and "A·) are perfectly labelled with the chimeric biosensor fluorescent protein. The other three species are not labelled at all (B. b, C, c, D, d).

When comparing this developed biosensor method with other detection techniques, the 8 min needed in this case for complete detection of the fluorescence signal are much faster than other methods as those based on DNA. For example, *E. coli* detection methods requiring DNA amplification of specific genes for this bacterium, as in the case of qRT-PCR, need at least 30 min manipulation plus 90 min amplification

assay. In the case of DNA hybridization methods, the complete assay last for at least 2 days, as genomic DNA must be obtained, then restricted, blotted by Southern Blot, and finally hybridized with the corresponding DNA probe. A disadvantage of our fast detection method, which uses the fluorescent chimera biosensor protein, in comparison with qRT-PCR technology is that this later one allows quantification of *E. coli* cells in the water sample, with a higher sensitivity (as low as 1 CFU), and larger linear detection range (up to several million cells). However, this higher accuracy of qRT-PCR assays are not useful when dealing with fast decisions on the potability of any tested drinking water, as regulatory frames just establish in 0 CFU for *E. coli* the permitted amount of cells in 100 mL of these drinking waters, and the extra accuracy obtained from qRT-PCR assay at higher cost (2-4 € per sample, 120 min) is not compensating the fast biosensor method described here (less than 0.5 € per sample, 8 min). This disadvantage is even worst for DNA hybridization assays (several tens of €, 2 days).

Near infrared spectroscopy assays (NIRS) are able to detect in a few seconds some known materials in a water sample, including cells and mineral salts. However, two main disadvantages are important with NIRS. On the one hand, NIRS equipments are very expensive (thousands of €), and on the other hand, NIRS technology requires, in order to identify the presence of cells from a given species, as *E. coli*, the construction of a huge reference database with signals arisen from as many differences in water with respect to chemical composition (which may vary between seasons or depending on rain/dry weather cycles) and microbiota composition of water sources and reservoirs, including also the effects of this changing microbiota (seasons, temperature, etc.) with respect to the presence in the water reservoir of microbial metabolites (toxins, polysaccharides, small molecular weight metabolites), which need to be in same concentrations in the reference signals database as in the experimental water sample to be analyzed each time, in order to obtain detection accuracy.

Main disadvantages of DEP-FFF (dielectrophoretic field-flow fractionation) methods for detection of *E. coli* is that the necessary equipment is also complex, difficult to convert into in portable version, and time consuming, as the assay usually takes fractions of hours. This electrophoretic technology relies on the rate of movement of particles along an electrical field inside a gel solution, and an association is made between displacement position and the type of particle. These analyzed particles can be also cells, but giving the natural variability of sizes and electric charges (both at the membrane and at the cytoplasm) in just a single species, as *E. coli*, with cell sizes ranging from less than a μm to several ones, depending on environment and cultivation conditions, the technique would provide data with difficulties for interpretation. Also, other bacterial cells from other species may eventually have DEP-FFF characteristics similar to reference *E. coli* tests, introducing a second bias.

Detection techniques based on immunoassays and immunomagnetic separation (as lateral flow tests) are very expensive in comparison with the fluorescence biosensor assay described in this article. Lateral flow tests are quite fast (around 10-15 minutes), but require expensive antibodies. Immunoassays, as FISH, are very tedious with respect to sample preparation (several hours or few days) and very expensive with respect to necessary equipment (which may include a confocal microscope or a flow cytometer: more than 200.000 €).

Other detection techniques for bacteria in water samples include methods which require incubation of the water samples in a given culture medium or under specified conditions. Incubation micro-chambers (as VITEK^{TM}) require low sample volume, but around 6 h incubation in order to produce results, with a cost per sample of several €.

β-D-glucuronidase assays make use of this specific enzyme produced by *E. coli* strains, which allows to determine if this bacterium is present in a water sample. The cost of this enzymatic assay is not very high, but in contrast to the 8 min required for the chimeric biosensor fluorescence detector shown in this work, the β-D-glucuronidase assay requires at least a few hours of incubation, in order to allow small water concentrations of *E. coli* cells to produce enough β-D-glucuronidases. The reason for this is that this method relies in the breakdown of substrates as p-nitrophenyl-β-D-glucuronide or 4-methylumbelliferyl-beta-D-glucuronide, which once hydrolyzed, gives rise to free p-nitrophenyl or 4-methylumbelliferyl end products, easy to detect by fluorescence or spectroscopically.

V. CONCLUSION

A synthetic gene has been created which codes for a chimeric protein formed by GFP and colicin S4 subunits. This chimeric protein is able to specifically recognize and bind to *E. coli* cells. At the same time a fluorimeter has been designed to specifically excite the GFP-S4 chimeric protein, as a method to identify present *E. coli* cells in the assay. In particular the chimeric protein is excited at 395 nm and the generated fluorescence is detected at 509 nm, converting this light signal into an output voltage. In this way, a linear detection range between 20 and 1000 *E. coli* CFUs per ml per assay has been obtained. The lowest amount of detectable CFUs, the sensitivity, is 3. The specificity of the method has been demonstrated to be high, as labeling with the chimeric biosensor protein has been observed only for *E. coli*, but not for other *Enterobacteriaceae* species as *Enterobacter cloacae*, *Se. marcescens* and *Sa. Enterica*.

This device allows for an easy and fast detection of *E. coli* CFUs as an indicator of water potability, in just 8 minutes (5 min for incubation with the chimeric protein and 3 min for filtering, pipetting and measurement). This is in contrast with current culture-dependent methods, where 24 hours analysis time is required. Also, this device allows carrying out the assay without the restrictions which a fully equipped laboratory would require. Potentially, these measurements could be carried out on site, and results could be sent by mobile network to the central offices of the corresponding water distribution company.

ACKNOWLEDGMENT

Financial support from IDEPA (Instituto de Desarrollo Económico del Principado de Asturias) and FICYT (Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología) through the projects IE09-106 and IDE2012/000452 is gratefully acknowledged.

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