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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.

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 non-target 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.

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Table 1
Bathing water directive *E. coli* and Enterococci limits [6].

	Fresh water			Marine water		
FI Bacteria	Excellent	Good	Sufficient	Excellent	Good	Sufficient
E. coli CFU 100 mL^{-1}	500 *	1000 *	900 **	250 *	500 *	500 **
Enterococci CFU 100 mL^{-1}	200 *	400 *	330 **	100 *	200 *	185 **

* 95th percentile evaluation
** 90th percentile evaluation.

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-Methyl-Umbelliferyl-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 (6-CMUG) 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, $K_{cat}=222\pm13.4\ S^{-1}$ for 4-MUG and $K_{cat}=207\pm8.5\ S^{-1}$ 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%) (6-CMU) 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®/Quanti-Tray 2000® 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

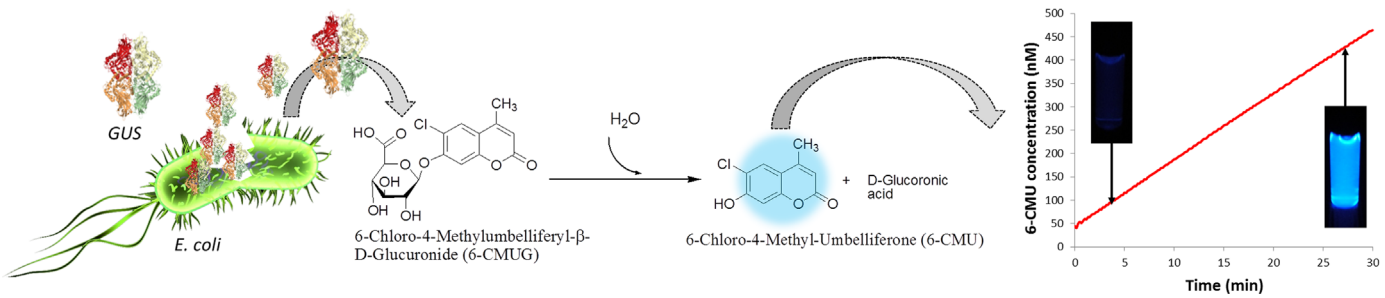

Fig. 1. Fluorescence based enzyme assay principle. Cell lysis and release of β-D-Glucuronidase (GUS), substrate: 6-Chloro-4-Methyl-umbelliferyl-β-D-Glucuronide (6-CMUG) hydrolysis to 6-Chloro-4-Methyl-umbelliferyl (6-CMU) catalysed by GUS, fluorescence increase over time.