

IN SITU DETECTION OF PATHOGEN INDICATORS USING LASER-INDUCED FLUORESCENCE

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INTRODUCTION

Background

Mandated by the Rivers and Harbors Act and Clean Water Act, the Corps of Engineers is responsible for the maintenance and monitoring of numerous reservoirs and river miles. Contamination of these resources is a real concern whether by anthropogenic or anthropocentric means. Of these means, the deliberate contamination of potable water resources by microbial pathogens is one of the greatest potential threats. The immense scale of many water resources and the heterogeneity of contamination make standard assays inadequate in emergencies and particularly watershed-based source tracking. There is a critical need for probe technologies that can offer a first alert using complimentary remote sensing to monitor water resources at the landscape level.

The adaptation of rapid chromogenic and fluorogenic assays to detect and track pathogen indicators in situ at synoptic scales is beginning to emerge. Recent advances for bacterial water quality monitoring take advantage of these techniques offering high quantum efficiency fluorophores and chromophores coupled with substrate media or imprinted into polymeric designs. Inoculated probes possessing fluorophores having high quantum efficiency can serve as sentinels that, once stimulated, are available for interrogation by remote sensing in the optical spectrum (Anderson et al., 2002). The distribution of such probes strategically throughout a watershed or reservoir could effectively provide data on potential threats. We report here the results of tests using defined substrates and adapted probes for the detection of pathogen indicators. This approach provides an example of a laboratory technology adapted to watershed-level sensing of contaminants.

METHODS

Defined Substrate Media

The enzyme β – glucuronidase is exclusive to *E. coli* within the Enterobacteriaceae 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 to 5 cm deep), unnamed stream with suspected inputs of bacteria (Van Pouke and Nelis, 1995). Four (Spectra Por) dialyzers possessing permeable (500 MW) membranes were inoculated with 25 ml of ECMUG and placed in various parts of the stream. The design of the dialyzer ensured that enzyme activity would occur under direct solar illumination due to the absorption of heat (optimal substrate conversion occurs above 30° C for enteric organisms).

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 48h 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-pound backpack connected via a network of cables to an optical head. The system was designed for distances of 1 - 2 meters and has an image field about 1 m² at that distance, maintaining good spatial resolution for objects of small size. The camera incorporates an intensified CCD using an RS-170 video format. The camera's shutter is timed to the light (excitation) source, a tripled Nd:YAG laser. A frame grabber 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. The multiband 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 (Fischer et al., 2000).

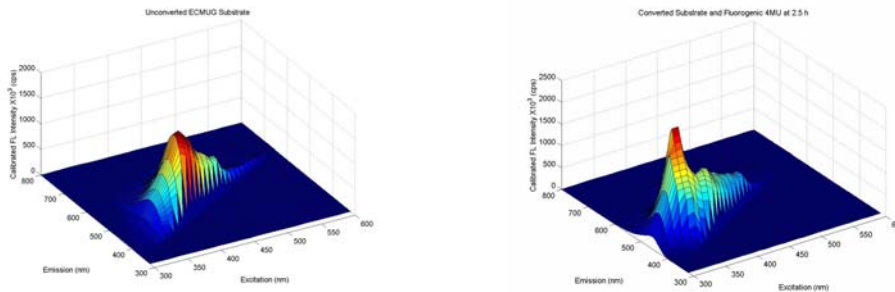
RESULTS

Conversion of ECMUG occurred during the early afternoon starting two 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 (Figure 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 observed, but in a diminished state of intensity (Figure 2).

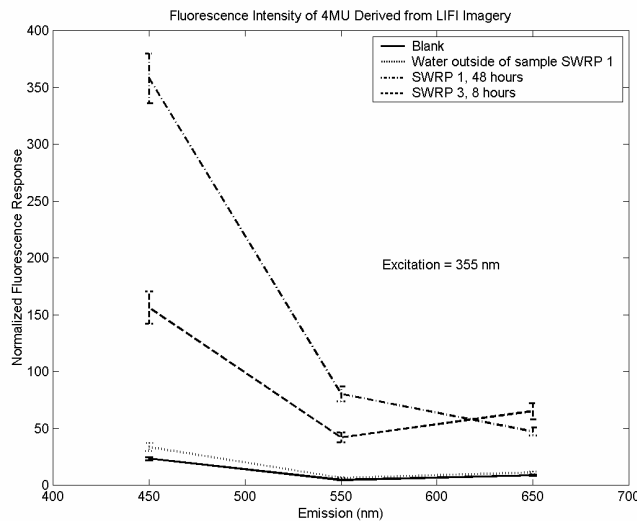
The wavelengths of excitation and emission for 4MU were measured at 350 nm and 445 nm, respectively. The fluorochrome remained stable until detection could be performed at dusk using the LIFI. In fact, over a 6h period, the samplers averaged 33° C while the water averaged 23° C.

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 dialyzers by LIFI recorded bright blue emissions against dark backgrounds in three spectral bands (Figures 3). 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 fluorescence spectral signatures measured for cube standards. The spectral emission signatures were extracted from the imagery and are presented in Figure 4. These signatures represent backgrounds and two sample intervals. Concurrent luminescence measurements are presented in Figure 5. Measurements for source water from the stream and dialyzers resulted in emission maxima at 445 nm for 8 h and 48 h readings.



Figures 1 and 2. Excitation-emission matrices for unconverted ECMUG (left) and converted ECMUG (right).



Figures 3 and 4. Fluorescence emission image of dialyzer probe under laser excitation (355 nm) (left) and spectral emissions derived from the spectral imagery.

Comparative measurements for ECMUG controls and stream water 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 ($r^2 = 0.85$) (Figure 6). This relationship is observed with the brightest intensities being associated with the highest cell number concentrations.

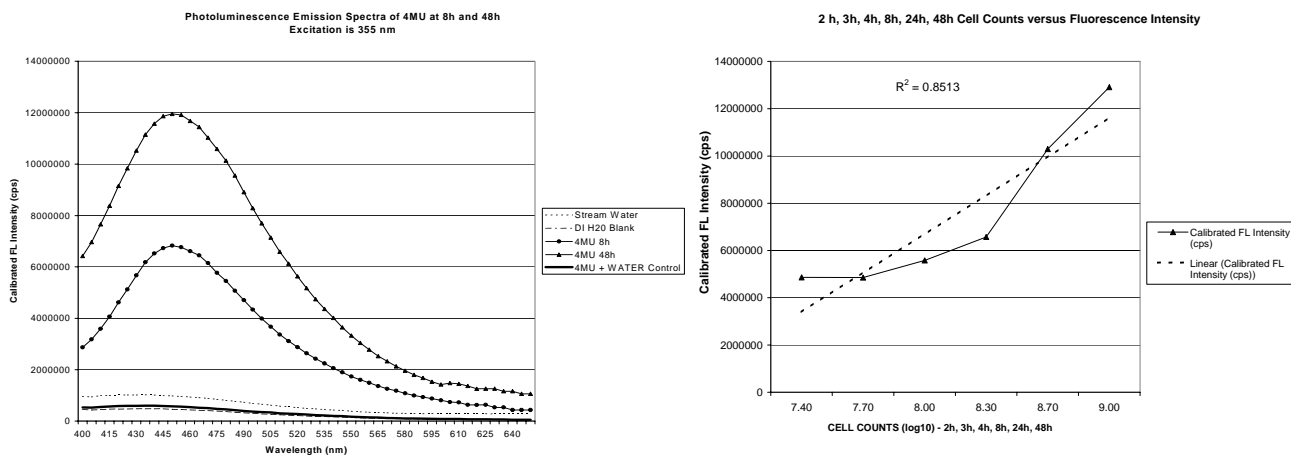


Figure 5 and 6. Concurrent luminescence measurements for substrate conversion over time (left) and cell counts for sampled time points (right).

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 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 detection of β - galactosidase positive organisms. These assays may include ECMUG (using fluorogenic 4MU) as well as chromogenic o-nitrophenyl- β -D - galactopyranoside (ONPG) or p-nitrophenyl- β -D galactopyranoside (PNPG) (Covert, 1989; Clark and El-Shaarawi, 1993). 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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