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In Situ Detection of the Pathogen Indicator *E. coli* Using Active Laser-Induced Fluorescence Imaging and Defined Substrate Conversion

J. Anderson,[1] S. Webb,[2] R. Fischer,[1] C. Smith,[1] J. Dennis,[2] and J. Di Benedetto[3]

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To remotely sense the pathogen indicator *E. coli* in aquatic systems, we used laser-induced fluorescence imaging to detect the signature produced by the *in situ* conversion of the defined substrate medium ECMUG. The presence of the enzyme β-glucuronidase, indicated by the activity of the fluorogenic 4-methylumbelliferyl (4MU)-β-D-glucuronide (MUG), is specific to the presence of this organism. Substrate conversion was accomplished in a small stream impacted by non-point source wastewater inputs. Sample chambers slowly inoculated source water with 100 ml of ECMUG media in direct sunlight. Luminescence spectroscopy monitored conversion activity and detected liberation of the 4 MU fluorochrome in 2.5 hours. Detection by laser-induced fluorescence imaging followed at dusk and indicated bright blue emissions typical for converted media. This technique lays the foundation for active remote sensing of source water contamination.

KEY WORDS: Laser-induced fluorescence; photoluminescence; defined substrates; *E. coli*.

INTRODUCTION

Contamination by microbial pathogens is the greatest threat to potable water resources. Assays of pathogenic activity using bacteria such as fecal coliforms, enterococci, and *E. coli* provide an estimate of contamination of source [1,2]. Because the threat of pathogenic organisms increases as both the developed and undeveloped world stress water resources, some of the key challenges to monitoring and source tracking have been improving specificity, reducing assay times, reducing labor intensity, and improving representation of contaminant spatial heterogeneity. Standard assays, largely restricted to point measurement, fail to characterize the spatial heterogeniety typical for non-point source contaminants in affected surface waters [3]. Additionally, they may take 18–48 hours to detect coliforms, are unable to detect stressed microorganisms, and can require large numbers of cells to convert target media to produce a detectable fluorescence signal. Standard assays are therefore inadequate in emergencies and in watershed-level source tracking. Sidorowicz and Whitmore [4] suggested that future methods developed for the detection of indicator bacteria from source waters should take 6–8 hours or less and improve sensitivity, speed, non-destructive sampling, and low-cost analysis of large volumes of water. Recent advances for bacterial water quality assessments emphasize chromogenic techniques and enzyme substrate conversion and fluorescence [5,6]. These (optical-based) assays may have an advantage over polymerase chain reaction (PCR) techniques because conversion of substrate by viable organisms is required for fluorescence, thereby eliminating false positives introduced by inactive or dead organisms.

Passive multi-spectral and hyperspectral remote sensing have been effectively used in assays of waters affected by acid mine drainage using specific signatures for microbial $Fe(OH)_3$ precipitates [7–9]. *In situ* fluorogenic assays provide a basis to detect pathogenic microbial indicators in the field using active remote sensing. Our approach utilized: (i) direct inoculation of source

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waters suspected of contamination with defined substrate media; (ii) *in situ* conversion of the fluorogenic substrate by pathogen indicators, and (iii) detection of the fluorochrome emission by laser-induced fluorescence imaging (LIFI). Samplers containing fluorogenic media provided the defined substrate for target organisms. Converted media, generated by the specific pathogen, expresses a characteristic fluorescence emission signature under UV excitation.

METHODS

Defined Substrate Media

The enzyme β-glucuronidase is exclusive to *E. coli* within the Enterobacteriacea family and a few isolates of *Shigella* and *Salomonella*. To take advantage of this exclusivity, we adapted the fluorogenic coumarin 4-methylumbelliferyl-β-D-glucuronide (ECMUG) to detect β-glucuronidase for *in situ* testing using a shallow (3–5-cm deep), unnamed stream with suspected inputs of bacteria [10,11]. Black metal samplers, constructed to hold approximately 1.5 L of water and 100 ml of ECMUG were placed in various parts of the stream. The sampler design ensured that enzyme activity would occur under direct solar illumination by the absorption of heat (optimal substrate conversion occurs above 30°C).

Photoluminescence Spectroscopy and Cell Counts

To monitor enzyme activity, 3 ml of source water was extracted hourly from the samplers and analyzed using a JY Horiba FluoroMax3 luminescence spectrometer. The spectrometer recorded total photoluminescence excitation (300–600 nm) and emission (350–800 nm) spectra associated with the conversion of the ECMUG substrate and subsequent liberation of the fluorogenic 4MU molecule. Log phase growth of *E. coli* was monitored using the optical turbidity measurement method for 2-, 3-, 4-, 8-, 24-, and 48-hour sampling periods. Cell counts were plotted against luminescence intensity to obtain a relationship between converted substrate and cell biomass.

Laser-Induced Fluorescence Imaging

The LIFI used for this experiment incorporates a power supply and computer in a 35-lb backpack connected via a network of cables to an optical head. The system was designed for distances of 1–2 m and has an image field about 1 m^2 at that distance, maintaining good spatial resolution for objects of small size. The camera incorporates an intensified CCD using an RS-170 video format. Typically, shutter times of less than 50 ns are used to reject ambient light to a tolerable limit. The camera's shutter is timed to the light source, a tripled Nd: YAG laser. An image capture card and associated computer process a real-time false color of the camera output. Data images are displayed in two forms: red, green, blue composites or real-time. The real-time display is a single-band, gray-scale image. A background subtraction is performed on the data image. A third image is collected with enough exposure to capture the ambient background. This image is used to create a gray-scale underlay reference. The background-subtracted, laser-illuminated data are typically shown in false color for clarity. Signature sensitivity is therefore accomplished through intensity information shown in the real-time display, which has proven highly effective for detecting both time-dependent phosphorescent and fluorescent materials. The multi-band imagery produced by the system is collected using a filter wheel incorporating blue, green, and red filter sets. These data are used to create color composite images of fluorescence phenomena for analysis [12].

Image Capture and Processing

Laser excitation was at 355 nm, and three separate wavelength emissions were recorded on an intensified CCD camera at 450 nm, 550 nm, and 650 nm (blue, green, and red regions, respectively). To increase signal-to-noise levels, each emission image is an average of 6–7 individual frames collected by the CCD camera. Three band "color" images are then developed by spatially registering the blue (450 nm), green (550 nm), and red (650 nm) emission bands. These three band images are next cross-calibrated making use of three UV fluorescent calibration standard cubes placed in each scene before laser excitation.

RESULTS

Conversion of ECMUG occurred during the early afternoon, starting 2 hours after inoculation of the samplers. Initially the media possessed excitation maxima at 400 nm and an emission maxima at 470 nm. This signature is largely due to the glucose substrate to which it is attached and the still-bound 4MU molecule (Fig. 1). Afternoon air temperatures exceeded 30°C as the 4MU fluorochrome was liberated. Upon synthesis by *E. coli*, the oxygen bond with glucose is broken and can still be

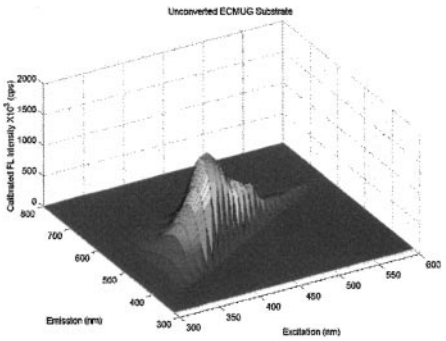

Fig. 1. Excitation and emission plot of unconverted ECMUG photoluminescence.

observed, but in a diminished state of intensity (Fig. 2). The wavelengths of excitation and emission for 4MU were measured at 350 nm and 445 nm, respectively. The fluorochrome remained stable until detection could be performed at dusk using the LIFI. In fact, over a 6-hour period, the samplers averaged 33°C and the water averaged 23°C. The affected stream also maintained a pH of 6.5, which is close to optimal for the performance of the ECMUG substrate.

Remote sensing of the stream and sample chambers by LIFI recorded bright blue emissions against dark backgrounds in three spectral bands (Fig. 3, 4). The emissions were consistent with converted ECMUG product and expression of 4MU. The resulting composite images were normalized to laboratory sample response, making use of the fluorescence spectral signatures measured for the cube standards. The spectral emission signatures were extracted from the imagery and are presented in Fig. 5. These signatures represent backgrounds and two sample intervals. Concurrent luminescence measurements are presented in Fig. 6. Measurements for source water from the stream and samplers resulted in emission maxima at 445 nm for 8-hour and 48-hour readings. Comparative measurements for ECMUG controls and stream water

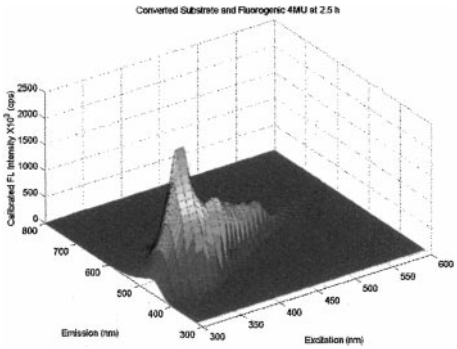

Fig. 2. Converted 4MU product after 2.5 hours.

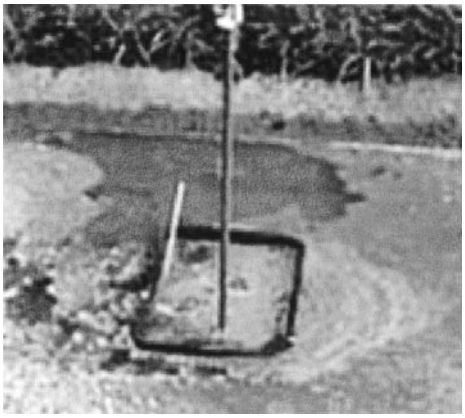

Fig. 3. In-stream sampler for ECMUG.

outside the samplers exhibited dark, flat background signatures. These spectra show the clear separation of the detectable 4MU signature against dark backgrounds. Cell numbers obtained from the samplers at specific time intervals and their relationship to fluorescence intensity show a linear relationship ($r^2 = 0.85$) (Fig. 7). This relationship is observed with the brightest intensities being associated with the highest cell number concentrations.

DISCUSSION

As a means to assist in source tracking, detection, and monitoring, we have demonstrated conversion of ECMUG by *E. coli* in 2.5 hours *in situ* and recorded the fluorogenic response both with non-imaging and imaging fluorescence spectroscopy techniques. An advantage of this technique will be improved spatial representation afforded by the distribution of target samplers in large watershed analyses. In fact, the LIFI system has been used

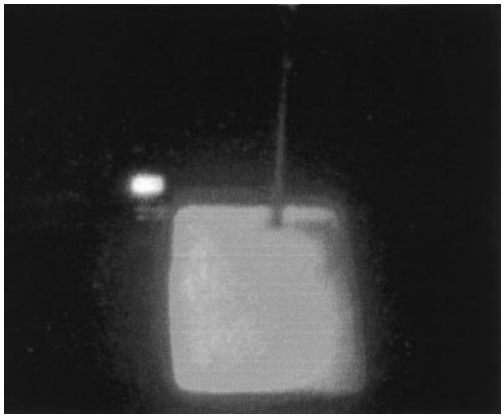

Fig. 4. LIFI imagery acquired for the same sampler.

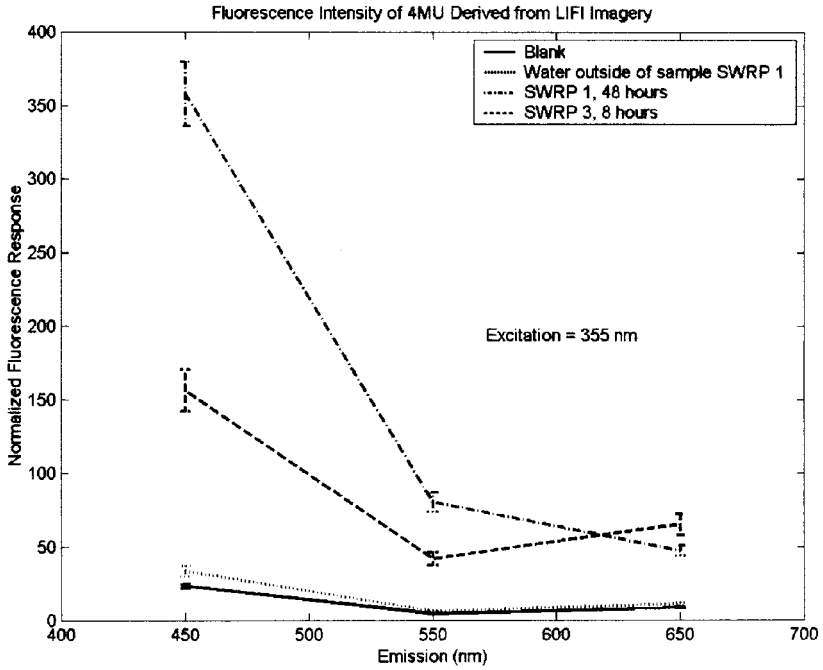

Fig. 5. Intensities derived from multi-band LIFI emission data showing converted and unconverted 4MU and background controls (blank and water outside samplers).

to collect data on vegetation fluorescence at distances of over 25 feet. In addition to expanded synoptic viewing, the simultaneous optical detection of *E. coli* and fecal coliforms would expand source water tracking for pathogen indicators. Combination detection strategies using fluorogenic and/or chromogenic media would improve

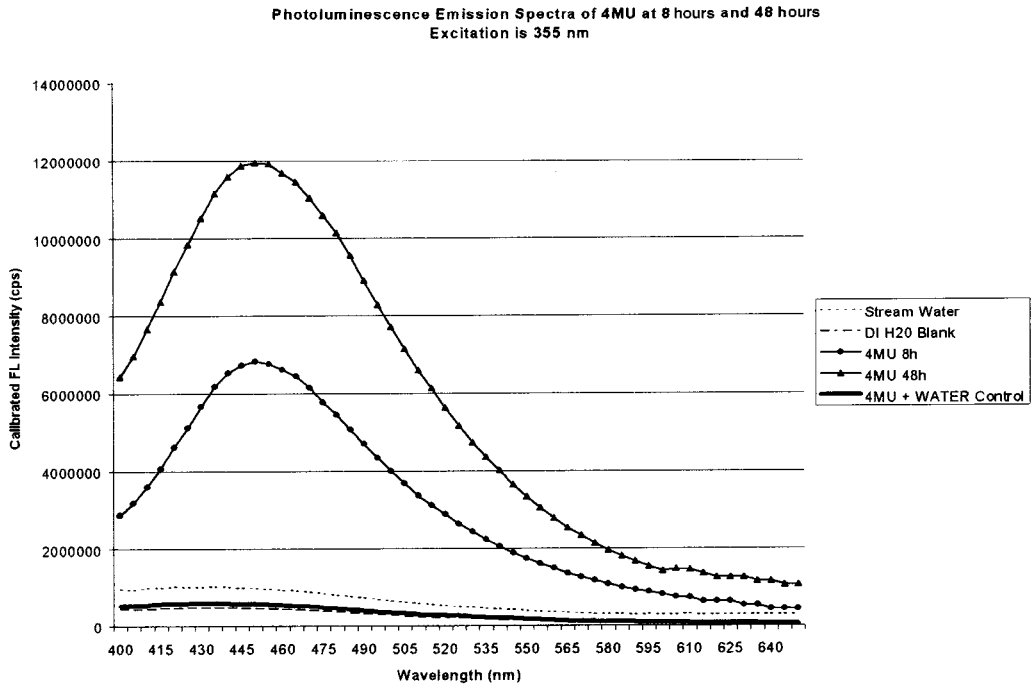

Fig. 6. Comparative 4MU emission at 8 hours and 48 hours by total luminescence spectroscopy.

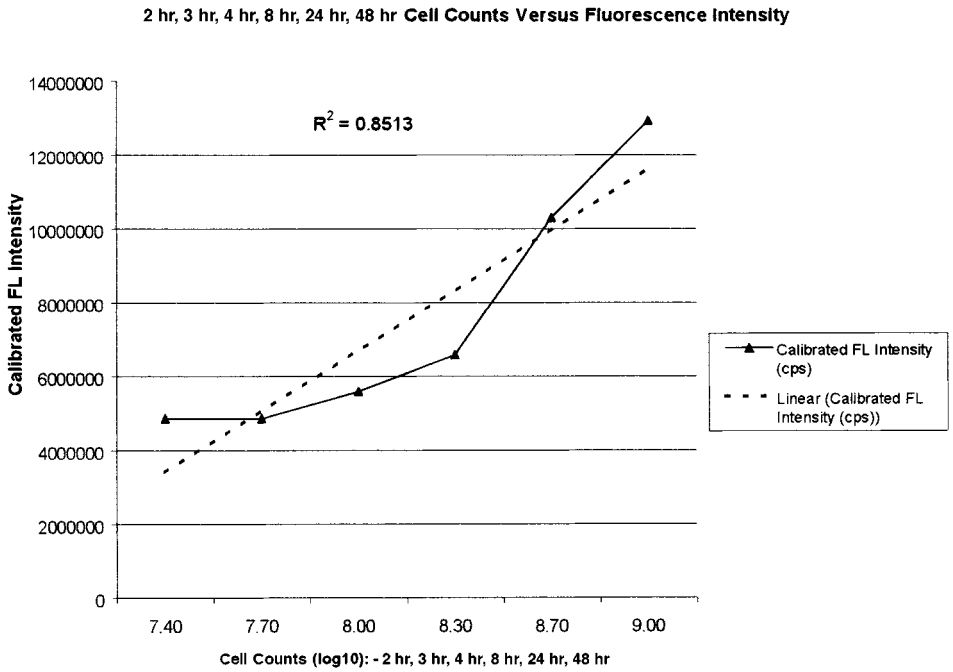

Fig. 7. Relationships between cell counts and fluorescence intensity for six sample intervals.

detection of β-galactosidase–positive organisms. These assays may include ECMUG (using fluorogenic 4MU) and chromogenic O-nitrophenyl-β-D-galactopyranoside (ONPG) or *p*-nitrophenyl-β-D-galactopyranoside (PNPG) [13,14]. Further development of this remote sensing technique should provide an additional analytical capability beyond current point measurement techniques for bacterial source water tracking and holistic watershed management.

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