Continuous fluorometric method based on β-D-Glucuronidase for rapid detection of *Escherichia Coli* in water.

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Introduction

*A*t present standard methods employed for the microbiological monitoring of bathing waters require at least 18 hours to perform and are based on culturing techniques. This is a huge drawback when immediate action is required. Real-time and on-line monitoring are key factors for consideration in current method development for continuous indicator organism detection in order to meet early warning requirements and water safety plans. The European Union has introduced a new Bathing Water Directive that is going to be implemented in all member states from 2008-2013. According to this directive classification of bathing water in "excellent", "good" and "sufficient" quality is based on the microbial indicators *E.Coli* and *intestinal enterococci.*

Methods utilising β-D-Glucuronidase (GUD) activity as an indicator of *Escherichia Coli* presence use labelled glucuronides to produce optical signals. Fluorometric assays for the measurement of *Escherichia Coli* GUD activity are traditionally performed using the fluorogenic substrate 4-methyl-umbelliferone-β-D-glucuronide (4-MUG) which upon hydrolysis releases the fluorophore 4-methyl-umbelliferone (4-MU). The major drawback of 4-MU is its high pKa (7.8), which causes only partial dissociation at pHs around the optimum pH for GUD activity (6.5-7.0). To overcome this issue researchers have employed discontinuous enzyme assays which require the addition of alkali.

In this context we explore the spectrophotometric properties of three fluorogenic substrates and their respective aglycons (Fig.1) for the continuous measurement of GUD activity and we apply the developed method for the rapid detection of Escherichia Coli in environmental water samples.

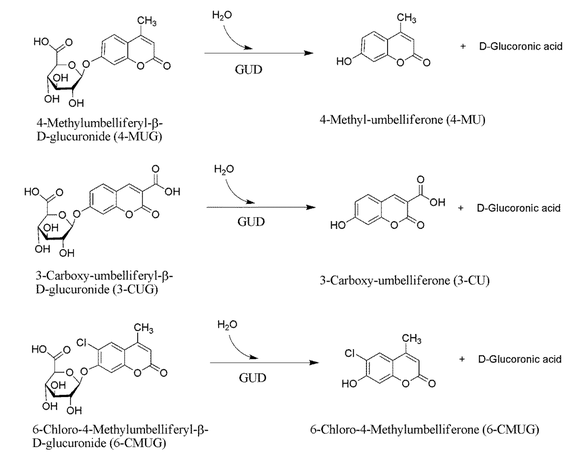


Figure 1. Fluorogenic substrates and their respective fluorophore upon enzyme mediated catalysis.

Results

UV-VIS Characterization

UV-VIS spectroscopy was used to determine the absorption λ_{max} for the fluorophores and substrates at different pH values and the protonation/deprotonation behaviour of the fluorophores (Fig 2.)

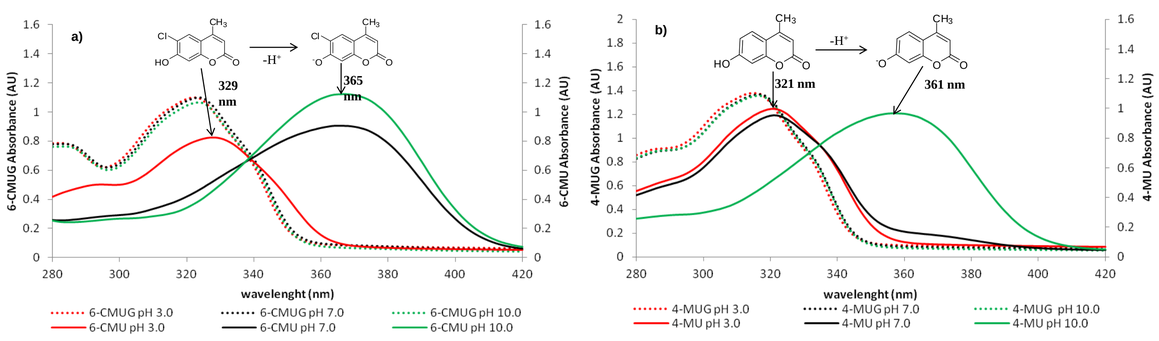


Figure 2. Absorption spectra of (a)100 μM 6-CMUG and 50 μM 6-CMU and (b)100 μM 4-MUG and 50 μM 4-MU , in acidic, neutral and alkaline conditions.

Fluorescence Spectroscopy Characterization

➢pH dependent fluorescence of the umbelliferone based fluorophores

When the excitation wavelength is selected to maximise the emission, the fluorescence intensity of 6-CMU in the 6.8-7.0 pH range is 6 times higher than that of 4-MU and 2.5 times higher than the fluorescence of 3-CU (Fig. 3).

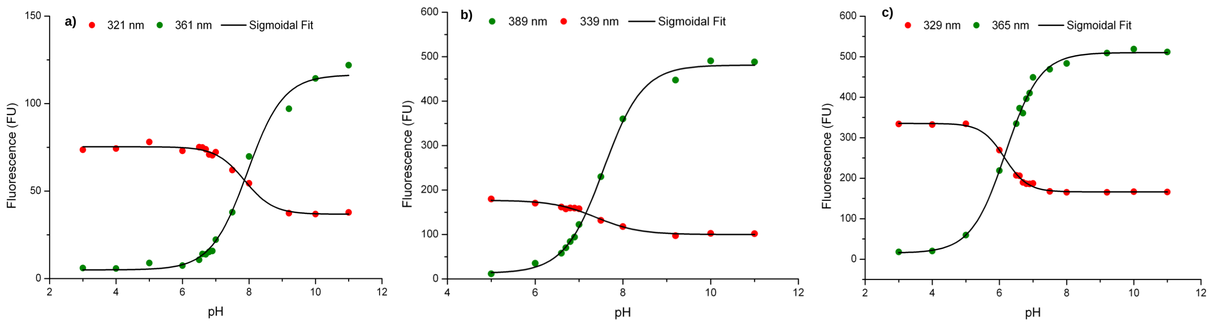


Figure 3. Nonlinear regression fitting of the experimental data to Boltzman Sigmoidal model; Experimental data and model line for 4-MU (a), 3-CU (b) and 6-CMU (c). Green series were obtain using the λ_{ex} for the anionic forms forms (361 nm for 4-MU, 339 nm for 3-CU and 365 nm for 6-CMU). Red series were obtained using the λ_{ex} for the neutral forms (321 nm for 4-MU, 339 for 3-CU and 329 for 6-CMU).

➢Influence of substrate on the excitation and emission spectra of the fluorophores

At pH 6.8, the excitation spectra of 3-CU and 4-MU are strongly affected by the presence of substrate due to inner filter effects (Fig. 4 a,b) while 6-CMU is not (Fig. 5 c).

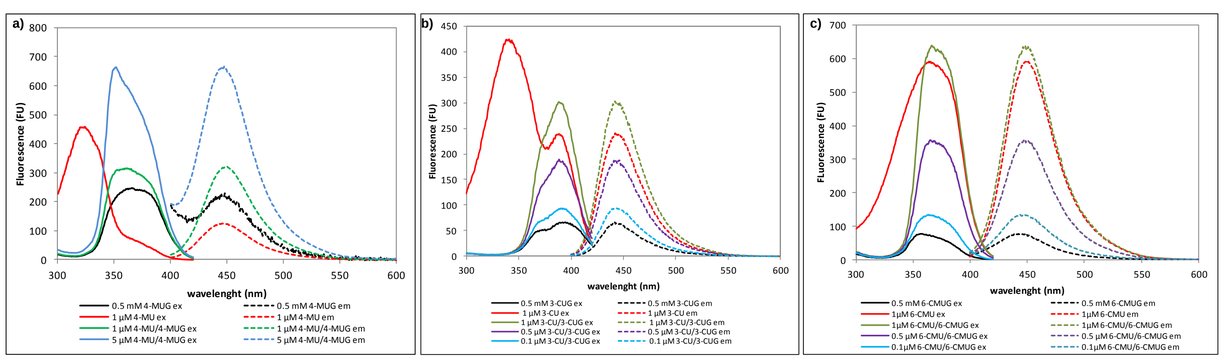


Figure 4. Excitation and emission spectra of (a) 4-MUG and 4-MU, (b) 3-CU and 3-CUG, (c) 6-CMU and 6-CMUG; (a) 4-MU em and 4-MU ex are the spectra of 4-MU; 4-MU/4-MUG ex and 4-MU/4-MUG em are spectra of 4-MU in the presence 0.5 mM 4-MUG; concentrations of 4-MU used are shown in the legend; emission wavelength: 446 nm; excitation wavelength: 351 nm; slit width: 5 nm (ex) and 2.5 nm (em).

GUD – Substrate Kinetics

One way to investigate the interaction between GUD and the three substrates is through the use of Michaelis Menten parameters: K_m and V_{max}. A comparison between these parameters for the three substrates can give insights into the GUD's preferred molecule, catalysis rates and optimal substrate concentration. By conducting studies in the same conditions (pH, temperature, GUD concentration) a comparison and a decision can me made regarding which of these substrates is optimal for continuous GUD assay.

Table 1. Michaelis-Menten kinetic parameters

Substrate	K_m (μM)	V_{max} (μM min^{-1})	V_{max} / K_m
4-MUG	70.82	2.56	0.031
3-CUG	479.28	0.99	0.002
6-CMUG	106.88	2.07	0.019

Initial reaction velocities were plotted against substrate concentration (Fig 5 a,b,c). Michaelis-Menten equation was used to estimate the K_m and V_{max} (Table 1).

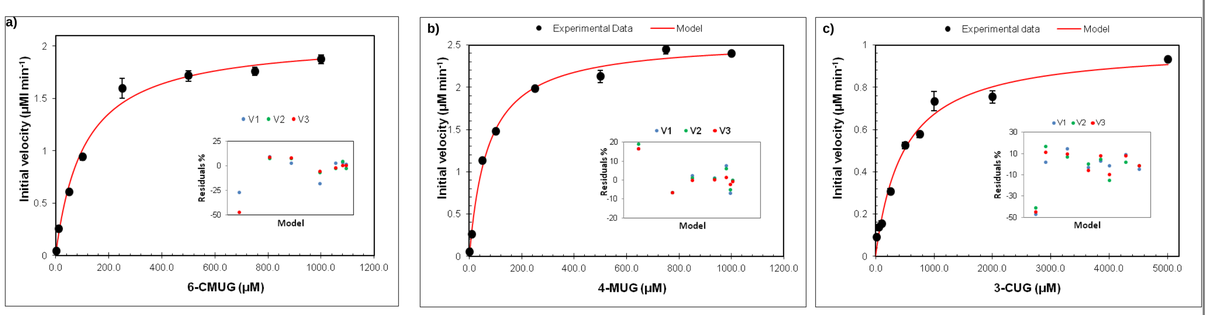
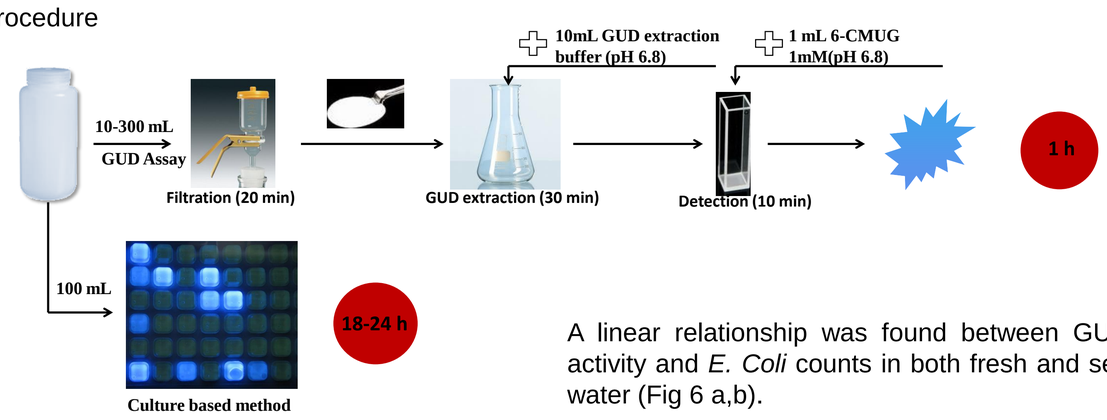


Figure 5. Michaelis-Menten models used to estimate Km and Vmax for the GUD catalysed hydrolysis of (a) 6-CMUG, (b) 4-MUG and (c) 3-CUG. The inset shows the mean residual analysis; V1, V2, V3 are the reaction velocities corresponding to the 3 runs. Reaction rates were followed for 10 min, with readings takes at each 5 s.

Rapid method for *E. Coli* detection-Proof of concept

➢Procedure



A linear relationship was found between GUD activity and *E. Coli* counts in both fresh and sea water (Fig 6 a,b).

➢Results

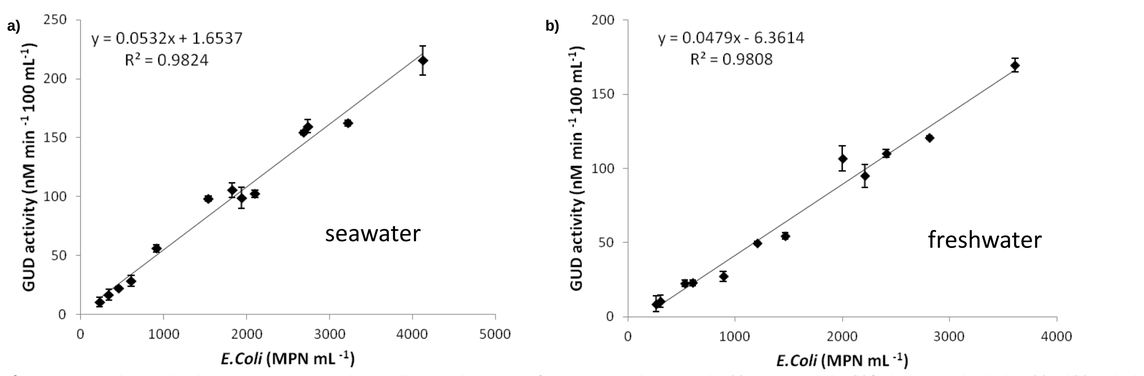


Figure 6. Linear regression between *E. Coli* concentrations determined using Colilert 18 and GUD activity from environmental water samples: (a) sea water samples, (b) fresh water samples. For both (a) and (b) 3 individual water samples were used from which different dilutions were prepared and assayed for GUD activity.

Conclusions

A continuous fluorometric method for the measurement of *E.Coli* GUD activity has been developed using 6-CMUG and offers a more straightforward approach for the evaluation of kinetic data. Benefits of this method as compared to a continuous one, include less sample manipulation, less reagent consumption, less experimental errors and better LOD.

The method was applied for the detection of *E. Coli from* environmental water samples and was successful in predicting *E. Coli* concentrations below the EU threshold for "excellent quality" , in 1h.

Acknowledgements

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