ColiSense, today's sample today: A rapid *on-site* detection of β-D-Glucuronidase activity in surface water as a surrogate for *E. coli*

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abstract

A sensitive field-portable fluorimeter with incubating capability and triplicate sample chambers was designed and built. The system was optimised for the *on-site* analysis of *E. coli* in recreational waters using fluorescent based enzyme assays. The target analyte was β-D-Glucuronidase (GUS) which hydrolyses a synthetic substrate 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6-CMUG) to release the fluorescent molecule 6-Chloro-4-Methyl-Umbelliferyl (6-CMU). The system was calibrated with 6-CMU standards. A LOD of 5 nM and a resolution of less than 1 nM was determined while enzyme kinetic tests showed detection of activities below 1 pmol min ^{1} mL ^{1} of sample. A field portable sample preparation, enzyme extraction protocol and continuous assay were applied with the system to analyse freshwater and marine samples. Results from a one day field trial are shown which demonstrated the ability of the system to deliver results *on-site* within a 75 min period.

Keywords:
Fluorescence Recreational water, *E. coli,* Enzyme assay, β-d-glucuronidase, On-site

1. Introduction

E. coli and Enterococci are widely used as bacterial Faecal Indicators (FI) for recreational waters [1,2]. Table 1 shows the specified maximum limits in Colony Forming Units (CFU) for marine and transitional waters as per the EU Bathing Water Directive 2006/7/EC. Standard culture based detection methods are slow to produce a result e.g. Colilert 18, a Most Probable Number (MPN) method, requires 18 h incubation and Petri-Film, a colony counting method, requires 22 h incubation. The incubation period, plus the time to take the sample and transport it to the laboratory, means that a result is not obtained until the following day. There is a demand for "Rapid" or same day test methods preferably *on-site* and autonomous [3,4]. Enzyme assays have been suggested as the best solution for this [5].

Enzyme assays have long been suggested as a rapid alternative to culture based FI assays. β-D-Galactosidase (GAL) and β-D-Glucuronidase (GUS) have been used as marker enzymes in assays for *E. coli* [7–13] while Glucosidase has been used in assays for Enterococci. Of these target enzymes GUS is the most specific, being present in 94–97% of *E. coli* strains tested [9,14].

There are a number of key differences between enzyme activity assays and culture based methods. Enzyme assays measure the activity of (i) Viable Culturable (VC), (ii) Viable But Not Culturable Bacteria (VBNC) plus dead bacteria, and (iii) free enzyme depending on the particular method used; whereas culture based methods count only the VC portion of bacteria present in a sample [13,15]. If clusters of aggregated or particle bound *E. coli* are present in a sample, culture based methods count clusters as single units thus underestimating the number of cells present whereas enzyme assays account for the activity of each cell thus giving a better representation of the total number of cells present. For these reasons it is difficult to correlate the two approaches although this is commonly done due to a lack of a practical alternative standard reference method [9,12,16]. There is a growing body of evidence pointing to the virulence of VBNC bacteria and suggesting the importance of measuring their numbers in environmental waters [17–19]. For this purpose enzyme assays are a measurement technology which could be implemented and optimised.

GUS activity assays for *E. coli* do not have a selective growth step (as culture-based methods do). Thus they are susceptible to interference from other GUS sources [9]. Sources include plant and algal biomass [20], free (extracellular) enzyme [21], dead target bacteria and GUS positive non-target bacteria. GUS positive nontarget bacteria generally have GUS activities which are several orders of magnitude less than those of GUS induced *E. coli,* thus present little interference unless at very high concentrations.

However certain species e.g, A. *viridans*, *Bacillus spp* [22] and *Vibrio harveyi* (particularly in the marine environment) [8] are highly GUS positive and may interfere with an assay if present at similar numbers to the target bacteria. Further interferences can occur from chemicals in the water matrix [23].

Chromogenic and fluorescent synthetic substrates have been used for enzyme assays and of the two; fluorescence offers much greater sensitivity by up to 1000 . As a consequence 4-MethylUmbelliferyl-β-D-Glucuronide (4-MUG) has been used extensively in discontinuous assays. Its fluorophore 4-Methyl-Umbellierone (4-MU) has a

pKa of 7.8 and is highly fluorescent at pH values over 9 [8,9,13]. Recent work in our research group [24] demonstrates the use of 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6CMUG) for continuous GUS assays with greatly reduced sample handling. Its fluorophore 6-Chloro-4-Methyl-Umbelliferone (6-MUG) has a lower pKa value (6.12) than 4-MU and at pH 6.8 is almost fully dissociated into its highly fluorescent anionic form. Furthermore GUS catalysis rates for the two substrates are quite similar, K_{cat} ¼ 222 7 13.4 S ^{1} for 4-MUG and K_{cat} ¼ 207 7 8.5 S ^{1} for 6-CMUG at 37 °C and pH 6.8 [24].

Enzyme assays typically involve sample filtration, lysing, incubation, and detection steps. Fig. 1 outlines the principle of the continuous 6-CMUG assay. *E. coli* cells are trapped and lysed, releasing GUS which catalyses the hydrolysis of 6-CMUG to a glucuronic acid and the fluorescent molecule 6-CMU. The amount of fluorophore released in a certain period of time is directly proportional to the number of *E. coli* cells trapped. The assay performs optimally at 44 °C and at pH 6.8 [24].

In the literature, GUS activities per *E. coli* are reported within the range of 0.1–100 fmol min ^{1} per culturable *E. coli* depending on method used. Garcia-Armisen [12] using a method from George [16] based on 4-MUG, reports GUS activities of approximately 100 fmol (4-MUG) min ^{1} per culturable *E. coli* for lightly contaminated freshwater samples (i.e. 100–1000 *E. coli*. 100 mL ^{1} as established by MPN method). Lebaron [25] using the same method reports GUS activities per culturable *E. coli* of approximately 20 fmol (4-MUG) min ^{1} per culturable *E. coli* for seawater samples.

Instrumental detection of hydrolysis products of assays has commonly been conducted using standard laboratory bench fluorimeters [8,16]. There have been a few attempts to conduct analysis *on-site* with portable fluorimeters [26,27], but there remains a need for a rapid, sensitive *on-site* test for FI bacteria.

This paper presents a field portable fluorimeter (called ColiSense) with built-in incubation and triplicate sample chambers..

This system was specifically designed for *on-site* fluorescent enzyme assays for *E. coli*. A field portable sample pre-concentration and lysing procedure was applied in the system with a continuous 6-CMUG based assay [24]. The system lysing procedure and assay were tested both in the laboratory and the field. Rapid, sensitive *on-site* detection of *E. coli* GUS activity is demonstrated.

2. Experimental/materials and methods

2.1. Chemicals and reagents

The fluorophore, 6-chloro-4-methylumbelliferone (97%) (6CMU) was obtained from CarboSynth, UK. The fluorogenic substrate, 6-chloro-4-methylumbelliferyl-β-D-glucuronide (97%) (6-CMUG) was obtained from GlycoSynth, UK. The enzyme: β-D-glucuronidase type VII-A (27%) from *E. coli* and 1,4-dithiothreitol (DTT) were obtained from Sigma Aldrich Ireland. The Colilert-18 ^{s}/Quanti-Tray 2000^{s} system from IDEXX Laboratories used for the enumeration of coliforms and *E. coli* was obtained from TechnoPath Ireland. Corning syringe filters with cellulose acetate surfactant-free membranes diameter 28 mm, pore size 0.45 μm were obtained from Sigma Aldrich, Ireland. Bacterial PELB buffer and PELB lysozyme were obtained from VWR Ireland. Water was passed through a Milli-Q water purification system. Stock solutions of fluorophore and substrate (100 mM) were prepared in 1 mL DMSO (99.5%) and stored at 4 °C.

2.2. Engineering components

Ultraviolet LEDs (FG360-R5-WC015) with peak emission wavelength at 361 nm were obtained from ATP, USA. Photodiodes (BPW21R), operational amplifiers (MCP601), voltage regulators (LM317), Darlington transistor array (ULN2803), digital temperature sensor (DS18b20) and silicone matt heater (1.25 W, 50 mmx25 mm) were obtained from Radionics Ireland. Optical filters (GG-420, Long Pass, diameter 12.5 mm) were obtained from Edmund Optics, UK. A Wixel micro-controller board was obtained from Cool-Components, UK. The instrument enclosure (Diatec S White) was obtained from OKW, UK. Glass sample vials (TVL-050-040) were obtained from SciChem Ireland. The heating block was machined in-house from aluminium.

2.3. Prototype design and construction

A portable incubating fluorimeter (ColiSense) was designed and built to conduct the detection step of the continuous, 6-CMUG based assay. See Fig. 2d. The system featured three sample chambers for performing assays in triplicate as recommended by Lebaron due to the high Coefficient of Variance (CV) (15%) of enzymatic methods [25]. Each chamber was set to incubate at 44 °C and control to within 0.5 °C. A fluorescence detection system with excitation at 362 nm and emission at 445 nm was integrated into each chamber.

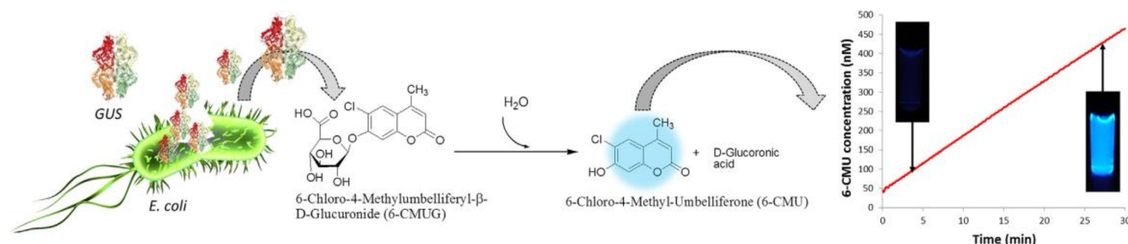

Fig. 1. Fluorescence based enzyme assay principle. Cell lysis and release of β-D-Glucuronidase (GUS), substrate: 6-Chloro-4-Methyl-umbelliferyl-β-D-Glucuronide (6-CMUG) hydrolysis to 6-Chloro-4-Methyl-umbelliferyl (6-CMU) catalysed by GUS, fluorescence increase over time.

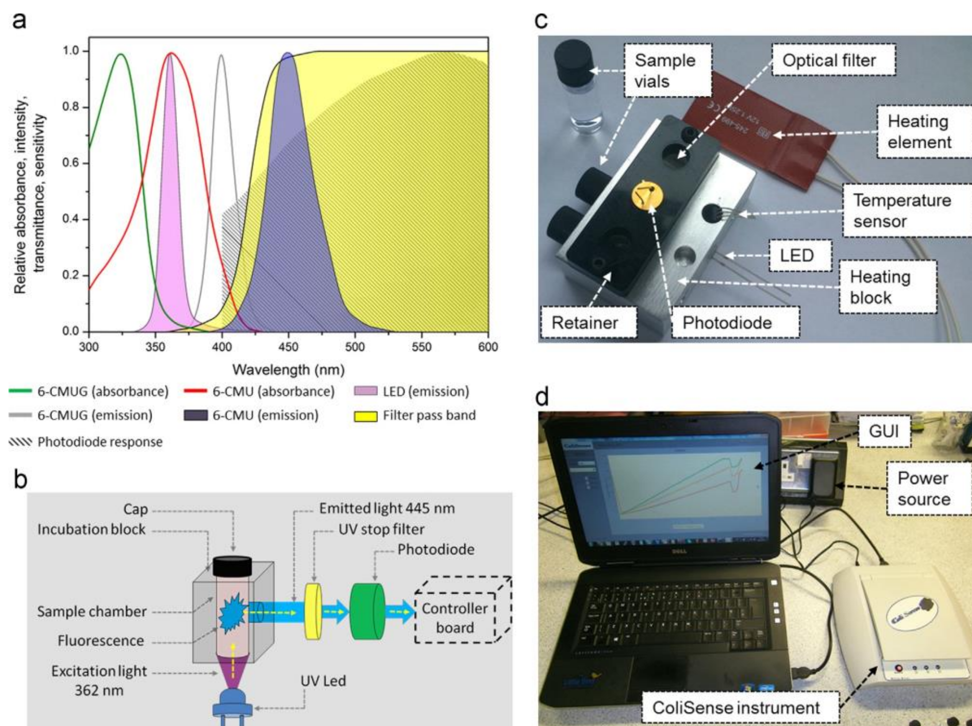

Fig. 2. ColiSense system design and construction. (A) Normalised spectra of chemical components of the assay and optical components of the fluorescence detection system. (B) Schematic of the incubation and fluorescence detection system. (C) Physical realisation of key system components. (D) ColiSense instrument with power source and Graphical user interface (GUI).

2.3.1. *Optical and mechanical design*

Fig. 2.a shows the normalised absorbance and emission spectra for the chemical components of the enzyme assay at neutral pH (pH 6.8) and shows the characteristic spectra of the optical components of the system. An LED with peak emission at 361 nm and spectral width of 20 nm was selected to excite the 6-CMU close to its maximum while exciting the 6-CMUG as little as possible. A photodiode with peak sensitivity at 570 nm and enhanced sensitivity in the blue region (65% of max at 445 nm) was selected as the detector. A high pass optical filter with 420 nm cut-off was selected to reduce any interference from substrate fluorescence and block the excitation light from the detector. Glass sample vials (2 mL) were used as cuvettes due to their disposable nature and low cost. The optical characteristics of the vials were tested and results are shown in supplementary information.

An incubation block, shown in Fig. 2c, with 3 detection chambers was machined from aluminium. A self-adhesive silicone foil heater was attached to this and cork insulation (6 mm) was applied to exposed surfaces of the heating block to increase thermal efficiency. A digital temperature sensor was inserted in the block and fixed in place with thermally conductive epoxy. The components of the fluorescence detection system were incorporated into the heating block in an off axis (90°) arrangement as shown in the schematic in Fig. 2b and the image in Fig. 2c. The glass sample vials were inserted into the incubation block where the LED excited the fluorescence from below and fluorescence was emitted at right angles, filtered and captured by the photodiode. The heating block and the fluorescence detection system were incorporated into the instrument enclosure shown in Fig. 2d.

2.3.2. *Electronics*

The electronic control system was designed around a Texas instruments CC2511F32 micro-controller based development board called Wixel. This was programmed in a variant of C via USB comms. The board offers features including a 3.3 V regulator, USB, low power radio, 12 bit differential Analog to Digital Converter (ADC). It has a small form factor and is highly versatile. The ADC with 2047 quantisation levels was set to use an internal reference of 1.25 V. Thus the resolution was 1.25 V /2047¼ 0.61 mV.

An LED light source was chosen for the device as it offers low power consumption, small size, low weight, high robustness and high monochromaticity. The emission spectrum is shown in Fig. 2a. To maximise sensitivity, the LED was powered at its maximum continuous rating (20 mA) though a constant current supply based on a LM317 regulator delivering a radiant power of 750 mW. To reduce any possible bleaching of the fluorophore by over exposure to the excitation source, the sample rate was set to
0.1 hz with a sample illumination duty cycle of 0.5%. I.e. the sample was illuminated for 50 ms each 10 s.

A photodiode was chosen as the optical detector for the device as it offers low power consumption, small size, low weight and high robustness. The acceptance spectrum of the chosen photodiode is shown in Fig. 2a. The photodiode was used in photovoltaic mode with a trans-impedance amplifier based on an MCP601 operational amplifier to convert its output to a voltage. The voltage was recorded by the 12 bit ADC on the controller board. Details of trans-impedance amplifier gain resistor selection are in supplementary information.

A silicone foil heater was selected as the heat source. This delivered 5 W of heat while powered with 24 V and drawing 200 mA. Temperature control was performed using a Dallas 1-wire digital temperature sensor (DS18B20) allowing control to within 0.5 °C. Details of temperature and power testing are presented in supplementary information. A Darlington transistor array (ULN2803) controlled by the Wixel was used to switch the LEDs and heater. This component can switch loads up to 500 mA per channel at up to 36 V. The system was powered by a 24 V switch mode plug top supply for laboratory use and by a 24 V battery for field use. Communications to the PC was via USB using serial protocol at 9600 Baud rate.

2.3.3. *Software*

The Wixel development platform around which the electronics of the system was based was programmed in C. Function prototypes were created to control each of the system components. Fluorescence levels in each sample chamber plus the temperature of the incubating block were transmitted to the PC and displayed on ExtraPutty terminal or graphed using a Graphical User Interface (GUI) designed in Java, while simultaneously being recorded in a log file in Comma Separated Value (CSV) format. The timestamp feature on ExtraPutty V0.26 was used to append a date and time to each reading stored in the log-file. The log-files were subsequently imported into Microsoft Excel for analysis. Fig. 3 shows a flow diagram of the firmware on the ColiSense instrument and software on the attached PC including the programmed Java GUI.

2.4. *System characterisation*

2.4.1. *Fluorescence calibration*

To select optimum gain settings for the photodiode trans-impedance amplifier and establish the range and sensitivity of the ColiSense system it was tested against a range of 6-CMU concentrations (0.1 nM to 130 mM in sodium phosphate buffer at pH 6.8). Details of these tests can be found in supplementary in-
formation. An optimum sensitivity setting with a trans-impedance amplifier feedback resistance of 100 MΩ was selected and the following calibration procedure was carried out.

Standards to give final 6-CMU concentrations from 0.1 nM to 10 mM were prepared in 2 mL glass vials also containing 0.5 mM 6-CMUG, 5% PELB (v/v), 0.05 mg mL ^{1} PELB lysozyme and 20 mM DTT. Analysis was carried out in triplicate. The three vials were placed in channel A, B, C of the ColiSense instrument, incubated at 44 °C and fluorescence response was recorded.

2.4.2. *Enzyme kinetics*

Commercial GUS at a range of concentrations (0.02– 0.42 ng mL ^{1}) was inoculated into 2 mL glass vials containing 2 mL of 500 mM 6-CMUG in phosphate buffer at pH 6.8. Analysis was carried out in triplicate. The three vials were placed in channel A, B, C of the ColiSense instrument where they were incubated at 44 °C and fluorescence increase was recorded during 30minutes at intervals of 10seconds.

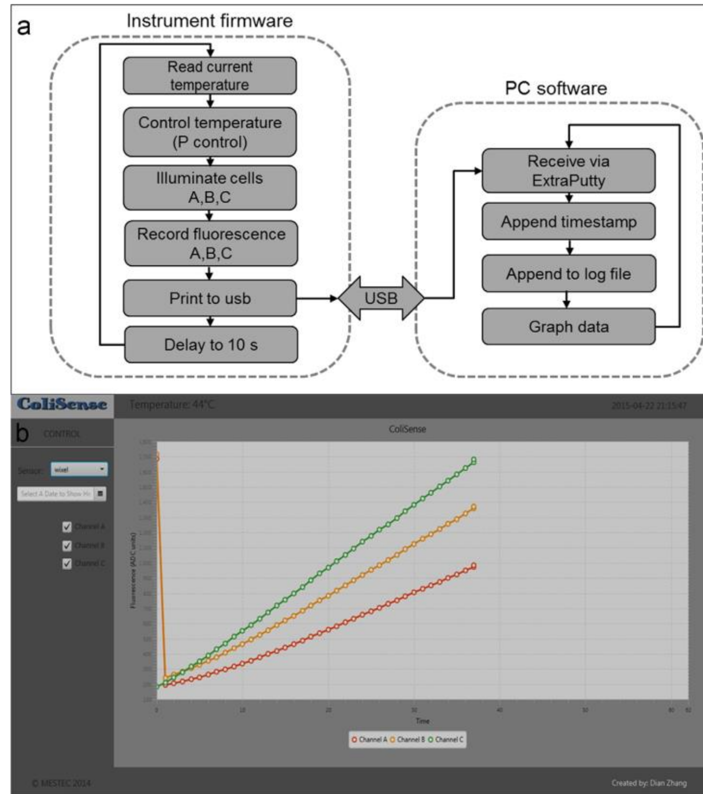

Fig. 3. ColiSense system software components. (A) Instrument firmware and PC software flow diagrams and (B) detailed view of Graphical user interface (GUI).

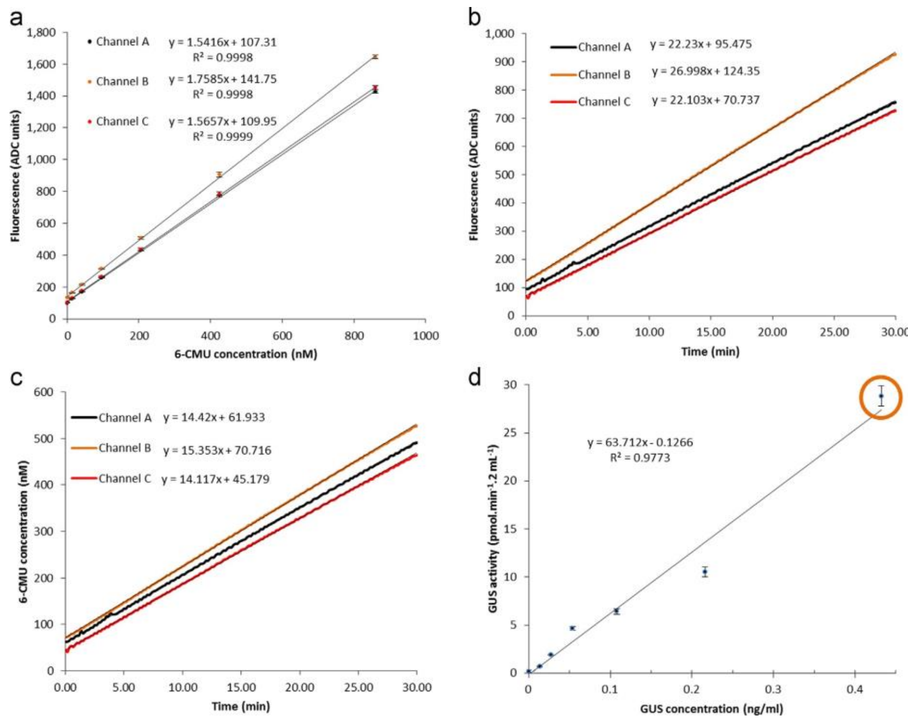

Fig. 4. System charachterisation. (A) Calibration of fluorescence response of ColiSense channels A,B,C with concentrations of 6-CMU up to 1000 nM in pH 6.8 phosphate buffer with 500 mM 6-CMUG and PELB (n¼3). Error bars show SD of triplicate measurements. (B) Progress curves for 0.43 ng L ^{1} GUS added to 500 mM 6-CMUG in pH 6.8 phosphate buffer with PELB in ColiSense Channels A,B,C. (C) Progress curves from panel B expressed as 6-CMU concentration. (D) Enzyme activity per 100 mL recorded by ColiSense for concentrations of GUS up to 1 ng L ^{1} (n¼3). Error bars show SD of triplicate measurements.

2.5. *Environmental sample testing*

Fresh water and salt water samples were collected in high density polypropylene (HDPP) bottles from the river Tolka and the river Liffey estuary respectively, both in Dublin, Ireland. Samples were transported to the lab on ice within 2 h and inoculated within 4 h after collection. Prior to any analysis the 1 L samples were allowed to settle for 30 min to remove heavy sediment.

For the determination of *E. coli* (MPN) the enumeration protocol was followed in accordance with manufacturer's instructions. Aliquots of 10 mL from the original water samples were diluted 1:10 with sterile deionised into 100 mL bottles. After the addition of Colilert-18, samples were inoculated into Quanti-Trays and sealed. For *E. coli* and coliform enumeration, samples were incubated at 37.0 °C for 18–20 h. Following incubation the Quanti- Tray wells were read visually for yellow colour indicating the presence of coliforms and for blue fluorescence indicating the presence of *E. coli.*

GUS activity was measured as described below by filtering various volumes of water sample (100–2 mL) to achieve varying concentrations of target bacteria trapped in the syringe filter.

Briefly the protocol involves two main steps: sample preparation and GUS quantification [24]. The sample was filtered through 0.45 μm syringe filters for bacteria capture and pre-concentration using 50 mL syringes, followed by lysing agent addition (100 mL).

In the next step, the filters were sealed using screw caps and incubated at 37 °C for 30 min. In the third step, 1.9 mL of buffer (pH 6.8) was flushed through the filter using 2.5 mL syringes and the samples were recovered in 2 mL glass vials. The vials were placed
into the ColiSense to allow pre-warming to 44 °C after which a 10 μL aliquot of 100 mM 6-MUG in DMSO was added and the vials were vigorously mixed. The vials were placed back into the Coli- Sense, allowed to equilibrate and GUS activity was monitored/recorded for 30 min. Triplicate blanks were also prepared by adding 1.9 mL of buffer, 100 μL lysing agent and a 10 μL aliquot of 100 mM 6-MUG in DMSO to 2 mL vials. These were placed in ColiSense and their activity was monitored for 30 min to detect auto-hydrolysis of 6-CMUG.

2.6. *Field trial*

On 27th February 2015a field trial was conducted to demonstrate the portability of the ColiSense system. The ColiSense system and miniature incubator for use in the lysing procedure were placed in a van for transport and powered from the van's battery source. Seven points along the Tolka River, Dublin, Ireland were sampled and analysed *on-site* in 1day. Sampling points 1–5 were within the urban area at approximately 2 km spacing's while points 6 and 7 were in the rural catchment 1 km apart with point 6 being 6 km west of point 5. The sample capture and testing began in the early morning downstream just above the tidal range and concluded in the evening in the upstream catchment.

GUS activity was measured in triplicate using the protocol detailed in Section 2.5, with filtered volume fixed at 50 mL Additional water quality parameters were measured including *E. coli* MPN and total coliforms MPN, pH, temperature, turbidity, conductivity measured by Hydrolabs DS5x multi-parameter sonde, while phosphate, nitrate and nitrite were measured using a Hach DR900 Nutrient Analyser.

3. Results and discussions

The ColiSense system was designed to perform *E. coli* enzyme assays *on-site* and was optimised for conducting a continuous 6-CMUG based assay. The system was designed with triplicate sample chambers to facilitate statistical analysis of results. Simultaneous assays were the only option for achieving triplicates as sequential assays were not possible in microbiological analysis of environmental waters due to samples aging.

To remain relevant to bathing water standards [6] the system was required to detect GUS activities in water with corresponding
E. coli concentrations below 250 culturable *E. coli* per 100 mL. Assuming GUS activity of 100 fmol (6-CMU) min ^{1} calculated per culturable *E. coli* [12], this meant detection of sample activities below (250 culturable *E. coli* per 100 mL) (100 fmol (6-CMU) min ^{1} per culturable *E. coli*) ¼ 25,000 fmol (6-CMU) min ^{1} 100 mL ^{1}. To achieve this, the system was designed with a nano-molar 6-CMU fluorescence detection range and sensitivity less than 1 nM 6-CMU.

3.1. *Fluorescence detection calibration*

Fig. 4a shows calibration curves for channel A, B, C of the ColiSense instrument for concentrations of 6-CMU up to 900 nM. Points represent the average of triplicate samples and vertical error bars represent the standard deviation of those samples. Channel A and C showed a sensitivity of 1.54 and 1.57 quantisation levels (ADC units) per nM 6-CMU. Channel B was slightly more sensitive at 1.76 quantisation levels per nM 6-CMU. Resolution was

less than 1 nM for each channel. The LOD for each channel was 5 nM 6-CMU.

In Fig. 4a each curve crosses the *Y* axis at 100 fluorescence units or more. This is due to a combination of leakage of the excitation source light to the detector, the fluorescence of the substrate (6CMUG) itself and the presence of small concentrations of fluorophore (6-CMU) from auto-hydrolysis of the substrate. The substrate, was present in the assay at much higher concentration than the fluorophore (500 mM vs r 1 mM respectively), thus any substrate fluorescence emitted was a significant interference. The substrate (6-CMUG) absorbed maximally at 325 nm and emitted maximally at 400 nm while its hydrolysed fluorophore (6-CMU) absorbed maximally at 365 nm and emitted maximally at 445 nm. The high pass optical filter with 420 nm cut-off reduced interference from substrate fluorescence and leakage of the excitation light to the detector while allowing the 6-CMU fluorescence to pass with minimal attenuation. Further calibration data and tests against a standard instrument are presented in the supplementary information.

3.2. *Enzyme kinetics*

Fig. 4b shows progress curves for 0.42 ng mL ^{1} GUS added to 500 mM 6-CMUG in Channels A, B, C of the ColiSense instrument. The curves were linear to 30 min. Channel B showed a larger value response than A or C due to its higher sensitivity, (see Fig. 4a). Fig. 4c shows the same progress curves converted to 6-CMU concentration by dividing each sample point by the channel sensitivity found from Fig. 4a. Points represent the average of triplicate samples and vertical error bars represent the standard deviation of those samples. The circled point in Fig. 4d shows the mean and standard deviation of the same progress curves converted to activity levels in picomoles 6-CMU per minute per 2 mL (cuvette volume ¼ 2 mL) and plotted against enzyme concentration. Fig. 4d shows further data obtained in the same manner for GUS concentrations from 0.02 to 0.42 ng mL ^{1}.

The system was shown to detect GUS activities as low as 0.1 pmol (6-CMU) min ^{1} ml ^{1} for the blank and up to 14.5 pmol (6-CMU) min ^{1} ml ^{1} for the highest enzyme concentration tested. The design requirement for the system set out previously (Section 2.3) i.e. detection lower than 25 pmol (6-CMU) min ^{1} 100 mL ^{1} equates to 0.25 pmol (6-CMU) min ^{1} ml ^{1}. The detection level achieved is lower than the design level thus the system can be used to analyse samples containing 250 *E. coli* per 100 mL without any pre-concentration. The activity of 0.1 pmol (6-CMU) min ^{1} ml ^{1} recorded for the blank (i.e. without the addition of enzyme) is due to substrate auto-hydrolysis where 6-CMUG spontaneously separates into 6-CMU and glucuronic acid in the presence of water.

The coefficient of variance (CV) for the method depended on enzyme concentration, with a CV of 14% calculated for the lowest GUS concentration (0.02 ng mL ^{1}) and a CV of 1.8% calculated for the highest concentration (0.42 ng mL ^{1}).

3.3. *Environmental samples*

An MPN of 3873 *E. coli* per 100 mL was recorded for the freshwater sample (salinity 4‰) while an MPN of 8164 *E. coli* per 100 mL was recorded for the seawater sample (salinity 32‰). By varying the volume of sample filtered, a range of MPNs from 193 to 3873 *E. coli* for freshwater and from 163 to 8164 *E. coli* for seawater were achieved. Fig. 5a for freshwater and 5.b for seawater show graphs of recorded GUS activity (pmol (6-CMU) min ^{1} 100 mL ^{1} of sample) vs *E. coli* MPN. Points represent the average of triplicate samples and vertical error bars represent the standard deviation of those samples.

Both samples show linear relationships between GUS activity and *E. coli* MPN down to *E. coli* concentrations lower than the excellent standard (MPN 250 *E. coli*) as stipulated by the BWD [6]. The slopes of the curves however differ significantly with the seawater sample having 4 times more GUS activity per *E. coli* than the freshwater. This may be attributed to a higher proportion of VBNC to VC *E. coli* in the marine environment than in freshwater. This is not proven here experimentally but it has been addressed adequately by other researchers [15,28,29]. Another potential influence is interference from GUS positive marine biomass including plant and algal matter as detailed by Davies [20]. Plant based interference occurs through the release of GUS into the water body. In this work a sample filtration step is included so this interference is removed. However, GUS positive algae which Davies states are more common in the Marine than in Freshwater [20] remain a potential interference as they are retained during filtration.

The coefficient of variance for the method varies depending on the *E. coli* concentration being measured with higher variability at lower concentrations. For the freshwater sample CV decreases from 6% at the lowest *E. coli* MPN (193) to 1.5% for the highest *E. coli* MPN (3873) while similarly for the seawater sample CV decreases from 23% at the lowest *E. coli* MPN (163) to 0.3% for the highest *E. coli* MPN (8164).

3.4. *Field trial*

The field trial began at 7 am and concluded at 7 pm on the same day. With 7 sites sampled this gave an average time per sample of 103 min including analysis (75 min) and transit between sites, thus demonstrating the field portable nature and rapidity of the device and assay.

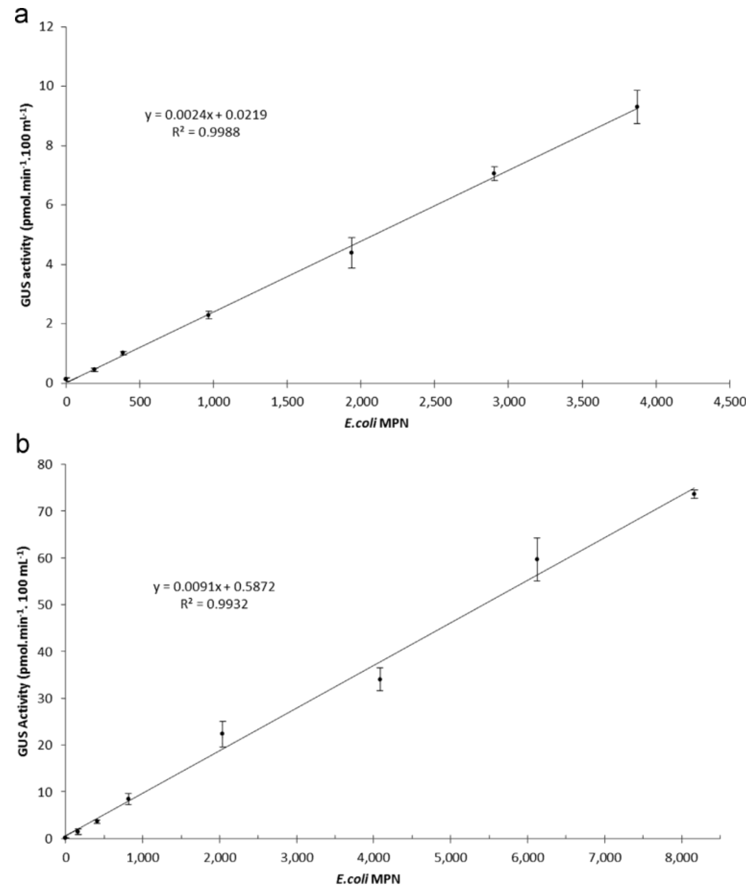

Fig. 5. Target analyte testing. (A) Enzyme activity per 100 mL sample vs *E. coli* concentration in River Water (Salinity¼ 4 ppt) (*n*¼ 3), Error bars show SD of triplicate measurements. (B) Enzyme activity per 100 mL sample vs *E. coli* concentration in Sea Water (Salinity¼ 32 ppt) (*n*¼ 3), Error bars show SD of triplicate measurements.

Fig. 6a shows the location of sampling points along the Tolka River and the GUS activities and *E. coli* MPNs recorded. Fig. 6b shows the collected *E.coli* MPN and GUS activity data. Error bars on MPN measurements represent the upper and lower 95% confidence limits of the method, while error bars on the activity measurements represent 95% confidence intervals for triplicate samples. The horizontal red line indicates the acceptable upper limit for recreational waters i.e. MPN 1000 *E. coli* per 100 mL.

Point 1 shows elevated *E. coli* MPN (15 times the acceptable upper limit for recreational waters) and GUS activity. There had been heavy rain in the hours prior to the sample being taken so it is supposed that the high levels were due to a combined sewer overflow (CSO) as is known to occur on the Tolka [30]. *E. coli* levels of this order have been recorded previously at the same site under similar conditions [31]. In the case of a CSO *E. coli* MPN would be expected to be high and GUS activity correspondingly high [15]. Points 2–5 show lower *E. coli* MPNs (3–6 times the acceptable upper limit for recreational waters) and correspondingly lower GUS activities. This decrease from point 1 agrees with previous research where it was shown that bacterial counts reduce with progress upstream in the river Tolka [31] and it is supposed that this is due to decreased urbanisation. Progress curves for point 1 are included in supplementary information.

Points 6 and 7, both in the agricultural catchment of the Tolka River, showed high levels of GUS activity relative to measured E. coli MPN. It is supposed that this was due to the recent application of farm slurry to land in the catchment. The slurry spreading seasons had opened on 15th February in the catchment area. Furthermore phosphate levels at points 6 and 7 were 0.26 and 0.25 mG L 1 (PO43) respectively. The highest of the other 5 points was point 3 at 0.01 mg L 1 (PO43), see supplementary information. Slurry on farmland would contribute a large pro- portion of VBNC compared to VC E. coli due to it being aged either on land or in the farmyard before reaching the river [29]. Thus a high level of GUS activity can be expected while the MPN of cul- turable E. coli is low.

The coefficient of variance for the method depended on the *E. coli* concentration being measured with higher variability at lower concentrations. CV decreased from 15.6% at the lowest *E. coli* MPN (624) to 2.6% for the highest *E. coli* MPN (14,136). These figures agree with the work of Lebaron who in a much more extensive study using the discontinuous 4-MUG method for measuring GUS activity reported CVs less than 15% [25]. By comparison,

culture based MPN methods typically report CVs of 15% to 30% [32].

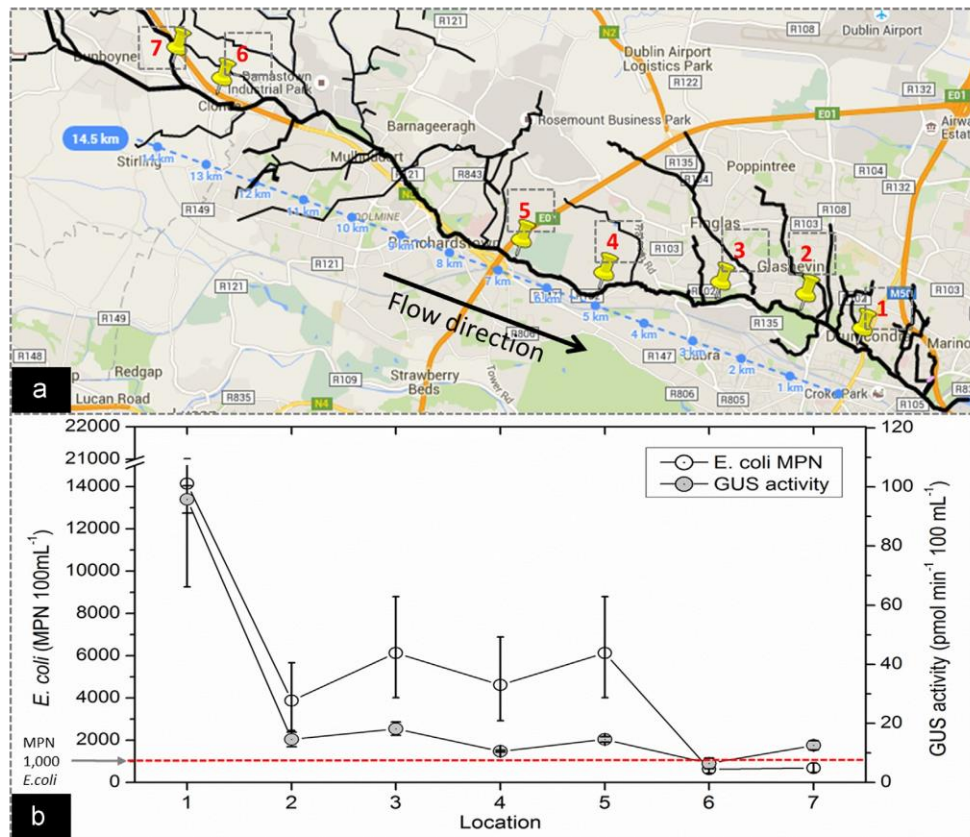

Fig. 6. Field Trial. (A) Locations sampled along the Tolka River (Dublin, Ireland) (B) *E. coli* MPN and GUS activity by location. Vertical error bars on GUS activity show the 95% confidence intervals of triplicate samples in each channel of ColiSense, Vertical error bars on *E. coli* MPN represent the upper and lower 95% confidence intervals for the Colilert 18 MPN method.

4. Conclusions

The aim of this work was to address the need for a 'Rapid' *onsite* test for microbiological quality of surface waters. Such a test is needed to allow same day action in the case of a pollution event. The ColiSense detection system detailed here and β-D-Glucuronidase (GUS) enzyme assay applied offer a rapid (75 min) *on-site* solution.

The ColiSense system is a sensitive purpose built fluorescence detection and incubation system with three sample chambers for triplicate analysis. When combined with an efficient GUS extraction protocol and a continuous fluorometric assay based on 6-Chloro-4-Methyl-Umbelliferyl-β-D-Glucuronide (6-CMUG) enzyme substrate, it provided a sensitive and rapid method of *on-site* analysis of surface waters for *E. coli*.

The system has demonstrated an LOD of 5 nM (6-CMU) with a resolution of less than 1 nM (6-CMU) and detection of GUS activities below 1 pmol (6-CMUG) min ^{1} mL ^{1}. This allowed detection of GUS activities for *E. coli* concentrations lower than MPN 250 100 mL ^{1} (the upper limit for excellent marine recreational water as per the Bathing Water Directive). The CV of the method has been shown to be dependent on *E. coli* concentration (23% at the lowest concentrations measured to o 1% at the highest concentrations). The triplicate analysis facility allows for greater confidence in results.

The system and assay detect VBNC along with VC *E. coli* and GUS from sources other than *E. coli* which leads to some disagreement with culture based methods but as an alternative to standard culture based methods of *E. coli* detection it offers greater rapidity and portability and is capable of meeting the need for rapid faecal indicator detection to ensure recreation water quality standards.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.10. 035.

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