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Evaluation of the Pathogen Detect® System and Anthracene-Based Enzyme Substrates for the Detection and Differentiation of *E. coli* and Total Coliforms in Water Samples

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Abstract

Indirect detection of *Escherichia coli* and total coliforms can be based on the enzymatic activities of *β*-glucuronidase (*β-glu*) and *β*-galactosidase (*β-gal*). These enzymes utilize the substrates *anthracene-β-d-glucuronide* and *pyrene d-galactopyranoside*, respectively. Substrate cleavage by the enzyme releases the soluble fluorescent molecules 2-*hydroxyanthracene* and 1-*hydroxypyrene*, which can then be detected by a fluorometer. The Pathogen Detect® system is an automated portable unit that can measure fluorescent enzyme products. In this report, we investigated the utility of the Pathogen Detect® system for potential automation of water quality monitoring. The PDS unit has the ability to detect *E. coli*, mean 14.7 h at a standard deviation of 1.5, when the sample mean is 9.1 cells in 100 mL with a standard deviation of 12.6. Similarly, total coliforms may be detected at mean 14.7 h with a standard deviation of 1.4 when the sample mean is 59.6 cells in 100 mL, with a standard deviation of 144.5. The PDS unit has the ability to detect single cells of either total coliforms or *E. coli* in 100 mL water sample within 18 hours. Turbidity and color of water samples have no impact on the detection of *E. coli* and total coliforms.

Keywords

***Escherichia coli*, Total Coliforms, Enzyme, Substrate, Water Quality**

1. Introduction

Coliform bacteria are characterized, based on their origin, as either total or thermo tolerant coliform. The total

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group includes fecal coliform bacteria such as *Escherichia coli*, as well as other types of coliform bacteria that are naturally found in the soil. Fecal coliform bacteria exist in the intestines of warm blooded animals and humans, and are found in their waste. *Escherichia coli* is a member of the Enterobacteriaceae, and is described as a facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacterium that elaborates *β*-glucuronidase activity. Members of the total coliforms (TC) group elaborate *β*-galactosidase activity.

Total coliforms do not necessarily indicate water contamination by fecal waste, however the presence or absence of these bacteria in drinking water is often used to determine whether water disinfection is working properly or not [1]. The presence of thermo-tolerant coliform in drinking water indicates contamination with human sewage or animal droppings which could contain other bacteria, viruses, or other disease causing microorganisms. Therefore, coliform bacteria are considered indicator organisms in the drinking water industry and their presence warns of the potential presence of disease causing microorganisms to take precautionary action for drinking water treatment facilities and recreational water.

Currently, membrane filtration (MF) is commonly used to detect *E. coli* and total coliforms in drinking water using presence-absence (P-A) test with DC differential coliform (DC) solid media for culturing. However this method needs qualified staff, expensive instruments and lab (Sterile environment) and incubation of minimum 24 hours at 37˚C. High turbidity can also interfere with the MF method. The retention of particulate matter by the filter can interfere with colony development and the production of surface sheens/fluorescence by presumptive coliforms or *E. coli*. Similarly, concentrations of HPC bacteria in excess of 500 colony-forming units (CFU) per milliliter can interfere with coliform recovery when using presumptive coliform media [2]-[4]. The MF method also is not conducive to field testing and remote monitoring. It is also necessary to reduce detection time to allow for timely reaction when such coliforms are detected. Remote monitoring detection system will have a huge cost savings for smaller municipalities. More recent developments in the area of coliform detection involve the utilization of the polymerase chain reaction (PCR) technique [5] [6]. These methods are reliable when the technique actually works. The PCR method can be sensitive but not more so than other enzyme based detection methods. The PCR method also has the draw-back of being too sensitive to dissolved and particulate matter in the water samples. The same material can contribute to water turbidity.

The defined substrate technology is based on the principle that only the target microbes (TC and *E. coli*) are selected for and no substrates are provided for other bacteria. During the process of substrate utilization, a chromophore or a fluorophore is released from a defined substrate and the release of such indicators highlights the presence of the target microorganisms. The defined substrate method was basically constituted as a presence/absence test [7].

The enzyme *β*-D-glucuronidase (GUR) catalyzes the hydrolysis of *β*-D-glucopyranosiduronic derivatives into their corresponding glycans and D-glucuronic acid. The bacterial *β*-D-glucuronidase enzyme was reported to be mostly elaborated by *E. coli* [8]. Other studies however have shown that out of 35 samples, a mean of 34% and a median of 15% were found to be GUR negative in lauryl sulfate tryptose broth with 4-methylumbelli-feryl-beta-D-glucuronide as a substrate. The *E. coli* from three samples were temperature dependent for GUR production: very weakly positive at 37˚C but strongly positive at 44.5˚C [9]. An earlier report has shown great promise for the recognition and enumeration of *E. coli* where 70% of environmental isolates tested positive for GUR production [8]. These results reflect differences between fecal and clinical *E. coli* populations, of diversity in GUR regulation and expression in natural populations of *E. coli*, and of the need for caution in using GUR for the detection of fecal *E. coli*.

The enzyme *β*-glucuronidase hydrolyzes *β*-glucuronides [10]. The enzyme produced by *E. coli* cleaves the substrateanthracene-*β*-d-glucuronide. Similarly, *β*-galactosidase produced by total coliforms cleaves the substrate pyrene *β*-d-galactopyranoside resulting in the fluorescent products2-hydroxyanthraceneand 1-hydroxypyrene, respectively. These substrates thus allow the development of sensitive fluorescent methods for the indirect detection of these bacteria. Physical and chemical parameters such as aluminum, fluoride, pH, color, and turbidity in water may affect the growth of *E. coli* and total coliforms and the activity or production of their enzymes. Therefore it is necessary to investigate further the effects of such physiochemical factors on the reliability of such enzyme based detection methods (EBDM). In this report, we evaluated the reliability of an automated EBDM using the Pathogen Detect© system (PDS) under the influence of these physiochemical factors.

2. Materials and Methods

2.1. Materials

Enzymes substrates, anthracene-*β*-D-glucuronide and pyrene-*β*-D-galactopyranoside, were synthesized in our laboratory as described elsewhere (PCT International Publication number WO 2004/027084 A1). Bacterial growth medium Luria-Bertani (LB) and all other chemicals, unless indicated otherwise, were purchased from Sigma (St. Louis, Missouri). Both *E. coli* ATCC 11303 and *Klebsiella pneumonia* (representing total coliforms) were obtained from American Type Culture Collection (ATCC). PDS unit hardware obtained from Pathogen Detection Inc., Kingston, Ontario and modified for the experiments as needed.

2.2. Preparation of Enzyme Substrate Anthracene-*β*-D-Glucuronide

This substrate (**Figure 1**) was prepared essentially as described by Brown *et al*. [11] with some modification. Briefly, a precipitate is produced by mixing 2-hydroxyanthracene (100 mL, 0.515 mmole) and dry pyridine (1.0 mg), chlorotrimethylsilane (120 µL, 0.845 mmole) in a 200 mL beaker. The precipitate is then stirred overnight (12 - 14 h) at ambient temperature. Then 3 mL of toluene is added and the mixture filtered through Whatman number 1 filter paper. The precipitate is further washed with toluene and then separated through filtration a second time. The precipitate is now an orange crystalline solid. An additional wash with toluene (5 mL) was used to remove trace pyridine hydrochloride. The Solvent is left to evaporate at ambient temperature and then vacuum dried. The dried precipitate remaining was 140 mg of trimethylsilyl ether. To this precipitate 2- hydroxyanthracene trimethylsilyl ether 10 (191 mg, 0.717 mmole) and imidate 9 (479 mg, 1.0 mmole) were added in a 25 mL pear shaped Pyrex flask and flushed with nitrogen. Then powdered activated molecular sieves (1.0 g) were added and flushed again with nitrogen and cooled by using dry ice. A Dry dichloromethane (8.5 mL) was added into the solution and stirred for 30 min on dry ice. Boron trifluoride etherate (85 µL, 0.67 mmole) was added and the solution turned into a grey-green color. The solution was then stirred overnight (12 - 14 h) in a nitrogen saturated chamber before being allowed to settle at ambient temperature. Methanol (2 mL) is then added and stirred for 10 min before the precipitate was filtered and washed with dichloromethane. The filtrate is

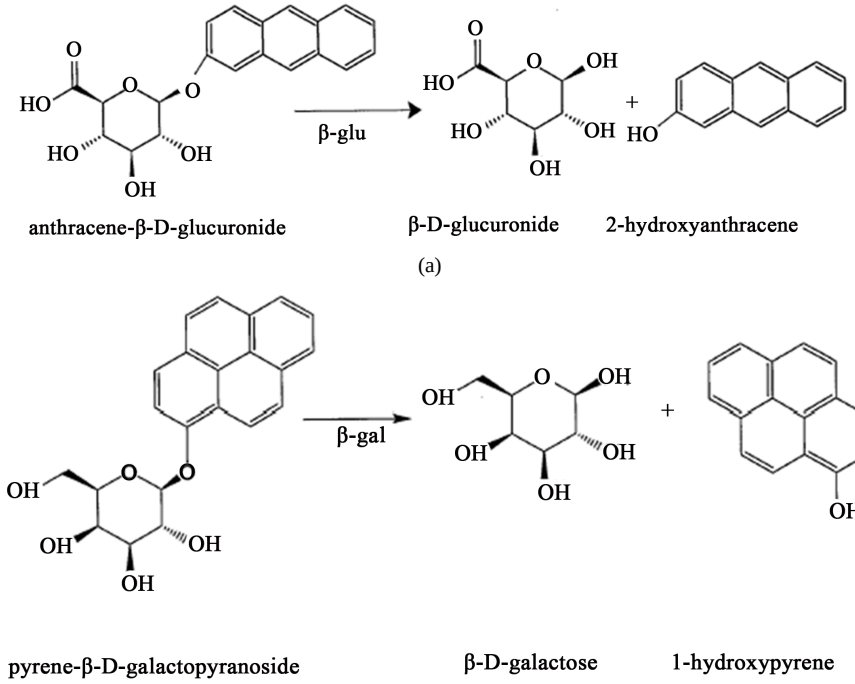

Figure 1. Activity of the enzymes (a) glucuronidase (*β*-glu) on the fluorogenic substrate anthracene-*β*-D-glucuronide and (b) galactosidase (*β*-gal) on the fluorogenic substrate pyrene-*β*-D-galactopyranoside (adopted from Brown *et al*., 2004).

allowed to air dry to produce a residue (brown colored foam). The residue is then dissolved in dichloromethane, treated with silica gel (1 g) and re-evaporated. The residue was then placed on the top of a silica gel column (20 g) before being dissolved in dichloromethane. This solution evaporated and formed a yellow solid (152 mg); then crystallized by using ethyl acetate (2.0 mL). The final result was a formation of 69 mg of a white solid (yield is 19%) [11].

2.3. Preparation of Enzyme Substrate Pyrene-*β*-D-Galactopyranoside

Pyrene-*β*-D-galactopyranoside is prepared by mixing and stirring Tetra-O-acetyl-1-bromo-1-deoxygalactose (205 mg, 0.5 mmole), 2-hydroxyanthracene (74 mg, 0.381 mmole), tetrabutylammonium hydrogen sulfate (100 mg, 0.294 mmole), dichloromethane (2.5 mL) and sodium hydroxide (0.5 mL, 0.75 mmole) vigorously at room temperature (21˚C) overnight (12 - 14 h). The solution is then diluted with ethyl acetate (20 mL), washed with water (3 × 10 mL), brine (10 mL), and dried with sodium sulfate two times. The resultant solvent is looks like a brown gum (249 mg). It was then chromatographed on silica gel using dichloromethane, and then eluted with 39:1, dichloromethane ethyl acetate to form golden foam (129 mg, 65% yield). The foam is then altered by preparing Tetra-O-acetyl-2-anthracenyl-*β*-D-galactopyranoside (30 mL, 0.057 mmole) in methanol (0.6 mL) and stirred at room temperature (21˚C) before sodium methoxide (in methanol) (100 uL, 0.0052 mmole) is added. After about 20 min a white precipitate will start to form. The solution was then stirred overnight and then filtered. It was then washed with methanol and vacuumed dried. It resulted in 14.8 mg (yield is 72.5%) of an off-white solid [11].

2.4. The PDS Unit

PDS Desktop Testing Unit (PDS-DTU-220) from Pathogen Detection Systems, Inc., (Kingston, Ontario) was used for our study. These units are configured to hold 100 mL samples of water in a sample detection cartridge. The cylindrical polypropylene cartridges have a proprietary, polymer-based optical sensor embedded in the test cartridge into which the released fluorescent reaction products are partitioned. The partitioned fluorophores are then illuminated and fluorescence detected by a fiber optic detector connected to a charge-coupled device. The parameters of the PDS unit during sample testing were excitation 345 nm for both target fluorophores; emission set at 385 nm (2-HA gate) for *E. coli* detection and at 405 nm (1 HP gate)for total coliforms detection. Our growth sample holder contained 0.75 mg of eachsubstrate anthracene-*β*-D-glucuronide and pyrene-*β*-D-galactopyranoside and 3 g of LB medium (40% tryptone, 40% sodium chloride, and 20% yeast extract) and 2 mg ofnon-target growth inhibitor cefsulodin. Water samples with residual chlorine (from treatment plants) also contained 0.8 mM of sodium thiosulfate to neutralize the chlorine. The strategy of this method is to be able to detect and differentiate between *E. coli* and coliforms in the same sample. The detection is based on having a fluorogenic cleaved product, 1-HP be released by the galactosidase produced by the bacterium. To be able to detect and differentiate the coliforms, the cleaved product 2-HA, is used. The growth of *E. coli* produces both the 1-HP and 2-HA, but the coliforms produce only the 1HP. For this to work, both substrates must be present in the growth medium.

2.5. Spectroscopic Measurements

Spectroscopic characterization of 2-HA and 1-HP and their cleaved fluorescent products, 2-hydroxy anthracene and 1-hydroxy pyrene, respectively, was performed using a QuantaMaster UV VIS-NIR spectrofluorometer. Samples (2.0 mL) typically contained 3.4 mM of the cleaved fluorescent products 2 hydroxy anthracene or 1 hydroxy pyrene in LB medium or appropriate buffer as indicated. Effect of fluoride, aluminum, and pH on the fluorescence of 2-hydroxy anthracene and 1-hydroxy pyrene were determined by adding the indicated amount of each to the samples.

2.6. Water Filtration Method

Routine detection and quantitation of *E. coli* and total coliforms were achieved using the standard filtration method. Briefly, water samples were filtered through a 0.22 µm cellulose acetate filter (45 mm diameter), which was subsequently placed onto the surface of Differential coliform (DC) media and incubated at 37˚C overnight. Colony count and color were then scored.

2.7. Effect of Water Quality on Cleaved Substrate Fluorescence

Since field samples will vary in their physicochemical properties, we investigated the effects of certain factors on the fluorescence of the cleaved substrates within the sample holder. The following parameters were modified: pH, fluoride and aluminum concentrations. Stock solutions were prepared using Hydrofluoro silicic acid (fluoride source) or Polyhydroxy aluminum sulphate (aluminum source) in water at 100 ppm concentrations. Serial dilutions were then prepared and mixed with 100 µL of culture supernatant in a total volume of 3.0 mL. The changes in the fluorescence of the cleaved substrates were then recorded using the Quanta Master UV VIS-NIR spectrofluorometer. For pH effects, samples were mixed in a total of 3.0 mL of 25 mM tris buffer, with pH of 5.5, 6.5, or 7.5. These are the pH values typically encountered in drinking water.

2.8. Effect of Water Quality on Enzyme Expression and Activities

The effect of the parameters outlined in section 2.4 on enzyme expression and activities were determined as follows: following an overnight growth in LB medium, the culture supernatant of *E. coli* or *K. pneumonia* were collected. Supernatant (100 µL) was mixed with serial dilutions of fluoride or aluminum in a total of 5.0 mL and incubated at 37˚C for 6 hours. Changes in the fluorescence of the samples were then recorded using the Quanta Master UV VIS-NIR spectrofluorometer. To determine the effects on enzyme expression, bacterial growth in the sample holder was performed in the presence of various concentrations of fluoride and aluminum. Following the growth, the bacteria were removed by centrifugation at 10,000 rpm for 5 minutes at 5˚C. The protein concentrations in each of the supernatants were determined using the BCA protein assay (Pierce Corporation, Rockford, IL). Cleavage of the substrates was then performed with the cell free supernatants in a total volume of 5.0 mL of 25 mM tris buffer, pH 7.2 containing 3.4 mM 2-HA or 1-HP and 100 µg total supernatant protein. The reactions were incubated at 37˚C for 6 hours and their fluorescence recorded using the QuantaMaster UV VIS-NIR spectrofluorometer.

2.9. Effect of Color and Turbidity on Detection Using the PDS Unit

Surface runoff samples of high color and turbidity were collected from near Ramsey Lake, Sudbury, Ontario beginning of spring season 2012. Color and turbidity of the filter-sterilized samples were recorded using HACH DR 2000 spectrophotometer and the HACH 2100 N laboratory turbidity meter, respectively. Two fold dilutions of the sterile water samples were prepared using sterile distilled water, inoculated with an average of 3.7 cfu and subjected to detection in the PDS unit.

2.10. Field Water Sample Collection

Water samples were collected from within the Greater Sudbury Area (Ontario, Canada). A total of 32samples were collected from the fresh water beaches, Wanapitei and David water treatment plant intake raw water pipes. Samples were placed in sterile containers until arrived in the laboratory.

2.11. Statistical Analysis

The data from **Table 1** was used to further investigate the relationship between variables and detection time for *E. coli* and total coliforms. The Pearson correlation coefficients were calculated (SPSS on a VAX 4000 computer, significance level 0.01, 2-tailed) for time taken to detect *E. coli* and total coliforms against; turbidity, color, and pH in order to find a relationship between each parameter. It also analyzed the relationship between each variable (**Table 2**).

3. Results and Discussion

The success of this method depends on the ability to detect and distinguish between the two fluorogenic cleaved products. The fluorescent properties of both cleaved products in LB broth were investigated and are shown in **Figure 2** and **Figure 3**. Excitation scan of the cleaved products mixture produced by *E. coli* showed a sharp excitation band at about 345 nm with a minor shoulder at about 384 nm. When the excitation of the mixture is selected at 345 nm and an emission scan is carried out, a relatively sharp emission maximum at about 385 nm is observed with a shoulder at about 405 nm (**Figure 2**).

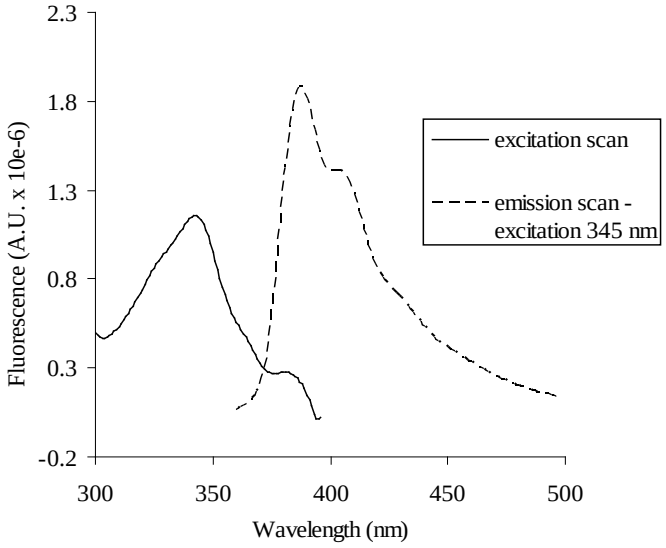

Figure 2. *β*-glucuronidase and galactosidase activity of *E. coli* in a mixture of 2-HA and 1-HP in LB broth. Cells were added to a mixture of the substrates (3.4 mM each) in growth media (LB) and incubated 24 h at 37˚C and cells free supernatant was scanned for fluorescence.

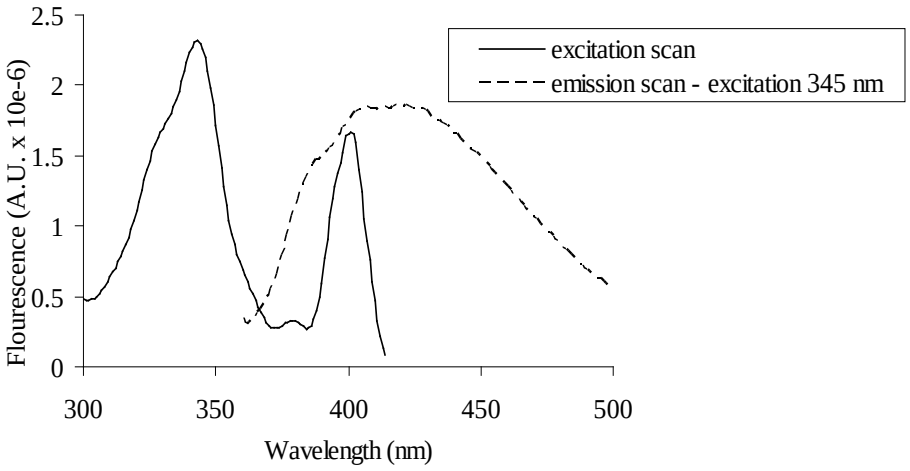

Figure 3. Galactosidase activity of *K. pneumoniae* on a mixture of 2-HA and 1-HP in LB broth. *K. pneumoniae* cells were added to a mixture of the substrates at an amount of 3.4 mM and growth media (LB) and incubated 24 h at 37˚C and cell-free supernatant was scanned for fluorescence.

Table 1. Effect of color and Turbidity on detection time.

Sample	Turbidity NTU	Color TCU	Detection Time HH:MM:SS	
			Escherichia coli	Total coliforms
A	18.12	173	Not Detected*	14:23:19
B	7.69	79	Not Detected*	13:39:31
C	1.89	25	6:57:23	13:39:50
D	0.926	13	6:07:56	10:2147
E	0.566	8	5:51:05	11:29:54

*Not detected during the standard 18 hours incubation period. However, presence of the bacterium was detected following an additional incubation period of 12 hours. TU: Nephelometric Turbidity Unit; CU: True Color Unit; HH:MM:SS—Hour:Minutes:Seconds.

Table 2. Test results from field samples.

Location	Turbidity	Color	pH	A EC	B TC	Detection Time HH:MM:SS	
						EC	TC
Bell Grove Beach	0.761	15	7.97	24	9	13:59:23	15:03:01
Bell Grove Beach	0.745	14	7.94	3	14	14:09:21	15:32:08
Capreol Beach	1.25	34	7.14	36	TNC	13:02:40	11:43:42
Capreol Beach	1.33	36	7.02	41	TNC	13:03:26	12:32:46
Bell Park Main Beach	0.694	12	7.8	13	26	16:07:01	14:25:04
Bell Park main Beach	0.556	13	7.88	2	TNC	13:44:49	12:40:26
Wanapitei River Intake	1.46	40	7.42	16	28	14:31:12	14:56:53
Wanapitei River Intake	1.16	35	7.42	6	32	13:58:14	14:59:11
Wanapitei River Intake	4.02	54	7.07	6	12	12:07:38	13:39:27
David plant Raw Tap	0.27	13	6.95	5	10	15:07:59	13:46:44
Moon Light Beach	0.753	18	7.36	4	20	15:15:16	15:50:34
Capreol Beach	0.92	42	7.06	41	17	11:59:47	12:44:15
Ella Beach	1.16	33	7.02	12	5	17:36:19	15:39:48
Centennial Park Beach	1.1	39	7.24	8	21	14:10:06	14:21:36
Whitewater lake Park	2.17	29	7.57	32	19	13:42:08	13:50:34
Meatbird Lake Park	1.42	24	7.41	1	7	17:15:37	13:38:41
Kalmo Beach (Sandy Beach)	1.03	41	7.15	3	12	13:57:05	14:35:24
Simon Lake Park	1.23	36	7.49	4	42	15:04:32	15:59:43
Bell Park Main Beach	0.684	25	7.55	1	15	14:23:54	14:17:46
New Beach	0.691	25	7.49	4	27	14:35:24	14:40:00
Amphitheatre Beach	1.88	32	7.58	4	53	14:20:27	14:25:49
Nepahwin Park Beach	0.783	3	7.87	22	16	13:31:01	13:47:06
Bell Grove Beach	0.662	4	7.59	ND	ND	Absent	Absent
New Beach	0.552	4	7.7	ND	2	Absent	17:45:31
Laurentian University Beach	0.776	5	7.76	1	1	16:32:18	16:23:06
Amphitheatre Beach	0.548	2	7.71	ND	ND	Absent	Absent
Canoe Club Beach	0.564	3	7.65	ND	ND	Absent	Absent
Ella Lake Beach	1.34	24	7.11	ND	2	Absent	Absent*
Meatbird Lake Park	0.691	4	7.18	ND	ND	Absent	15:54:21
Simon Lake Park	1.1	20	7.56	1	7	15:27:32	16:12:45
Centennial Park Beach	1.05	34	7.56	ND	4	17:07:57	15:45:55
Wanapitei Intake	1.06	43	7.42	2	5	16:02:02	16:48:48

Time: HH:MM:SS [Hour: Minutes: Seconds]; ND: Not Detected; *Absent: Not detected within 18 hours. But detected after 27 Hours incubation; A EC: Number of *E coli* colonies in 100 mL filed sample; B TC: Number of Total coliforms colonies in 100 mL field sample; TNC: Too numerous to count; EC: *Escherichia coli*; TC: Total coliforms.

When the cleaved products mixture is investigated using a coliform, *K. pneumoniae*, two excitation maxima are observed, at 345 nm and at 405 nm. Using the latter to excite the mixture did not produce a useful emission pattern. However, when the 345 nm wavelength is used for the excitation, a broad emission maximum is observed (**Figure 3**). From the two figures, it was concluded that the 345 nm wavelength can be used for the excitation of the fluorescent mixture and the ability of this system to discriminate between *E. coli* and the coliforms will depend on using different emission wavelength detection. In our experimental set up, we used the 345 nm for excitation in both cases and used the 385 nm emission maximum to confirm presence of *E. coli* and the 405 nm emission maximum to confirm the presence of the coliforms.

To apply this method in the field, the effects of a number of physical and chemical properties of water had to be investigated. For example, depending on the source of the water sample, which can be well water, surface water, recreational water, or drinking water from various treatment stages, such properties as pH, color, turbidity, presence of fluoride, and aluminum will affect the specificity and sensitivity of our method. Fluoride is often added to water to control its quality and its presence in the water (at levels comparable to those used in the industry) and its effect on microbial detection was investigated. As **Figure 4** shows, the presence of Fluoride had no significant negative effects on the fluorescence of the cleaved products mixture. In fact, Fluoride was found to enhance the fluorescence/enzymatic activity of the bacterial supernatant at 0.87 ppm. Effect of aluminum on the fluorescence of the cleaved products was also investigated (**Figure 5**). Aluminum was found to drastically

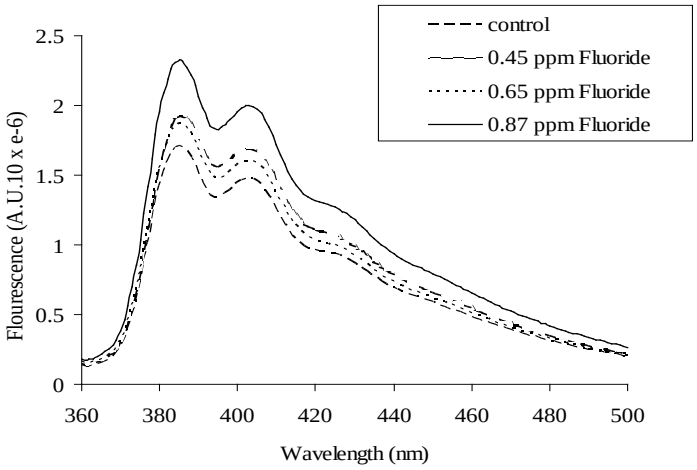

Figure 4. Effect of fluoride on the fluorescence mixture of the 2-HA and 1-HP.Samples were prepared by adding different concentrations of fluoride to the cell-free culture supernatant post incubation with *E. coli* in LB broth containing 3.4 mM of each of the substrates. Excitation wavelength was 345 nm.

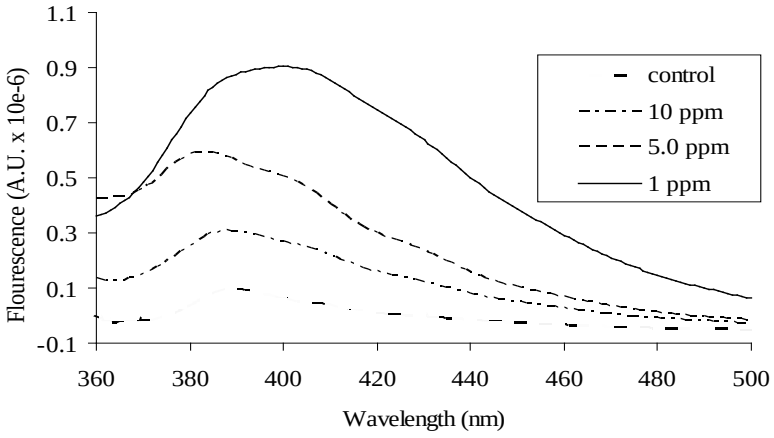

Figure 5. Effect of aluminum on the fluorescence mixture of the 2-HA and 1-HP.Samples were prepared by adding different concentrations of aluminum to the cell-free culture supernatant post incubation with *E. coli* in LB broth containing 3.4 mM of each of the substrates. Excitation wavelength was 345 nm.

reduce the fluorescence efficiency of the mixture. It is not clear how aluminum produces this effect. However, since it is added to control the quality of the water in the form of polyhydroxy aluminum sulfate, it causes coagulation of the medium. This has several secondary effects and could produce a change in pH, an increase in turbidity, and/or precipitation of the substrates. To gain an understanding of effect of the aluminum on the fluorescence of the cleaved products, the effect of pH on their fluorescence was determined. As seen in **Figure 6**, an increase in the fluorescence of the cleaved products was observed when the pH of the medium was reduced, showing highest fluorescence at pH 5.5.

We can therefore conclude that the effect of aluminum on the fluorescence is not due to changes in the pH as acidification does not reduce the fluorescence. But does pH reduce the levels of enzymes produced by the bacteria? pH was found to affect the rate of bacterial growth but was not found to significantly affect the levels of enzyme activities (**Figure 7**). The effect of fluoride on levels of enzyme production was also investigated. Fluoride was found to reduce the levels of enzymes produced by the bacteria (**Figure 8**). Similar effects of aluminum

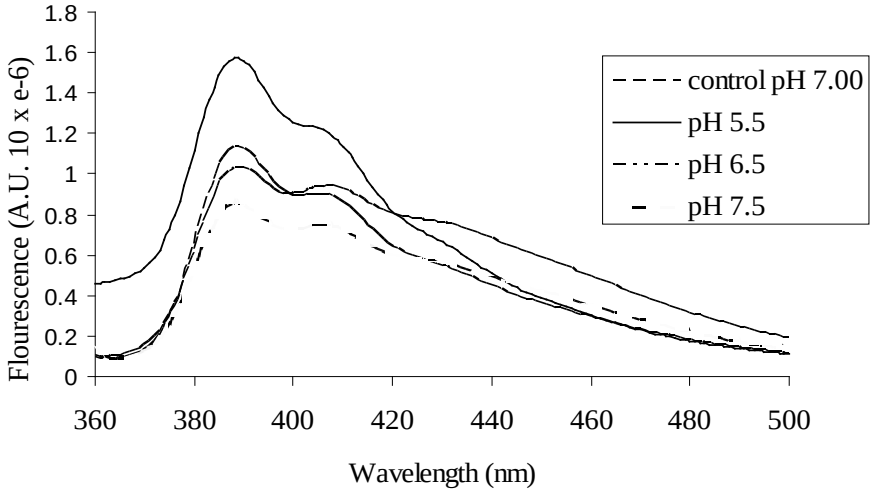

Figure 6. Effect of pH on the fluorescence mixture of the 2-HA and 1-HP. Samples were adjusted to varying pH and cell-free culture supernatant post incubated with *E. coli* in LB broth containing 3.4 mM of each of the substratesin 25 mM Tris buffer, pH 6.8. Excitation wavelength was 345 nm.

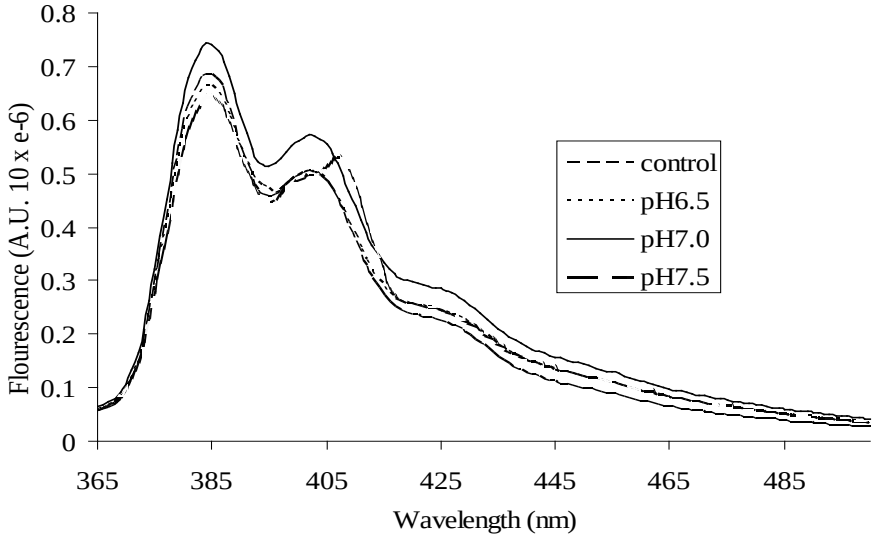

Figure 7. Effect of pH on the expression of galactosidase and glucuronidase activities during the growth of *E. coli*. Cell-free culture supernatant (100 µg) was mixed with each substrate (3.4 mM final concentration) in 50 mM Tris buffer, pH 7.2 and incubated for 6 h at 37˚C before being scanned for fluorescence. Excitation wavelength was 345 nm.

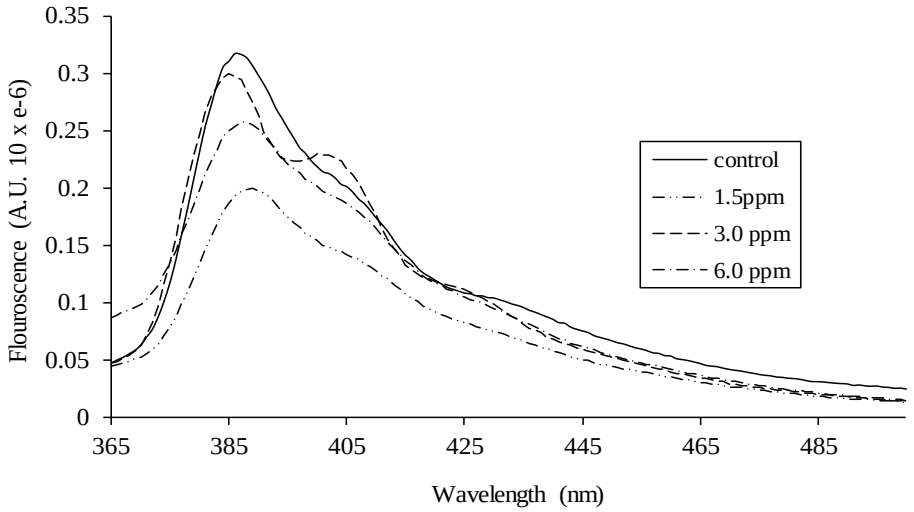

Figure 8. Effect of fluoride on the expression of galactosidase and glucuronidase activities during the growth of *E. coli*. Cell-free culture supernatant (100 µg) was mixed with each substrate (3.4 mM final concentration) in 50 mM Tris buffer, pH 7.2 and incubated for 6 h at 37˚C before being scanned for fluorescence. Excitation wavelength was 345 nm.

were investigated. When present during bacterial growth, aluminum produced a drastic reduction in enzyme activity at concentrations higher than 0.5 ppm (**Figure 9**). When this effect was investigated further, it was found that aluminum, through coagulation of the medium and potentially through other effects, significantly reduced bacterial growth. Additionally, presence of aluminum was shown to reduce the rate of enzymatic hydrolysis of the substrates (**Figure 10**). Further complicating the picture was our finding that aluminum, at the same levels, enhances the fluorescence of the cleaved products (**Figure 10**). Therefore, we may conclude that aluminum produces its effects not directly on the fluorescence of the cleaved products but rather on the bacterial growth and on enzyme activities. Color and turbidity may also affect the ability of our method to detect and differentiate between *E. coli* and coliforms. We started with a turbid water sample and diluted it with clear distilled water to give a gradient of color and turbidity (**Table 1**). At a turbidity of 1.89 NTUs or less, our method was able to detect *E. coli* in less than seven hours and coliforms in less than 14 hours. Typical water samples from surface or drinking water have NTU values of 1.0 or less. At higher turbidity and color levels, detection of *E. coli* required more than 18 hours, whereas the detection of coliforms at those same levels was achieved at about 14 hours (**Table 1**).

The results of our investigations of the physicochemical parameters of water as reported above were used to generate a final detection protocol. This protocol was used to compare our method to the classical plating method for detecting *E. coli* and coliforms in field samples. Water samples were collected from a number of sites within the Greater Sudbury Area and were subjected to microbial testing using the filter plate method and our detection method. The results are summarized in **Table 2**. The PDS unit has the ability to detect *E. coli*, mean 14.65 h at a standard deviation of 1.477, when the sample mean is 9.125 cells in 100 mL with a standard deviation of 12.574. Similarly, total coliforms may be detected at mean 14.709 h with a standard deviation of 1.395 when the sample mean is 59.563 cells in 100 mL, with a standard deviation of 144.476. However, these results may not reflect the sensitivity of the PDS unit because of the presence of outliers during sample analysis. Further investigation into the validity of these outliers is necessary. However, the PDS unit has the ability to detect single cells of either total coliforms or *E. coli* in 100 mL water sample within 18 h of incubation at 37˚C (**Table 2**). Turbidity and color has no relation or impact on the detection of *E. coli* and total coliforms (**Table 3**). The number of *E. coli* and total coliforms cells in 100 mL sample has a strong inverse relationship with time for detection. As the number of cells increases in the sample the detection time decreases; whereas if the number of cells in the sample decreased, the detection time length increased. A significant relationship was also established between total coliforms and *E. coli* in the same sample. It is obvious any microbial contamination from raw sewage has both *E. coli* and total coliforms. Our method detects β-galactosidase enzyme activity, indirectly. Both *E. coli* and total coliforms express β-galactosidase enzymes and therefore detection is enhanced when both

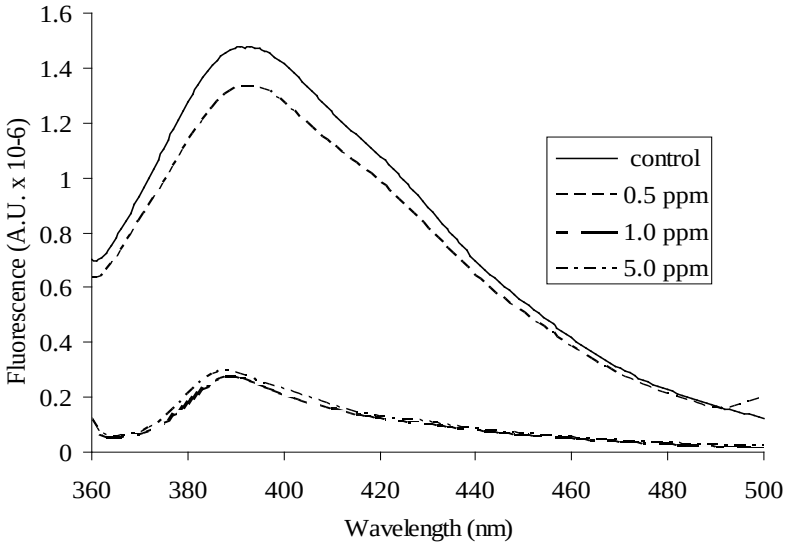

Figure 9. Effect of aluminum on the expression of galactosidase and glucuronidase activities during the growth of *E. coli*. Cell-free culture supernatant (100 µg) was mixed with each substrate (3.4 mM final concentration) in 50 mM Tris buffer, pH 7.2 and incubated for 6 h at 37˚C before being scanned for fluorescence. Excitation wavelength was 345 nm.

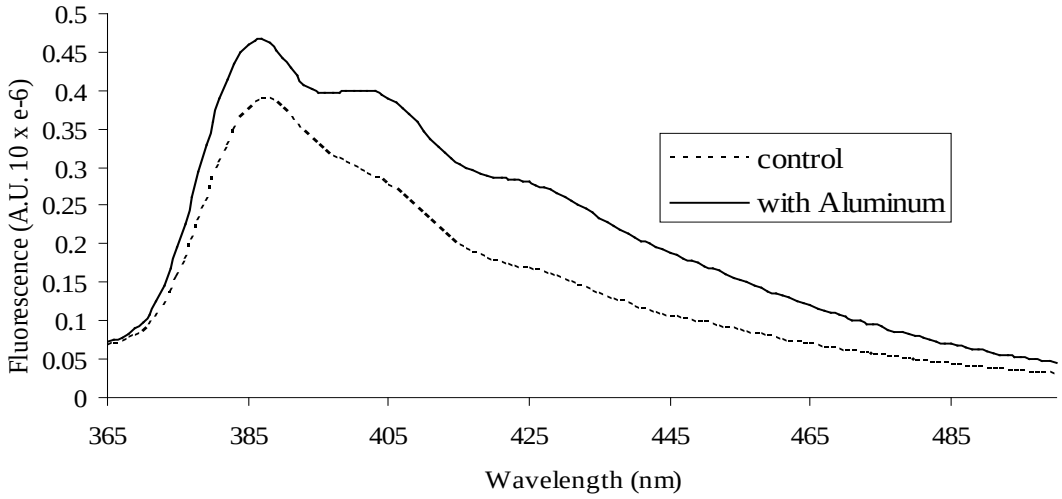

Figure 10. Effect of aluminum on the catalytic activity of *E. coli* glucuronidase and galactosidase. Cell-free supernatant (100 µg) was added to the substrates (1-HA and 2-HP) in 50 mM Tris buffer, pH 7.2 or in the presence of 1.0 ppm aluminum and incubated for 6 h, after which the supernatant was scanned for fluorescence. Excitation wavelength was 345 nm.

Table 3. Pearson correlation coefficients.

	Turbidity	Color	pH	A Ec	B c	Tec	Ttc
Turbidity	1.0000	0.6485**	−0.3210	0.1762	0.0068	−0.3177	−0.2238
Color	0.6485**	1.0000	−0.5717**	0.3103	0.1256	−0.2770	−0.2601
pH	−0.3210	−0.5117**	1.0000	−0.2327	−0.1194	0.1468	0.3011
A Ec	0.1762	0.3103	−0.2327	1.0000	0.4616**	−0.5506**	−0.6252**
B c	0.0068	0.1256	-0.1194	0.4616**	1.0000	−0.3626	−0.6210**
Tec	−0.3177	−0.2770	0.1468	−0.5506**	−0.3626	1.0000	0.6264**
Ttc	−0.2238	−0.2601	0.3011	−0.6252**	−0.6210**	0.6264**	1.0000

**Significance level at 0.01, 2-tailed. A Ec: Number of *E. coli* cells in 100 mL samples; B c: Number of coliforms (non *E. coli*) in 100 mL samples; Tec: Time taken to detect *E. coli* hours; Ttc: Time taken to detect Total coliforms hours.

microorganisms are presents in a sample. Detection time was insensitive to pH for both *E. coli* and total coliforms. This method is able to detect single cells of *E. coli* or total coliforms in no longer than 18 h with incubation at 37˚C. In raw water samples collected from Ella Lake beach (b), 2 CFU/100 mL coliforms were found (MF Method). However, the PDS unit was not able to detect the bacteria within 18 h of incubation. When the sample cartridge was incubated further it was able to detect total coliforms after 27 h. Ella lake beach (b) water sample had a turbidity of 1.34 NTU, color 24 TCU and pH 7.11 which is similar to sample Ella Lake Beach (a) in which total coliforms (5 CFU/100 mL) was detected after 15.67 h of incubation (having Turbidity 1.16 NTU, color 33 TCU and pH 7.02). This discrepancy may be due to external environmental factors and should be investigated further.

4. Conclusions

Rapid enzyme base detection techniques on the basis of indirect measurement of glucuronidase or galactosidase activity can be used for estimating the level of coliform bacteria and *E. coli* in water samples. This method is useful because it is simple, easy to use, and cost effective and makes field testing possible.

Glucuronidase and galactosidase enzymes are found in numerous microorganisms and are not specific for coliform bacteria and *E. coli*, and therefore may give false positive results. However false positive coliforms results due to the presence of *Aeromonas spp.* can be eliminated by using cefsulodin, a growth inhibitor of *Aeromonas* spp.

Detection time length needs to be minimized and evaluated further in order to be used for monitoring of the microbial water quality of drinking water. Development of more sensitive detection techniques may improve detection limits. Studies on selective optimization of substrate hydrolysis by enzymes of target bacteria, and on inhibition of enzymes of non-fecal origin, could help improving sensitivity and specificity of enzyme based detection methods.

Glucuronidase and galactosidase activities are more resistant to disinfection stress (e.g. UV light) than cultivability. UV disinfection is more commonly used in the drinking water industry. Enzyme activity that is detected from presumably dead *E. coli* that has been treated with UV light may result in false positives because although the enzymes are present the bacteria are no longer of concern. Therefore this method is questionable for evaluating the quality of UV disinfected water.

However, enzyme base detection methods can be useful for early warning of fecal contamination in water, and represent an alternative indicator concept since the glucuronidase and galactosidase activities are more persistent to environmental stress than the cultivability of coliforms and *E. coli*. It is well known that some pathogens, for example viruses and protozoa, may survive longer in the environment than *E. coli*, and further research is needed to better understand the relationship between results from rapid enzyme methods and human health risks associated with these pathogens. Including a rapid enzyme base detection methods in further research, may also be useful for determining the risks associated with microbiological quality of drinking water.

Acknowledgements

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