Covalently-linked immunomagnetic separation/adenosine triphosphate technique (Cov-IMS/ATP) enables rapid, in-field detection and quantification of *Escherichia coli* and *Enterococcus* spp. in freshwater and marine environments

Christine M. Lee[1], John F. Griffith, William J. Kaiser[2] and Jennifer A. Jay[1]

Abstract

Developing a rapid method for detection of fecal pollution is among the critical goals set forth by the Environmental Protection Agency (EPA) in its revision of water quality criteria. In this study, we examined the efficacy of using covalently-linked antibody-bead complexes to measure *Escherichia coli* or Enterococcus spp. in fresh and marine water. Water samples analyzed using a covalenty-linked immunomagnetic separation/adenosine triphosphate quantification technique (Cov-IMS/ATP) and culture-based methods yielded good correlations for *E.coli* ($R = 0.87$) and *Enterococcus* spp. ($R = 0.94$), with method detection limits below EPA single-sample recreational water quality standards. Cov-IMS/ATP was also used as a field method to rapidly distinguish differential loading of *E.coli* between two stream channels to their confluence and may also have utility as a source-tracking tool. Due to its portability and economy, Cov-IMS/ATP is a potential alternative rapid detection method to expensive, laboratory intensive methods such as quantitative polymerase chain reaction (qPCR), particularly in circumstances where beach sites are distant from the laboratory and resources are scarce.

Introduction

Rapid detection methods and alternative indicators are at the forefront of developing initiatives to ensure clean and safe water quality (USEPA 2007). Pollution from diverse and multiple sources have historically confounded efforts to preserve coastal and freshwater environments. The development of rapid detection assays, allowing enumeration of microbial contaminants as quickly as one hour and enabling a more diverse suite of organisms to be studied, has progressed significantly in recent years (Griffith and Weisberg 2006). Among these explored technologies are qPCR (Haugland *et al*. 2005, Caldwell *et al*. 2007, Shanks *et al*. 2008), antibody fluorescence (Garcia-Armisen *et al*. 2005, Zimmerman *et al*. 2009), enzymatic methods (Lebaron *et al*. 2005), flow cytometry (Caruso *et al*. 2008, Hammes *et al*. 2008), and immumomagnetic separation/ATP (IMS/ATP) quantification (Lee and Deininger 2004, Bushon *et al*. 2009a). Immumomagnetic separation /ATP is particularly advantageous because it is field-portable (eliminating travel time), the most rapid of the mentioned processes (less than one-hour processing time), and viability-based.

Immunomagnetic separation has been used as a selection step in measuring protocols for *Giardia* (USEPA 2005, Hsu and Huang 2007), *Cryptosporidium parvum* (Rochelle *et al*. 1999), and *E.coli* O157 (Tomoyasu 1998). Complexes, comprised of antibodies sorbed to magnetic beads, are used to capture and separate out a target population with the application of a magnetic field. After targets are isolated, cells are ruptured and ATP is quan-

[1] *University of California, Civil and Environmental Engineering Department, Los Angeles, CA*
[2] *University of California, Electrical Engineering Department, Los Angeles, CA*

tified through the addition of luciferin/luciferase. Luciferase, in the presence of luciferin and oxygen, catalyzes a reaction that consumes ATP and emits light as a by-product. Intensity of this light emission is measured by a luminometer and correlated to cell concentration. Immumomagnetic separation /ATP has been the basis for quantifying pathogenic or bacterial activity and/or contamination in numerous applications such as in the food industry (Siragusa *et al*. 1996, Tu *et al*. 2000, Murphy *et al*. 2007,) in veterinary applications (Watarai *et al*. 2005), in drinking water (Deininger and Lee 2001, Delahaye *et al*. 2003), and in wastewater (Allegra *et al*. 2008, Bushon *et al*. 2009b). Though an IMS/ATP-based assay has numerous advantages (portable, specific, and economical), this method has been relatively understudied.

Recently, significant progress has been made to translate the applicability of IMS/ATP to recreational water quality monitoring; notably, in 2004, Lee and Deininger were the first to publish such findings, which describe the development of this assay for monitoring *E.coli* in recreational freshwater environments. Bushon *et al*. were able to adapt this work further, optimizing Lee and Deininger's protocol to measure *Enterococcus* spp. in freshwater (Bushon *et al*. 2009a) and both *E.coli* and *Enterococcus* spp. in wastewater influent (Bushon *et al*. 2009b). The selective magnetic bead-antibody complex used to isolate target organisms out of an environmental sample utilizes hydrophobic sorption as the primary attachment mechanism. This complex, however, can be destabilized fairly easily, even with physical (pipetting/vortexting) or chemical (use of a nonionic detergent) treatment, which may explain why the method has not been validated in a marine system.

The presented work achieves three significant steps towards using an IMS/ATP-based assay in recreational water quality monitoring. We developed a more robust antibody-bead complex that is covalently linked. When this complex is coupled with ATP quantification (Cov-IMS/ATP), we show that it can be used for marine and fresh water quality analysis for *E.coli* and *Enterococcus* spp. and instrumented as a field-portable system that adaptively tracks faecal indicator bacteria (FIB) in a local impaired watershed. These combined improvements are also examined in the context of a more efficient and effective tiered source-tracking strategy.

Methods

Antibody-Dynabead Biosorbent

Antibodies specific to each FIB were used to generate biosorbents that targeted *E.coli* (Cat#B65001R) or *Enterococcus* spp. (Cat#B65173R, ent; Meridian Life Sciences 2009a,b). *Escherichia coli* antibodies are selective to *E.coli* with an O or K antigen, which represent a broad range of *E.coli* serotypes (Ørskov *et al*. 1977) in the environment. The manufacturer's datasheet reported potential cross-reactivity with *Enterobactericeae*, such as *Shigella* and *Salmonella*. This cross-reactivity was tested by Lee and Deininger (2004), and on average accounted for ~10% of the bound population and, with respect to *Enterococcus* spp. antibodies, minimal cross-reactivity was detected in a similarly constructed polyclonal immunoglobulin (IgG) by Caruso *et al*. 2008. For these reasons, specificity was not independently explored in this work.

Dynabead particles (Invitrogen, 142.04, M-280) are uniform, superparamagnetic, polystyrene beads coated with a polyurethane layer. Dynabeads are functionalized with sulphonyl ester surface groups that permit chemical covalent attachment (amino or sulfhydryl) of ligand proteins such as IgG. The typical particle diameter is 2.8 µm and has an average density of 1.4 g/cm^3. The hydroxy groups on the Dynabeads are activated using p-toluensulphonyl chloride.

Coupling Protocol

Three-hundred fifty µl Dynabeads were washed in borate buffer (0.1 M, pH 9.5) and separated from solution using a rare earth magnet. After the second wash, clean Dynabeads were added to the 55 µl IgG solution. The final suspension was incubated at 37^{o}C on a rotating mixer for 16 to 24 hours. The final mixture was washed and stored in Bovine Serum Albumin (BSA) buffer at continuous rotation at 4^{o}C.

Adsorption-based IMS/ATP protocol was obtained from previous works (Bushon *et al*. 2009a, Lee and Deininger 2004). Robustness of the covalently-coupled antibody-bead complexes presented in this work was compared to that of adsorption-based antibody-bead complexes. This was accomplished by measuring the integrity of the attachment that was sustained after two mixing treatments: pipette-mixing and vortex-mixing, each for 10 seconds and for 2 washes. Uncoupled or detached IgG in the supernatant was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Cat# 23225).

Culture-Based Methods (IDEXX and EPA Methods 1600/1603)

Standard methods of measuring FIB concentrations were IDEXX and membrane filtration. These protocols were used to calibrate Cov-IMS/ATP to standard units; IDEXX Quanti-Tray and Colilert-18 (*E.coli*) or Enterolert (enterococci) yielded values of *E.coli* or enterococci concentrations in Most Probable Number (MPN)/100 ml in freshwater. Environmental Protection Agency membrane filtration methods 1600 and 1603 were used for marine sample measurements of enterococci (USEPA 2006a) and *E.coli* (USEPA 2006b), respectively, and yielded values in colony forming units (CFU)/100 ml. Both IDEXX and membrane filtration methods were used because there is considerable interference when using IDEXX to enumerate marine sample concentrations (Pisciotta *et al*. 2002, Griffith *et al*. 2006) and were conducted on the resuspension and converted to per 100 ml original sample volume for analysis. Previous works have shown that IDEXX and membrane filtration methods are comparable (Noble *et al*. 2003a,b; 2004).

Cov-IMS/ATP Target ATP Assay

Samples were incubated with anti-*E.coli* or anti-ent biosorbent for 30 minutes at ambient temperature. A magnetic separator was used to separate biosorbent with bound targets from remaining solution. The bound complexes were washed with phosphate buffered saline (PBS) followed by 200 µl of Somatic Cell-Releasing Agent to eliminate materials that could cause interference. The bound product was lysed using 50 µl Bacteria Cell-Releasing Agent (BRA) and treated with 50 µl luciferin/luciferase to initiate the light-emitting reaction. A luminometer (New Horizons Diagnostics, model 3550) was used to quantify the intensity of emitted light. A minimum of three measurements was collected per sample, consisting of a 30-second integration average for each value of light intensity in relative light units (RLU). Processing time of each sample was approximately 36 minutes each, as numerous samples were processed concurrently.

Field Application

Several field-adapted components were integrated into the assay protocol to facilitate on-site processing. A rapidly deployable field station consisted of a portable lab bench (Coleman Model 2790-490), deep-cycling battery, AC/DC inverter, Millipore vacuum/pressure pump (#WP6111560), cordless power drill (DeWalt DC987K2), Rotomix Orbital Shaker (Fisher Scientific #12-815-2D), filtration apparatus, several small coolers, luminometer, and general supplies. Antibody-bead complexes were kept in continuous suspension manually or using the shaker. Mixing was achieved through repeated pipetting. Constant rotation was achieved by using a battery-powered drill and, while revolutions per minute (rpm) may have varied somewhat (55 to 65 rpm), care was taken to ensure that bead-containing tubes were being gently mixed. All equipment was able to be packaged into a Pelican case (Pelican Products Model 1620) and transported in a standard hatchback vehicle or four-door sedan.

Field Sampling

Freshwater Site

Will Rogers State Beach is located in Santa Monica, California. Two urbanized channels, West Channel and Entrada, drain into WRSB through the Santa Monica Canyon channel system. During dry season (April-October), a low-flow diversion is used to help mitigate impact to the beach by diverting channel flow to a wastewater treatment plant. Potential sources of FIB include significant bird populations, algae lining the channel, and storm drains (0.3 to 1.2 m diameter openings) with dry and wet weather runoff. There is often a pool of standing water at the WRSB channel outlet.

Samples were collected on multiple field days during August 2008–February 2009 from 11 sites in the Santa Monica Canyon channel system; 6 sites from Entrada (E1 - E6), 4 from West Channel (W1 - W4), and 1 from the confluence (C1), were sampled at different times throughout the day (Figure 1). A varying subset of the total collected on each field day were analyzed using both Cov-IMS/ATP and standard culture-based methods.

Coastal Survey

Anti-*E.coli* or anti-ent biosorbents were tested in marine water samples from beaches in Santa Monica Bay and/or Orange County. Field sites were determined by evaluating historical FIB data obtained from Heal the Bay (www.healthebay.org). Several beaches (denoted below with an *) were on average in exceedance of the health standard (single sample standards at 104 MPN/100 ml for enterococci and 400 MPN/100 ml for *E.coli*) for FIB (USEPA

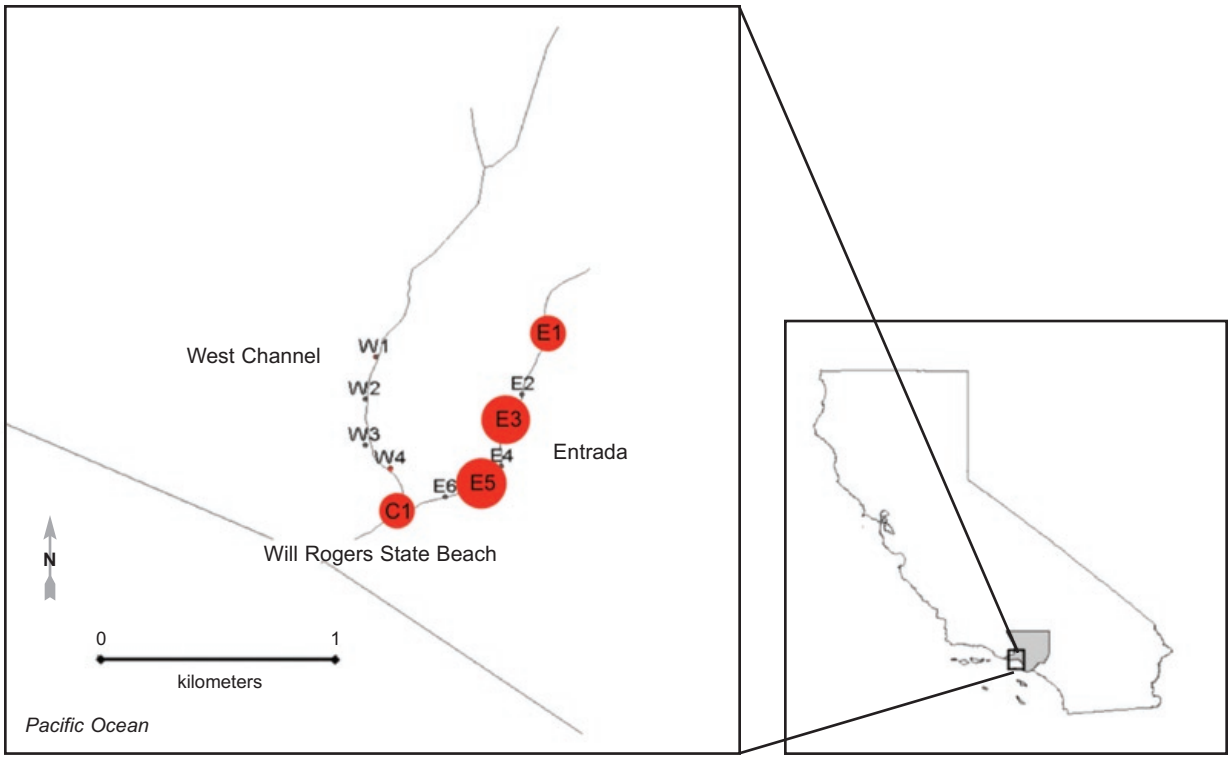

Figure 1. Map of sampling locations in the Santa Monica Canyon Channel with relative concentrations of *E. coli.*

1986). The sampled sites included *Puerco Beach, Surfrider Beach, Topanga Canyon Beach, *Dockweiler Beach, *Santa Monica Beach at the pier, and Doheny State Beach in South Orange County during the Southern California Coastal Water Research Project (SCCWRP) epidemiological study in summer 2008.

Sample Processing

Approximately 500 to 1000 µl per sample were filtered-concentrated and analyzed using Cov-IMS/ATP and culture-based methods. Samples were filtered once through a 20-µm pore size filter (Millipore, NY2004700) to remove large particles and debris followed by a 0.45-µm filter (Millipore, SA1J792H5) to capture bacteria. Bacteria were extracted into 5 to 10 ml of 0.1 M, pH 7.4 PBS from the 0.45-µm filter through resuspension by manual shaking or vortex for 1 minute. For a subset of samples, the 20-µm filter was enumerated using a membrane filtration method to account for organisms that could possibly be attached to the surfaces of trapped debris. One to 1.5 ml of the resuspension was added to the anti-*E.coli* or anti-ent biosorbent, incubated on a rotating mixer for 30 minutes at ambient temperature, and processed according to methods described previously. Remaining resuspension was analyzed using IDEXX or membrane filtration for *E.coli* or enterococci, respectively. Field blanks were prepared by sterilizing samples from the field site and processed after filtering and resuspending in PBS, while lab blanks consisted of measuring ATP in sterilized PBS or milli-Q water.

Luminometer Calibration

The luminometer was calibrated using standard ATP solution (Sigma Aldrich, FLAAS-5VL) diluted to 0.22 to 43 µM (actual mole amount measured ranged from 11 to 434 picomole in 50 ml) to account for shift in signal due to inherent variability of the instrument.

Effect of Hold Time

One sample at site E5 was taken at approximately 10:00 a.m.; 500 ml of sample were filtered and resuspended into 10 ml of PBS. One ml of resuspension was measured after 0 minutes, 15 minutes, 2 hours, and 6 hours of incubation on ice to determine the effect of holding time on ice on of target cell ATP.

Statistics

Spearman rank correlation coefficients were calculated in Excel. SPSS statistical software was used

to determine correlation coefficients as well as p-values for the relationship between culture-based method results and Cov-IMS/ATP results.

Results

Covalent (COV) and Hydrophobic Adsorption (ADS) and Antigen Capture Efficiency

Attachment efficiency between the IgG antibodies and magnetic beads were comparable between COV and ADS-based complexes (~80% attachment, when comparing concentrations of antibody prior to and post coupling). Retention of antibodies to the magnetic beads after undergoing vortexing or pipetting treatments was also compared by measuring IgG concentrations in the supernatant of the respective complexes (Figure 2). For ADS complexes, the complex retained 41.6% (pipette-mixing) and 11% (vortexing) of the antibodies that attached, whereas there was no detectable loss when COV-biosorbents underwent the same treatment. Percent retention for COV-biosorbents was calculated based on one half the detection limit of the ligand assay used to measure antibody concentrations, which corresponded with a 1.1% loss of antibody. The percent loss values were averaged from duplicates. On a separate of set of complexes (that did not undergo physical treatments, only sample processing), *E.coli* concentrations were also measured using IDEXX prior to and after incubation; COV-beads bound more than twice as many *E.coli* cells as ADS-beads; 67% (COV) versus 31% (ADS) of the added *E.coli* population were measured by mass balance to have attached to the antibodies $(E.coli_{added} - E.coli_{supernatant})/(E.coli_{added})$.

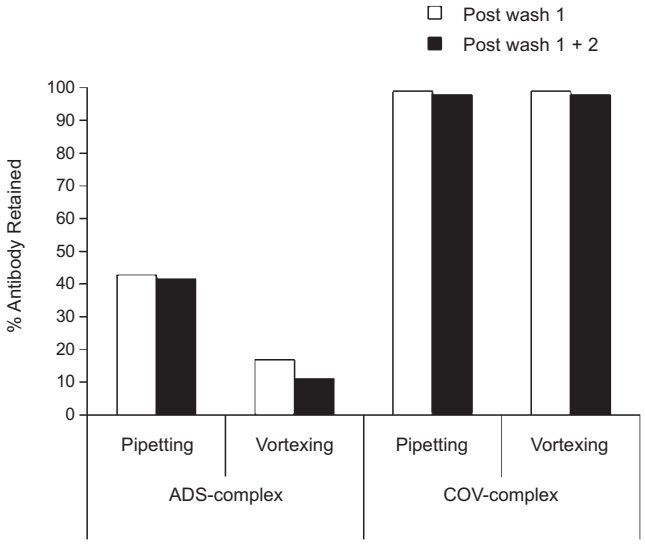

Figure 2. Physical treatments (mixing via pipetting or vortexing) destabilises the antibody-bead complex that is generated through hydrophobic sorption.

Effect of Hold Time on Sample ATP

The amount of measured ATP per culturable *Enterococcus* spp. cell decreased when the sample resuspension was held on ice. Solution temperature was also recorded each time a sample was analysed. Results reveal that target cellular ATP measurements decreased by 26% within 15 minutes of being stored on ice and 30% after 2 hours (Figure 3). At 6 hours, a decrease of 38% from the 0 time point of sample ATP values was observed (Figure 3).

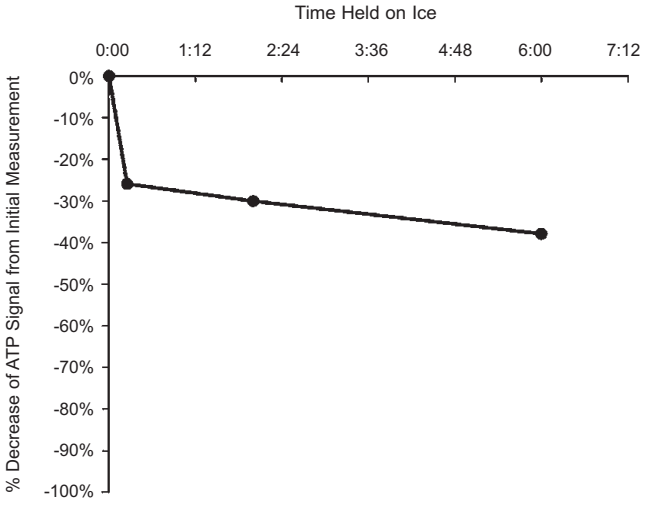

Figure 3. Decrease in relative light units signal as a function of hold time on ice.

E.coli and *Enterococcus* spp. Calibration between Cov-IMS/ATP and Traditional Methods

Freshwater samples were measured using Cov-IMS/ATP and IDEXX while marine samples were enumerated with Cov-IMS/ATP and membrane filtration. Both analyses were conducted on filtered resuspension of the sample; plotted values were adjusted to units of 100 ml original sample using amount of volume filtered and quantified using each respective method. The calibration relationships are summarised as follows:

E.coli:

$$\text{Log RLU/100 ml} = 0.66 \text{ Log MPN or CFU/100 ml} + 3.48,\ R = 0.87 \qquad \text{Eq.1a}$$

Enterococcus spp:

Log RLU/100 ml = 0.69 x Log MPN or CFU/100 ml + 3.93, $R = 0.94$ Eq.1b

Cov-IMS/ATP (RLU/100 ml) correlates well with both *E.coli* and *Enterococcus* spp. in both environmental water samples ($R = 0.87$ and $R = 0.94$, respectively; Figures 4 and 5). The lower detection limit of *E.coli* based on the combined dataset of freshwater and marine samples was approximately 38 cells per 100 ml sample and the calculated limit for *Enterococcus* spp. is 25 cells per 100 ml. These detection limits were determined using the method detection limit document generated by the EPA (USEPA 1986b). Calibration of instrument with ATP standards was conducted frequently to ensure consistency of measurements by luminometer.

Water Quality Classification

We used Equations 1a and 1b to evaluate how consistently Cov-IMS/ATP values predicted exceedances and non-exceedances in water quality standards. Correct exceedance (Quadrant II) and correct non-exceedance predictions (Quadrant III) are specified by the points in the upper right and lower left quadrants of the plot, respectively (Figures 5 and 6). False positives (Quadrant I) occurred when Cov-IMS/ATP correlation indicated a CFU or MPN/100 ml value that exceeded the health standard when culture-based methods did not, and false negatives (Quadrant IV) occurred when Cov-IMS/ATP indicated a CFU or MPN/100 ml that did not exceed the health standard when culture-based methods did

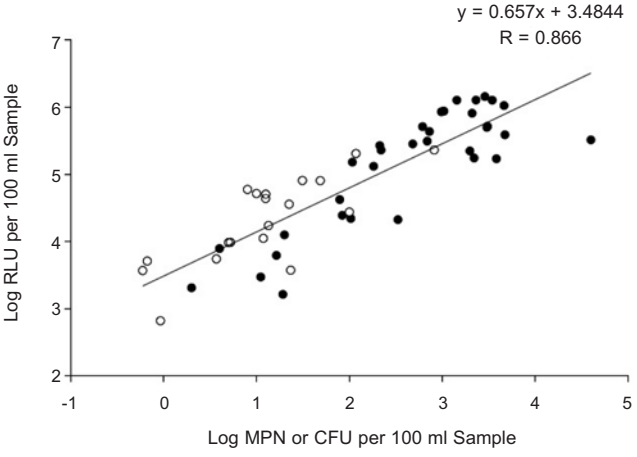

Figure 4. *E. coli* correlation between culture-based methods (IDEXX or membrane filtration, MPN or CFU/100 ml sample) and Cov-IMS/ATP (Relative Light Units – RLU/100 ml sample). $R = 0.87$.

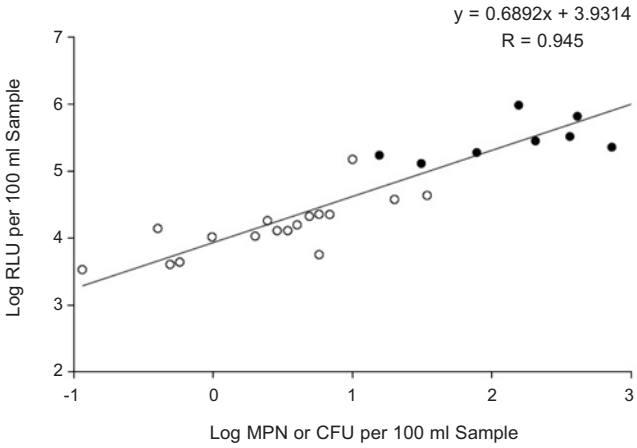

Figure 5. *Enterococcus* spp. correlation between culture-based methods (IDEXX or membrane filtration, MPN or CFU/100 ml sample) and Cov-IMS/ATP (Relative Light Units – RLU/100 ml sample). $R = 0.94$.

(Figures 5 and 6). The percent correct classification ranged from 87 and 94 for *E.coli* (n = 52) and *Enterococcus* spp. (n = 29), respectively. The solid and dashed lines represent the intersection of sample exceedance with respect to the single standard in freshwater and marine recreational waters, respectively (USEPA 1986; Figures 6 and 7).

Filter Recovery and Analysis

There were no colonies on the enumerated 20-µm filters that were used for pre-filtration, though solids and particulates were visibly collected and separated from the volume that underwent the 0.45-µm filtration. Recovery (comparing the resuspension to the original sample) was determined for 10 samples and ranged from 60 to 85%. Several low-concentration

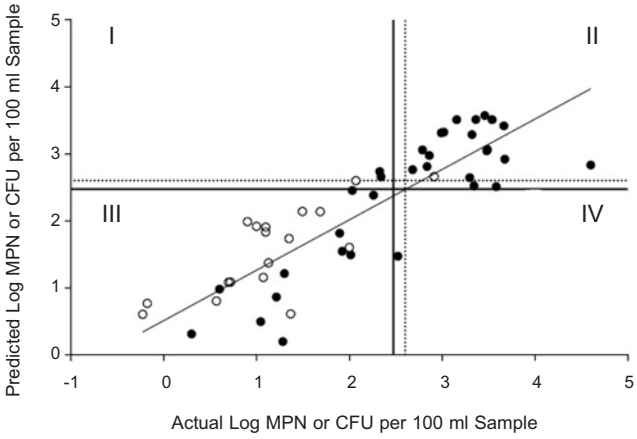

Figure 6. *E. coli* concentrations, as predicted by Cov-IMS/ATP, have a 92% correct classification rate (n = 52).

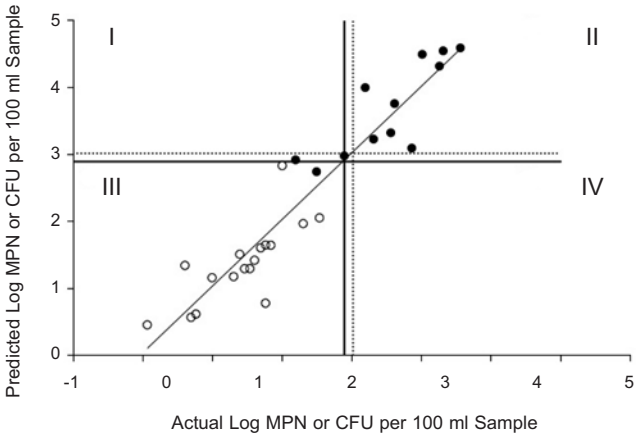

Figure 7. *Enterococcus* spp. concentrations, as predicted by Cov-IMS/ATP, have a 94% correct classification rate (n = 31).

samples appeared to have a >100% recovery efficiency, though this may be accounted for due to an increased detection limit from concentrating a larger volume. It is important to note that, while filter-concentration can still be further optimized, it significantly improves the detection limit of the method.

Cov-IMS/ATP as a Tool for Adaptive Sampling

When this method was used in-field, we were able to determine differences in the contributing loads at the channels confluence: the *E.coli* concentration at sampling site E6 was approximately 20-fold greater than that of W4 (Figure 1). When this model was applied retrospectively to theremainder of samples on that date, there were significantly higher inputs of *E.coli* from Entrada than from West Channel. Further analysis of Entrada inputs revealed that the concentration of *E.coli* increased nearly 1.4 times between sampling sites E1 and E3 (from 921 to 1,254 MPN/100 ml) and increased slightly (~ 4%) at E5 (from 1,254 to 1,300 MPN/100 ml) before reaching the confluence C1 (at 913 MPN/100 ml).

FIB results from Santa Monica Canyon channel system; results from sites W4, E5, and C1 were also applied to a weighted average mixing equation as conservative tracers. Flow calculations were based on measurements taken on the channel in-field (depth at multiple points along the flow, width of the flow stream, velocity), which were highly variable due to the heterogeneities in surface roughness and the presence of debris. Both assays (IDEXX and Cov-IMS/ATP) predicted C1 values that were within close range at +14% and -28%, respectively, of actual values as measured by each technique.

Statistical Analysis and Reproducibility

The Spearman rank correlation coefficients were calculated for log-transformed values of RLU and MPN or CFU and significantly correlated for *E.coli* ($\rho = 0.94$) and *Enterococcus* spp. ($\rho = 0.95$). Spearman correlations were conducted on all samples, including blanks. These values were considered at a significance level of 0.01 for the respective sample set. Regression coefficients also reflected a good fit to the data for *E.coli* ($R = 0.87$) and *Enterococcus* spp. in ($R = 0.94$) with p-values <0.001 for both *E.coli* and *Enterococcus* spp. Reproducibility was also examined in controlled lab experiments, in which four samples replicates were conducted. Samples were comprised of a diluted lab culture (*E.coli*, ATCC#12014) and were processed according to the target ATP assay. The sample values exhibited a 6.8% error (ratio of standard deviation to mean) for four samples. Additional field samples and duplicates reflected a range of variability from 6 to 25%.

Discussion

COV-Biosorbents and ADS-Biosorbents Robustness

Cov-IMS/ATP has an added robustness over previous antibody-bead complexes employed in recreational water quality measurements with IMS/ATP (Lee and Deininger 2004; Bushon *et al*. 2009a,b). Because antibodies are covalently linked to paramagnetic beads, they are less likely to disengage during sample processing than are adsorption-based complexes. More specifically, we observed a minimal loss of antibody attachment in COV-beads (<1.1%) in comparison to ADS-beads (89% for vortex-mixing and 57.5% for pipette-mixing). Previous documentation is consistent with this finding, whether the complexes are undergoing physical (Bangs Laboratory 1999; 2002a,b) or chemical (Meisenberg and Simmons 2006; Bushon *et al*. 2009a,b) treatments. It is possible that this detachment contributed to the high percentage of false negatives observed in previous work (Griffith *et al*. 2004) as well as the poorly correlated measurements between IMS/ATP and culture-based methods, likely due to the presence of interfering materials, from one of the wastewater treatment sites reported in Bushon *et al*. 2009b.

Variability and Use of Cov-IMS/ATP Technique as a Classification Tool

One reason for apparent overestimation of cells (false positives) with Cov-IMS/ATP compared to culture-based methods may be non-specific binding that results in the capture and quantification of organisms that are cross-reacting with the antibody. Another possible factor is that Cov-IMS/ATP is capable of measuring viable but non-culturable cells, which are not accounted for in membrane filtration or IDEXX. Recent work with the use of antibodies has shown that they can be used to visualize both culturable and non-culturable target organisms (Zimmerman *et al*. 2009). Furthermore, Cov-IMS/ATP can potentially be used for monitoring in marine waters, which is a significant addition to the capacity of previous IMS/ATP assays. Validation in marine samples may be largely due to the added robustness of the biosorbent as well as the filtered resuspension into phosphate buffer; filtering improves the detection limit and allows quantification of targets even in low concentrations in marine water.

Adsorption-based IMS/ATP has recently expanded to measurement of enterococci in freshwater (Bushon *et al*. 2009a) and FIB in wastewater samples (Bushon *et al*. 2009b) and this work has shown that Cov-IMS/ATP can be applied in marine systems for *E.coli* and *Enterococcus* spp. *Enterococcus* spp. has recently become the preferred indicator (with regards to standard FIB) for marine sites (Noble *et al*. 2003a,b), though *E.coli* is still relevant in freshwater (Kinzelman *et al*. 2003) and may still be useful in marine systems receiving significant, urban-impacted freshwater inputs (Noble *et al*. 2006, He and He 2008).

In-Field Processing Improves Efficacy in the Tiered Approach through Rapid Adaptive Sampling

A rapid, portable method has been identified as an important tier to the multi-tiered approach to source-tracking (Field and Samadpour 2007) which has conventionally relied on day-old culture-based results (Boehm *et al*. 2003, Noble *et al*. 2006) or on hydrologic events as the intermediate tier. The inefficiencies of traditional tiered approach may arise from the temporal variability of FIB in aquatic environments (Boehm *et al*. 2002, Boehm and Weisberg 2005, Boehm 2007) as well as differences in the survival behavior of FIB and the pathogens they proxy (Geldenhuys and Pretorius 1989, Lund 1996, Lemarchand and Lebaron 2003, Harwood *et al*. 2005). After determining that a higher *E.coli* input was originating from Entrada than from West Channel, we were able to assess load distribution throughout Santa Monica Channel (Figure 1). A map such as this, which can be generated on-site, can provide important, near real-time insight to where additional samples should be collected. This adaptive sampling approach may significantly improve the efficiency of a tiered method, especially as Cov-IMS/ATP is further streamlined and field-packaged, and assays for additional targets are developed.

Processing a sample on-site also minimizes differences in ATP concentration arising from varying sample exposure to ice. Hold time could possibly be controlled and optimized , depending on the target organisms' varying response to temperature and ATP synthesis/degradation. Previous work has shown that ATP concentrations are stable when cells are at steady-state (Schneider and Gourse 2004); in this context, holding on ice may likely impact the kinetics between the antibody and the target rather than internal ATP values. Observed variation in environmental sample duplicates could also be the result of keeping samples on ice. Cov-IMS/ATP is a good alternative to other proposed methods for laboratories and agencies that do not have the resources to support a full-scale laboratory.

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