

**Final Report**

**Rapid Determination of Pathogenic Bacteria in Surface Waters**

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## **Executive Summary**

The beaches in Michigan, both on inland lakes and on the Great Lakes have encountered numerous beach closings in the past years due to high levels of *E. coli* in the beach water. The method of testing for *E. coli* is slow and requires 24 hours before the results are known. The consequence of this is that beaches are closed too late, and the opening of them is delayed. A method that would do the test in less than an hour will allow personnel responsible for the safety of the beach to test the beach early in the morning before people arrive. The test method developed in this study will allow this and although it is still a bit cumbersome, it provides a much more timely testing. The method has been tested on four beaches in Michigan.

Further work is necessary to simplify the method, and it needs to be tested on a larger database, i.e., on a larger number of beaches. Some training of the personnel is also necessary.

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## Statement of the Problem

The purpose of this project was to develop a fast and reliable method for testing river and lake water samples for pathogenic bacteria onsite and in a very short time. There should be no need to bring the water samples to the laboratory.

The current test methods take from one to two days. The closure of beaches based upon the test results is sometimes too late, and the delay in opening the beaches is not in the interest of the public. More timely information needs to be available to the responsible Health Departments and the general public.

The outcome of the project is a set of test procedures that can be used by personnel responsible for the safety of the beaches in the Great Lakes area. The focus was on the Southeastern part of Michigan due to logistic and financial considerations. The results of the test procedure are available almost immediately to the local health department.

## Review of the Literature

Culture-based tests require at least 18 to 24 hours for completion and are just too slow.. There are technologies emerging for the rapid detection of *E. coli* in water. More recently rapid assays for detecting *E. coli* without cultivation have been explored.

### 1. Solid phase cytometry & enzymatic method

Van Poucke et al. (2000) evaluated an enzymatic membrane filtrate technique using a laser-scanning device to reduce the analysis time. The procedure they proposed is as follows. Water samples are filtered on a 0.4- $\mu\text{m}$  pore-size filter. The retained bacterial cells are treated with reagents to induce the enzyme  $\beta$ -D-glucuronidase (3 hrs at 37°C) and label (0.5 hour at 0°C) the induced cells. The principle of the method is that only the  $\beta$ -D-glucuronidase of viable *E. coli* can be induced and therefore only these bacteria cleave the non-fluorescent substrate (fluorescein-di- $\beta$ -D-glucuronide) while retaining the fluorescent end product inside the cell. The fluorescence of a cell is detected by the ScanRDI device.

### 2. Solid phase cytometry & immunomagnetic separation (IMS)

Pyle et al. (1999) used a combination of IMS and solid phase laser cytometry for the detection of *E. coli* O156:H7 spiked in water. Concentration steps use magnetic beads coated with anti-O157 rabbit serum and a magnetic separation. Various analyses such as enumeration of culturable cells and respiring cells were performed. Culturable cells were counted by membrane filtration and identified by an immunofluorescence assay using a scanning device. This approach applied to spiked water samples showed higher sensitivity than a culture-based method.

### 3. Polymerase chain reaction (PCR)

PCR allows a DNA target sequence to be amplified by cycling replication using DNA polymerase (*Taq* polymerase). The cycling of PCR results in an exponential amplification of the amount of the target sequence and thereby significantly increases the chance of detecting low numbers of target organisms in a sample (Bej et al. 1990). In order to detect the target sequence from an environmental sample, the concentration step is necessary, followed by cell lysis and chemical extraction. The concentration step can be performed using membrane filter (Bej et al., 1991; Iqbal et al., 1997). Briefly, the PCR amplification steps are as follows: 1) a DNA denaturation from double- to single-stranded DNA, 2) annealing primers to the single-stranded DNA at a specific hybridization temperature, 3) primer extension by a DNA *Taq* polymerase. Amplification of a target sequence by PCR requires 20 to 40 cycles. For the detection of *E. coli*, the proposed target sequences are a region of *malB* gene and *uidA* gene which encodes for a maltose transport protein and  $\beta$ -D-glucuronidase enzyme, respectively (Bej et al., 1990, 1991; Tsai et al., 1993). The *malB* region includes the *lamB* gene which encodes a surface protein recognized by an *E. coli*-specific bacteriophage. However, *Shigella* and *Salmonella* genera were detected using this primer set. PCR products are detected after electrophoresis on agarose gel and after staining of amplification products by a fluorochrome dye or by hybridization with a labeled probe.

PCR-based assays have difficulty in the quantification of microorganisms, and most of the PCR studies were performed on water samples spiked with cultured strains of *E. coli* (Rompre et al., 2002). Another limitation in using PCR for the analysis of environmental samples is the frequent inhibition of the enzymatic reaction by the substances that are present in the samples, such as humic substances and colloid matter (Way et al., 1993). The procedure does not differentiate between dead and alive organisms.

### 4. Fluorescent In situ hybridization (FISH)

The FISH method uses fluorescent-labeled oligonucleotide probes to detect complementary nucleic acid sequence (mainly 16S and 23S rRNA). The procedure of FISH includes cell fixation, hybridization, washing and detection. Hybridized cells are detected by epifluorescence microscopy and counterstaining, such as DAPI or acridine orange, is used to determine the total number of cells (Amann et al., 1995). FISH technique has been used for the detection of *E. coli* in spiked microcosm (Shi et al., 1999), and urine, rivers, sewage and food samples (Regnault et al., 2000). The rRNA content of a bacterium does not completely reflect its physiological status because rRNA molecules can remain for a relatively long period after the loss of culturability (McKillip et al., 1998). However, FISH is currently considered as a highly specific detection method, and as relatively easy to perform (Rompre et al., 2002).

In summary, the above methods are highly specific but can only be performed in a laboratory with well-trained staff.

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### ***Approach used in this study***

The project used several techniques and the literature list following cites some of the most recent publications describing the techniques in more detail.

### ***Immunomagnetic Separation***

There have been numerous studies about the bacteriological quality of recreational water. Most of these studies were epidemiological analyses based on most probable number, membrane filtration and plate count methods (Prüss, 1998; Cabelli *et al.*, 1982; Fleisher *et al.*, 1996). Traditional culture methods for examining water generally require enrichment followed by an identification of the bacteria. Due to the incubation time or an enrichment step in order to reach the detectable numbers, there is a considerable time delay from sampling until the results are available. The need for rapid and direct methods to assess active target bacterial population in water has been widely acknowledged. The use of more rapid methods for detecting pathogens, including immunomagnetic separation (IMS), has become more common (Wright *et al.*, 1994; Fratamico *et al.*, 1992; Restaino *et al.*, 1996). The IMS uses uniform superparamagnetic polystyrene beads coated with antibodies. The antibody coated beads bind to the desired bacteria population, forming a bead/bacteria complex that is easily separated from a heterogeneous bacteria suspension by exposure to a magnetic field. It has been known that IMS is useful tool for downstream applications such as DNA analysis (Höller *et al.*, 1999), flow cytometry (Pyle *et al.*, 1999) and plate count (Tan *et al.*, 1999).

### ***ATP Bioluminescence***

In our study, ATP bioluminescence was used to estimate the bacteria in a sample after the target pathogens were separated by IMS. The estimation of bacterial numbers with the results of an ATP bioluminescence method is known to be highly correlated with the current plate count method (Lee *et al.*, 1999; Van der Kooij *et al.*, 1995). The ATP method allows an estimate of the number of bacteria to be done within minutes. An additional advantage of the method is that it only counts viable bacteria.

### ***Riboprinter***

The ribotyping technique, which uses restriction fragments of nucleic acids from bacterial genomes to characterize organisms, was used in the proposed study to confirm bacterial strains that were separated by IMS. It has been shown that the pattern of distribution of DNA fragments is unique and highly conserved, and the genetic pattern is not affected by environmental conditions (Sethi, 1996). It is useful to discriminate among many of the

bacterial strains below the species level, which allows insight into the origin of the contamination (Ralyea *et al.*, 1998; Wiedmann *et al.*, 1997).

Cabelli, V. J. *et al.* 1982. Swimming associated gastroenteritis and water quality. *American Journal of Epidemiology*. 115:4:606-616.

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Höller, C., S. Koschinsky, D. Witthuhn, 1999. Isolation of enterohaemorrhagic *Escherichia coli* from municipal sewage. *Lancet*. 353:9169:2039.

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Sethi, M. Fully automated microbial characterization and identification for industrial microbiologists. *American Laboratory*. May 1997, pp31-35.

Tan, W., L. A. Shelef, 1999. Automated detection of *Salmonella* sp. in Foods. *Journal of Microbiological Methods*. 37:87-91.

Wiedmann, M. *et al.* 1997. Investigation of a Listeriosis epizootic in sheep in New York state. *American Journal of Veterinary Research*. 58:733-737.

Wright, D. J., P. A. Chapman, and C. A. Siddons, 1994. Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. *Epidemiological Infection*. 113:31-39.

Van der Kooij, D. et al. 1995. Biofilm formation on surfaces of glass and teflon exposed to treated water. *Water Research*. 29:1655-1662.

## Methodology

### *Introduction*

The current procedure for checking the bacteriological quality of bathing beaches is to take a 100ml sample at 3 locations on a beach, bring the samples to a laboratory, filter the samples through a membrane filter, and then place the membrane filter on mTEC agar that is specific for *E. coli*, and count the number of colonies after an incubation time of 22 hours.

The current standards for beach water are that the geometric average of the 3 samples shall not exceed 130 CFU/100 ml, and that no single sample should exceed 300CFU/ml. The current practice is to take a sample at the beaches in the morning, and bring the samples to the laboratory for analysis in the afternoon. Some departments contract the analysis out to certified laboratories, and the results are available in 2-3 days. Thus beaches may be closed too late, or their opening may be delayed. This project was designed to do the analysis in minutes, directly at the beach, and thus allow more timely decisions.

The picture below shows that the entire test equipment can be put onto a clipboard to carry easily to the field (Fig. 1). It includes all the necessary equipment and supplies. In the center are the luminometer, the battery power supply and a micropipet. The small bottles are lysing agents and enzyme/substrate (luciferine/luciferase).

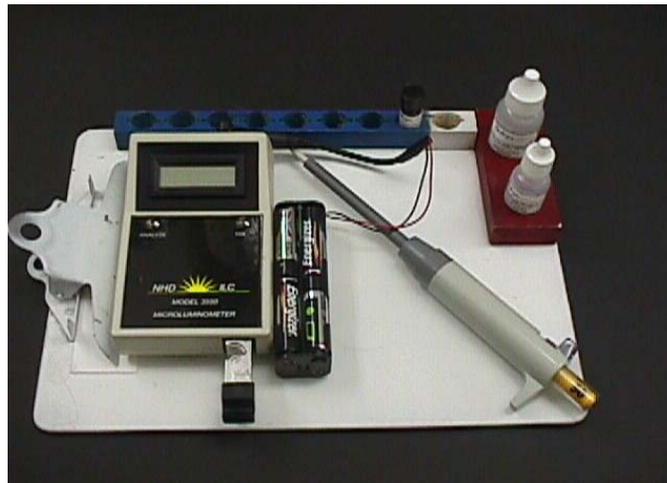


Fig. 1. A luminometer and other equipment.

### *Preparation of the antibody coated magnetic beads*

Magnetic beads coated with antibodies for *E.coli* are not commercially available. They must be made in the laboratory.

#### 1) Selection of antibodies

Antibodies for *E. coli* are available from several vendors. A list of the manufacturers is as follows:

<u>Vendor</u>	<u>Web address</u>
Biodesign	<a href="http://www.biodesign.com">www.biodesign.com</a>
Chemicon International	<a href="http://www.chemicon.com">www.chemicon.com</a>
Maine Biotech	<a href="http://www.mainebiotechnology.com">www.mainebiotechnology.com</a>
ViroStat	<a href="http://www.virostat-inc.com">www.virostat-inc.com</a>

We chose the antibodies based upon the following criteria: 1) range of specificity, 2) type of antigen to raise antibodies, 3) cost. A polyclonal antibody contains a mixture of antibodies and is able to bind to a number of sites on the antigen. A monoclonal antibody is able to bind only to one of the binding sites on the antigen so it potentially offers greater specificity. Antibodies targeted against all environmental strains of *E. coli* do not exist because the types of *E. coli* in natural environment is quite diverse. Having the aim of the study detecting *E. coli* in beach water, it was decided to use polyclonal antibody instead of monoclonal antibody to capture a broader range of target organisms. We purchased the antibodies from BioDesign because the type of antigen to raise antibodies was heat-killed sonicate of whole cell *E. coli*, rather than specific antigen such as lipopolysaccharide, O antigen, or K antigen. They targeted a broader range of *E. coli* in the environment. The cost of the antibodies was reasonable. The manufacturer mentioned that the antibodies may cross react with *Enterobacteriaceae* such as *Shigella* and *Salmonella*. Thus, some of the bacteria captured may not be *E. coli*, but other enteric bacteria. Since the *E. coli* are indicator organisms of fecal contamination, a few other species captured do not change the intent of the test.

#### 2) Selection of beads

Magnetic microspheres are available from several vendors. A list of the vendors is as follows:

<u>Vendors</u>	<u>Web address</u>
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Bangs Laboratories  
DynaL  
Miltenyi Biotech

[www.bangslabs.com](http://www.bangslabs.com)  
[www.dynalusa.com](http://www.dynalusa.com)  
[www.miltenyibiotec.com](http://www.miltenyibiotec.com)

We chose beads from Bangs Laboratories based upon the size of the beads (<1  $\mu\text{m}$ ), the ease of handling, and the availability of technical support.

### 3) Disinfection of beads

The magnetic beads were disinfected with 0.1% sodium azide. The disinfected beads were rinsed with sterile distilled water three times before they were mixed with antibodies.

### 4) Calculation of the amount of beads and antibodies for coating

There are currently several means of attaching antibodies to the magnetic beads including adsorption, covalent bonding, and attachment to beads that are pre-coated with a generic binding protein, such as Streptavidin or Protein A. We used adsorption techniques for our purpose. The adsorption method is widely used today for attaching proteins to microspheres due to the simplicity and flexibility of this method. The mechanism of adsorption is based primarily on hydrophobic attraction between the hydrophobic portions of the adsorbed ligands and the polymeric surface of the microspheres (TechNote #204, Bangs Laboratories, Fishers, IN). The amount of antibodies and beads to achieve surface saturation was calculated using the following equation:

$$S = (6/\rho D)(C)$$

where  $S$ = amount of antibodies needed to achieve surface saturation  
(mg protein/g of microspheres)  
 $C$ = capacity of beads surface for give protein  
(mg protein/ $\text{m}^2$  of polymer surface)  
 $\rho$ = density of beads ( $\text{g}/\text{cm}^3$ )  
 $D$ = diameter of beads, in microns

In order to ensure the correct spatial orientation and decrease the likelihood of non-specific binding, the manufacturer recommends adding antibodies in a 3-10X excess of the calculated monolayer. We added 3 times more antibodies to the microspheres. The diameter of the chosen beads was 0.6 microns.

### 5) Coating antibodies onto beads

Magnetic beads (0.2ml of 10% (wt) solid contents) were coated with 0.14ml of anti-*E.coli* antibodies (the original concentration of antibodies was 3mg/ml) and 0.66 ml of PBS (pH 5.5). The adsorption procedure was adapted from the passive adsorption method (TechNote #204, Bangs Laboratories, Fishers, IN). The suspension was incubated for 1 hour and mixed at 60 rpm at room temperature. The beads were removed from the solution with a magnet and resuspended in 0.8ml PBS and then rinsed again in 0.2ml PBS (pH 7.4). The antibody-bead complexes were stored in 0.2ml PBS with 1% BSA at

4°C until they were used. The storage concentration of the beads was 100 mg bead per ml. The shelf life is about 2 weeks.

## *Analysis of Beach Water Samples*

### **1) Concentration of bacteria by serial filtration**

Prefiltration was used to remove large particles from the water sample, which will interfere in the further analysis such as immunomagnetic separation. Various filtration methods and setups were investigated to find the simplest and most effective method for prefiltering the water samples. The prefilter material used for the first testing included a glass fiber filter (Whatman GF/D, 47 mm, Cat. No 1823047), 5micron pore size nylon filter and a nylon filter with a nominal pore size of 20 microns (Osmonics, Magna, Cat No. R22 Sp04700). The glass fiber has no nominal pore size rating, which means that there is no measured particle that it will allow through. The diameter of the filter depends on the amount of material present. A typical 47 mm filter was used. A hand held turbidity meter was used to measure turbidity in a sample.

During the early phase of the study, one method selected was the use of a Pall Magna funnel (Fig 2); the second method was to attach a Millipore funnel to a Pall Filter Holder (Fig 3). The water sample was drawn through the filters by either a hand-powered vacuum pump (Fig 4) or an electric vacuum pump set for a vacuum of 15 in either by 110 Volts or a portable battery (Fig 5). The tested water volume during the early phase of the study was between 500ml and 1L. Later we found that filtration volume of 100 to 500ml was enough for the analysis. It was due to the improvement in the recovery method and the separation step. The final filtration was for concentrating bacteria that passed through the prefilter. It was accomplished with a rated membrane with a pore size of 0.45 $\mu$  to retain *E. coli* as well as a number of other organisms. The filter membrane was held in a filter holder that was directly connected with the prefiltration device

### Pall Magna Funnel

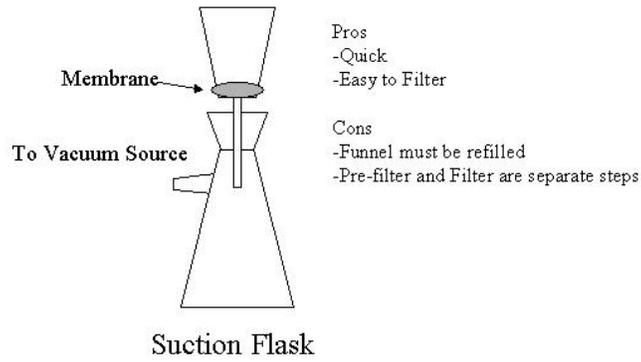


Figure 2. Pall Magna Funnel

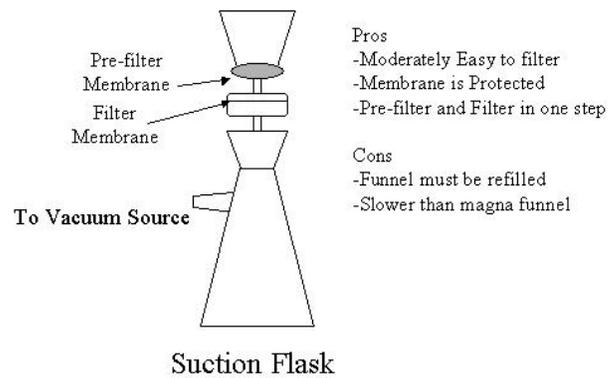


Figure 3. Pall Magna Funnel/Pall Filter Holder Hybrid



Figure 4. A filtration unit with a hand pump.



Figure 5. A filtration unit with a battery-operated pump.

One concern of prefiltration is the number of *E. coli* that would be lost by prefiltration from the original sample. Following is a chart on the possible loss of bacteria during filtration procedure (Figure 6). It shows that only a small amount of bacteria are left on the prefilter membrane and the majority of bacteria pass through the prefilter.

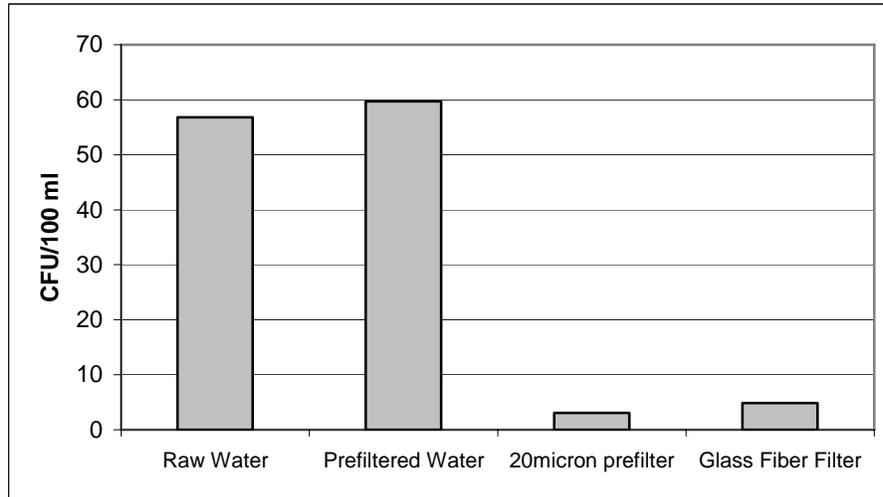


Figure 6. Examination of bacterial loss during filtration procedure. The values are the average of 10 tests.

Later on we modified the prefiltration/filtration step. It includes using a disposable prefiltration device to make the procedure more field-applicable (Fig 7).



Figure 7. A serial filtration unit using a disposable prefiltration device.

## 2) Selective capture and measurement of *E. coli*

The organisms trapped on the membrane were removed from the final membrane either via resuspension in Tween 20-containing PBS (PBST) or back flushing by a syringe while still in the filter holder. Magnetic beads that are coated with *E. coli* antibodies were added to that tube, and the tube was mixed for a short period of time (60rpm for 15min). For the mixing, the Dynal sample mixer was used for coating the beads with antibodies and testing the beach water samples that were brought to the laboratory (Fig. 8). In order to do the test procedure in the field, we constructed a portable sample mixer (Fig. 9). At this stage, the antibody-coated beads bind the target bacteria and form beads/bacteria complexes (Fig. 10-11). Using a magnetic separator, these complexes were concentrated to the magnet side of the tube wall (Fig. 12). The supernatant was discarded. After removing the magnetic separator, the bead-bacteria complexes are resuspended in a PBS solution (10ml). This separation and washing step was repeated twice.

At the final washing step, the entire pellet was suspended into 1ml of PBS and transferred to a microcentrifuge tube. After magnetic separation, the buffer was discarded and 50  $\mu$ l of somatic cell releasing agent (SRA) was added to remove any possible remained non-bacterial cells. The SRA was removed by pipetting and the pellet was washed with PBS. After magnetic separation, the buffer was discarded. Bacterial releasing agent (50  $\mu$ l) was added to rupture bacterial cells and magnetic separation was done to remove the magnetic beads. At this stage all the ATP that was derived from *E. coli* was collected into the liquid portion. The desired amount of liquid (< 50  $\mu$ l) or the entire liquid was transferred to a filtravette. The enzyme/substrate, luciferin and luciferase, for light development was added and the result was recorded as relative light unit (RLU). The RLU value is due to the *E. coli*, which were captured with antibody-coated magnetic beads.

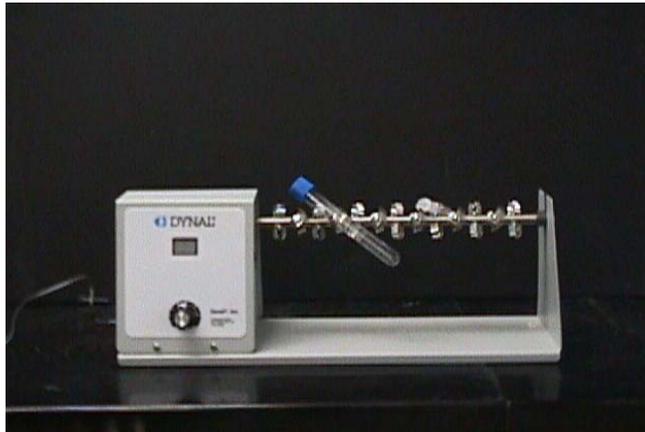


Figure 8. A sample mixer used in laboratory.

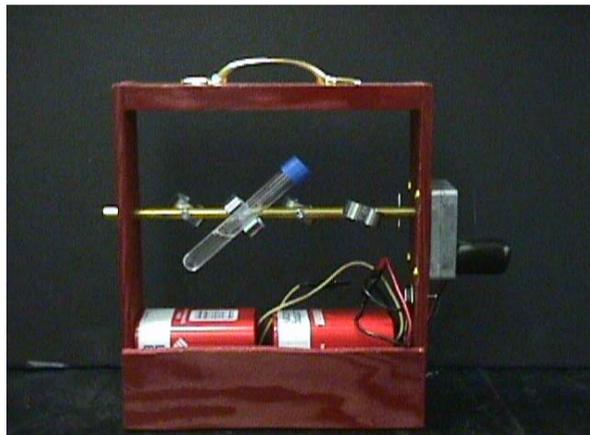


Figure 9. A portable mixer for field application.

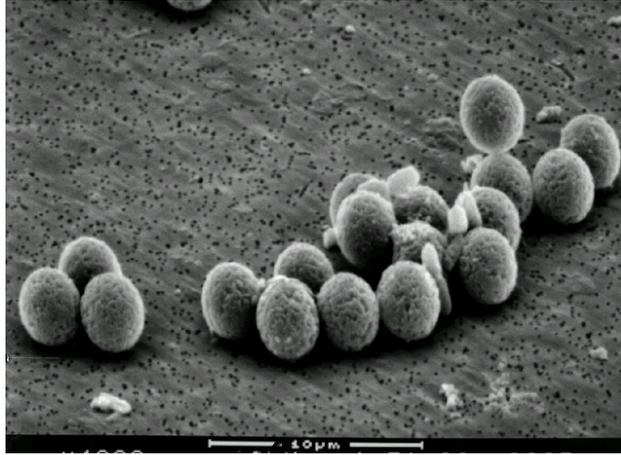
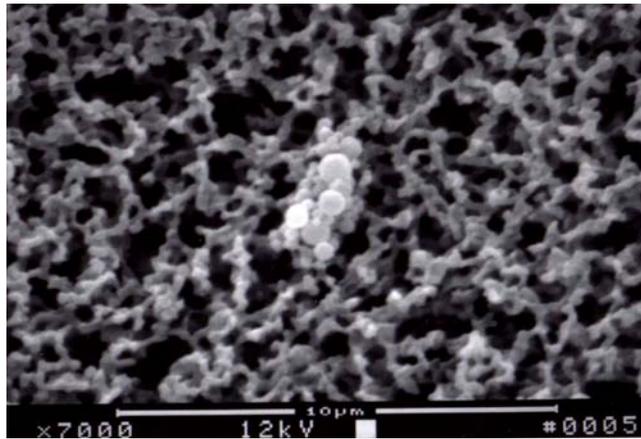
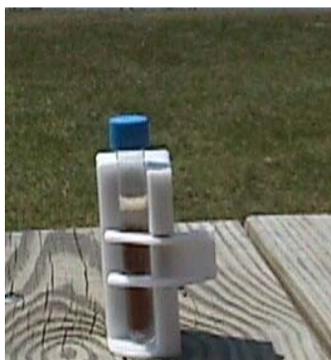


Figure 10. Target bacterial capture by antibody-coated magnetic beads. The tested organism was *E. coli* O157:H7. The bead size was 2.8 μm.



**Figure 11. E. coli captured by antibody-coated magnetic beads.**

**The bead size was .6 µm.**



(a)

(b)

Figure 12. Separation of bacteria-antibody-bead complexes from the suspension using a magnetic separator.

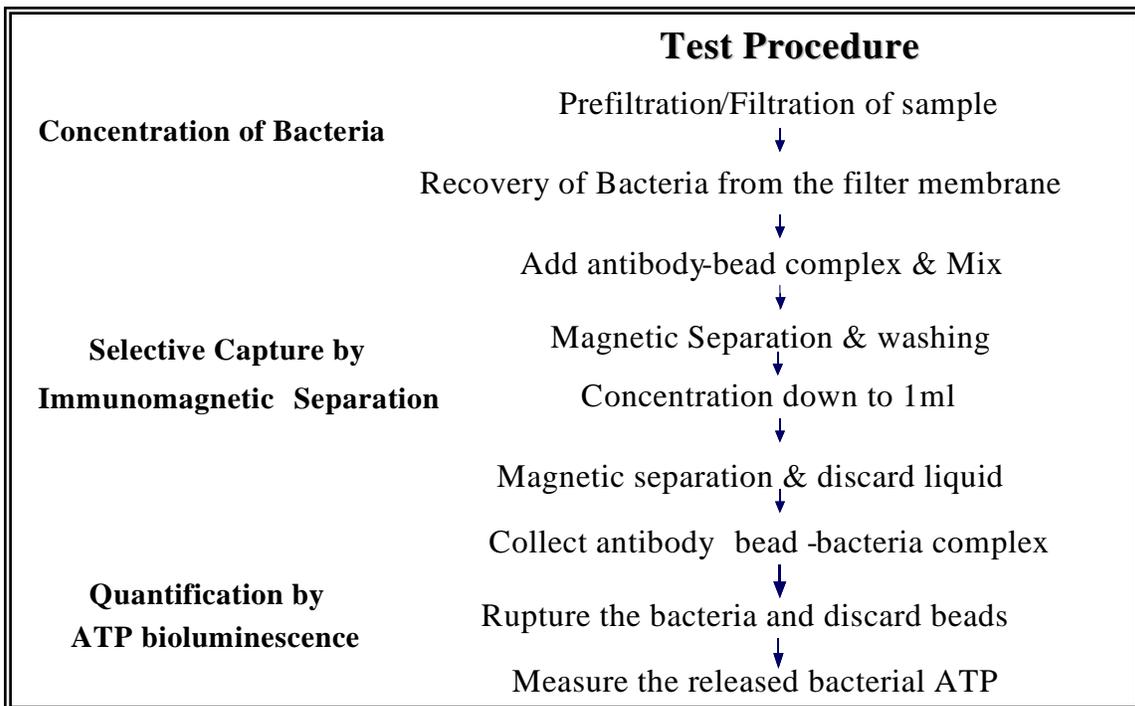


Figure 13. Summary of the analysis procedure for E. coli detection in a beach sample.

## Results of the Investigation

The sampling personnel of four health departments at four beaches in Southeastern Michigan took samples on their regular sampling schedule with additional sample for our study. We picked up the samples and delivered them to the laboratory within four hours. In the laboratory two methods were performed: the traditional plate count method (m-TEC) and the IMS-ATP bioluminescence. The table following shows the results of the analyses (Table 1). Two aspects are of concern. First, did our analyses of plate counts show the same results as the health departments? Figure 14 shows the data comparison between the laboratories and indicates that there is an excellent agreement.

Table 1. Comparison of the E. coli analyses of health departments and UM laboratory.

Location	Sampling Date	Plate Count (CFU/100ml)				ATP (RLU/100ml)
		Health Dept. (average)	Health Dept. (individual)	U of M (average)	U of M (individual)	
Memorial Park (Lake St. Clair)	5/21/01	116		115	128, 148, 130, 80, 110	129000
	5/29/01	86		700	524, 910, 720	3997
	6/4/01	47		77	74, 84, 70, 80	90
	6/11/01	11		15	10, 16, 20	1050
	6/18/01	2		3	2, 4	450
	6/25/01	5		2	2	18250
Byram Lake (Inland lake)	5/22/01	142	172, 199, 83	100	118, 88, 120, 80	81200
	5/29/01	234	167, 365, 209	160	178, 164, 140, 240	75
	6/4/01	10	9, 10, 10	6	4, 8	56.3
	6/11/01	28	26, 26, 30	43	40, 48, 40	132.5
	6/18/01	28	20, 28, 36	18	24, 12, 20	12
	6/25/01	11	7, 18, 10	33	48, 32, 20, 40	20700
Sterling Park (Lake Erie)	5/20/01	9		22	16, 30	2500
	5/29/01	3		1	1, 1	12.5
	6/4/01	0		2	2	10
	6/11/01	80		10	0, 0, 10	15
	6/18/01	52		33.2	18, 28, 20, 120	50
	6/25/01	43		24	20, 18, 24, 40	17250
Independence Lake (Inland lake)	5/22/01	16		53	46, 52, 80, 40	63400
	5/29/01	32		21	20, 19, 24, 20	175
	6/5/01	8		16	16, 12, 20	35.4
	6/12/01	8		22	18, 28	179
	6/19/01			12	18, 8	50
	6/27/01	<4		7	2, 16, 10	16650

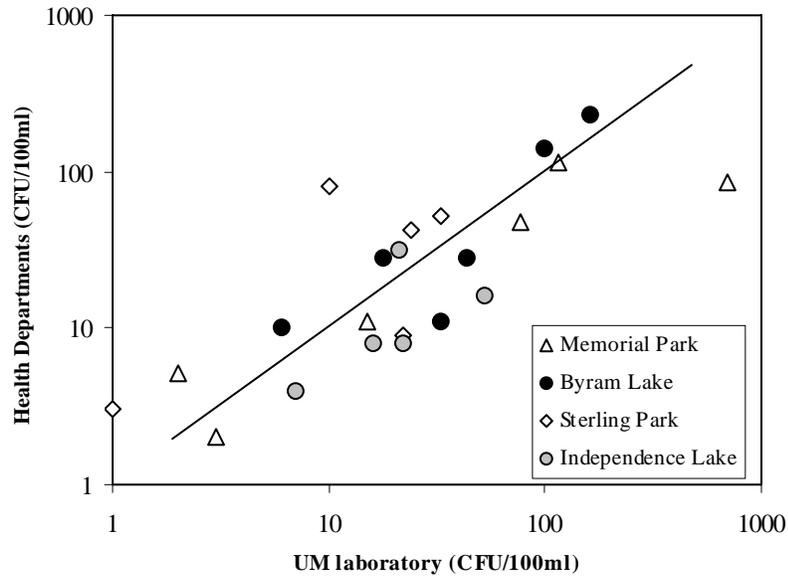


Figure 14. The relationship between the *E. coli* plate counts between the health departments and the University of Michigan.

The next figure shows the relationship between the ATP measurements (RLUs) and the plate counts (Figure 15). Since during the sampling period the beaches were in good conditions (little rain), a few samples were taken from the Huron river to supplement the high bacterial count area. The relationship between the ATP assay and the plate counts is:

$$\log \text{CFU}/100\text{ml} = 0.91 * \log \text{RLU}/100\text{ml} - 0.503$$

Using this relationship for a prediction of the *E. coli* level, the predictions are summarized in Table 4 and 5. The latter shows the RLUs to be expected for the concentration of 130 and 300 *E. coli*/100ml.

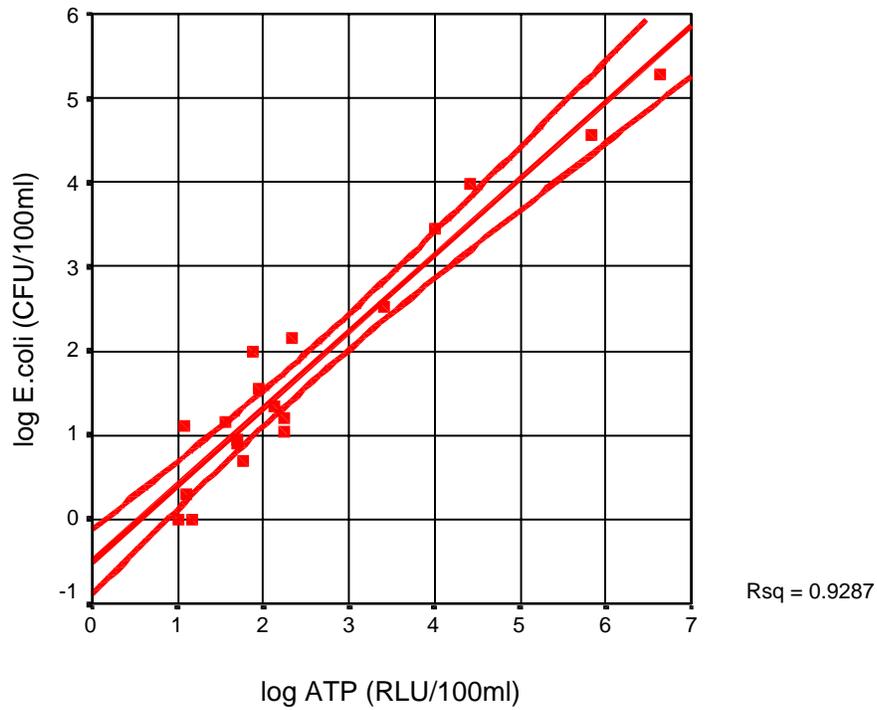


Figure 15. The relationship between ATP (RLU) and plate count (prefiltered).

Table 2. . Expected *E. coli* counts based on the ATP analysis (CI=confidence interval).

RLU/100ml	CFU/100ml	lower CI	upper CI
10	3	2	3
100	21	14	31
500	90	55	145
1000	168	101	280
1500	244	144	411
2000	317	186	540
3000	458	265	792
10000	1369	760	2465
100000	11125	5756	21501
1000000	90396	43878	186231

Table 3. The expected RLU's for a concentration of 130 and 300 *E. coli*/100ml.

<b>E. coli CFU/100ml</b>	<b>ATP RLU/100ml</b>
130	751
300	1883

### ***Determination of the Antibodies Specificity***

There is more than one way to test the specificity of the antibodies. One way is to test the IMS procedure with different antigens that show possible cross-reactivity. In this case of using *E. coli* antibodies, it is thinkable that the antibodies may react not only with *E. coli*, but also with other members of *Enterobacteriaceae*. However, this protocol did not seem to be a right choice because the duration of the project was too short to test all the possible bacteria. Another way is identifying the bacteria after IMS in beach samples. For this, the bacteria captured by IMS were identified using the genetic fingerprinting method or biochemical analysis method. The methods were Riboprinter (Qualicon, Wilmington, DE) and rapid API 20E (bioMerieux, Hazelwood, MO), respectively. Beach samples were filtered with the same procedure of IMS. After IMS, the suspension was filtered through 0.45 µm filter membrane. The filter membranes were put on m-TEC plates and incubated (44.5°C for 2 hours then 35°C for 18 hours). All the yellow colonies from the m-TEC plates, which contained about thirty colonies or so, were streaked onto nutrient agar plates and serially subcultured to check purity. From 20 to 24 hour cultures, colonies were inoculated into provided buffer (Riboprinter) or 0.85% NaCl solution (rapid API 20E). The remaining procedure followed the manufacturers' instruction. The flow chart of the specificity test is shown in Fig.16. The beach sample for the specificity test was randomly selected among the collected samples.

The bacteria captured by IMS were identified as *E. coli*, *Yersinia enterocolitica*, *Vibrio alginolyticus*, *Shigella spp*, and *Serratia plymuthica* using the rapid API 20E method. They are all enteric group bacteria. Another test using Riboprinter showed that the isolated bacteria by IMS procedure were *E. coli*, *E. coli* O157:H7, and some of them were not identifiable.

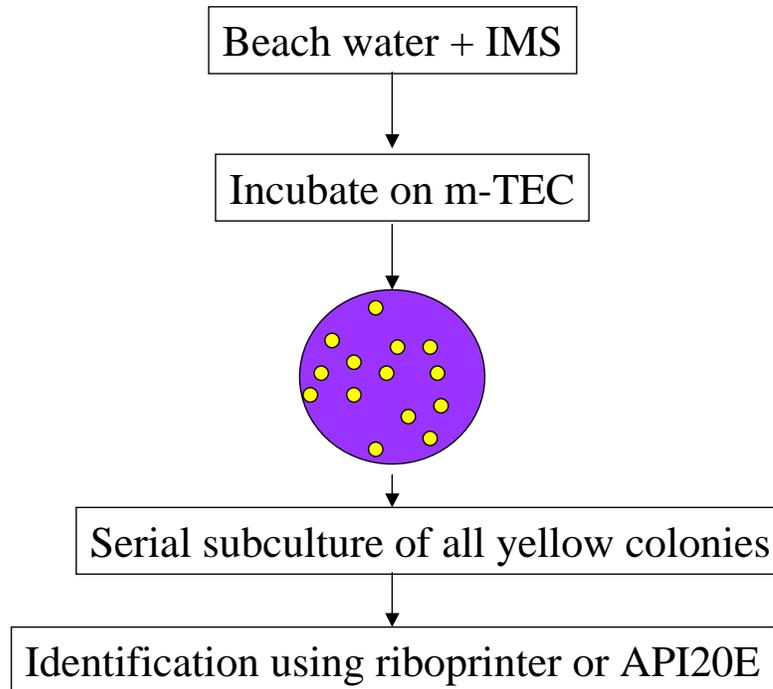


Figure 16. A scheme of identification procedure.

	1kbp	5	10	15	50
1	M62501-3	<None>	<None>	<None>	ECOR 202-101-S-1
2	I61901-3	DUP-3064	0.86	Escherichia coli ser. O157:H7 #	ECOR 202-101-S-2
3	B62501-1	DUP-3006	0.94	Escherichia coli ECOR	202-48-S-2
4	B62501-2	DUP-3006	0.94	Escherichia coli ECOR	202-48-S-2
2	S62601-1	DUP-3006	0.96	Escherichia coli ECOR	202-48-S-2 0.0
3	Ecoli	DUP-3002	0.80	Escherichia coli ECOR	202-104-S-3 0.07

Figure 17. An example of riboprinter results.

## Results of Pseudomonas testing

*Pseudomonas aeruginosa* is considered to exist ubiquitously in the environment. The illnesses most often associated with *P. aeruginosa* are dermatitis and folliculitis.

The purpose of this test was to examine the feasibility of IMS-ATP bioluminescence procedure as a platform technology to selectively determine a target biological agent in water. For this antibodies against *P. aeruginosa* were coated onto magnetic beads.

### 1) Bacteria, antibodies, and paramagnetic beads

*P. aeruginosa* strain (ATCC 27853) was acquired from MicroBioLogics, Inc. (St. Cloud, MN). Anti-*P. aeruginosa* Guinea pig serum and anti-*L. pneumophila* rabbit antibody were obtained from BioDesigns International (Saco, ME). Superparamagnetic beads with a mean diameter of 0.6  $\mu\text{m}$  were purchased from Bangs Laboratories, Inc. (Fishers, IN).

## 2) Preparation of antibody-magnetic bead complex

The entire preparation procedure was similar to the preparation step of *E. coli* antibody coated beads. There was minor modification in calculation of the ratio between the amount of antibodies and the amount of beads to achieve proper saturation. This was because the concentration of *P. aeruginosa* and *E. coli* were different. Briefly the procedure is as follows. Superparamagnetic beads were coated with anti-*P. aeruginosa* serum. The adsorption procedure was used according to the manufacturer's instruction (Bangs Laboratories). The bead suspension was diluted 5 times (original concentration 10% solid) and then the beads (0.2ml) were removed from the suspension with a magnet. They were rinsed with sterile distilled water twice and then resuspended in 0.68 ml of PBS (pH 5.5) containing 0.12ml of antiserum or antibody. The remaining procedure was the same as *E. coli* antibody coating procedure.

## 3) Sample preparation

*P. aeruginosa* were grown at 35°C in Tryptic Soy Broth (Difco Laboratories, Detroit, MI). Tryptic Soy Agar was used for the plate counts (Difco).

## 4) Efficiency of IMS

An overnight culture of *P. aeruginosa* was collected by centrifugation (2500 rpm for 2 min), resuspended in PBS or 0.1% peptone water and vortexed for 1 min. The bacterial concentration was adjusted to about  $10^8$  CFU/ml. The cell suspension (0.1ml) was inoculated into water sample (20ml).

To test the efficiency of magnetic capture, the inoculated water sample was serially diluted (10 fold) with either PBS or 0.1 % peptone water. Immediately after dilution, the efficiency of magnetic separation was tested by adding 5  $\mu\text{l}$  of antibody-bead

complex per ml of water sample and mixing for 15 min. After the mixing, the tubes were placed in a magnetic particle separator (Dynal) for 10 min in order to separate the magnetic beads from the sample. The beads were washed twice with PBS and resuspended in PBS. The immunomagnetically separated bacteria were measured by both the ATP bioluminescence method and also compared with the plate count method. All the tests were done in triplicate and a blank control was done with PBS.

### **5) ATP bioluminescence**

The number of immunomagnetic captured bacteria were determined with a microluminometer (model 3550, New Horizons Diagnostics [NHD], Columbia, MD). The sample was filtered (0.05-1ml) through a Filtravette (NHD), which is a combination of a filter and a cuvette. The Filtravette was inserted into the microluminometer, and 50 µl of the bacterial cell-releasing agent (NHD) was added to lyse bacterial cells retained on the surface of the filtravette. The released bacterial ATP was mixed with 50 µl of luciferin-luciferase (NHD), and the light emission was recorded. The unit of measurement used was relative light units (RLU), and the result was expressed as RLU/ml after dividing by the filtered volume.

### 6) Sensitivity of the IMS procedure

The sensitivity of the IMS procedure for detecting *P. aeruginosa* was measured by two methods. The sensitivities measured by ATP bioluminescence and plate count are presented in Figure 18 and 19, respectively. The comparison of the level of *P. aeruginosa* captured by IMS with the initial level of bacteria followed a very similar pattern in both cases. However, the cell measurements of *P. aeruginosa* after IMS were slightly higher than the initial level of bacteria. It might be due to the cell growth during the procedure. These results suggested that the magnetically captured bacteria can be measured rapidly with the ATP bioluminescence method.

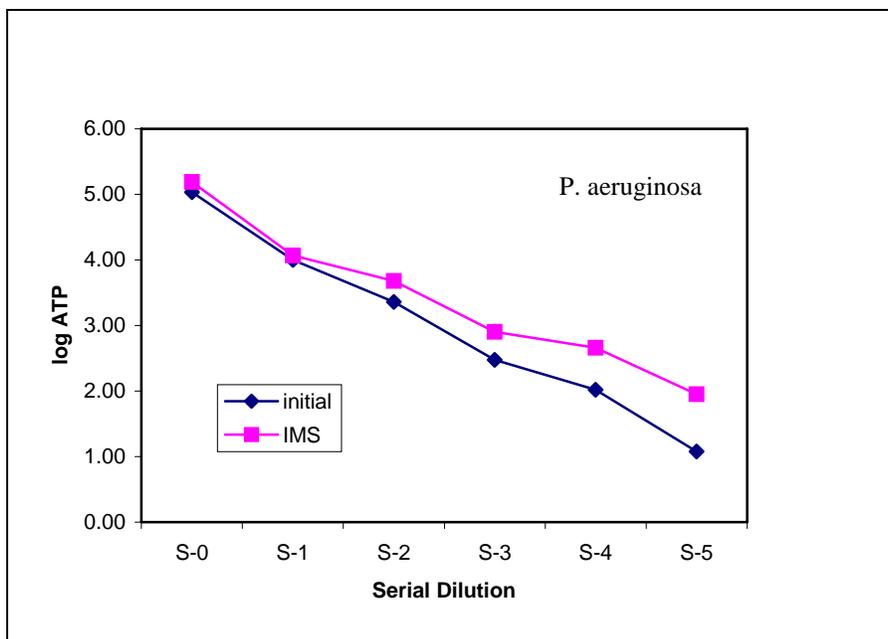


Figure 18. Determination of the sensitivity of detecting *P. aeruginosa* by IMS. The bacterial level was measured by ATP bioluminescence.



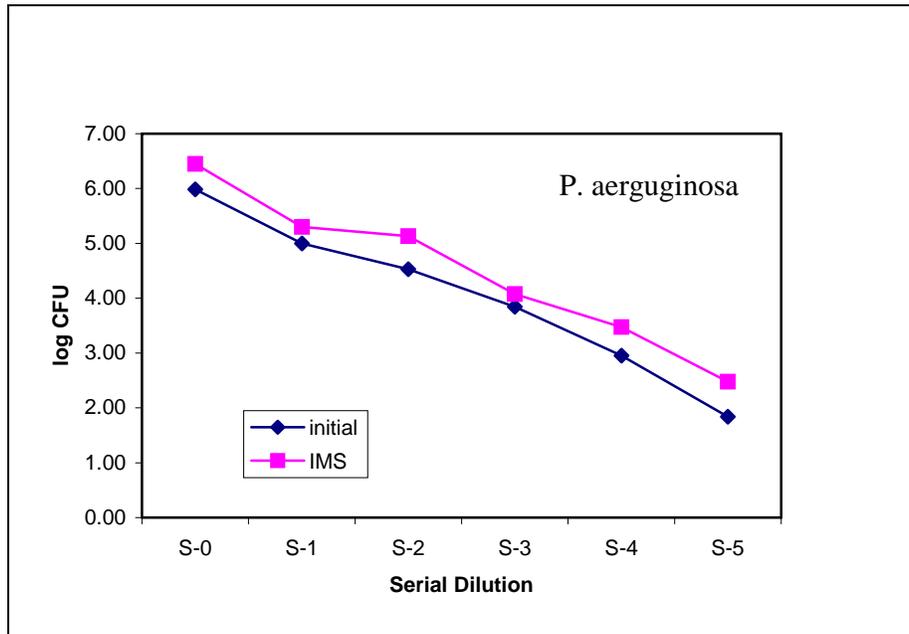


Figure 19. Determination of the sensitivity of detecting *P. aeruginosa* by IMS. The bacterial level was measured by plate counts. The unit is CFU/ml.

## Conclusions

This was the first study that investigated the feasibility of IMS and ATP bioluminescence to detect *E. coli* in beach samples rapidly. The entire procedure can be done within one hour without an enrichment step. It showed a good correlation with the traditional plate count method. The method was sensitive enough to detect the level of *E. coli*, which is of interest (130, 300 CFU/100ml).

In order to make this method more feasible in the field and to increase sensitivity while having good specificity, we recommend the following for further study:

- Expand database, i.e., more samples
- Simplify the procedure
- Use mixed antibodies from different vendors
- Coat beads with different ratio of antibodies to beads
- Specificity test with other enteric groups

## Appendix A

### Rapid *E. coli* Test Procedure

#### **Step 1: Concentration of bacteria by serial filtration**

After vigorous shaking a sample bottle, pour 100 – 500 ml of a beach sample into a combined unit of prefiltration and filtration, which is connected with a vacuum pump (See Fig. 7). After a complete filtration, add 10ml of PBS (phosphate buffered saline) to rinse the combined filtration unit.

#### **Step 2: Recovery of deposited bacteria**

Two methods can be used to resuspend the deposited bacteria into a buffer solution. One is removing the membrane and shaking in buffer. The other is backflushing. For the removing membrane method, open the final filter holder and take out the filter membrane with sterile forceps. Put the membrane into a sterile small jar and add 10 ml of PBS with Tween 20 (PBST). Shake the jar vigorously and transfer the entire liquid into a sterile test tube. Rinse the jar with 2ml of PBS and transfer into the same test tube.

For the backflushing method, push the same amount of PBST using a sterile syringe through the final filter holder reverse direction. Collect the liquid in a sterile test tube. Rinse the final filter holder with 2ml of PBS and collect the rinsed buffer into the same test tube.

#### ***Step 3: Addition of antibody-coated beads and mixing***

Add the prepared antibody-coated magnetic beads (100  $\mu$ l) into the test tube. Mix it for 15 min at 60 rpm at ambient temperature (not in direct sunlight).

#### ***Step 4: Magnetic separation***

Put the test tube in a magnetic particle concentrator (MPC) for 10 minutes. After the magnetic separation, discard the liquid and resuspend\* into new PBS buffer (10ml). Repeat the same procedure.

**Step 5: Concentration to a small working volume**

At the final stage, resuspend\* the pellet, which is bacteria-antibody-bead complex into 1ml PBS buffer and transfer to a microcentrifuge tube (\*while resuspending, the tube is removed from the MPC).

***Step 6: Removal of any remaining unwanted cells***

Put the microcentrifuge tube in a small MPC. After the magnetic separation, discard the liquid. Take off the tube from the MPC and add 50  $\mu$ l of SRA (somatic cell releasing agent) to remove any possible remaining nonbacterial cells. After magnetic separation, discard the SRA. Rinse the pellet with 0.2ml of PBS and discard the PBS.

**Step 7: Lysing of the captured bacteria**

Take the tube from the MPC and add 50 µl of BRA (bacterial cell releasing agent) and mix well. After magnetic separation, transfer the entire liquid portion into a cuvette. At this stage, the ATP derived from the captured bacteria is released into the liquid portion.

***Step 8: Addition of luciferin-luciferase and measurement of light emission***

Put the cuvette into the drawer of a microluminometer. Add 50 µl of LL and close the drawer. Record the light emission.

***Step 9: Estimation of plate count using correlation equation***

Use the RLU and the correlation equation to get the estimate of CFU and the lower and upper confidence interval.

***Necessary equipment and materials for testing E. coli from a beach water sample***

Sampling bottle (1L)

Microfil V: A disposable filtration device that comes with 0.45 µm pore size filter membrane. Replace the membrane with 20 µm pore size filter membrane for prefiltration.

Filter holder (Ø 47mm)

20 µm pore size filter membrane (Ø 47mm)

0.45 µm pore size filter membrane (Ø 47mm)

Filtering flask (1L)

Vacuum pump

Mixer

Small jar with a lid

Pipettes (10ml)

PBS

PBS with Tween 20

Test tubes

Microcentrifuge tubes

Micropipette

Micropipette tips

E. coli antibody coated magnetic beads

Magnetic particle collector (2)

Microluminometer

Luciferin/luciferase

SRA/BRA

Forceps

Container for collecting liquid waste

## Appendix B

### Estimated Cost of the Test

We have been frequently asked by health department personnel and other people who are interested in our method on how much the rapid *E. coli* testing costs. The exact cost of a test is hard to calculate. However, following is an estimate of the major consumable items that are needed for a test. The estimated cost per test is about \$8. It does not include items that most laboratories commonly have such as gloves, pipettes, tips, tubes and buffers. It does not include the cost for the time of the technician. Nor does it include the time for coating the beads.

<u>Item</u>	<u>Cost per package</u>	<u>No. of tests per pk.</u>	<u>Cost per test</u>
Cuvette & Luciferin/luciferase	\$ 300	\$ 100	\$ 3
Antibody	130	14	.09
Magnetic beads	202	50	.04
Microfil V	69	24	3
Prefilter membrane	100	100	1
Filter membrane	73	100	.70
			<hr/>
			~ \$ 8