

**Research Article** 

# Continuous Real-time Detection of Microbial Contamination in Water using Intrinsic Fluorescence

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#### Abstract

With the rapid increase in global population, geographically changing drought conditions and the ensuing potential water shortage, water quality has become a major concern. In some extreme cases, such as Arizona, the population may have to switch and use recycled toilet water for potable use in the near future. However, our current monitoring methods for drinking water do not provide fast and reliable results to deal with these challenges. By using intrinsic fluorescence, microbial contamination in water can be monitored in real-time, continuously, without sample collection or contact and at very low concentration. The detection limit of the instrument designed specifically for this purpose and reported here is ~50 bacterial cells/L. By monitoring the fluorescence of cellular components of microorganisms, their concentrations and metabolic states (live, dead, spores) can be determined. These fluorophores include reduced pyridine nucleotides (RPNs), flavins, and cytochromes to distinguish live cells; cytochromes for dead cells; and calcium dipicolinic acid (DPA) for spores. By using this method, a wide range of microorganisms such as bacteria, protozoa, amoebae, fungi and other microorganisms can be detected.

**Keywords:** Intrinsic fluorescence of microbes; Real-time monitoring; Microbe detection; Water monitoring

**Abbreviations:** DPA: Dipicolinic Acid; RPN: Reduced Pyridine Nucleotides; LED: Light Emitting Diode; PMT: Photomultiplier Tube; UV: Ultraviolet Light

## Introduction

Clean water is crucial for human health. With the growth of the global population, the scarcity of our most precious natural resource, clean water has become an issue. However, this importance is at a very different magnitude and scale in different parts of the world. In the southwestern part of the United States, this problem is imminent. Over 30 million people in California, Arizona and Nevada depend on the Colorado River for their water supply [1]. However, the lower Colorado River basin is facing a water shortage [2,3]. One of the solutions to this problem is to treat and reuse water of impaired quality (e.g., wastewater for potable use). One of the obstacles to direct potable reuse of wastewater is the need for rapid assessment of the microbial contamination of the treated water. Domestic wastewater contains a wide variety of waterborne pathogens (viruses, bacteria, and protozoa). These pathogens include Vibrio cholerae, Shigella dysenteriae, Escherichia coli 0517:H7, Salmonella typhi, Hepatitis A virus, Cryptosporidium parvum oocysts, and Giardia lamblia cysts [4]. Most analytic methods for the assessment of these pathogens require 18 hrs or longer and none can be used continuously. More rapid methods for microbial load and pathogen detection are needed to ensure the quality of the treated water if it is to be used for potable purposes.

While there is a wide range of methods that have been developed to detect microbes in water, they all suffer from a number of limitations. One of the most rapid methods available for detection of microbial contamination in drinking water is PCR (polymerase chain reaction). PCR suffers from a number of limitations: 1) does not determine viability of the organism, 2) interference from solutes in the water, 3) only small volumes can be assayed (10 to 100 microliters), 4) specific primers required for each organism or groups of organisms, and 5) recovery and purification of DNA from low numbers of microorganisms is problematic [5-7]. Another limitation is the sampling method.

Currently, we do a blind sampling or subsample from high or flowing volumes, which may not be representative of the water contamination.

#### Intrinsic fluorescence

The use of intrinsic fluorescence to study cellular metabolic components has been explored since the 1950s [8-10]. Remote, realtime detection and quantification of live cells, dead cells, and spores in fluids (air, water) and on surfaces and sub-surfaces (like those of food, surgical theaters, soils, rock, ice) at low concentrations has been demonstrated [11-13]. Some microbes have demonstrated specific fluorescence signatures that depend on their environment or growth conditions [14], e.g., Escherichia coli, Enterococcus faecalis and Staphylococcus aureus in otitis media and contamination source tracking of several Pseudomonas spp. in dairy products [15]. Our approach makes use of the following metabolic intrinsic fluorescence signatures to detect the presence of microbes that are present under physiological conditions: reduced pyridine nucleotides (RPNs), flavins, cytochromes, calcium dipicolinic acid (DPA) and others [11-13,15-17]. Metabolic signals, which are indicators of live cells, fluoresce in the bluegreen region with 340-360 nm excitations. The fluorescence emitted is directly proportional to the concentration of metabolites and thus to the average number of live cells [11]. Flavins and protoporphyrin IX, which also fluoresce in the red region upon 565-595 nm excitations, are found in both live and dead cells. Cytochromes fluoresce in the near IR upon 610-640 nm excitations. Excitations in the deep UV (e.g., 250-300

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Received February 27, 2013; Accepted March 14, 2013; Published March 16, 2013

**Citation:** Kilungo AP, Carlton-Carew N, Powers LS (2013) Continuous Real-time Detection of Microbial Contamination in Water using Intrinsic Fluorescence. J Biosens Bioelectron S12: 002. doi:10.4172/2155-6210.S12-002

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nm) produce fluorescence from a variety of biological molecules (e.g., aromatic amino acids), which are also found in dust, pollen, smog, and other environmental substances. While similar instruments using this approach have been used to demonstrate detection and quantification of microorganisms on surfaces [13], this paper will discuss the proof-of-concept for using this technology to continuously monitor drinking water in real-time as a public health measure.

Intrinsic fluorescence technology has several advantages over other rapid methods. It is less costly than PCR, no sample contact is required and water monitoring can be done continuously and in real-time with high sensitivity. Based on our initial experiments with a prototype instrument reported here, the detection limit for viable bacteria is ~50 cells/L. While the method does not identify the microbes, it immediately identifies changes in total microbial load and hence the water quality caused by environmental conditions or failure in the water distribution system. This system can also direct where and when to sample when such events occur. There are technologies that can be used simultaneously with the intrinsic fluorescence technology for identification of microorganisms of interest. One such method that has been demonstrated is the use of cell-capture technology using surfacebound ligands [13,18].

# Methods

# Intrinsic fluorescence technology

The fluid prototype instrument, shown in Figures 1 and 2 is similar







in design to those reported previously [11-13] for surfaces except that the optical arrangement places the excitation light at right angles to the fluorescence collection for optimum signal-to-noise ratio. Light emitting diodes (LEDs) are used for the excitations and the intensity of each is modulated at a different frequency so that only a single photomultiplier tube is needed for fluorescence detection. Fourier transformation of the data isolates each component, separating the intrinsic fluorescence contributions resulting from the excitations. It is important to note that viable cells, dead cells, spores, and even media can be distinguished by these methods [11].

This instrument for fluids has a total of 10 LEDs-4 amber, 4 red and 2 UV (Figure 1). The number and position of these LEDs with respect to the photomultiplier tube (PMT) are designed to give similar intensities of each fluorescence emission at the photomultiplier tube for a sample containing an equal amount of live cells and dead cells. Red and amber LEDs are positioned closer to the PMT since the absorption of their light in water is considerably higher than for UV. As sample flows through the instrument, the fluorescence emission is measured by the PMT, which uses an excitation rejection filter [13] to discriminate the fluorescence emission from the excitation light produced by the LEDs. The output of the photomultiplier tube is sent to the electronics, which analyzes the data as described above and communicates it to the GUI [13].

## **Methods and Measurements**

#### Preparation of live bacteria

*Bacillus thuringiensis* (ATCC10792) was used to demonstrate realtime continuous detection of live and dead bacteria, as well as spores. For the preparation of live bacteria, bacteria were grown in Luria-Bertani broth (LB) (Sigma-Aldrich, MO) and sampled at the mid-log growth phase. Bacteria were centrifuged at 10,000 rpm for 5 minutes to obtain a pellet and subsequently washed three times in phosphate buffered saline solution (PBS) to remove any residual growth medium. To determine the total bacterial count, bacterial cells were suspended in PBS and counted using the Petroff-Hausser method [19] (Hausser Scientific, PA) as shown in Figure 3d. 1:10 serial dilutions were performed to obtain different concentrations of bacteria. Bacterial measurements were taken by sampling 1 ml from each dilution into a water circulator containing 6 L of deionized water (DI water). A total of 25 measurements were averaged to obtain a single measurement for each dilution.

## Preparation of dead bacteria

For the detection of dead bacteria, the same protocol for growth and sample preparation was used. To kill the bacteria, bacteria were treated with 10% bleach for 10 minutes after the three washes. Counts were performed as described above. However, instead of measuring 1 ml aliquots, 0.2 ml aliquots were dispensed into a water circulator. The measurements were performed as described above for live bacteria measurements. To confirm dead cells, a 1:10 serial dilution was performed and bacteria were plated in LB agar plates overnight at 37°C.

## Spore preparation

Bacillus thuringiensis was inoculated into  $100 \ \mu$ l of Difco sporulation medium and supplements (DSM+S), and this was spread on a DSM+S plate. The plate was incubated for 6 hours at 37°C. 5 ml of DSM+S was added to a light lawn, which was then scraped. Absorbance was measured at 600 nm to determine cell density, followed by inoculation of 0.1 ml into a 1000 ml flask and incubation overnight at 37°C at 150 RPM. Spores were collected by centrifuging and re-suspending in

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cold distilled water, and by washing 6 times using cold distilled water. Samples were stored in PBS at 4°C. Crystal violet was used to stain the spores. Under the microscope, spores appeared to have a halo, as shown in Figure 5d and were also counted using the Petroff-Hausser method described above [19]. The measurements were performed in the same manner as the dead cell measurements, except the water temperature in the circulator was kept at 4°C. In order to determine spore viability, spores were plated in triplicates after a 1:10 serial dilution and incubated overnight in LB agar plates at 37°C.

#### Results

The results of the measurement of the intrinsic fluorescence as a function of concentration are shown below. Note that the excitation wavelength is shown in blue and DI water in red as annotated on the right of each graph.  $R^2$  is the square of the Pearson product moment correlation coefficient and is a measure of the goodness of the fitted line to the data. One standard deviation is indicated by the associated error bar for each point.

UV excitation, which is used primarily to monitor the increase in total microbial load, showed a linear increase in the log of the intrinsic fluorescence with the log of the live bacteria concentration (Figure 3a). The detection limit for live bacteria is ~50cells/L. There was no change in the intrinsic fluorescence produced by red excitation within the error with the addition of live bacteria (Figure 3b) because this channel detects only spores and dead bacteria (Table 1). Amber excitation produces fluorescence from both live and dead cells (Table 1) and the results (Figure 3c) show a small increase in intrinsic fluorescence with the increase in live bacteria concentration (end points are outside 3 standard deviations).









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Excitation/Emission	Contribution
UV (Ex 365 nm/Em 440 nm)	0.7 [live cells]+0.15 [dead cells] 0.15 [spores]
amber (Ex 590 nm/Em 675 nm)	0.5 [live cells]+0.5 [dead cells]
red (Ex 635 nm/Em 770 nm)	0.5 [dead cells]+0.5 [spores]

Table 1: Sources of contributions to intrinsic fluorescence signatures [13].

The detection limit for dead bacteria was much higher compared to that of live bacteria. This is likely due to fact that the harsh treatment used to kill the cells has damaged the fluorescent molecules and that the cells are not intact. No fluorescence below a concentration of ~10<sup>8</sup> dead bacteria/L was observed (Figures 4a, b and c). The UV fluorescence contains ~15% dead cells (Table 1) while red and amber contain 50% dead cells. The observed changes in fluorescence with all three excitations are ~3 standard deviations of each other indicating little change.

Intrinsic fluorescence contribution from spores should be detected by the red excitation (50%), but Figure 5b shows no change larger than a single standard deviation. Some change may be observed with UV excitation (only 15% of which is due to spores while 70% is due to live cells, (Table 1)) as the first and final points (Figure 5a) are greater than 3 standard deviations. In the amber excitation (50% live), the fluorescence of the beginning and end points in Figure 5c is is greater than 3 standard deviations. Taken together, these would support a conclusion that the spores had largely begun to germinate before and/or during the measurements.

#### Discussion

The results for live and dead bacteria measurements demonstrate the capability of the prototype instrument to detect live and dead cells in flowing water in real-time with a detection limit of ~50 bacterial





(DI reading 0, 1.95)







cells/L. There is a significant and reproducible increase in the UV excited intrinsic fluorescence (70% of which is due to live cells) with the increase in the concentration of live bacteria. A small increase is observed in the amber-excited fluorescence (50% of which is due to live cells). Killed bacteria may have had damaged fluorophores as little fluorescence change within the error is observed with concentration. While changes outside the error are observed in the fluorescence for spores, they are observed for the UV excitation (15% of which is due to spores, 70% to live cells) and not for the red excited fluorescence (50% of which is due to spores). This suggests that the spores had largely begun to germinate before and/or during the measurements.

There are two main reasons why the detection of spores as well as of dead bacteria in water may be difficult. First, the absorption coefficient











of red light in pure, particle-free water is ~1000 times higher than the absorption coefficient of UV/blue light [20]. This means that most of the red light used for excitation and emitted as intrinsic fluorescence is absorbed by the water. Second, spores change morphology slowly compared to their metabolism. In our experiments in cold water (4°C), we have observed spores to change metabolism to resemble that of a vegetative cell even though the morphology still superficially resembled that of a spore.

Even though amber excitation produces fluorescence from both live and dead bacteria (50% each), this fluorescence is in the red and reduced by a factor >100 by water absorption. While the intensities Citation: Kilungo AP, Carlton-Carew N, Powers LS (2013) Continuous Real-time Detection of Microbial Contamination in Water using Intrinsic Fluorescence. J Biosens Bioelectron S12: 002. doi:10.4172/2155-6210.S12-002

Figure 5d: *Bacillus thuringiensis* spores stained with crystal violet dye and viewed with a light microscope.

of the amber and red LEDs are significantly higher than that of the UV LEDs, this is not enough to compensate for the effects of water absorption in this prototype.

The use of intrinsic fluorescence has been demonstrated for the presence of live bacteria in water with a detection limit of ~50 cells/L. We were able to quantify the total microbial load and differentiate between dead and live bacteria for water-monitoring purposes. This technology, used together with direct microbial cell capture onto a ligand-coated surface may allow us to monitor our drinking water along the distribution water system continuously and determine the presence of pathogenic microbes [11,13,18] in real-time.

#### Acknowledgments

A. Kilungo was supported in part by the Sloan Foundation and NSF BioME. The authors thank Dr. Walther Ellis for his critical discussions and advice on this project; Dr. Charles Gerba for the help and support and Miriam Eaton for her help with laboratory procedures. Thanks to David L. William for sharing his expertise in spore preparation; and Ambie Mobula for her general assistance.

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J Biosens Bioelectron

ISSN:2155-6210 JBSBE an open access journal

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