The U.S. Environmental Protection Agency (EPA) Workshop on Innovative Approaches for Detecting Microorganisms in Water

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Introduction

Welcome to The U.S. Environmental Protection Agency (EPA) Workshop on Innovative Approaches for Detecting Microorganisms in Water. The mission of the EPA is to protect public health and to safeguard and improve the nation's natural environment—air, water, land—upon which life depends. Success at EPA is dependent, in large part, on our ability to make credible environmental decisions based on solid scientific information and technical methodologies. This workshop, co-sponsored by EPA's Office of Research and Development and the Rapid Detection of Microbial Contaminants of Water Work Group in EPA's Environmental Technology Center (ETC; http://www.epa.gov/etop/etc/index.html), is intended to facilitate progress on the quantitative assessment of microbial agents in water. Reliable, sensitive, robust, and versatile detection and monitoring tools are needed to address the risk assessment and management of known and emerging microbial contaminants in source water, treated water, and/or in distribution systems.

This workshop brings together Science To Achieve Results (STAR) recipients of the National Center for Environmental Research's "Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens in Drinking Water" research grant (project summaries are available at: http://es.epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html) as well as scientists and policy-makers from other research entities, EPA, states, local agencies, and other stakeholders. EPA invites one and all to collaborate on furthering the development of cost-effective, timely, innovative technology solutions in assessing and managing environmental risks to human health.

Innovative Pathogen Detection in the Context of the National Program for Drinking Water Research

Audrey D. Levine
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Presentation Abstract

The need for pathogen quantification is a high research priority for the U.S. Environmental Protection Agency (EPA) Office of Research and Development (ORD) National Research Programs (Drinking Water, Water Quality). The EPA-ORD National Program for Drinking Water Research is designed to provide research support for review, revision, and implementation decisions associated with regulations and rules pertaining to source water, treatment, residuals disposal, distribution systems, and water use. The focus of the EPA-ORD Water Quality Research Program is on water quality criteria, watershed management, and source control and management.

This presentation will provide an introduction to the workshop and an overview of the research needs for developing pathogen detection and monitoring tools that are reliable, sensitive, robust, and versatile. Examples of pathogen-related research topics in the EPA-ORD National Research Programs (Drinking Water, Water Quality) will be presented. Key research themes include pathogen detection and control, source tracking, indicator/pathogen relationships, exposure assessments, and relationships between pathogen (and indicator) occurrence and health outcomes.

Use of Innovative Detection Methods for Detecting Contaminant Candidate List Pathogens

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Presentation Abstract

Microbial contaminants to be considered for possible regulation in drinking water are listed on the U.S. Environmental Protection Agency's (EPA) drinking water Contaminant Candidate List (CCL). Information is needed in several areas, including occurrence of the contaminants in drinking water, to make a decision to regulate or not to regulate CCL contaminants. Analytical methods for pathogens of concern should be sensitive, detect viable and infective microorganisms, and detect particular species or strains that cause adverse health effects, to provide occurrence information that is useful for making regulatory determinations. Existing methods for pathogens of the CCL may lack one or more of the needed capabilities, and often are expensive and labor intensive. Because of these methodological deficiencies, the National Research Council (NRC) noted that a "bottleneck" exists in that few microorganisms are considered for regulatory determinations because of a lack of information. NRC recommended that EPA consider the use of genetic or other innovative methods for detection of emerging pathogens as a means of speeding up the collection of occurrence information and for screening potential drinking water contaminants. Other experts have made recommendations on how EPA could incorporate innovative methods into the Unregulated Contaminant Monitoring Regulation, which is designed to collect occurrence information on CCL contaminants in drinking water. EPA is considering these recommendations. Several workshops have been held on different aspects of innovative methods, and EPA has funded investigations into the development of these methods. Discussions are ongoing on how methodological needs can be met with the use of genetic or other types of innovative methods for detection of CCL or other emerging pathogens that occur in drinking water.

LATE-PCR: Maximizing Detection Information From a Single Tube

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Presentation Abstract

Real-time polymerase chain reaction (PCR) assays offer quantitative detection of known microbes, but the number of surveyed organisms is usually limited to the four to six fluorophores that can be simultaneously detected by the thermal cycler. Some methods may also fail to detect organisms that have even minor variation from the targeted sequence. These limitations are overcome by Linear-After-The-Exponential (LATE)-PCR, an advanced form of asymmetric PCR invented in our laboratory. LATE-PCR assays efficiently generate single-stranded DNA amplicons, which can be probed for sequence variants using mismatch tolerant probes over a broad range of temperatures (25-60ºC). Fluorescence ratios between two or more probes that hybridize to each amplicon provide a "fluorescence signature" for each sequence variation and make it possible to distinguish hundreds, or potentially even thousands, of sequence variations. Unknown viral or bacterial strains having a previously undetermined fluorescent signature could be readily detected and identified by "Dilute-'N-Go" sequencing, another convenient feature of LATE-PCR. These properties will be demonstrated with assays developed for Hepatitis C virus and other viruses and bacteria. The new platform technologies are applicable to detection of a wide range of microorganisms in water by amplifying highly conserved genes.

Advanced Oxidation Technologies and Nanotechnologies for Water Treatment: Fundamentals, Development, and Application in the Destruction of Microcystin LR

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Presentation Abstract

The enormous diversity of toxic and organic pollutants of different chemical composition eliminates the possibility of using a universal treatment method for water decontamination and has led to the development of special treatment methods. Advanced Oxidation Technologies (AOTs) and Advanced Oxidation Nano-technologies (AONs) are among the most promising emerging chemical oxidation processes, and are anticipated to play a crucial role in water treatment as stand-alone processes or in combination with conventional technologies. Among AONs, TiO_2 photocatalysis is of particular interest because of its environmentally friendly features. TiO_2 photocatalysis is characterized by a specific oxidation pathway, which includes the formation of hydroxyl radicals (·OH) or other powerful oxidizing species. The hydroxyl radicals are extremely reactive and readily attack most organic contaminants. As a result, the organic contaminants are sequentially transformed to simpler organic molecules that are eventually mineralized to CO_2, H_2O, and mineral species (i.e., Cl). In general, due to rapid hydroxyl-radical-based oxidation reactions, AONs are characterized by high reaction rates and short treatment times.

Dr. Dionysiou will discuss some general aspects of AOTs, Environmental Nanotechnology (fundamentals, applications, implications), and specific examples of destruction of organic contaminants using hydroxyl radicals as well as TiO_2-based AONs for the treatment and purification of water in general and, in particular, for the destruction of cyanobacterial toxins. He will discuss the development of a TiO_2 photocatalytic technology using novel nanotechnology and self-assembling strategies. Results also will be presented on the synthesis of TiO_2 catalyst in the form of films, and membranes and the application of these materials on the destruction of microcystin-LR, a potent cyanobacterial hepatotoxin.

Development of Gene Microarray Assays for Risk Assessments

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Presentation Abstract

Macroorganism bioindicators have been used extensively as assessment tools for aquatic ecosystems. There is great potential value in using prokaryotic and eukaryotic microorganisms as bioindicators, however, as the tools of molecular biology have been adapted for use in natural systems. The approach can be used not only to detect specific organisms of interest, such as cyanobacteria or pathogens, but also to provide general assessments of water quality, including monitoring for pollutant inputs and evaluation of remediation processes. This approach relies on several fundamental assumptions, however, and must deal with the issue of the "underexplored 'rare biosphere.'" The first, and generally accepted, assumption is that microorganisms respond rapidly to their environment. The second is that many, if not most, microbial taxa are widely distributed. These lead to a third assumption: the abundance of microbial taxa will vary widely due to their environment. Our work, and that of others, suggests these assumptions are generally valid. In turn, they lead to the hypothesis that one need only monitor a subset of the overall microbial diversity to characterize the current and predicted health of an aquatic ecosystem. Our current projects are focused on building an appropriate subset of bioindicators for such assessments.

Characterization of Naturally Occurring Amoeba-Resistant Bacteria

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Presentation Abstract

Bacteria that are able to replicate and survive within amoeba hosts have been termed "amoeba-resistant bacteria" (ARB). Some of the bacteria are lytic for their amoebal hosts. These bacteria include members of the genus *Legionella* as well as other *Legionella*-like amoebal pathogens (LLAPs). Serological evidence suggests that these LLAPs may be a significant cause of respiratory disease and, because many do not grow on conventional laboratory media, they may be overlooked.

The objectives of this study are designed to address the protection of human health through clean and safe water and healthy communities. The objectives are to continue the biological cleanup of previously collected water samples containing infected amoebae, continue the phylogenetic and phenotypic characterization of the bacteria, and isolate additional ARB from both environmental and human-constructed water sources.

The cleanup of the samples to remove contaminating bacteria will involve axenic co-culture with amoebae and extensive washing and dilution of these cultures. The 16S rRNA genes of the bacteria will be sequenced for phylogenetic comparisons and construction of phylogenetic trees. Also, we will use unique sequences from the bacteria to probe additional water samples to determine the distribution of the ARB in the environment. Phenotypic studies of these organisms will include determining how long bacteria released from infected amoebae can remain infectious, and whether these bacteria can survive dessication and chemical treatment when protected by vesicles from ciliates. We also will use histochemical and viability staining to determine whether these bacteria are cytopathogenic for human cell lines. We will continue to use methods developed in our laboratories, which have led to the successful isolation of ARB, to screen additional water samples and collect more of these novel organisms.

Preliminary results have already identified novel ARB with characteristics, such as replication in the nucleus, which have not been previously described. The results of this work will not only expand knowledge of the types or organisms that are ARB but also provide genetic sequences that will allow assessment of the distribution of these organisms in both natural and human-constructed water environments. Studying the survivability of these organisms in the environment and their ability to infect human cells also will help to identify previously undescribed organisms as potentially pathogenic for humans.

Biofilm Sampling and Screening Techniques for Amoeba-Related Biofilm Pathogens

Nick Ashbolt
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U.S. Environmental Protection Agency, Washington, DC

Presentation Abstract

A risk-based management approach directed to pathogens within distribution systems is proposed via sampling biofilm-related microbiota. Justification of this approach will be provided with examples from a recent European Union project (MicroRisk) that identified "intrusion" events within distribution systems as one of the largest uncertainties in undertaking a quantitative microbial risk assessment (QMRA) of whole systems. Sampling biofilms may provide a "historic" view of water quality and a more timely assessment of short-duration events within distribution. The role that amoeba may play in the sequestering of pathogens, and, in some cases, amplification of pathogens, will be discussed in relation to frank pathogens and those indigenous to biofilms (e.g., *Legionellae*, *Mycobacteria*, and the novel giant amoeba virus, mimivirus). Lastly, the potential for a common "virulence" factor for amoeba-hosted pathogens will be discussed.

Overview of the U.S. Environmental Protection Agency's Office of Research and Development and the Science To Achieve Results (STAR) Program

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Presentation Abstract

The National Center for Environmental Research (NCER) is part of the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (ORD). ORD's research results are invaluable to its "customers": EPA program offices and regions; other agencies and policy partners; place-based customers (states, tribes, and local communities); the academic research community; and environmental technology providers. The mission of NCER is to augment the Agency's in-house research capabilities through support for high-quality research conducted outside of the Agency by the nation's leading scientists. Supported external research improves the scientific basis for decisions on national environmental issues and helps EPA achieve its goals. NCER is responsible for the Science To Achieve Results (STAR) grants program, which funds research grants and fellowships in numerous environmental science and engineering disciplines through a competitive solicitation process and independent peer review. The program engages the nation's best scientists and engineers in targeted research that complements EPA's own intramural research program. Additionally, NCER periodically establishes large research centers in specific areas of national concern, such as children's health, hazardous substances, and particulate matter. Each year, NCER awards about 180 research grants, 40 research grants jointly with other federal and private-sector partners, and 125 graduate fellowships, in approximately 280 universities and nonprofit research institutions. On an annual basis, NCER's technical staff, with backgrounds in engineering, economics, and the ecological and health sciences, manages 650–750 active research grants and 300 fellowships, as well as organizes peer review activities for the Center and, as requested, for selected projects elsewhere in EPA. Research grant recipients provide annual progress reports and final reports along with any papers resulting from the research conducted. NCER communicates research results through their Web Site (www.epa.gov/ncer), ORD laboratories, program office and regional meetings, and publications.

Overview of Methods for Simultaneous Detection of Pathogens and Introduction to a Highly Multiplexed Nucleic Acid-Based Assay

R. Paul Schaudies and Doreen A. Robinson
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Presentation Abstract

We will provide a summary of current methods for collection, extraction, and identification of multiple organisms that present water hazards. Promising technologies, as well as current technical limitations, will also be discussed.

GenArraytion, Inc. has developed a rapid, cost-effective microbial genotyping method based on identification of a unique genomic sequence that discriminates between microbial species and strains. We have developed a bioinformatic and laboratory-based method that efficiently identifies genomic-unique regions by comparing a genome of interest against all publicly available information with subsequent laboratory validation. Different filters can be created to identify sequences that are unique at the strain, species, and genus level, increasing confidence and allowing characterization of microbial species and strains for which genomic sequence is not available. The unique nature of the sequence identified using this approach has been validated by hybridizing oligonucleotides from the unique regions against the target organism, its nearest neighbors, more distantly related species, and mammalian genomes using a microarray format. Based on these results, we have developed microbial genotyping with strain-level discrimination of microorganisms for medical diagnostics, environmental monitoring, and food and water safety testing. We will present data identifying and characterizing bacteria on the basis of unique genetic regions, as well as the presence of virulence factors and antibiotic resistance genes. Using a microarray format, we have the capability to look for multiple organisms from a single sample in a massively parallel fashion. Data will be presented using arrays containing unique sequence from more than 20 different microbial organisms.

Robust Piezoelectric-Excited Millimeter-Sized Cantilever Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter

Raj Mutharasan
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Presentation Abstract

The goal of the proposed research is to develop antibody-immobilized piezoelectric-excited millimeter-sized mechanically robust cantilever (PEMC) sensors for detecting pathogenic agents (PA) such as *Cryptosporidium* and *Giardia* and others in drinking water systems and source waters without a concentration or filtration step.

The project has three main objectives: (1) explore and establish experimentally piezoelectric-actuated millimeter-sized cantilever sensors suitable for detecting one pathogen in 1 L of water using new cantilever oscillation and measurement modalities; (2) develop a flow cell-PEMC sensor detection assembly for testing sample volumes of 10–100 L, and characterize the response of the sensor to samples containing a known number of *Cryptosporidium* and *Giardia*; and (3) develop a PEMC sensor for confirming pathogen identity by its DNA signature, immobilize known 38-mer oligo (Gene Bank: L16997), and use DNA extracted from PEMC collected cells to verify the identity of the pathogen (*Cryptosporidium*).

We will fabricate PEMC sensors and chemically immobilize antibodies against *Cryptosporidium* and *Giardia* on the sensor surface. Spiked samples of water with the pathogen in a concentration range of 0.1 to 10 cells/L will be passed through the PEMC sensor, and resonance frequency measured continuously. Binding of pathogen decreases resonance frequency quantitatively. New oscillation modes that show promise of higher sensitivity (1 cell per mL) will be investigated. A new flow cell will be developed for passing 10 L or a larger amount for detection in 10 minutes. The sensor surface acts as a "filter," and thus after a sensing cycle, the attached cells will be released, DNA extracted, and then exposed to another PEMC sensor that has an immobilized DNA sequence that is unique to *Cryptosporidium*. The reduction in resonance frequency will indicate a positive detection.

We have shown that the mass change sensitivity of our current PEMC sensor is in the range of 10–100 picograms. Through the proposed innovation, we expect to improve the sensitivity to 1 picogram. This will be accomplished through the use of a new oscillation modality (twist or flex, or bending at 150 to 250 kHz). The novelty of the proposed modality of sensing is the elimination of the filtration step—it is one-step sensing.

Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water

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Presentation Abstract

This project will develop, evaluate, and validate an integrated, rapid method for the quantitative assessment of pathogens/indicator organisms from large volumes of source and drinking water. Microorganisms of concern will include protozoa (*Cryptosporidium*, *Giardia*, *Microsporidium*); bacteria (*Escherichia coli*, *Legionella*, *Helicobacter pylori*, *Aeromonas hydrophila*, *Micobacterium avium* intra-cellular); algae (*Cyanobacteria*); and viruses (adenoviruses, caliciviruses, coxsackieviruses, echoviruses). We propose integrating the technologies of concentration, purification, detection, and testing for viability/ infectivity into a universal, simplified, economic, and user-friendly methodology. The proposed concentration by continuous flow centrifugation (CFC) and a detection of small number of microorganisms from large volumes of water, using multiplex miniaturized fiber bead microarrays, is a rapid, cost-effective method involving robust and portable equipment and employing simple procedures. In a previous EPA Science To Achieve Results (STAR) project, we developed, optimized, and validated a novel method, the CFC, for the concentration of three protozoa (*Cryptosporidium* spp. *Giardia*, *Microsporidia*) from large volumes of water (1000 L). In this project, we propose to expand this approach to include selected Contaminant Candidate List (CCL) pathogens (i.e., bacteria, algae and viruses) and integrate the pathogen concentration by CFC with a DNA bead microarray technique recently developed for the detection of selected CCL microorganisms.

We will integrate the concentration of protozoa, bacteria, algae, and viruses from water into a single concentration procedure. The CFC will then be fine-tuned for its ability to concentrate each of the CCL pathogens. We will focus on detection and quantitative identification of CCL pathogens in water, using multiplex miniaturized fiber optic bead microarrays, coupled with a compact confocal-type imaging system, and compare it with conventional methods.

In this study, we propose to combine continuous flow centrifugation and fiber optic bead microarray, both cutting-edge technologies, for rapid and accurate detection of waterborne pathogens. The major tasks involved with this approach include: (1) integrating the concentration of protozoa, bacteria, algae, and viruses from water into a single concentration procedure; (2) selecting sequences, developing assays, preparing bead microarrays, and testing both synthetic and spiked samples; and (3) validating the combined method with complex water matrices.

At the completion of laboratory and field validation studies, we anticipate that the proposed approach will enable the detection of small numbers of waterborne pathogens from large volumes of various water matrices in less than 4 hours.

Development of High-Throughput and Real-Time Methods for the Detection of Infectious Enteric Viruses

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Presentation Abstract

Improved methods for rapid and reliable detection of infectious viruses are required to enable rapid and quantitative determination of their presence for public health assessments. Current methods to detect infectious viruses are based on mammalian cell culture and rely on the production of visible cytopathic effects (CPE). For hepatitis A virus (HAV), viral replication in cell culture has been reported to be nonlytic and relatively slow. It may take more than 1 week to reach maximum viral production and the subsequent visualization of CPE.

A molecular beacon (MB), HAV1 (5'– FAM – CTTGGGCCGCCGCTGTTACCCTATCC CCCAAG – DABCYL – 3'), specifically targeting a 20 bp, 5' noncoding region of HAV was designed and synthesized. MB HAV1 was introduced into fixed and permeabilized fetal rhesus monkey kidney (FrhK-4) cells infected with HAV strain HM-175. Upon hybridizing with the viral RNA, fluorescent cells could be easily visualized under a fluorescence microscope. Discernible fluorescence was detected only in the infected cells by using specific MB HAV1, and nonspecific MB (5'– FAM – CGCTAT GCATCCGGTCAGTGGCAGTATAGCG – DABCYL – 3'), which is not complementary to the viral RNA sequence, produced no detectable fluorescence. The number of fluorescent cells enabled the direct quantification of viral dosages by direct counting of fluorescent foci. A detection limit of 1 PFU was obtained at 6 hour post infection (PI). MBs provide a label-based, and separation-free, detection scheme that produces fluorescence upon target binding. By directly visualizing the fluorescent hybrids with newly synthesized viral RNA, the combined cell culture-MB assay provides rapid and sensitive detection of infectious viruses.

For real-time studies in living cells, however, the durability of MBs is affected due to cellular nuclease degradation. The current method requires cell fixation and permeabilization to denature and crosslink the proteins to maintain cellular architecture and to protect the injected MB structures. It is essential to improve the efficacy of MBs to probe and quantify infectious viruses for *in vivo* studies. We developed nuclease-resistant MBs for the detection of coxsackievirus B6 Schmitt strain in buffalo green monkey kidney (BGMK) cells via TAT peptide delivery. An MB CVB6, specifically targeting the 18 bp 5' noncoding region of the viral genome, was designed and synthesized. The MB structure was modified by combining 2'-O-methyl RNA bases with phosphorothioate internucleotide linkages to allow MB CVB6 to be resistant to cleavage by DNase I and the hybrids with viral RNA to become refractory to digestion by RNase H. Cell-penetrating TAT peptides were conjugated to MB CVB6 using thiol-maleimide linkages to introduce MB CVB6 into living cells without permeabilization. At 5 µM, MB CVB6 demonstrated self-delivery within 20 minutes with nearly 100 percent efficiency. Confluent BGMK monolayers were infected with 10-fold serial virus dilutions and discernible fluorescence was observed around 30 minutes PI, suggesting that the entry and uncoating of virions occurred within 30 minutes after infecting the cells. The degree of fluorescence was monitored, and it was observed that each viral reproductive cycle was completed between 10 and 12 hours PI. The number of fluorescent cells increased in a dose-responsive manner, enabling the direct quantification of infectious viral dosages. The validation of the fluorescence assay for viral quantification was also demonstrated by comparing with traditional plaque assays.

Enteric viruses are easily transmitted through the fecal-oral route and cause a diverse array of clinical manifestations. Recent outbreaks associated with viral contamination in aquatic environments have called for the development of a more efficient and accurate surveillance system for infectious viruses. We established two *in vivo* reporter systems for the development of a high-throughput protocol for identifying infectious enteric viruses. A genetically engineered cell line expressing a fluorescent indicator for active virus replication was generated as a cellular platform for the detection of enteric viruses. A novel quantum dot-based method

was developed to monitor viral RNA production within the infected cells in real-time. Both methods allow the rapid detection within the first round of virus replication cycle and can be modified for automatic sample processing to achieve high-throughput analyses of infectious enteric viruses.

Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water and Its Application for the Detection of Contaminant Candidate List Organisms in Water

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Presentation Abstract

Although numerous technologies have been developed [e.g., polymerase chain reaction (PCR)] or are under development (e.g., microarrays) for the detection of as few as one microorganism, the practical application of these methods to the detection of pathogens in water is problematic. These technologies can only be used to analyze from 10 to 1,000 µL volumes. Therefore, their application will never be fully realized unless rapid and economic (in terms of cost and manpower) methods are available to concentrate pathogens into a volume small enough to be assayed. Thus, this study focuses on the development of an application for the rapid and economic concentration of enteric viruses, parasites, and bacteria from water.

Recently, surface-modified nanofiber carbons have been developed that are capable of removing all classes of pathogens from water at high flow rates (> 10 L per minute). These filters are capable of removing more than 10^8 organisms per gram of adsorbent from highly turbid waters. Because the adsorbent material is made of carbon, it also removes chlorine, preventing inactivation of the collected organisms and reducing the need to add a chlorine neutralizer prior to sample processing. This universal microbial collector (UMC) will be evaluated with waters of various physical/chemical qualities to determine precision, robustness, and accuracy. The U.S. Environmental Protection Agency's (EPA) Contaminant Candidate List (CCL) organisms coxsackievirus B5, adenovirus 40, *Encephalitozoon intestinalis*, and *Helicobacter pylori* will be used to statistically compare (students t-test) the UMC with the recovery from four or more types of currently available collector media using EPA-approved methods (for viruses and protozoa). Finally, the UMC will be applied to determine the occurrence of study organisms in distribution systems and groundwater at several different locations in the United States.

The UMC modified carbon filters will be optimized to recover the greatest number of viable organisms with the least amount of material that may interfere with molecular (e.g., PCR, immunological) detection methods. The goal is to produce a final microorganism concentrate of 1 to 5 mL. It is expected that the recovery of pathogens and CCL organisms from water will be substantially higher using the UMC than from currently available collector/concentration media, including those utilizing EPA-approved methodologies.

Timely Multi-Threat Biological, Chemical, and Nuclide Detection in Large Volume Water Samples

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Presentation Abstract

The use of chemical, biological, and radioactive agents, combined with rapid global transport of diseases, have transformed Jimmy Carter's "moral equivalent of war" into an infectious equivalent of war. There is clearly a need for a rapid, accurate, threat-vector detection platform that can handle many threats, sample types, and physical scales (nano to macro). A new agent identification solution capable of quickly detecting multiple threats is required, as is a new metric to quantify the effectiveness of the new solution. We have made progress on a multi-agent detection platform capable of sensing multiple threat agents (chemical, biological, and nuclear) simultaneously in diverse media with the ability to analyze large fluid samples, and a performance model of this detection platform. A new performance metric, "time-to-identify" (TTI), has been developed to assess the time required to identify the presence of a given target analyte at a given concentration. We use our model and metric to predict the detection platform's sensitivity and speed for several CONcepts of Operation (CONOP): (1) agent detection (chemical, viral, or bacterial) in a 1 mL clinical sample; (2) botulinum toxin detection in the milk; and (3) a high flow rate liquid sample ($1m^3$/hr). Application of the model in these CONOPs indicates that the proposed platform can be optimized to dramatically reduce TTI, thereby minimizing the impact of natural and manufactured chemical, biological, and radiological events.

On-Chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens

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Presentation Abstract

The objective of this research is to develop and validate a highly parallel, sensitive, specific, and quantitative biochip combining the principles of polymerase chain reaction (PCR) and microarrays for the simultaneous detection of 20 waterborne pathogens. Establishing highly parallel and specific methods are essential to reduce the health risk from microbial pathogens present in source and drinking waters.

More than 250 virulence and marker genes (VMGs) serving as signatures for 20 selected pathogens and 30 potential indicator organisms will be assayed in parallel using a novel on-chip PCR assay. The on-chip PCR device will be capable of amplifying multiple targets and samples with a high level of sensitivity, specificity, and quantitation. A highly efficient cross flow microfiltration process for sample concentration also will be incorporated to provide the sample processing step from surface water to the chip and add to the sensitivity. The list of 20 pathogens includes 3 bacterial candidates included in the U.S. Environmental Protection Agency's Contaminant Candidate List (*Aeromonas hydrophila*, *Helicobacter pylori*, and *Mycobacterium avium* intercellulare) as well as 17 other organisms, including *Cryptosporidium* and *Giardia*. The detection method will be validated by spiking various concentrations of relevant organisms in a number of source and treated drinking water samples. The method also will target selected marker genes from potential indicator organisms. The proposed method is expected to have an overall detection limit of 1 target cell per 100 mL of source or drinking water, be performed in less than 4 hours, and employ a quantitative strategy similar to real-time PCR. A small part of the project also is devoted to developing viability assays using a nanoparticle-based technology capable of detecting a change in bacterial cell concentration of 10 to 100 cells in less than 1 hour.

The developed method will provide a high-throughput tool capable of quantitatively monitoring multiple waterborne pathogens using their signature VMGs. The method, when developed, can be used as an economical tool to screen for many pathogens with high specificity and sensitivity in the water and wastewater industry.

A Novel Molecular-Based Approach for Broad Detection of Viable Pathogens in Drinking Water

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Presentation Abstract

The overall objective of this project is to develop and evaluate a novel, molecular-based approach for broad detection and enumeration of viable pathogens in drinking water. The specific objectives of the proposed project are to: (1) develop and evaluate cutting-edge filtration and microfluidic methods for pathogen concentration and purification from drinking water; (2) develop a two-step detection approach, based on general amplification of the extracted metagenome and metatranscriptome, followed by pathogen-specific detection by viable RT-PCR methods; and (3) evaluate the performance of the developed method for a variety of spiked and unspiked samples from regional water utilities.

Organisms selected for this study include several Contaminant Candidate List (CCL) organisms (*Mycobacterium avium*, *Aeromonas hydrophilla*, echovirus, and adenovirus). Filtration using novel positively charged filter media will be evaluated in seeded studies for concentration and recovery of organisms in comparison to hollow fiber ultrafiltration. Also, novel microfluidic methods will be evaluated in seeded studies against commercial spin column kits for nucleic acid extraction. For the first step in the detection approach, novel whole genome and whole transcriptome amplification methods will be adapted and applied to total nucleic acid extracts from water concentrates and short-term enrichments to achieve a limited initial amplification. As a second step, fluorescent RT-PCR methods will be developed targeting specific sequences in target organisms indicative of viability (e.g., precursor ribosomal RNA for bacteria, replicative forms for echovirus, or mRNA for adenovirus). The composite method (consisting of the best performing concentration and purification methods with the combined two-step detection method) will be evaluated on seeded and unseeded samples obtained from regional utilities.

The proposed research will develop a novel approach for concentration, purification, and detection of viable pathogens in drinking water that is broadly applicable across pathogen classes (e.g., DNA and RNA viruses, and gram negative and acid fast bacteria). The overall method developed in this study will improve the ability to assess risk associated with microbial contamination of drinking water by providing a rapid, sensitive, and quantitative method for the detection of known and emerging pathogens in drinking water. Furthermore, the proposed study will offer a novel approach for both prospective and retrospective investigation of waterborne infectious disease outbreaks.

Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis

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Presentation Abstract

This project addresses critical issues in rapid pathogen detection methods by advancing sample processing, concentration, and nucleic acid extraction techniques so that high throughput sample interrogation tools, like microarrays, can be used to their full potential.

The ultimate objective of this project is to develop an innovative approach for detecting multiple waterborne bacterial, protozoan, and viral pathogens utilizing large volume (100–1,000 L) ultrafiltration (UF) as a universal pathogen concentration technique, direct extraction of nucleic acids, whole sample genome amplification (WSGA), and hybridization to a multi-pathogen, water quality microarray.

Cryptosporidium parvum, *Salmonella typhimurium*, and human adenovirus 2 are used as model pathogens for UF method development, infectivity assays, and real-time quantitative polymerase chain reaction to assess the efficiency of DNA extraction and WSGA procedures. Microarrays will be used for various aspects of the project to: (1) capture specific target-pathogen sequences on an array-based, solid-phase substrate to improve amplification sensitivity; (2) assess the efficiency of WSGA techniques using an *Escherichia coli* genomic array; (3) measure host cell response to pathogens as a rapid and sensitive infectivity detection assay; and (4) develop a multi-pathogen, multi-target, water quality microarray. The final detection array will target organisms on the U.S. Environmental Protection Agency Candidate Contaminant List, other potential water-borne pathogens, and traditional microbial indicators.

We anticipate that these experiments will clearly establish that microarray technology can be useful for the water industry and provide direction for future methods development. The ability to detect pathogens using several different methods, including cell culture infectivity, host response microarrays, and pathogen-specific microarrays, will improve the water industry's ability to protect consumers from potential microbiological threats.

Simultaneous Concentration and Real-Time Detection of Multiple Classes of Microbial Pathogens From Drinking Water

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The objectives of this research are to: (1) develop, collaboratively test, and field evaluate new and improved, rapid ultrafiltration methods and electropositive filter adsorption-elution methods to concentrate viruses (and cellular pathogens such as bacteria and protozoan parasites with the ultrafilter) from waters of different qualities (i.e., particulate and dissolved organic matter); (2) compare recovery efficiencies to the U.S. Environmental Protection Agency's (EPA) existing 1MDS filter method; (3) evaluate rapid polyethylene glycol (PEG) precipitation for postfiltration sample processing to further concentrate viruses and improve large volume nucleic extraction methods to remove inhibitors for molecular detection of viral nucleic acids; (4) optimize viral nucleic acid amplification by real-time reverse transcriptase polymerase chain reaction (RT-PCR) for rapid detection of low virus concentrations; and (5) collaboratively (round-robin) test developed methods to validate their performance at selected water virology laboratories.

Current EPA methods for recovery of viruses use a sole-source, expensive filter with variable performance for some viruses and waters (poor for some enteroviruses and adenoviruses, and for waters with high salinity, particulates, and organic matter). We will evaluate two new, improved, and cost-effective filters for virus concentration from water: a disposable, hollow fiber ultrafilter (HFUF) for unified concentration of all microbial pathogen classes and a new, thin-sheet positively-charged filter medium (TSM) fabricated from glass wool, prepared in the laboratory, and optimized for performance. Properties and preparation methods of the new TSM will be provided to commercial entities for manufacture and mass production. Optimum eluents and elution conditions for a range of enteric viruses will be determined and specified. Subsequent virus concentration and purification from filter eluates and retentates will employ a rapid PEG precipitation method applied to water concentrates seeded with low levels of a suite of viruses (adenoviruses, enteroviruses, and noroviruses) representing those that exist in the United States. Nucleic acid extraction techniques will be optimized for applicability to large sample volumes (1–4 mL) and for effective removal of inhibitors of real-time RT-PCR detection. Real-time RT-PCR will be optimized for detecting target viruses as broad groups in addition to select specific viruses (e.g., adenovirus types 40 and 41), with the option to genotype resulting products. The developed methods will be provided to four water virology laboratories recruited for collaborative testing.

Key Contaminant Candidate List viruses will be rapidly detected at low levels in water samples concentrated by a rapid HFUF, or a new TSM electropositive filter adsorption-elution method, and compared with the approved EPA method (1MDS VIRADEL). A unified and rapid virus concentration, nucleic acid extraction, and real-time, quantitative RT-PCR amplification technique will be of great value to the water industry because it will provide rapid and sensitive virus recovery and concentration, simplified nucleic acid extraction processes, and robust detection of multiple virus types by rapid real-time PCR and RT-PCR assays.

Identification of Bacterial DNA Markers for the Detection of Human and Cattle Fecal Pollution

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Presentation Abstract

Technological advances in DNA sequencing and computational biology allow scientists to compare entire microbial genomes. However, the use of these approaches to discern key genomic differences between natural microbial communities remains prohibitively expensive for most laboratories. Here, we report the application of a genome fragment enrichment (GFE) method that identifies genomic regions that differ between the metagenomes of different fecal microbial communities. In two separate experiments, either human or cow fecal microbial community DNA was hybridized against a pig fecal DNA background. A total of 819 individual clones were sequenced and screened for redundancy. Dot blot analysis of 677 non-redundant sequences confirmed that 97.7 percent of the sequences were specific for respective human or cow fecal microbial communities. Bioinformatic analyses of non-redundant sequences indicated a preponderance of *Bacteroidales*-like regions (41.3%) predicted to encode membrane-associated proteins (52.3%). Oligonucleotide primers capable of annealing to 29 of these sequences did not amplify pig fecal DNA and exhibited different levels of specificity with fecal DNA from other animal sources. Eight polymerase chain reaction (PCR) assays exhibited extremely high levels of host-specificity (> 99%), including four human-specific and three cow-specific assays. These assays also demonstrated a broad distribution of genetic markers among respective host source populations ranging from 61 to 100 percent of samples tested. Host-specific assays were then challenged against water samples collected from fecal impacted streams to explore their potential for water quality monitoring in ambient waters.

Detection of Waterborne Pathogens Using Real-Time PCR and Biosensor Methods

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Presentation Abstract

Improved methods for monitoring pathogens in the environment are needed to address an important component of the risk assessment framework, including hazard identification and exposure assessment. In addition, during extreme events (intentional contamination or flooding), rapid and less expensive tests are needed to triage numerous samples. This research is focused on two types of technologies for application to water contamination: (1) the development of a nano-wire enabled antibody-based conductometric biosensor; and (2) a real-time qPCR assay for detecting bacterial pathogens, such as *Helicobacter pylori* and *Escherichia coli* O157:H7. The biosensor consists of two components: an immunosensor that is based on electrochemical sandwich immunoassay, and a reader for signal measurement. The architecture of the immunosensor utilizes the lateral flow format. The biosensor provides a cost-effective, low volume, and real-time detection mechanism.

Initial results show that the biosensor can detect as low as 10^2 bacterial cells/mL in 6 minutes from sample application to final results. Design and performance will be presented to highlight the robustness of the biosensor in detecting the targeted organism in pure culture and water samples. In addition, the ability to change the specificity of the antibodies will enable the biosensor to be used as a semi-quantitative detection device for other types of waterborne pathogens. Such a device can be used to enhance environmental quality monitoring and policy implementation. The real-time qPCR, a modification of polymerase chain reaction (PCR) that can simultaneously quantify and amplify a specific part of a given DNA molecule, is a valuable tool to detect waterborne pathogens. A real-time qPCR technique was developed and used for determination of *H. pylori* concentrations in water and for investigation of the occurrence of the bacteria in sewage. Conventional culture was compared to conventional PCR and to the real-time qPCR approach for quantification of the bacterium. Real-time qPCR demonstrated a 100-fold greater sensitivity for detection of *H. pylori* DNA in comparison to conventional PCR. This assay provided a specific, sensitive, and rapid method for quantitative detection of *H. pylori* in sewage with cells ranging from x to y in untreated sewage. The quantitative detection of *H. pylori* by rapid and less expensive methods than the TaqMan assay using SYBR green could be an important tool to monitor infection in a community by measuring the concentrations in sewage and to meet the new regulatory and risk-based frameworks for water supplies.

The goals are to contrast and compare the applicability of these two molecular approaches and demonstrate their value in studying the prevalence of two important bacterial pathogens in various sources and waterbodies (*H. pylori*, *E. coli* O157:H7) as well as other waterborne pathogens as suggested by the U.S. Environmental Protection Agency Contaminant Candidate List. Types of environments to be studied include nondisinfected ground waters as one of the key exposure sites, as well as sewage and manures as a source of the contaminants.

Microarray Detection of Human Viruses From Community Wastewater Systems

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Presentation Abstract

Human viruses are responsible for a number of disease idiopathies ranging from mild gastroenteritis to more severe neurological symptoms. Their presence at high numbers in human excreta has been well documented. Current detection methods are limited in their ability to detect multiple virus types from single samples. Microarray technology has been used in the clinical arena to screen patient samples for many hundreds of pathogenic viruses in a single reaction. Adapting microarray technology to screen environmental samples for multiple pathogens has been suggested as an efficient tool with many potential applications.

This study describes the novel use of a viral microarray to screen municipal wastewater for the presence of circulating human viruses. RNA viruses were more frequently detected compared to DNA viruses. Some seasonality among certain viral groups was observed, and other viral groups were shown to be ubiquitously present in sewage. Microarrays are able to serve as a primary screening tool for community waste and show the presence of certain circulating viral types. Potential applications of environmental microarrays include environmental monitoring and public health monitoring through community waste screening.

Quantitative Assessment of Pathogens in Drinking Water

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Presentation Abstract

A major limiting factor in assessing the human health risk of microbial pathogens in raw and finished drinking water is the lack of robust, efficient methods for concentrating, identifying, and quantifying low levels of bacteria, viruses, and protozoa simultaneously, effectively, and rapidly. We have initiated the development of a microbial isolation and detection protocol capable of qualitative and quantitative identification of waterborne microbial pathogens by combining filtration followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), fluorescent *in situ* hybridization (FISH), and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) detection of target microorganisms. By combining an efficient membrane filtration recovery method with advanced molecular detection protocols, this study will provide a tool for obtaining quantitative data on human exposures to pathogenic viruses, protozoa, and bacteria present in raw and finished drinking water. As such, the study will enable regulatory agencies to make better informed risk management decisions.

Development of an Infectivity Assay for Noroviruses in Cells

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Presentation Abstract

Lack of suitable tissue culture methods hampers the ability to determine the viability and infectivity of certain U.S. Environmental Protection Agency (EPA) Contaminant Candidate List (CCL) pathogens isolated in source and drinking water supplies. In addition to gathering occurrence data in water supplies, these infectivity assays assist water utilities and the EPA in determining the risk of infection if these pathogens are detected by nucleic acid methods (e.g., polymerase chain reaction [PCR] and its variant, quantitative real-time PCR) in public water supplies.

Since its recognition more than 30 years ago, *in vitro* infectivity and cultivation assays, and animal infectivity assays have proved to be problematic for the waterborne CCL pathogen, human norovirus (NoV). Recent advances in engineering *in vitro* three-dimensional tissue assemblies, which recapitulate many of the physiological features of their *in vivo* counterparts, is the central enabling technological platform that allowed us to overcome the NoV cell culture infectivity problem. Our recent publication in *Emerging Infectious Diseases* (2007;13(3):396-403) documents the methods and data demonstrating the success of developing an infectivity assay for these viruses. Recently, we have optimized quantitative reverse transcription real-time PCR to determine the degree of propagation that is occurring within these systems. With training and technology transfer, waterborne virology laboratories should be able to replicate these techniques. Although the methods for generating these novel assemblies are initially complex to learn, the infectivity assays are fairly straightforward (e.g., standard $TCID_{50}$ assays and reverse-transcription real-time PCR). This advance will now allow for research studies to better assess human health risks of NoV in water supplies.

An Overview of Pathogen Research in the Microbiological and Chemical Exposure Assessment Research Division

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Presentation Abstract

The Microbiological and Chemical Exposure Assessment Research Division of the U.S. Environmental Protection Agency's National Exposure Research Laboratory has a robust in-house research program aimed at developing better occurrence and exposure methods for waterborne pathogens. In particular, research is aimed toward developing and improving occurrence methods so that they are more rapid, sensitive, and inexpensive. To conduct this research, a diverse array of detection technologies is employed, including real-time polymerase chain reaction, microarrays, proteomics, cell culture, and others. In addition, the Division has invested in evaluating new approaches to collecting and concentrating samples more effectively. The long-term goal of this project is to enable the Agency to conduct better, more accurate risk assessments that will aid regulatory decision-making.

Rapid and Quantitative Detection of *Helicobacter pylori* and *Escherichia coli* O157 in Well Water Using a Nano-Wired Biosensor and QPCR

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Poster Abstract

The hypothesis of this project is that a disposable biosensor and quantitative polymerase chain reaction (QPCR) can be combined seamlessly to develop a unique biosensor-QPCR as a tool for near real-time determination of contaminant occurrence in drinking water. The specific objectives are to: (1) develop a protocol for processing water samples for the biosensor and QPCR; (2) assess the performance of the biosensor and QPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *Escherichia coli* O157:H7 and *Helicobacter pylori* in groundwater samples from the field; (3) develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by QPCR using bacteria isolated and screened by the biosensor system; and (4) validate a method for testing the viability of *E. coli* O157:H7.

The first step in our experimental approach is to evaluate the performance of the biosensor and QPCR separately. Once confirmed, a method to seamlessly integrate these two devices into a biosensor-to-QPCR field-laboratory technique, coupled with a viability test, will be developed.

This project is expected to advance the use of antibody-based methods and molecular techniques for application to drinking water supplies. The expected deliverables are: (1) proof of concept and assessment of biosensor and QPCR techniques for recovery, detection, and quantitation of bacteria in water; and (2) development of a unique attribute that is not in the literature at the moment—the capability for direct confirmation through QPCR of presumptive results from the biosensor, thereby minimizing false positive and false negative analysis.

Development and Evaluation of a Microarray Approach To Detect and Genotype Noroviruses in Water

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Poster Abstract

Noroviruses are the leading cause of nonbacterial gastroenteritis outbreaks in the United States, some of which are caused by the ingestion of contaminated water. These viruses are usually detected and genotyped using reverse transcription-polymerase chain reaction (RT-PCR) based methods followed by sequencing. Unfortunately, the accurate detection of noroviruses in environmental samples is often hindered by the co-amplification of non-specific DNA, which can result in the need for further purification of PCR products before accurate sequence information can be obtained. As an alternative to direct sequencing, a generic microarray was evaluated for its ability to genotype norovirus RT-PCR products by probe hybridization. With this approach, RT-PCR amplicons were first mixed with a range of genotype-specific probes and then single base extension (SBE) reactions were run. This resulted in the labeling of those probes that have sequences complementary to specific RT-PCR products. These genotype-specific probes were then hybridized to an Affymetrix GenFlex Tag Array for detection. Using a standardized, multiplex SBE reaction, the genotyping of representative strains was accomplished and resulted in the generation of specific hybridization patterns, or fingerprints, on the microarray that were diagnostic for the genotype of norovirus detected. Furthermore, the SBE-GenFlex array method was shown to be successful in the genotype identification of noroviruses seeded into tap and Ohio River water samples. This study demonstrates the utility of using a microarray to genotype noroviruses in complex environmental matrices.

Pathogen Monitoring: Unique Challenges for Contaminant Sampling and Analysis Within EPA's Water Security Initiative

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Poster Abstract

The Water Security (WS) initiative is a U.S. Environmental Protection Agency (EPA) program that addresses the risk of intentional contamination of drinking water distribution systems. Initiated in response to Homeland Security Presidential Directive 9, the overall goal of the WS initiative is to design and deploy contamination warning systems for drinking water utilities. In 2005, EPA documented the conceptual design for contamination warning systems and began implementation of the first water security contamination warning system (WS-CWS) pilot in partnership with the City of Cincinnati at the Greater Cincinnati Water Works. This presentation describes unique challenges associated with pathogen sampling and analysis as part of the contamination warning system with an emphasis on activities conducted in collaboration with the Cincinnati Health Department Laboratory (CHDL). This presentation will also provide an overview and current status of the CHDL Pilot Project including: initial laboratory evaluation and laboratory enhancements, assay evaluation, validation and implementation efforts, and a summary of lessons learned.

Pathogen monitoring for the WS-CWS pilot involves coordinated sampling and analysis between the pilot utility and the analytical support laboratories. Drinking water samples (20–100 L) are concentrated at the utility using a hollow-fiber ultrafiltration procedure developed and validated by the Centers for Disease Control and Prevention (CDC) for recovery of biothreat (BT) agents ("Select Agents") from large volume water samples. The procedure utilizes a 30,000 Dalton molecular weight exclusion dialysis filter to effectively recover and concentrate pathogens and toxins for subsequent analysis. The ultrafiltration process was initially designed to accompany the real-time polymerase chain reaction and culture analyses performed by approved Laboratory Response Network (LRN) member laboratories. In cooperation with, and in support of, EPA's WS-CWS pilot, CDC has enabled the use of this sample processing technology at the pilot utility allowing onsite sampling and sample concentration by utility personnel under the direct supervision of the Ohio Department of Health (ODH). Concentrated samples (i.e., ultrafiltration retentates) are transported to the ODH Laboratory, where all downstream sample processing and analytical procedures are performed. At present, these procedures include rapid screening and confirmatory analyses for a panel of biothreat agents.

In addition to the existing laboratory capabilities for BT agent analyses, EPA, in collaboration with other agencies, is evaluating analytical methods for "non-select" agents in drinking water to support the WS-CWS. The deployment of standardized analytical methods that can be utilized for rapid screening, as well as presumptive and confirmatory analyses, is a priority effort. EPA anticipates that these analytical methods may be integrated into the Water Laboratory Alliance to improve and expand on the laboratory infrastructure supporting WS drinking water monitoring and surveillance efforts.

The current pilot project at CHDL includes activities relating to the development of enhanced laboratory capability and capacity for non-select agents: (1) evaluation of existing and required technical capabilities; (2) procurement of laboratory equipment and supplies related to sampling and analysis; (3) development of method- and process-specific standard operating procedures; (4) evaluation and implementation of analytical methods; (5) development of appropriate quality assurance/quality control procedures to evaluate and monitor overall performance of sampling and analysis; and (6) assistance with logistical issues and planning in, and among, support facilities and agencies.

CHDL currently analyzes clinical, food, and environmental samples for a variety of bacterial and viral agents of concern in these matrices, using both immunoassay and nucleic acid (amplification and hybridization) detection systems. By supplementing current instrumentation and technology, CHDL is expanding these capabilities to include rapid detection assays for contaminants of concern in drinking water. These same techniques can also be used for the analysis of clinical specimens and food samples, thereby promoting a sustainable investment with dual-use applications.

Examination of the Protein Profile of *Helicobacter pylori* Under Different Growth Conditions Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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U.S. Environmental Protection Agency, Cincinnati, OH

Poster Abstract

Helicobacter pylori is a human pathogen implicated in peptic ulcer disease and gastric cancer. Currently, the U.S. Environmental Protection Agency (EPA) is interested in *H. pylori* because it has been designated as an emerging contaminant and methods are needed to detect its occurrence in drinking water. *H. pylori* is an interesting microorganism for two reasons: (1) the lack of a selective culture medium; and (2) its ability to change from a metabolically active helical morphology to a coccoid morphology, and to simultaneously enter a state of dormancy. Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) was used to look at protein profiles of *H. pylori* as well as other related species over the course of 18 days. After 5 days on blood agar, more than 50 percent of the cells were coccoid and non-culturable, and a noticeable shift of protein expression was observed. This altered protein profile was continuously observed up to Day 18, at which all culturability was lost, but ATP was still present. Efforts are currently focused on identifying the proteins that have appeared in the spectra on Day 5 and observed in the spectra taken after 18 days of growth. It is our hope to use these proteins as possible makers of viability and to develop more sensitive methods using these markers as targets.

The Genus *Aeromonas*

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Poster Abstract

The genus *Aeromonas* contains virulent bacteria implicated in waterborne disease, as well as avirulent strains. One of my research objectives was to identify and characterizize host-pathogen relationships specific to *Aeromonas* spp. *Aeromonas* virulence was assessed using changes in host mRNA expression after infecting cell cultures and live animals. Messenger RNA extracts were hydridized to murine whole genome microarrays. Initially, these two model systems were infected with two virulent *A. hydrophila* strains, causing more than 250 genes to be upregulated in animal and cell culture tissues, respectively. Twenty-six genes were common between the two model systems.

The live animal model was used to define virulence for a variety of *Aeromonas* spp. Strains that demonstrated mortality and produced an average upregulation of greater than or equal to three-fold, at challenged doses of 10^7–10^8 CFU, were considered virulent. Mortality results correlated well with dose and transcript upregulation.

Cell cultures were then infected with representative virulent and avirulent *Aeromonas* strains. Transcriptional response from live animal and cell culture models were compared to find common transcripts unique to virulent infections. Two genes with potential for predicting virulence (Jun and Fos) were identified. Quantitative reverse transcriptase-polymerase chain reaction confirmation testing indicated that the Jun oncogene is potentially predictive of *Aeromonas* virulence using cell culture.

A. caviae is associated with gastrointestinal disease but lacks obvious virulence factors (VFs). Microarray profiling of neonatal mouse intestinal extracts after *A. caviae* infection produced a Th1-type immune response characterized by gamma-interferon (γ-IFN) induced genes. This suggests *A. caviae* causes a dysregulatory cytokine response leading to an inflammatory bowel-like disease. This could explain waterborne outbreaks attributed to *A. caviae.*

To evaluate loss of single VFs, isogenic mutants were produced using a transposable element. Using a virulent *A. hydrophila* isolate as the wild-type, mutations in VFs associated with lateral flagella, O-antigen, and secretion systems were created. Swarming motility was eliminated in a lateral flagella mutant, a trait associated with intestinal colonization. Colonization testing using streptomycin-treated mice was inconclusive. Murine cell monolayers demonstrated no difference in gene expression after infection with lateral flagella mutant and wild-type organisms.

Real-Time Quantitative PCR Detection of *Mycobacterium avium* Complex Organisms in Drinking Water

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Poster Abstract

The *Mycobacterium avium* Complex (MAC) includes the species *Mycobacterium avium* (MA), *Mycobacterium intracellulare* (MI), and others. MAC are listed on the U.S. Environmental Protection Agency's Contaminant Candidate List (CCL) due to their association with human disease and occurrence in public drinking water systems. Current methods for detecting MAC organisms in drinking water are culture-based. Evidence suggests, however, that culture-based methods have severe limitations, including long incubation periods, loss of target due to overgrowth of background organisms, up to 70 percent loss of target due to harsh decontamination techniques, and inability to recover MAC in a viable-but-non-culturable state. Because of these drawbacks and the need for more accurate and comprehensive occurrence data, we have developed real-time quantitative polymerase chain reaction (QPCR) assays for detection and quantification of MA, MI, and MA subspecies *paratuberculosis* (MAP) in drinking water. Real-time QPCR assays were developed using primers and TaqMan probes designed to amplify a region of the 16S rDNA in MA and MI, and regions of IS*900* and Target 251 in MAP. Primer/probe sets were found to be highly specific when compared to sequences in nucleotide databases and confirmed experimentally by screening 104 MAC strains. No false negatives occurred when each species was tested with its own primer/probe set, 2.3 percent (1/42) of MA strains were false positive with the MI primer/probe set, and 2.5 percent (1/40) of MI strains were false positive with the MA primer/probe set. No false positives were obtained when nine non-MAC species were screened with all primer/probe sets. Quantification is linear over a minimum range of six logs of target concentration in all four assays. Additionally, a control has been developed to measure PCR inhibition due to compounds in the water matrix. We are currently evaluating the QPCR assays for use on actual drinking water samples as a rapid alternative to culture methods to generate a more complete understanding of MAC occurrence in drinking water.

Identification of *Naegleria fowleri* in Warm Groundwater Aquifers

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Poster Abstract

Naegleria fowleri, a free-living amoeba, was the etiological agent of primary amoebic meningoencephalitis (PAM) that resulted in the death of two children in Peoria, Arizona, during the autumn season of 2002.

In this study, the source water from the greater Phoenix metropolitan area was tested for the presence of *N. fowleri* and *Escherichia coli* O157:H7 using molecular methods, such as polymerase chain reaction, cloning, and sequencing. Results of the study showed the presence of *N. fowleri* in 26 percent of samples. All samples tested negative for *E. coli* O157:H7. Phylogenetic analysis of bacterial communities in the source water seemed to indicate that there was no correlation between dominant bacterial communities in the source water and the presence of *N. fowleri*. The occurrence of *N. fowleri* in the 26 percent of water samples indicates a significant risk to consumers that needs to be addressed by the utilities in the area.

Nano-Intelligent Detection System

Matthew Odom
ANP Technologies, Inc., Newark, DE

Poster Abstract

ANP Technologies, Inc. has developed a field portable sample concentration system that is integrated with its Nano-Intelligent Detection System (NIDS®), consisting of hollow fiber filtration, immunoassays, and an automated reader, to make a complete detection and quantification system. The concentrator is the first stage in a complete detection system that can be used to monitor water supplies for a wide variety of biological agents and waterborne pathogens.

Characterization of Viral RNA Extraction Efficiency From Environmental Waters

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Poster Abstract

Inhibition of polymerase chain reaction (PCR) by environmental factors is a common problem affecting the sensitive detection of pathogenic microorganisms in environmental waters. This inhibition is caused by one of three mechanisms: (1) failure to lyse the microorganism; (2) degradation or sequestering of the nucleic acid following lysis; or (3) inhibition of DNA polymerase during amplification. One solution to overcome these problems is the use of commercially available kits designed for highly efficient extraction and purification of nucleic acids. These kits are designed to overcome these three inhibitory mechanisms by using: (1) optimized chemicals and procedures to ensure maximum lysis of microorganisms; (2) concentration columns that bind released nucleic acids and allow them to be washed to remove inhibitory substances; or (3) a combination of these two approaches. Although several commercial kits are available for the extraction of viral nucleic acids, none are designed or optimized for use in environmental water samples. Previous research has also shown that extraction efficiency in different water sources can be quite variable and affect detection efficiency by molecular techniques, such as RT-PCR. To characterize the extraction efficiency of five commercial RNA extraction kits, environmental water samples (drinking, surface, ground, sea, and sewage) from six geographically diverse regions of the United States were obtained and spiked with a known concentration of poliovirus 2, reovirus 1, and bacteriophage MS2. Water samples were taken and extracted using each RNA extraction kit and analyzed by real-time RT-PCR to determine extraction efficiency. In subsequent experiments, 10 L water samples were concentrated using a hollow-fiber ultrafiltration system and spiked with poliovirus 2, reovirus 1, and bacteriophage MS2. As with initial testing, water samples were taken, extracted using each kit, and analyzed by real-time RT-PCR. The objective of this research project is to characterize the efficiency of various commercial viral RNA extraction kits and their ability to produce high-quality nucleic acids suitable for molecular techniques and under various water conditions.

Phylogenetic Analysis of 16S rRNA Gene Sequences Reveals the Prevalence of *Mycobacteria* sp., Alpha-Proteobacteria, and Uncultured Bacteria in Drinking Water Microbial Communities

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Poster Abstract

Previous studies have shown that culture-based methods tend to underestimate the densities and diversity of bacterial populations inhabiting water distribution systems (WDS). In this study, the phylogenetic diversity of drinking water bacteria was assessed using sequence analysis of 16S rDNA clone libraries. Total community DNA was extracted from water samples collected at different times and locations in a metropolitan distribution system. Phylogenetic analyses of 990 clones revealed that actinobacteria and proteobacteria were the most predominant bacterial groups in the samples analyzed. Within the actinobacterial group, approximately 60 percent of the sequences were identified as mycobacterial species, with clones closely related to *Mycobacterium gordonae*, *M. sacrum*, and *M. mucogenicum* showing sequence similarities of 98 to 100 percent. Although members of the *Mycobacterium* genus are known to be pathogenic and have been isolated from drinking water samples, the public health relevance of these mycobacterial species in drinking water has yet to be determined. Proteobacterial sequences were obtained in all of the clone libraries with approximately 19 percent of all sequences being closely related to alpha-proteobacteria, whereas, beta- and gamma-proteobacteria were only 2 percent and 3 percent, respectively. A significant portion of the sequences (i.e., 18%) showed less than 97 percent identity with sequences present in public databases. Most of the latter sequences are closely related to drinking water sequences retrieved from previous studies, suggesting that these bacteria are normal WDS inhabitants. The results of this study, along with those from earlier studies, are helping us to better understand the molecular diversity and population dynamics of WDS microbial communities.

Development of an Internal Control for Standardization of a Quantitative PCR Assay for Detection of *Helicobacter pylori* in Water

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Poster Abstract

To get an accurate estimate of low numbers of *Heliocobacter pylori* in drinking water, proper internal controls (IC) are needed. A TaqMan quantitative Polymerase Chain Reaction (qPCR) assay was described that detected 10 *H. pylori* cells from 1 L of water using a FAM labeled probe (McDaniels et al., *Water Research* 2005;39:4808-4816). In this study, the 135 bp amplicon from the *ureA* gene, described by McDaniels et al., was modified by four bases at the probe binding region using PCR mutagenesis. The modified fragment was incorporated into a single copy plasmid and used as a PCR positive control. It was detected by a VIC labeled probe at a detection limit of five copies. The fragment was further cloned into *Escherichia coli* cells and used as a matrix spike. A DNA extraction kit was optimized that allowed sampling of an entire liter of water. Water samples spiked with the recombinant *E. coli* were shown to behave like *H. pylori* cells in the qPCR assay and were optimized to be used at 10 cells/L of water, where it was shown not to compete with 5–3,000 cells of *H. pylori* in a duplex qPCR assay. When four finished water samples were spiked with the surrogate *E. coli* (10 cells) and *H. pylori* (100 cells), and evaluated by the complete PCR method, the Ct values obtained were seen to be similar for the different samples, if the chlorine residual was first neutralized by sodium thiosulfate.

Internal Amplification Control for Use in Quantitative Polymerase Chain Reaction Fecal Indicator Bacteria Assays

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Poster Abstract

Quantitative polymerase chain reaction (QPCR) can be used as a rapid method for detecting fecal indicator bacteria. Because false negative results can be caused by PCR inhibitors that co-extract with the DNA samples, an internal amplification control (IAC) should be run with each sample. Currently available controls used in QPCR analyses for the fecal indicator bacterial groups *Enterococcus* and *Bacteroidetes* were designed primarily to determine variability in DNA yields from environmental samples and cannot be used to directly demonstrate PCR inhibition. Therefore, a competitive IAC plasmid DNA was constructed to detect the presence of PCR inhibitors in QPCR assays for both *Enterococcus* and *Bacteroidetes* rRNA gene targets.

The IAC was designed to contain a single site for hybridization with a unique probe sequence that is flanked by multiple primer-hybridizing sites that corresponded to the same primers used in the *Enterococcus*, *Bacteroidetes,* and several additional QPCR assays. The IAC construct was prepared by overlap extension PCR, inserted into the pCR4®TOPO plasmid vector (Invitrogen) and cloned. Gel electrophoresis, QPCR, and sequencing analyses were performed to confirm the presence of the correct IAC sequences in the plasmid. Slope and intercept values of standard curves generated from genomic DNA in simplex analyses were not significantly different ($p > 0.05$) from the values generated during multiplex analyses with a fixed number of 25 IAC plasmid copies. Ranges of genomic DNA concentrations that did not significantly affect the IAC results under the same conditions also were established.

Multiplex analyses with the IAC were used in a study of the relative levels of *Enterococcus* and *Bacteroidetes* DNA in fecal samples from cattle. In these analyses, the *Enterococcus* IAC assay results showed highly consistent cycle threshold values (mean = 34.15, standard deviation = 0.69, N = 159) where only three results failed to occur within the 95 percent confidence interval established from analyses of control samples with IAC plasmid but no fecal extracts present. Greater variability in the *Bacteroidetes* IAC assay results was consistent with the relatively high levels of genomic DNA from these organisms in the samples. These studies indicate that the IAC plasmid DNA performs well as an inhibition control and also may be useful as an alternative to genomic DNA standards for quantifying fecal bacteria target DNA sequences.

Appendixes

The U.S. Environmental Protection Agency (EPA) Workshop on Innovative Approaches for Detecting Microorganisms in Water

June 18-20, 2007
U.S. Environmental Protection Agency
Andrew W. Breidenbach Environmental Research Center
26 W Martin Luther King Drive
Cincinnati, OH

AGENDA

Goals:

- Provide a forum to discuss proposed solutions to the methodological challenges in the search for better methods of detection and assessment of waterborne microbial contaminants.
- Facilitate collaboration and cooperation among scientists and policy-makers from research entities, EPA, states, local agencies, and stakeholders.
- Formally kick off the start of research by the recently awarded Science To Achieve Results (STAR) grants. This workshop will provide a forum to facilitate information sharing and cultivation of collaborations between both the STAR grantees and scientists within EPA's Office of Research and Development, Office of Water, and the Regions. Summaries of the grantees' projects can be found at: http://es.epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html
- Assist EPA in identifying further research or technologies needed to address decisions and/or policy-making issues associated with the assessment of microorganisms in water.

Monday, June 18, 2007 (Auditorium)

Moderator: **Barbara Klieforth**, EPA, Office of Research and Development (ORD)

12:30 – 1:00 p.m.	**Registration**
1:00 – 1:15 p.m.	***Innovative Pathogen Detection in the Context of the National Program for Drinking Water Research*** **Audrey Levine**, EPA, ORD, Drinking Water National Program Director
1:15 – 1:30 p.m.	***Regulatory Perspective From the U.S. EPA's Office of Water*** **Phil Oshida**, EPA, Office of Water (OW), Office of Ground Water and Drinking Water (OGWDW), Standards and Risk Management Division (SRMD)
1:30 – 1:55 p.m.	***Use of Innovative Detection Methods for Detecting Contaminant Candidate List Pathogens*** **James Sinclair**, EPA, OW, OGWDW, Technical Support Center (TSC)
1:55 – 2:20 p.m.	***LATE-PCR: Maximizing Detection Information From a Single Tube*** **Kenneth Pierce**, Brandeis University
2:20 – 2:45 p.m.	**Break**

Monday, June 18, 2007 (Continued)

2:45 – 3:10 p.m.	***Advanced Oxidation Technologies and Nanotechnologies for Water Treatment: Fundamentals, Development, and Application in the Destruction of Microcystin LR*** **Dionysios Dionysiou**, University of Cincinnati
3:10 – 3:35 p.m.	***Development of Gene Microarray Assays for Risk Assessments*** **Parke Rublee**, University of North Carolina at Greensboro
3:35 – 4:00 p.m.	***Characterization of Naturally Occurring Amoeba-Resistant Bacteria*** **Anthony Farone**, Middle Tennessee State University
4:00 – 4:25 p.m.	***Biofilm Sampling and Screening Techniques for Amoeba-Related Biofilm Pathogens*** **Nicholas Ashbolt**, EPA, ORD, National Exposure Research Laboratory (NERL)
4:25 – 5:00 p.m.	***Overview of the U.S. EPA's Office of Research and Development and the Science To Achieve Results (STAR) Program*** **Barbara Klieforth**, EPA, ORD, National Center for Environmental Research (NCER)
5:00 p.m.	**Adjourn**
6:00 p.m.	**Dinner at the Kingsgate Marriott**

Tuesday, June 19, 2007 (Auditorium)

Posters will be up all day and are attended during the hour-long afternoon break.

Moderators: **James Owens**, EPA, ORD, NERL; and **Sandhya Parshionikar**, EPA, OW, OGWDW, TSC

8:30 – 9:00 a.m.	***Overview of Methods for Simultaneous Detection of Pathogens and Introduction to a Highly Multiplexed Nucleic Acid-Based Assay*** **R. Paul Schaudies**, GenArraytion, Inc.
9:00 – 9:25 a.m.	***Robust Piezoelectric-Excited Millimeter-Sized Cantilever Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter*** **Raj Mutharasan**, Drexel University
9:25 – 9:50 a.m.	***Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water*** **Saul Tzipori**, Tufts University
9:50 – 10:05 a.m.	**Break**
10:05 – 10:30 a.m.	***Development of High-Throughput and Real-Time Methods for the Detection of Infectious Enteric Viruses*** **Yu-Chen Hwang**, University of California at Riverside

<u>Tuesday, June 19, 2007</u> (Continued)

10:30 – 10:55 a.m. ***Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water and Its Application for the Detection of Contaminant Candidate List Organisms in Water***
Kelly Bright, University of Arizona

10:55 – 11:20 a.m. ***Timely Multi-Threat Biological, Chemical, and Nuclide Detection in Large Volume Water Samples***
Paul Galambos, Sandia National Laboratories

11:20 a.m. – 12:30 p.m. Lunch (in cafeteria)

Moderators: **Sam Hayes**, EPA, ORD, National Risk Management Research Laboratory (NRMRL); and **Shay Fout**, EPA, ORD, NERL

12:30 – 12:55 p.m. ***On-Chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens***
Syed Hashsham, Michigan State University

12:55 – 1:20 p.m. ***A Novel Molecular-Based Approach for Broad Detection of Viable Pathogens in Drinking Water***
John Scott Meschke, University of Washington

1:20 – 1:45 p.m. ***Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis***
Anthea Lee, Metropolitan Water District of Southern California

1:45 – 2:05 p.m. ***Simultaneous Concentration and Real-Time Detection of Multiple Classes of Microbial Pathogens From Drinking Water***
Otto (O.D.) Simmons III, University of North Carolina at Chapel Hill

2:05 – 3:05 p.m. Attend Poster Session

Moderators: **Nicole Brinkman**, EPA, ORD, NERL; and **Hiba Ernst**, EPA, OW, OGWDW, TSC

3:05 – 3:30 p.m. ***Identification of Bacterial DNA Markers for the Detection of Human and Cattle Fecal Pollution***
Orin Shanks, EPA, ORD, NRMRL, Water Supply and Water Resources Division (WSWRD)

3:30 – 3:55 p.m. ***Detection of Waterborne Pathogens Using Real-Time PCR and Biosensor Methods***
Sangeetha Srinivasan, Michigan State University

3:55 – 4:20 p.m. ***Microarray Detection of Human Viruses From Community Wastewater Systems***
Mark Wong, Michigan State University

4:20 – 4:45 p.m. ***Quantitative Assessment of Pathogens in Drinking Water***
Kellogg Schwab, Johns Hopkins University

<u>Tuesday, June 19, 2007</u> (Continued)

4:45 – 5:10 p.m.	***Development of an Infectivity Assay for Noroviruses in Cells*** **Timothy Straub**, Pacific Northwest National Laboratory
5:10 p.m.	**Adjourn**

<u>Wednesday, June 20, 2007</u> (Rooms 120 and 126)

8:30 – 9:00 a.m.	***An Overview of Pathogen Research in the Microbiological and Chemical Exposure Assessment Research Division*** **Ann Grimm**, EPA, ORD, NERL, Microbiological and Chemical Exposure Assessment Research Division (MCEARD)
9:00 – 11:40 a.m.	**Panel Discussion** ***Co-Leads:*** **Keya Sen**, EPA, OW, OGWDW, TSC; and **Ann Grimm**, EPA, ORD, NERL, MCEARD Panel of federal, commercial, and academic scientists
11:40 a.m. – 1:00 p.m.	**Lunch** (in cafeteria)
1:00 – 3:30 p.m.	**EPA Laboratories and Facilities Open House**
3:30 p.m.	**Adjourn**

The U.S. Environmental Protection Agency (EPA) Workshop on Innovative Approaches for Detecting Microorganisms in Water

June 18-20, 2007

U.S. Environmental Protection Agency
Andrew W. Breidenbach Environmental Research Center
26 W Martin Luther King Drive
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Innovative Methods for Pathogen Detection: Workshop Introduction

Dr. Audrey D. Levine, P.E.
National Program Director

Rationale for Research Workshop

- Increasing demand for real-time assessment of pathogens
  - –Drinking water safety
  - –Water Security
  - –Recreational Water
  - –Irrigation Water
  - –Food Processing and Production
  - –Reclaimed/Recycled Water
- Development of new tools for identification, quantification, and monitoring of pathogens

Pathogen detection research needs

- Sample handling and processing
- Potential interferences
  - –Microbial
  - –Chemical: Salts, Metals, Organics
  - –Particulate matter (organic, inorganic, nanoparticles)
- Viability/Infectivity
- Detection limits
- Relationship to indicators (microbial, chemical)
- Simultaneous detection of multiple pathogens
- Robust methods
- Rapid turn-around time

Overview of EPA-ORD Research Programs

- EPA Strategic Directions
- Science Questions
- Research Questions
- Research Program Design
  - –Core Research
  - –Problem-Driven Research
- Research Planning
  - –Outcome oriented
  - –Provide research support for regulatory decisions

National Research Programs

Goal 1 - Air (Dan Costa)
- NAAQS
- Air Toxics

Goal 2 - Water
- Drinking Water (Audrey Levine)
- Water Quality (Chuck Noss)

Goal 4 – Safe Communities and Healthy Ecosystems
- Homeland Security (John Hermann)
- Human Health (Hugh Tilson) & Ecosystems (Rick Lindthurst)
- Human Health Risk Assessment (John Vandenberg)
- Computational Toxicology (Robert Kavlock)
- Endocrine Disrupting Chemicals (Elaine Francis)
- Global Change (Joel Scheraga)
- Pesticides & Toxics (Elaine Francis)
- Fellowships

Goal 3 - Land (Randy Wentsel)
- Land Protection & Restoration
- Environmental Technology Verification
- SITE Program

Goal 5 - Stewardship
- Economics & Decision Science (Joel Scheraga)
- Sustainability & P2 (Alan Hecht)

National Programs and Directors

D.C.	RTP
Drinking Water: Audrey Levine	**Water Quality**: Chuck Noss
Resource Conservation/ Contaminated Sites: Randy Wentsel	**Human Health**: Hugh Tilson
Pesticides, Toxics and EDCs: Elaine Francis	**Ecological Systems:** Rick Linthurst
Global Climate Change and Mercury: Joel Scheraga	**Air**: Dan Costa

Planning and Development of Research Programs within ORD

- Research Coordination Team (RCT) or similar group
- Annual research planning
- Multi-Year Plans
  - Long-Term Goals
  - Annual Goals and Measures
- External Reviews

Legislative Authorities for Water

- **Safe Drinking Water Act**
  - Requires EPA to set maximum levels for contaminants in water delivered to users of public water systems
  - Sound science and risk-based standard setting

- **Clean Water Act**
  - Sets water quality criteria and guidelines and technology-based standards for ambient water

Safe Drinking Water Act

- Drinking Water Standards
  - Primary
  - Secondary
- Source Water Protection
- Underground Injection Control
- Total Coliform Rule
- Contaminant Candidate List (CCL)
- Unregulated Contaminant Monitoring
- Health Advisories

Clean Water Act

- Effluent Guidelines for the regulation of point sources
- Combined Animal Feeding Operations Rule
- Human Health and Aquatic Life Criteria

Drinking Water Research Program

Vision

Ensure that the United States has drinking water that is safe, sustainable, and affordable through application of sound and innovative science in partnership with the Office of Water, Regions, and other stakeholders

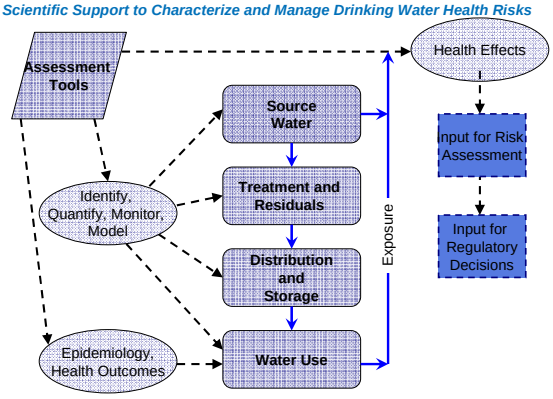
Long Term Goals

Characterize Risks

Manage Risks

Drinking Water Research Program Overview:

Scientific Support to Characterize and Manage Drinking Water Health Risks



Pathogen Detection Needs

- Assessment tools/Health Effects
  - –Methodology for quantifying pathogens
  - –Monitoring tools
- Source Water/Water Resources
  - –Prevalence/persistence of pathogens in Surface water and groundwater
  - –Effectiveness of management practices for controlling pathogens
  - –Source tracking

Pathogen Detection Needs: Treatment and Residuals

- –Fate of pathogens in treatment systems
- –Optimization of disinfection
- –Monitoring approaches to ensure safe drinking water

Pathogen Detection Needs: Distribution and Storage

- –Role of biofilms and inorganic deposits
- –Effectiveness of disinfectant residuals
- –Effect of hydraulics and water age on pathogen survival
- –Intrusion, pipeline integrity

Pathogen Detection Needs: Water Use/Health Outcomes

- Exposure pathways
  - –Drinking water
  - –Food preparation
  - –Non-potable water uses
  - –Irrigation
  - –Cooling water
  - –Recreational Waters
- Epidemiological studies
  - –Effectiveness of regulatory approaches

Key research topics in ORD Research Programs

- Development of methods for detection (viability, infectivity, virulence),
- Treatment systems (for small systems and point-of-use/point-of-entry (POU/POE) needs),
- Alternative approaches for disinfection
- Understanding the impacts of distribution system operation on controlling the risk of exposure to pathogens
- Source tracking,
- Indicator/pathogen relationships,
- Exposure assessments
- Relationships between pathogen (and indicator) occurrence and health outcomes.
- health effects
- Security of water systems

Example Research Topics: Source Water

- LTG1
  - – Characterize water quality (chemicals, pathogens, etc.)
    - Surface water
    - Ground water
  - – Watershed models
  - – Monitoring tools
- LTG2
  - – Best management practices for non-point discharges
  - – Source water protection (surface water, ground water)
  - – Carbon sequestration impacts
  - – Aquifer storage and recovery
  - – Ground water recharge

Example Research Topics: Treatment

- LTG1
  - Monitoring tools
  - CCL contaminants (chemicals and pathogens)
  - Pathogen identification and characterization
  - Characterize disinfection efficacy and disinfection reactions
- LTG2
  - Technologies for removal/control of contaminants
  - Simultaneous compliance
  - Residuals management

Example Research Topics: Distribution Systems

- LTG1
  - Characterize biofilms
  - Characterize chemical release from pipelines
  - Hydraulic models
  - Infrastructure assessment tools
  - Exposure models for pathogens and chemicals
- LTG2
  - Management of distribution systems
  - TCR; LCR; control of DBPs
  - Infrastructure rehabilitation

Example Research Topics: Water Use/ Health Outcomes

- LTG1
  - Health effects of chemicals and mixtures
  - Health effects of pathogens
  - Mode of action/dose response
- LTG2
  - Epidemiology studies
  - Water borne disease outbreak assessment
  - Potable/non-potable health outcomes

Water Quality Research Program

- Improve Water Quality on a Watershed Basis
  - Strengthen the Water Quality Standards Program
  - Improve Water Quality Monitoring
  - Develop Effective Watershed Plans and TMDLs
    - Control Nonpoint Source Pollution
    - Strengthen the NPDES Permit Program
    - Support Sustainable Wastewater Infrastructure
- Improve Coastal and Ocean Water Quality
  - Assess Coastal Conditions
  - Reduce Vessel Discharges
  - Implement Coastal Nonpoint Source Pollution Programs
  - Manage Dredged Materials
  - Manage Invasive Species
  - Support International Marine Pollution Control

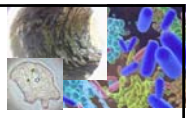
Current Research Related to Distribution Systems and TCR

- ***Distribution Systems and Water Infrastructure***
  - Condition assessment technology, repair, or rehabilitation techniques.
  - Water quality
    - modeling and monitoring
    - management and control of water quality
  - Water security
  - Biofilm pathogen sampling for use in water distribution systems
  - Corrosion studies
- ***Microbiological studies***
  - Genetics of the biofilm amoeba-bacterial-mimivirus environment
  - Virulence factor tools for quantifying infectivity and disinfection efficacy
  - Quantitative Microbial Risk Assessment
  - Microbial source tracking
  - Microbial characterization tools

Health related distribution system studies

- Gastrointestinal illness
  - Incidence
  - Health risks associated with distribution system vulnerability and water quality and identifying risk factors that impact distribution system water quality
  - Relationships between measured & modeled parameters of water distribution systems
  - Exposure, transmission and dose-response models
- Chloraminated water
- Exposure assessment to viruses and protozoan pathogens
- Waterborne Disease Outbreaks

Goals of workshop



- Learn about research efforts pertaining to innovative pathogen detection
- Identify potential research collaborations
- Identify research gaps/needs

Regulatory Perspectives of the Drinking Water Program

U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms in Water

June 18 – June 20, 2007

Phil Oshida, Deputy Division Director
Standards and Risk Management Division
Office of Ground Water and Drinking Water

Divisions of Office of Ground Water and Drinking Water (OGWDW)

- Standards and Risk Management Division
- Drinking Water Protection Division
- Water Security Division

Safe Drinking Water Act (SDWA) Regulatory Process

Based on Sound Science and New Information

1) **SDWA Priority Contaminants**:
- Microbials
- Disinfection Byproducts
- Ground Water Rule
- Arsenic
- Radon
- Radionuclides

3) **Six Year Review**
Review and revise, as appropriate, existing NPDWRs every six years.

(2) **Contaminant Candidate List (CCL) and Regulatory Determination Process**

Statutory Requirements for the Various Drinking Water Regulatory Processes

(1996 SDWA Amendments)

1) **Contaminant Candidate List (CCL)** – SDWA requires EPA to develop a list of contaminants that are known or anticipated to occur in drinking water and to publish the list every five years.

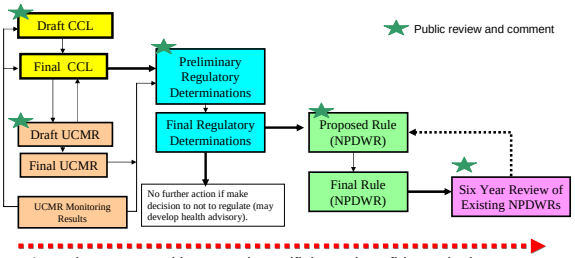
2) **Unregulated Contaminant Monitoring** – SDWA requires EPA to establish criteria for a program to monitor unregulated contaminants, and to identify no more than 30 contaminants to be monitored, every five years.

3) **Regulatory Determination for CCL** – EPA must decide whether to regulate at least five CCL contaminants with a national primary drinking water regulation (NPDWR) after evaluating three statutory criteria; Publish determinations on a five year cycle.

4) **Regulation Development** - If EPA decides to regulate a contaminant, the Agency has 24 months to propose and 18 months to finalize the Maximum Contaminant Level Goal (MCLG) and the NPDWR. SDWA requires that we evaluate a number of components as part of the standard setting process.

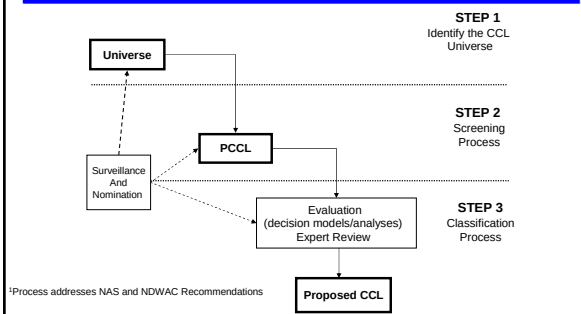
5) **Six Year Review** – Once a contaminant is regulated, every six years EPA is required to review and, if appropriate, revise the NPDWR.

Generalized Temporal Flow of Regulatory Processes



At each stage, need increased specificity and confidence in the type of supporting data used (e.g. health and occurrence).

CCL3 Classification Process[1]



[1]Process addresses NAS and NDWAC Recommendations

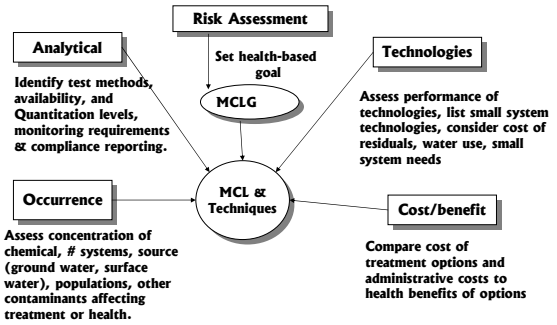
Safe Drinking Water Act -
Making Regulatory Determinations for CCL

SDWA requires EPA to publish a Maximum Contaminant Level Goal (MCLG) and promulgate a National Primary Drinking Water Regulation (NPDWR) for a contaminant if the Administrator determines that -

- *The contaminant may have an adverse effect on the health of persons;*
- *The contaminant is known to occur or there is substantial likelihood that the contaminant will occur in public water systems with a frequency and at levels of public health concern; and*
- *In the sole judgment of the Administrator, regulation of such contaminant presents a meaningful opportunity for health risk reduction for persons served by public water systems.*

SDWA Section 1412(b)(1)

SDWA Standards Setting Process



Current Challenges in monitoring

- Outbreaks
  - Indicator and treatment system although good is not always adequate
    - Outbreaks continue
  - Treatment failures
  - Disinfection resistant pathogens
- Existing tests may not allow timely response to indicator or treatment failures
  - Need for rapid test
    - Coliform indicator bacteria
      - Multiple tube fermentation - 96 hours
      - Colilert – 18 hours or more
  - Do not always give strain level identification
    - Cryptosporidium detection under LT2
    - Human versus animal strains

Outbreaks of Disinfection-Resistant Protozoa

Outbreaks from 1971- 2000 (Craun et al 2003)

Agent	Outbreaks	Illnesses
Giardia	83	25,001
Cryptosporidium	11	420,856

Outbreaks of Bacteria which Co-Occur with Indicators

Outbreaks from 1971- 2000 (Craun et al 2003)

Agent	Outbreaks	Illnesses
E. coli O157:H7	4	451
Shigella	14	5715
Salmonella, nontyphoid	11	3044
Campylobacter	9	5353

Innovative Methods

- OW programs evolve to be responsive to new challenges to drinking water safety
- Innovative methods may offer advantages over current methods
  - Speed, results in 1 to 6 hours
    - Allows response to contamination events before it is too late
  - Detects contaminants that don't co-occur with indicators
  - Specificity
  - Less laborious than microscopic methods

Possible Applications of Innovative Methods

- Unlikely to replace indicators, but could supplement
- Field portable devices
  - Detects pathogens at changing locations in the distribution system
  - Avoids transportation time back to the lab
- On-line monitoring
  - Continuous or frequent analyses from distribution system
    - Detects treatment failures
    - Detects cross-connections in distribution system
- Faster, cheaper screen for *Cryptosporidium, Giardia* than current methods

Possible Applications of Innovative Methods - continued

- Airline Rule
  - Microbial indicators found in airline water
    - Small water tanks, may be replenished with drinking water from other countries
  - Rule being developed to control airliner water quality
  - Desirable method capabilities
    - Conduct test at airport
    - Fast results, within 1 hour if possible
      - Need results before airplane departs

Possible Applications (continued)

Research surveys

- "Omics" for screening purposes
  - Known pathogens
    - Conduct exposure studies
  - Emerging pathogens
    - Assist where occurrence poorly characterized for known emerging pathogens
    - May identify unrecognized pathogens by their virulence factors

Use of Innovative Methods for Detecting CCL Pathogens June 18, 2007

James L. Sinclair, Ph.D.
U.S. EPA, OGWDW, TSC
Cincinnati, OH

EPA's Program for Regulating Emerging Pathogens

- EPA's approach for controlling pathogens in drinking water
  - Water treatment
  - Coliform indicator monitoring
- Some emerging pathogens not controlled by treatment, coliform indicator monitoring
  - Require individual regulations
- Process for regulating unregulated contaminants- Contaminant Candidate List

Drinking Water Contaminant Candidate List (CCL)

- Contaminants to be considered for regulation are listed
- CCL contaminants needing more information become research priorities

Drinking Water Contaminant Candidate List (CCL)

- Criteria for listing contaminants
  - Not regulated or anticipated to be regulated
  - Likely to occur in drinking water
  - Cause adverse health effects

Contaminant Candidate List Microorganisms

- To make regulatory decision information needed:
  - Health effects
  - Treatment
  - Occurrence in water
    - Analytical methods needed

Occurrence Surveys

- Source of occurrence information for regulatory determination
  - Unregulated Contaminant Monitoring Rule (UCMR)
    - EPA's regulation for getting occurrence info for drinking water
  - Other surveys
    - Can be used depending on information provided

Unregulated Contaminant Monitoring Rule (UCMR)

- For CCL or other contaminants needing occurrence information
- 1 year survey of selected drinking water systems
- 3 monitoring options based on method availability

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Unregulated Contaminant Monitoring Rule

- Monitoring options
  - Assessment monitoring
    - Established methods available, commonly used by drinking water laboratories
    - ≈ 4000 systems, enough statistical power for regulatory determinations
  - Screening survey
    - Newly developed methods, not commonly used in drinking water laboratories
    - ≈ 1200 systems, enough statistical power for regulatory determinations depending on results
  - Prescreen survey
    - Methods in early stages of development, specialized, limited applicability
    - Up to 200 systems

8

Types of Methods Needed for Occurrence Surveys

- Methods would ideally produce the following information:
  - Occurrence in drinking water at levels of concern
  - Detect viable, infective organisms
    - Nonviable, infective organisms not a health risk
  - Detect disease-causing species or strains
    - Avoid basing risk on types that don't cause disease

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Existing Methods for Emerging Pathogens

- Often culture methods
- Problems facing existing methods
  - May not distinguish between pathogenic and non-pathogenic forms.
  - Some pathogens not culturable
  - May only have research methods available. Can be:
    - Expensive
    - Slow
    - Labor intensive
- Deficiencies may limit collection of occurrence info.

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Evaluating Occurrence of CCL Microorganisms

- National Research Council (NRC), 2001, noted "bottleneck" in evaluating drinking water pathogens
  - 1 or 2 microorganisms evaluated in 5 to 10 years currently
- Recommended using different tools to determine occurrence of waterborne pathogens.

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NRC Recommendation for Detection of CCL Microorganisms

- Use genetic detection (especially PCR) to identify microorganisms
  - Nonculturable species
  - Viable but nonculturable forms
  - Distinguish pathogenic, nonpathogenic species or genotypes
  - Detect RNA to distinguish live from dead organisms

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NRC Recommendation-continued

- Use genetic detection of virulence factor genes to identify virulence in microorganisms
  - Virulence Factor Activity Relations (VFAR); relates biological characteristics of microorganism to potential for causing harm
    - Estimate potential virulence of microorganism
  - Could be used to detect unrecognized pathogens

NRC Recommendation-continued

- Recommend detecting virulence factor genes with microarrays
  - Use array to capture target nucleic acid for later PCR amplification
  - Use array to detect target nucleic acid via a fluorescent signal
    - Usually requires PCR amplification of sample before testing on the array

Other Recommendations for use of Innovative Methods- UCMR Monitoring

- UCMR 1 comment:
  - EPA needs to define method objectives and use molecular methods if they meet those objectives for a particular UCMR survey option
- Full method capability may not be needed for all 3 UCMR monitoring options

Comment on Suggested UCMR Survey Data Objectives

Assessment Monitoring	Screening Survey	Prescreen Survey
Infectivity (viability) highly desirable	Viability, infectivity not as critical	May not indicate viability or infectivity
Quantitation highly desirable	Quantitation desirable	Presence/absence methods acceptable
Specificity highly desirable	Specificity highly desirable	Specificity highly desirable

Comment on Suggested UCMR Survey Data Objectives

- Molecular methods could be used for the prescreen survey
  - Used to determine presence or absence only
  - Would indicate if further method development or Assessment Monitoring surveys needed or not
  - Would save money by eliminating work on pathogens that are not found in water

Other Suggestions for Use of Innovative Methods for Pathogen Monitoring

- Combine molecular methods with culture methods
  - Use simultaneously to determine specificity and viability
  - Use sequentially with molecular methods as a screen and culture to determine viability for positive cultures.

Steps Taken to Consider Recommendations

- Workshops
  - PCR QA workshop and QA guidance January 2003
    - www.epa.gov/safewater/ucmr//pdfs/meeting_ucmr1_january2003.pdf
    - www.epa.gov/nerlcwww/qa_qc_pcr10_04.pdf
  - VFAR workshop October 2004
    - http://oaspub.epa.gov/eims/xmlreport.display?deid=89544&z_chk=3923
  - Microarray workshop March 2005
    - www.epa.gov/safewater/ucmr/pdfs/summary_workshop_microarrays.pdf
- Research
  - Microarray projects
  - Virulence factor projects
  - Molecular method detection projects

Steps Taken to Consider Recommendations

- Environmental Technology Council (ETC)
  - Congressional mandate, 2003
    - Promote innovative technology for environmental problems
  - Implemented by EPA in 2004
  - Identifies where technology is a critical factor in providing a cost-effective solution
  - Leverage existing resources to promote innovative technologies including NCER grants programs
    - Includes recommending development of new technologies that can be used to detect emerging pathogens in drinking water

Steps Taken to Consider Recommendations

- ETC Program
  - EPA Members from ORD, program offices, Regions
  - 11 action teams, one focuses on detection of microbial contaminants in drinking water
  - Microbial team information: http://www.epa.gov/etop/forum/
  - Team leads: Keya Sen, OW, Sam Hayes, ORD
    - Participation by innovative methods researchers encouraged

Linear-After-The-Exponential (LATE) – PCR:
Maximizing Detection Information from a Single Tube

Kenneth Pierce, Ph.D.

Cristina Hartshorn, Ph.D.

Arthur Reis, Ph.D.

John Rice, M.S.

J. Aquiles Sanchez, Ph.D.

Lawrence J. Wangh, Ph.D.

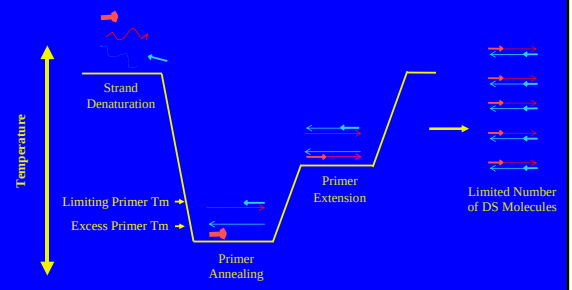
Laboratory of Molecular Diagnostics and Global Health
Brandeis University, Waltham, Massachusetts

LATE-PCR

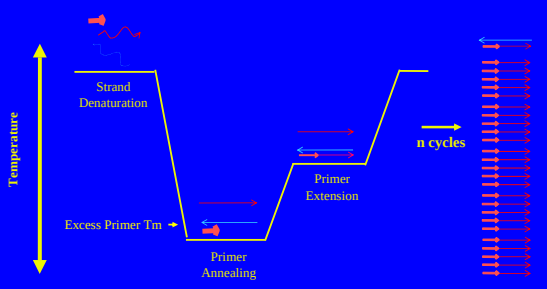
Linear After the Exponential-PCR

an advanced form of Asymmetric PCR

LATE-PCR phase I
Exponential Amplification of Double-Stranded DNA



LATE-PCR phase II:
Linear Amplification of Single-Stranded DNA



LATE-PCR: Axiom 1 $(Tm^L - Tm^X) \geq 0$

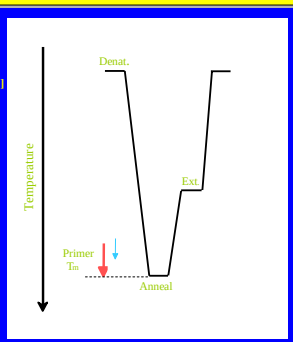
$Tm = [\Delta H/(\Delta S + R \ln (C/2))] - [273.15 + 12 \log [M]]$

LATE-PCR

Modifies Limiting Primer So That Limiting Primer T_m Is Above Excess Primer T_m

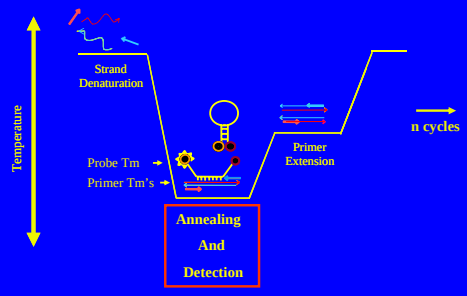
$(T_m^L - T_m^X) \geq 0$

Efficient!

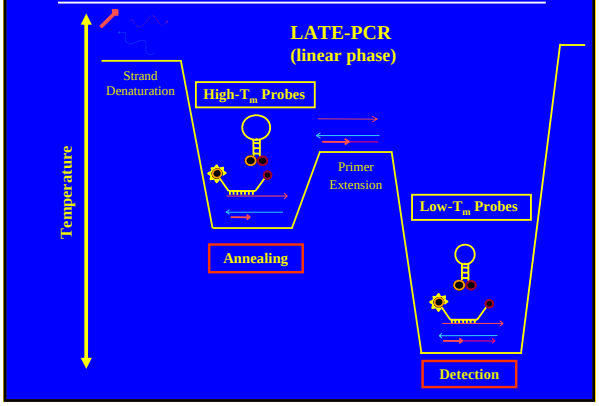


Sanchez *et al.* (2004) PNAS 101:1933-1938

Primer Annealing and Probe Detection are Linked In Symmetric PCR

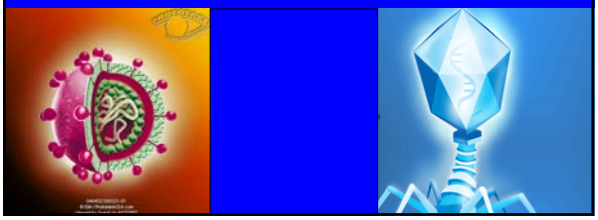


LATE-PCR Uncouples Annealing and Detection



Detection of Hepatitis C Viral RNA:

A demonstration of "virtual sequencing" using LATE-PCR and mismatch-tolerant probes



HCV Genome

- Positive Strand RNA
- 9,646 nucleotide length
- Proteins are synthesized from viral RNA
- No DNA intermediate

Armored RNA® from Asuragen

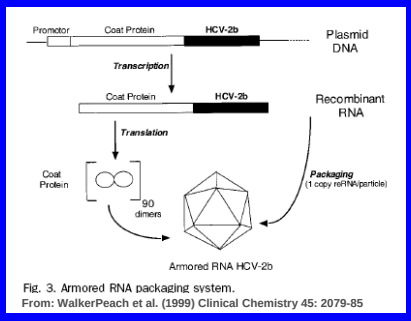
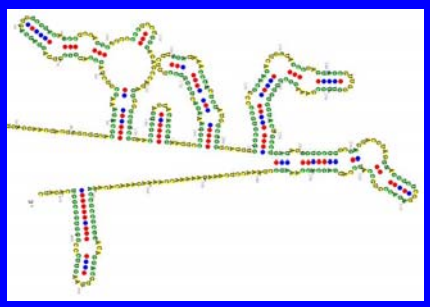
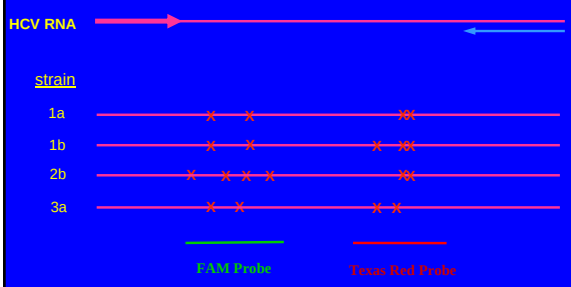


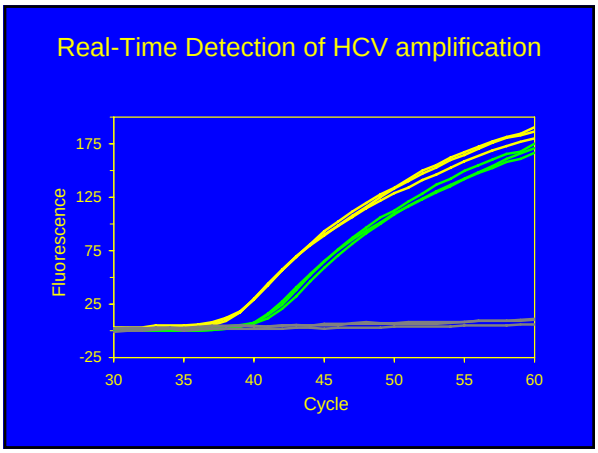
Fig. 3. Armored RNA packaging system.
From: WalkerPeach et al. (1999) Clinical Chemistry 45: 2079-85

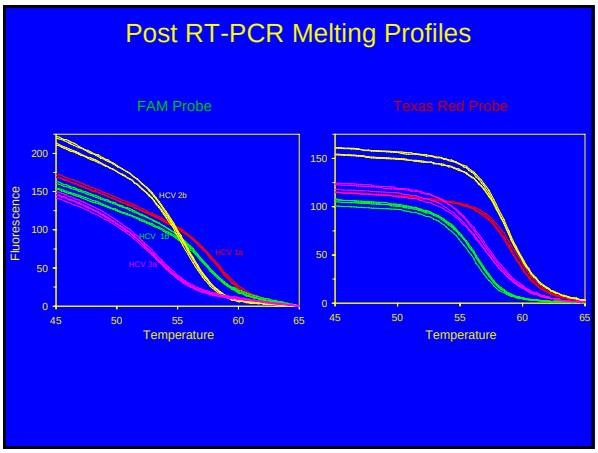
HCV 5' UTR secondary structure

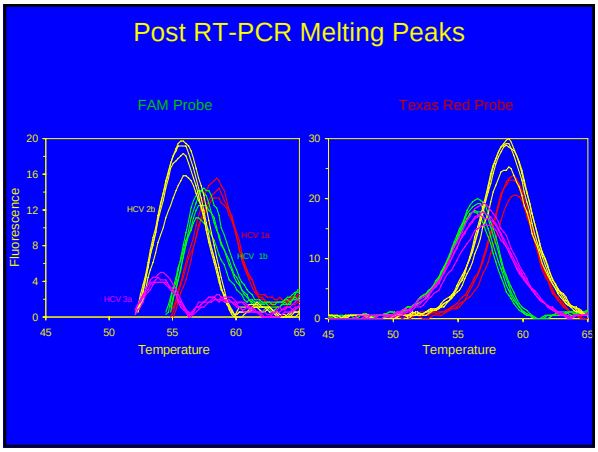


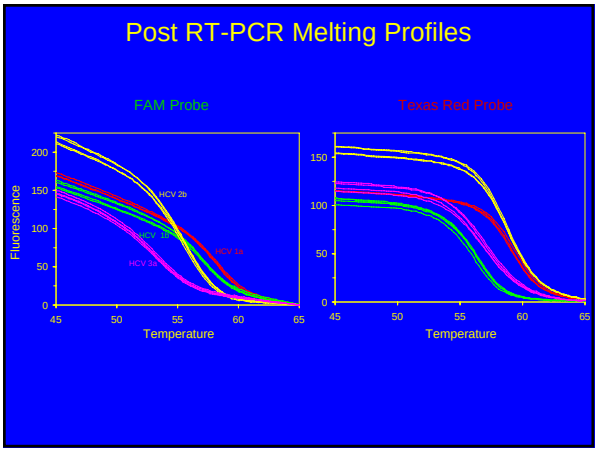
Primer and Probe Design

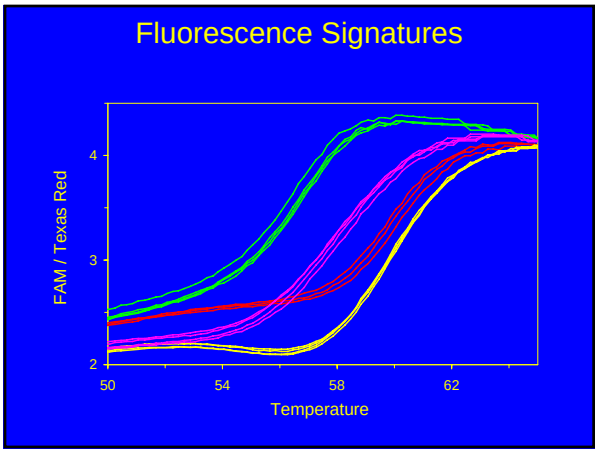


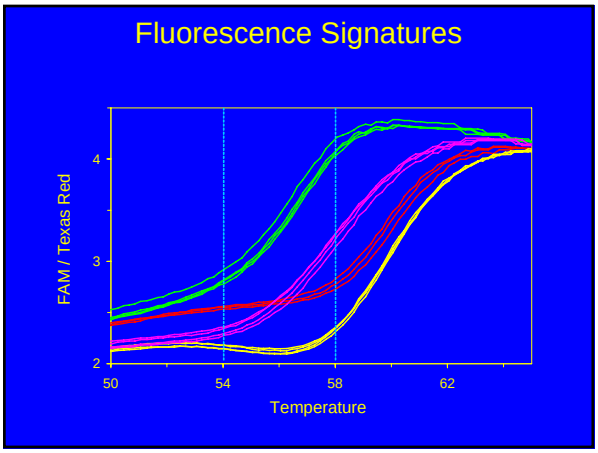

Real-Time Detection of HCV amplification
Fluorescence
175
125
75
25
-25
30
35
40
45
50
55
60
Cycle


Post RT-PCR Melting Profiles
FAM Probe
Texas Red Probe
Fluorescence
HCV 2b
HCV 1b
HCV 1a
HCV 3a
Temperature
Temperature

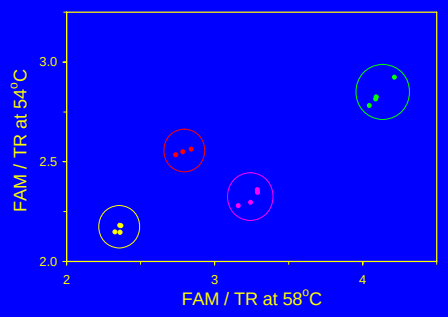

Post RT-PCR Melting Peaks
FAM Probe
Texas Red Probe
Fluorescence
HCV 2b
HCV 1a
HCV 1b
HCV 3a
Temperature
Temperature


Post RT-PCR Melting Profiles
FAM Probe
Texas Red Probe
Fluorescence
HCV 2b
HCV 1b
HCV 1a
HCV 3a
Temperature
Temperature

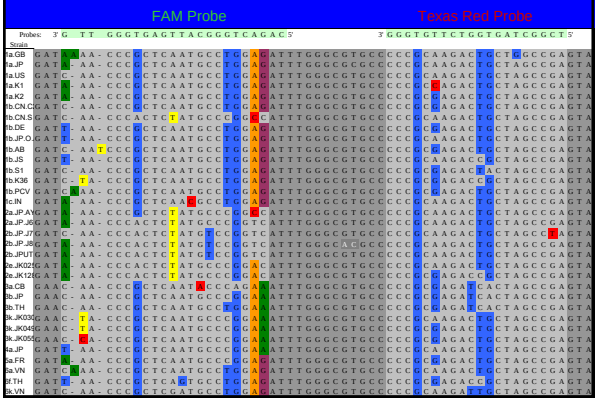

Fluorescence Signatures
FAM / Texas Red
4
3
2
50
54
58
62
Temperature


Fluorescence Signatures
FAM / Texas Red
4
3
2
50
54
58
62
Temperature

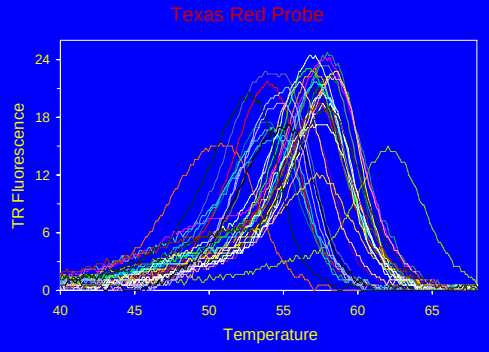
Fluorescence Signatures



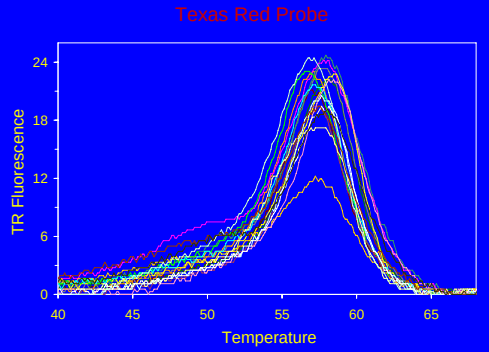
HCV sequence variations



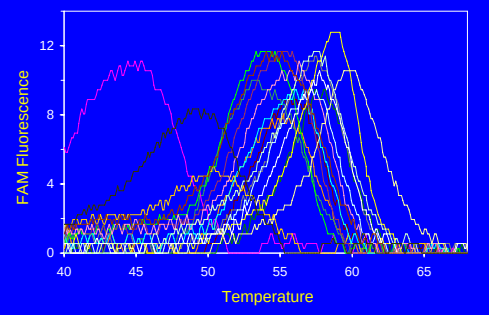
Probe – Target Melting Profiles



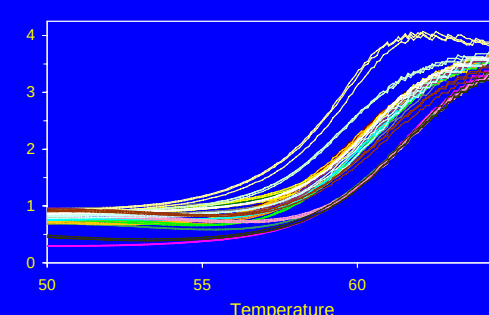
Probe – Target Melting Profiles

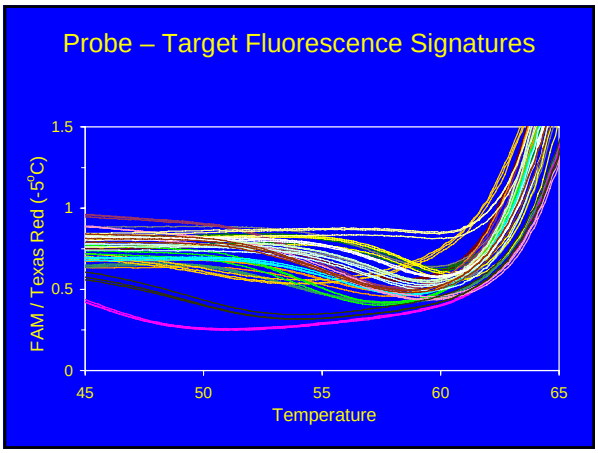


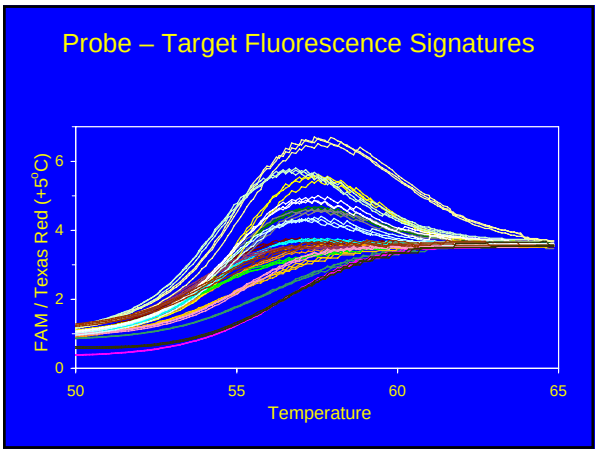
Probe – Target Melting Profiles

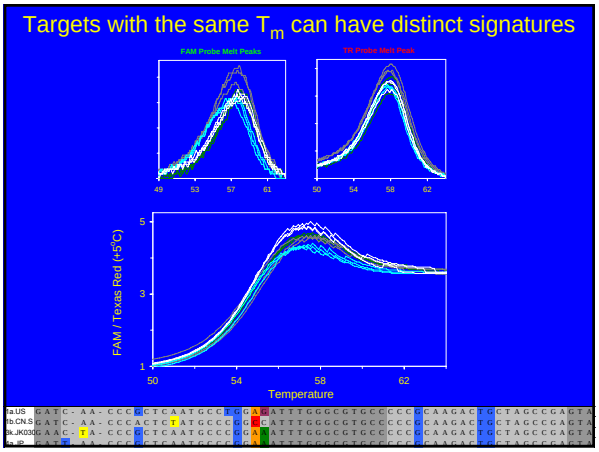


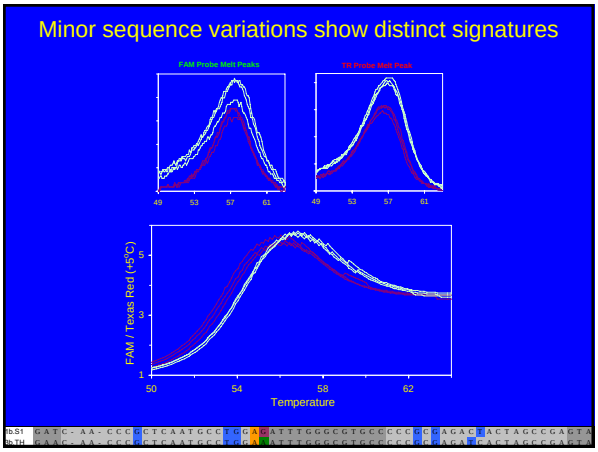
Probe – Target Fluorescence Signatures

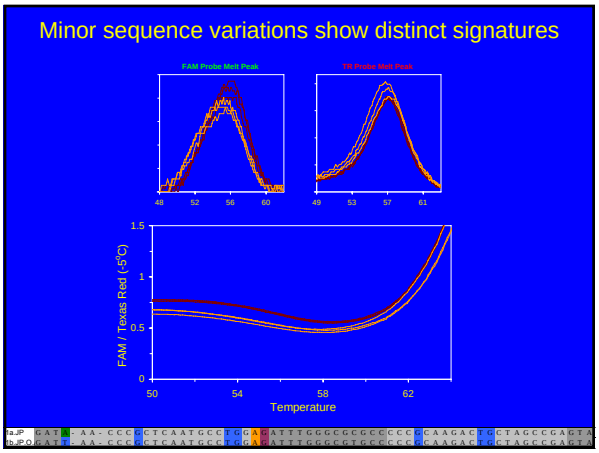


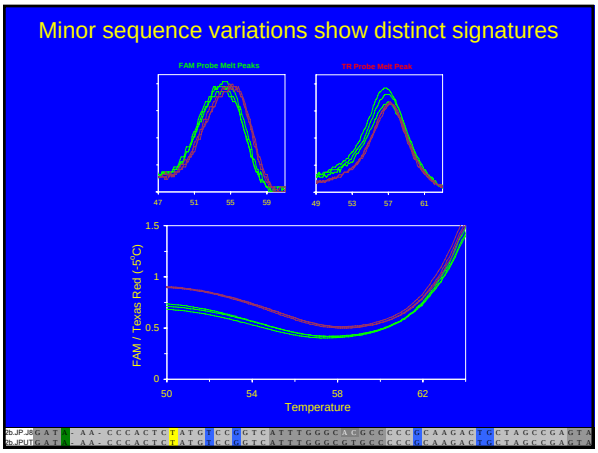

Probe – Target Fluorescence Signatures
FAM / Texas Red (-5°C)
Temperature
1.5
1
0.5
0
45
50
55
60
65


Probe – Target Fluorescence Signatures
FAM / Texas Red (+5°C)
Temperature
6
4
2
0
50
55
60
65

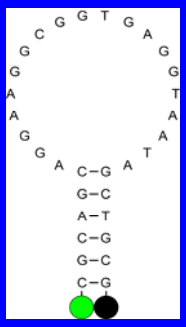

Targets with the same Tm can have distinct signatures
FAM Probe Melt Peaks
TR Probe Melt Peak
FAM / Texas Red (+5°C)
Temperature
5
3
1
50
54
58
62

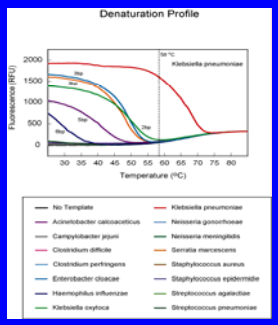

Minor sequence variations show distinct signatures
FAM Probe Melt Peaks
TR Probe Melt Peak
FAM / Texas Red (+5°C)
Temperature
50
54
58
62


Minor sequence variations show distinct signatures
FAM Probe Melt Peak
TR Probe Melt Peak
FAM / Texas Red (-5°C)
Temperature
1.5
1
0.5
0
50
54
58
62


Minor sequence variations show distinct signatures
FAM Probe Melt Peaks
TR Probe Melt Peak
FAM / Texas Red (-5°C)
Temperature
1.5
1
0.5
0
50
54
58
62

Finicky Molecular Beacons and LATE-PCR



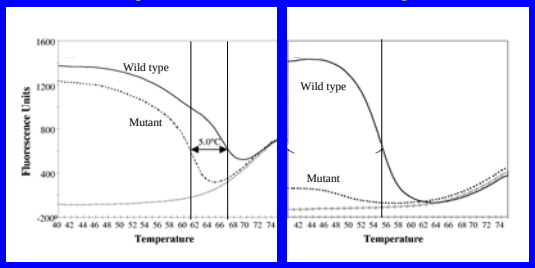


Fred Kramer, personal communication

Advantages of Low-T_m Probes: Increased target specificity

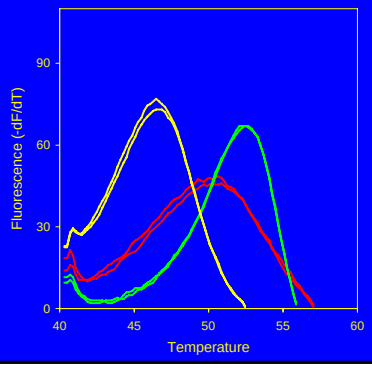
High-T_m Molecular Beacon Melting Curve

Low-T_m Molecular Beacon Melting Curve

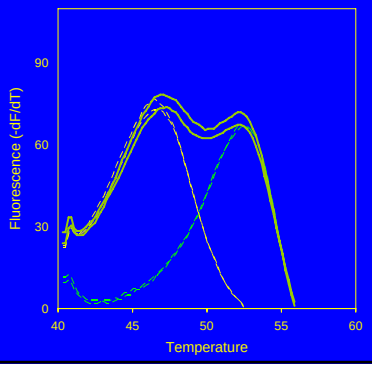


Sanchez *et al.* (2003) *Proc. Natl. Acad. Sci.*

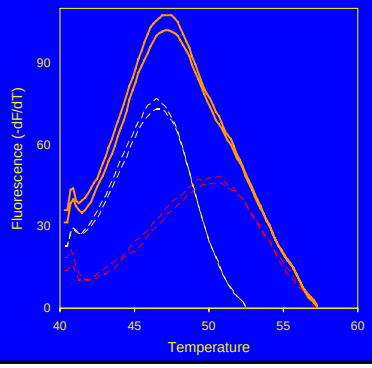
Specific probes with the same fluorophore can detect multiple targets in a single mixture



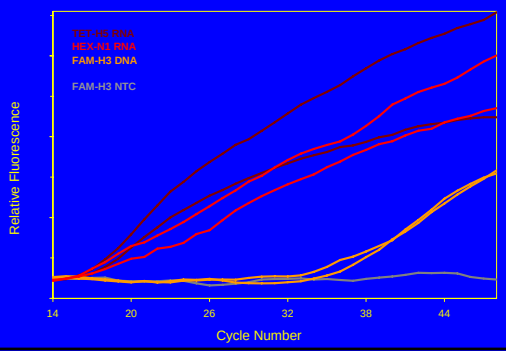
Specific probes with the same fluorophore can detect multiple targets in a single mixture



Specific probes with the same fluorophore can detect multiple targets in a single mixture



Specific probes can detect multiple targets at vastly different initial concentrations



Advantages of Low T_m Probes

Mismatch Tolerant Probes

- Strong signals with LATE-PCR products
- Potential to detect extremely high number of sequence variations
- Signal ratios ("fluorescence signatures") can distinguish most nucleotide variations within the region hybridized by the probes

Sequence Specific Probes

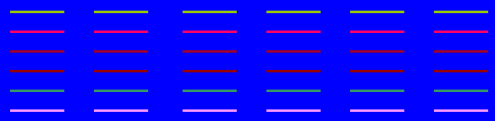
- Strong signals with LATE-PCR products
- High number of unique targets can be detected
- Low temperature enhances sequence discrimination
- Multiple target types in the same sample can be identified

A Look to the Future

Multiple Pairs of LATE-PCR Primers

6 Probes for each of 6 Colors

1 Specific Sequence Per Probe

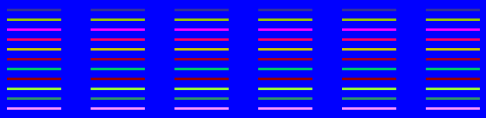


A Look to the Future

3 Pairs of LATE-PCR Primers – 3 Amplicons

6 Mis-match Tolerant Probes in 6 Colors

10 Sequence Variants Per Probe

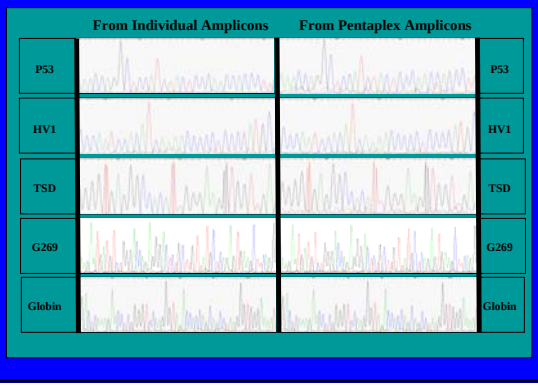


1,000,000
possible variants in a single closed tube

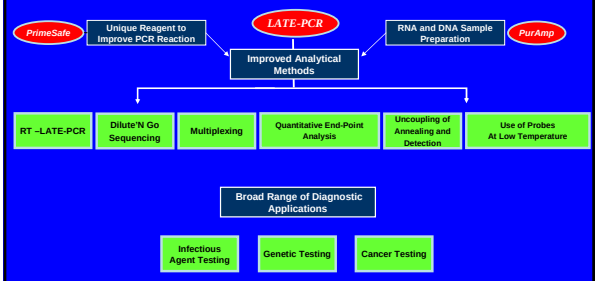
1,000,000
possible variants in a single closed tube

And if that is not enough
You can use Dilute-'N'-Go Sequencing
for all amplicons from the same tube

Dilute-'N'-Go Sequencing for Product Detection following Multiplexing



A Whole System Approach



BioSeeq2 from Smiths Detection

- Rapid thermocycling (fast time to result)
- Each thermocycler independently programmable
- Wireless communication for new assay formats
- Fully automated (minimal user intervention)
- Touch screen, no buttons
- Active cooling and up to 6 colors to take full advantage of LATE-PCR capabilities
- Ruggedised Field Portable PCR
- Decontamination by immersion in disinfectant

U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms in Water
U.S. EPA, Cincinnati, OH, June 18-20, 2007

Advanced Oxidation Technologies and Nanotechnologies for Water Treatment: Fundamentals, Development and Application in the Destruction of Microcystin LR

*Dionysios D. Dionysiou[1]**
Hyeok Choi[1], Maria G. Antoniou[1], Armah A. de la Cruz[2], Jody A. Shoemaker[2],

[1]Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, OH, USA
**dionysios.d.dionysiou@uc.edu*

[2]Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268, USA

Cyanobacteria and their toxins*

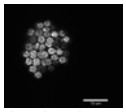
- The eutrophication of water resources, favors the formation of cyanobacteria harmful algal blooms (cyano-HABs)
- Overgrowth of cyano-HABs causes:
  - Green like bean soup color, taste and odor [geosmin, 2-methylisoborneol (MIB)]
  - Production and release of bioactive compounds (~ 50 genera) which are harmful to humans and the ecosystem:
    - Irritant toxins, Dermatotoxins, Hepatotoxins and Neurotoxins

The most commonly found cyanotoxin during cyano-HABs is a hepatotoxin, microcystin-LR

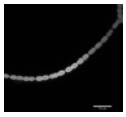
* Antoniou *et al.*, *J. Environ. Eng.* 131 (2005) 1239; Carmichael, *Scient. Amer.* 270 (1994) 78.

Microcystin-LR (MC-LR)

- Hepatotoxin, Protein Phosphatase (PP) Inhibitor, Tumor Promoter
- Chemical Structure
  - 5 invariant modified amino acids and
  - 2 variant amino acids (> 80 MCs isoforms)
  - MC –LR (L= Leucine and R= Arginine)
- High chemical stability (cyclic structure)
- Very Soluble in water (functional groups)
- LD_{50} = 50 µg/Kg (mouse bioassay)
- World Health Organization (WHO) 1 µg/L (provisional concentration limit in Drinking Water)
- Not regulated in terms of MCL and BAT

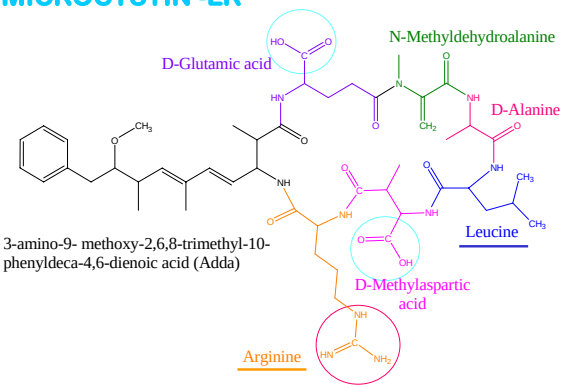


Microcystis aeruginosa



Anabaena

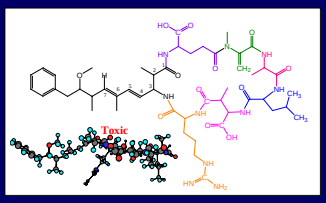
MICROCYSTIN -LR



Toxicity of MC-LR

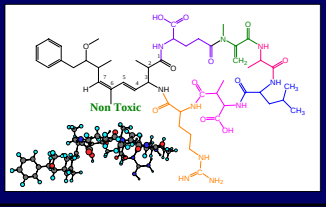
- The successful attachment of MC-LR in the receptor of the protein phosphatase is directly related to the 3-D configuration of the toxin.

Goldberg *et al.*, *Nature* 376 (1995) 475



- So far, two derivatives of MCs (LR and RR) where the bond at C_6-methyl and C_7-hydrogen was in *cis* configuration have been found to be non-toxic.

Harada *et al.*, *Chem. Res. Toxicol.* 3 (1990) 473



Health Incidences

- Health episodes regarding cyanobacterial contamination of animals and humans are found worldwide
  Carmichael W.W., *Sci. Am.*, 270 (1994) 78
- First scientific report on cyanobacteria: 1878
  Francis, G. Poisonous Australian lake. *Nature (London)* 18, (1878) 11-12
- Some Affected Countries:
  - USA (Florida)
  - Australia (Red Tides; Palm Island Mystery, 1979)
  - China (high occurrence of liver failure)
  - Mexico Bandala E. *et al*. *Toxicon*, 43 (2004) 829
- Fatal incident was reported in Brazil (1996) where more than fifty dialysis patients died due to the use of MC-LR contaminated water (CARUARU SYNDROME) Pouria S. *et al*. *Lancet*, 352 (1998) 21

Regulations

- Cyanobacteria and their toxins are part of the "The Drinking Water Contaminant Candidate List" (CCL 1&2).

  USEPA (2005) , *EPA 815-F-05-001*

- Microcystin has not yet been regulated for:
  - maximum contaminant level (MCL),
  - best available technology (BAT).

  USEPA (2003) , *EPA-816-R-03-XXX*

- World Health Organization (WHO): limit of MC-LR to potable water to 1 µg/L

- January 7th 2007: Peer review panel recommendation for MC-LR (USEPA, Cincinnati) to lower MC-LR limit to 100 ng/L.

- Cyanotoxins can bioaccumulate in fish tissues and shellfish, therefore WHO also established a tolerable daily intake (TDI) of 0.04 µg kg^{-1} day^{-1}.

  WHO (1999) http://www.who.int/docstore/water_sanitation_health/toxicyanobact/begin.htm

MC-LR Treatment

- Conventional treatment processes remove algal cells and part of the soluble toxin. Schmidt *et al.*, *Environmental Toxicology*, 17 (2002) 375
- Advanced Oxidation Technologies (AOTs) were tested

  Lawton and Robertson, *Chem. Soc. Rev*, 28 (1999) 217; Song et al., *Environ. Sci. Technol*, 40 (2006) 3941

- TiO_2 Photocatalysis as Technology
  - Water Purification-Complete mineralization and Disinfection
  - No addition of other chemicals
  - No production of hazardous wastes
  - Can perform detoxification of water as well

➢ TiO_2 nanoparticles in slurry have been used for the degradation of MC-LR successfully

Robertson *et al.*, *Chem. Commun. (Cambridge)* , 1994 (1997) 393

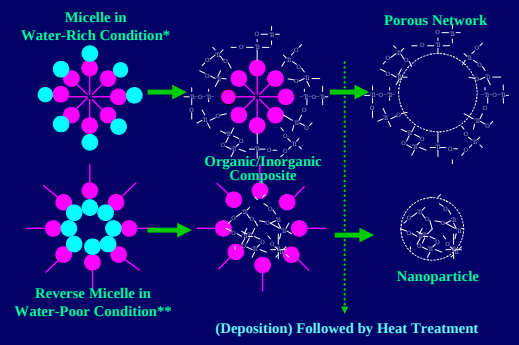
TiO_2 Photocatalysts: Desirable Properties

- Immobilization* onto Substrates: Films and Membranes
  - (TiO_2 nanoparticles in suspension: Possible toxicity)**
  - No need of TiO_2 separation
  - Multifunction of TiO_2 membrane: Photocatalysis and separation***
  - Uniformity and pore structure controllability
- Improvement of Physicochemical Properties
  - Active anatase phase and high surface area
  - High catalytic activity (in case of limited mass of immobilized TiO_2)

"Can be Achieved by Nanotechnology-based Methods and Procedures"

* Balasubramanian G. et al., J.-M., *Journal of Materials Science*, 38 (2003) 823; Chen and Dionysiou, *Applied Catalysis B: Environmental*, 62 (2006b) 255. **Wiesner *et al.*, *Environ. Sci. Technol*, 40 (2006) 4336; Long *et al.*, *Environ. Sci. Technol*, 40 (2006) 4346. *** Anderson *et al.*, *J. Membr. Sci*, 39 (1988) 243.

Synthesis Approach: Surfactant-Templates

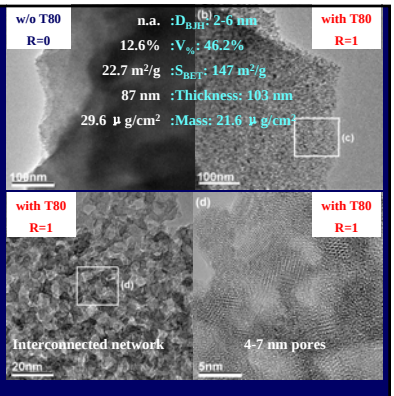


*Boss *et al.*, *Chem. Mater*, 15 (2003) 2463.
**Piling, *Langmuir* 13 (1997) 3266; Stathatos *et al.*, *Langmuir* 13 (1997) 4295.

TiO_2 Films (Surfactant Effect)*

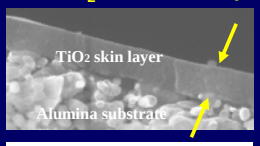
Formulation:
Tween80** (or others): R
iPrOH: 45
Acetic Acid***: 6
TTIP: 1

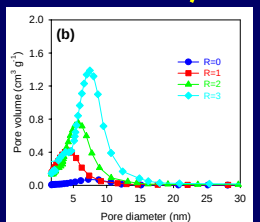
R=1 TiO_2 film has 4 times higher photocatalytic activity than R=0 TiO_2 film (MB degradation).

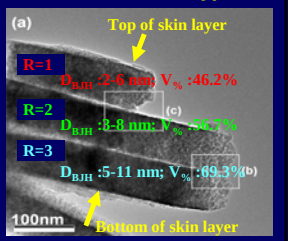


*Choi *et al.*, *Appl. Catal. B* 63 (2006) 60; *Thin Solid Films* 510 (2006) 107.
**Polyoxyethylenesorbitan monooleate.
*** Stathatos *et al.*, *Micro & Mesoporous Mater*. 75 (2004) 255; Wang *et al.*, *Inor. Chem*. 40 (2001) 5210.

TiO_2 Membranes (Pore Size Controllability)*







Multifunction*:
Photocatalysis: Creatinine, MC-LR
Disinfection: *E. coli*
Separation: PEG, activated sludge
Anti-biofouling: Less adsorption fouling

*Choi *et al.*, *Adv. Func. Mater*. 16 (2006) 1067.

Analytical Methods for the quantification of MC-LR and detection of intermediates

LC/MS/MS Analysis

- **LC: Agilent 1100 series***

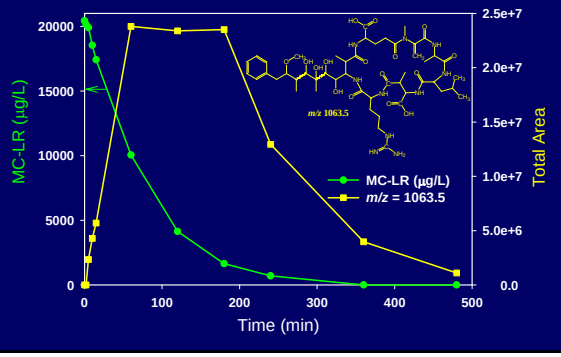
Column: Supelco C18, 150x4.6 mm, 5 µm particle size
T_{column}= 40 ºC
Flow rate = 1.0 mL/min
A= Water with 0.1% Formic Acid
B= Acetonitrile with 0.1 % formic acid

- **MS/MS: Thermo Finnigan LCQ DECA**

Ion Trap Mass Spectrometer Electrospray
Positive Ion Mode

*Modified method proposed by Liu *et al.*, ES&T, 37 (2003) 3214

Formation of *m/z* = 1063.5 reaction-by-product from the degradation of MC-LR with TiO_2 photocatalytic films



Chemical Mechanism of TiO_2 Photocatalysis

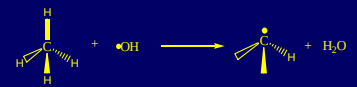
- **Step 1:** **Formation of reactive (oxidizing/reducing) species:**

$$^{\bullet}OH,\ e^-_{CB}$$

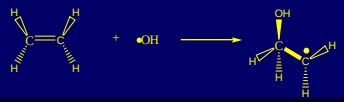
- **Step 2:** **Reaction of •OH with organic compounds and formation of carbon centered radicals.**

i.e., in the case of oxidation by hydroxyl radical:

Hydrogen abstraction:



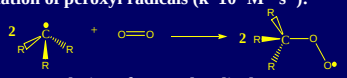
Hydroxyl ion addition:



* Al-Ekabi, 1997

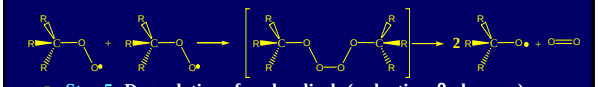
Cont.

- **Step 3:** **Addition of oxygen to carbon centered radicals: Formation of peroxyl radicals (k~10^9 M^{-1} s^{-1}):**



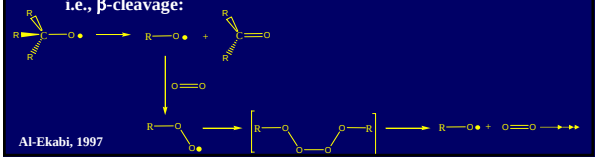
- **Step 4:** **Degradation of peroxyl radicals:**

i.e., bimolecular decay (formation of oxyl radicals):



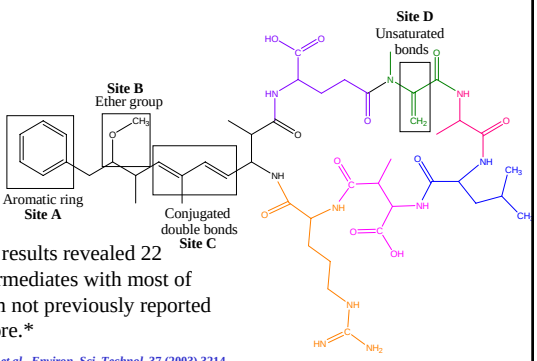
- **Step 5:** **Degradation of oxyl radicals (reduction, β-cleavage):**

i.e., β-cleavage:



Al-Ekabi, 1997

Sites of Attack of OH radicals on MC-LR

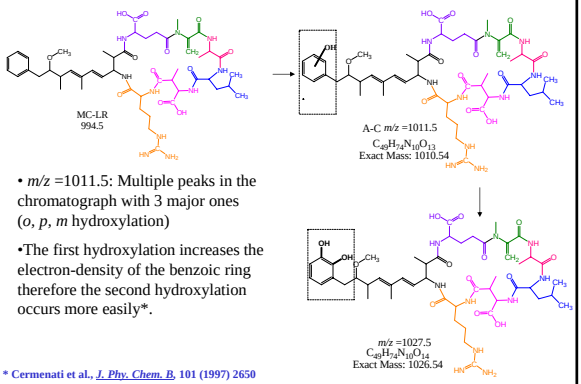


Our results revealed 22 intermediates with most of them not previously reported before.*

*Liu *et al.*, *Environ. Sci. Technol.* 37 (2003) 3214

*Song *et al.*, *Environ. Sci. Technol.* 40 (2006) 3941

Site A: Aromatic Ring



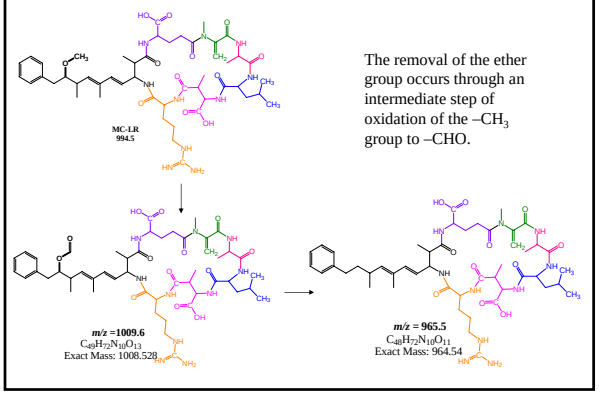
• *m/z* =1011.5: Multiple peaks in the chromatograph with 3 major ones (*o*, *p*, *m* hydroxylation)

•The first hydroxylation increases the electron-density of the benzoic ring therefore the second hydroxylation occurs more easily*.

* Cermenati et al., *J. Phy. Chem. B*, 101 (1997) 2650

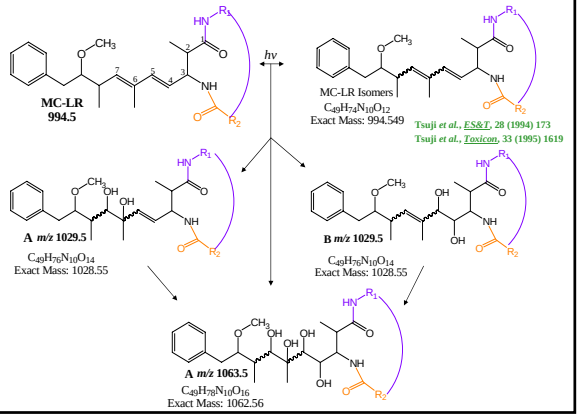
* Song *et al.*, *Environ. Sci. Technol.* 40 (2006) 3941

Site B: Ether Group

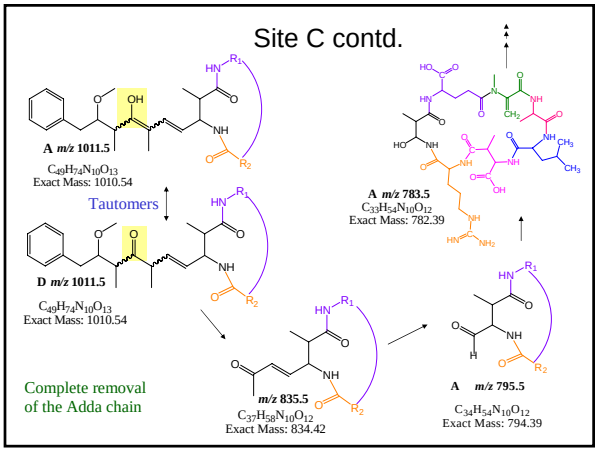


The removal of the ether group occurs through an intermediate step of oxidation of the $-CH_3$ group to $-CHO$.

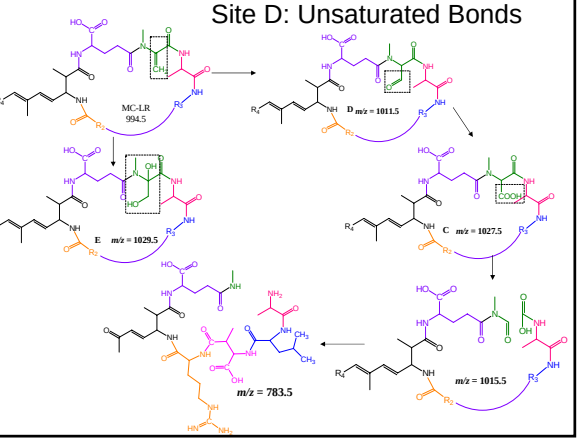
Site C: Diene Bonds



Site C contd.

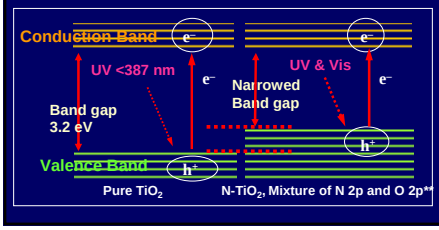


Site D: Unsaturated Bonds



Visible Light Activation of TiO_2

- **Explore Solar Driven TiO_2-based Water Treatment Technologies**
  - Solar light: sustainable source of energy
- **Band-Gap Narrowing of TiO_2 for Visible Light Activation***
  - Impurity doping (transition metals, non metal species like N, S)
  - N-TiO_2: Stable material and reproducible synthesis methods

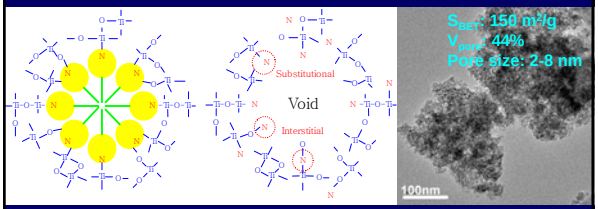


*Sato, *Chem. Phys. Lett* 123 (1986) 126; Burda *et al.*, *Nano Lett.* 3 (2003) 1049; Bacsa *et al.*, *J. Phys. Chem.* 109 (2005) 5994; Wu *et al.*, *Appl. Phys. A* 81 (2005) 1411.

**Asahi *et al.*, *Science* 283 (2001) 269; Irie *et al.*, *J. Phys. Chem. B* 107 (2003) 5483

Nanotechnological Approach for N-TiO_2 Synthesis

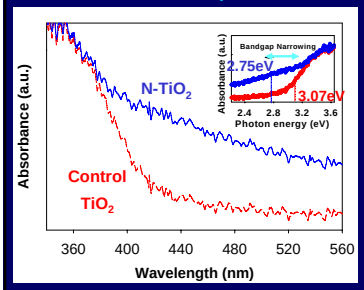
- **Use of Nitrogen-Containing Surfactant (NCS)**
  - NCS: pore template & nitrogen dopant
  - Synthesis of TiO_2, N doping, porous structure
  - → One step preparation



S_{BET}: 150 m^2/g
V_{pore}: 44%
Pore size: 2-8 nm

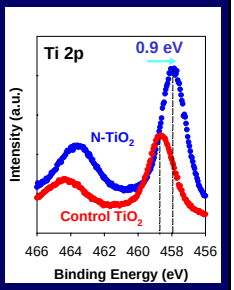
Band Gap Narrowing of N-TiO_2

UV-Vis Absorption



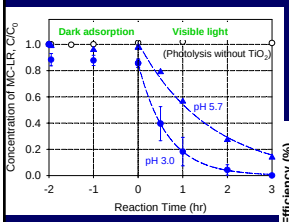
Bandgap narrowing, approx. 0.3 eV

XPS

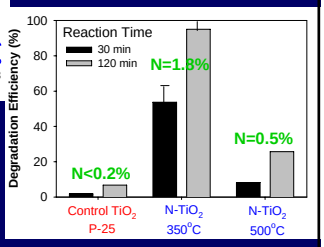


Less binding energy of electrons in Ti 2p state for N-TiO_2

MC-LR Degradation Using N-TiO_2 Under Visible Light



$C_{o,\ MC\text{-}LR}$ = 5 mg/L
TiO_2 = 0.5 g/L
Visible Light > 420 nm



Summary

- TiO_2 Material Synthesis *via* Surfactant Templating Approaches
  - Controllability of the structural properties: Target specific applications
  - High catalytic activity per unit TiO_2 mass
  - Immobilization as films and membranes: Process integration and sustainable and engineered approach
- Mechanistic Studies with MC-LR and HRs

HPLC/MS/MS: Identification of four positions on which HRs generated with TiO_2 photocatalysis can initially react with MC-LR
- Sites A, B, C : intermediates where the Adda chain is affected
- Sites D : intermediates of OH attack on the cyclic structure

Summary

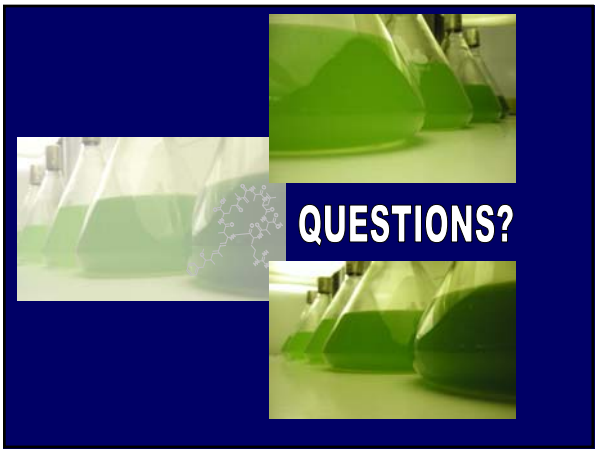
Main Mechanistic Steps:

- Hydroxylation (Substitution/ Addition)
- Oxidation
- Bond cleavage

- **Visible Light Activated N-TiO_2**
  - High catalytic activity under visible (and UV) irradiation: Use of sustainable and renewable solar energy
  - Promising for the remediation of water resources contaminated with biological toxins and other chemicals of concern using solar light

Acknowledgements

- US EPA (Drinking Water Education Center)
- NSF-CAREER Award (BES-0448117)
- NASA (Grant Number NAG 9-1475)
- Center of Sustainable Urban Engineering, University of Cincinnati
- Sigma Xi Grants-in-Aid Research Award for Maria G. Antoniou



Development of Gene Microarray Assays for Risk Assessment

Parke Rublee[1], Vincent Henrich[1], JoAnn Burkholder[2]
[1]University of North Carolina at Greensboro
[2] North Carolina State University

This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program Grant # RD-83162701

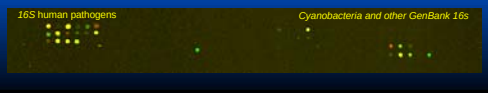
Relative proportion of cyanobacteria to total phytoplankton cell number in North Carolina waters (from Glasgow and Burkholder 2003).

Microcystins (WHO guideline for drinking water = 1 µg l^{-1})

2002 – 0.19 – 0.48 µg l^{-1} in twelve NC reservoirs
2005 – generally below detection limit (0.05 µg l^{-1}) except for bloom in one lake (up to 26 µg l^{-1} in thick cell mass)
2006 – 0.16 – 0.27 µg l^{-1} in five NC reservoirs

Goal - Approach

- Sample collections across NC reservoirs
- Extract genomic DNA – amplify with cyanobacterial primers
- Generate clone libraries – sequence and identify
- Generate primers and 50-mer probes to cyanobacteria taxa (to operational taxonomic units [OTUs] ≈ species)
- Generate probes to known cyanotoxin genes
- Spot oligonucleotide probes on microarray slides for rapid cyanobacteria assessment

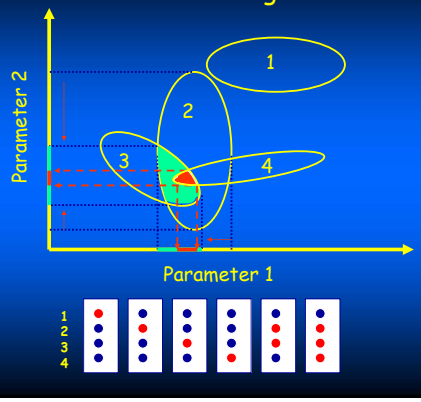


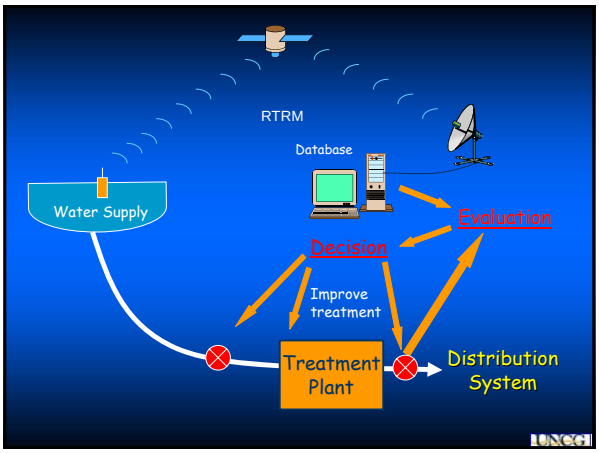
The Larger Context:

Environmental Monitoring and Assessment

- Macroorganisms as bioindicators
- Microorganisms as bioindicators
  - Size / activity / distribution
- Molecular diagnostics - metagenomics

Bioindicator organisms





Uses – not just pathogens

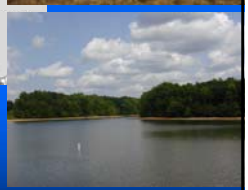
- Water quality assessment for municipal and industrial systems
- Environmental assessments of aquatic ecosystems
- Monitoring bioremediation
- Synoptic detection of pollutants / toxins including biological or chemical weapons (dual-use)

Fundamental questions:

- Target: How many targets?
  How to acquire targets?
  Structural or functional genes?
  DNA or RNA?
- Spatial Variability - are lakes different?
- Replication and sample size?
- Temporal Variability – how important are diurnal to seasonal time scales?
- Biogeography – is everything everywhere?
- Sensitivity: DNA extraction, PCR bias

Where do microbial bioindicator markers come from?

- Autecological approach: determine the environmental tolerance cultured species (not cost effective)
- Data Mining: Literature + GenBank
- Empirical field testing: Discovery of unique signatures from well-defined sites – including "unknown" species.
- Microcosms and mesocosms



16S and 18S libraries (50 clones)

Toolik Lake, AK

City Lake, NC

Lake Townsend (3 samples)

BLAST Results

- 24% of 18S clones (60/250) had GenBank matches
- 55% of 16S clones (136/247) had GenBank matches
- Most GenBank matches were from uncultured or unidentified clones.
- Many matches were from freshwater research studies including Crater Lake, the Colombia River and the Changjiang River, China
- The largest OTU, including 11 clones from City Lake, produced similarities with *Zooglea ramigera*.

Eukaryotic (18S) results

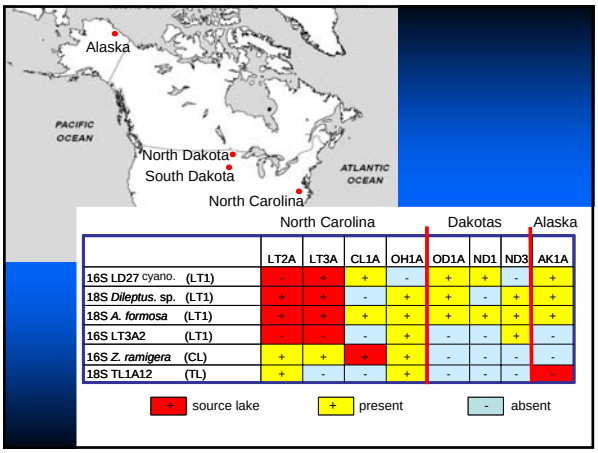
Shared genotypes in clone libraries

99% identity

	LT1	LT2	LT3	CL	Toolik
LT1	-	5	3	1	1
LT2		-	2	2	1
LT3			-	1	2
CL				-	0

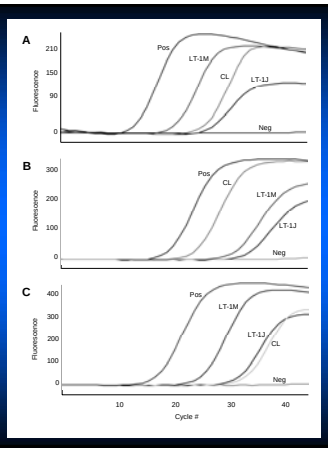
98% identity

	LT1	LT2	LT3	CL	Toolik
LT1	-	8	10	5	3
LT2		-	7	7	2
LT3			-	7	3
CL				-	2



		LT2A	LT3A	CL1A	OH1A	OD1A	ND1	ND3	AK1A
		North Carolina				Dakotas			Alaska
16S LD27 cyano.	(LT1)	-	+	+	-	+	+	-	+
18S *Dileptus.* sp.	(LT1)	+	+	-	+	+	-	+	+
18S *A. formosa*	(LT1)	+	+	+	+	+	+	+	+
16S LT3A2	(LT1)	-	-	-	+	-	-	+	-
16S *Z. ramigera*	(CL)	+	+	+	+	-	-	-	-
18S TL1A12	(TL)	+	-	-	+	-	-	-	-

+ source lake + present - absent



Unknown cyanobacteria
(LD27 AJ007876)

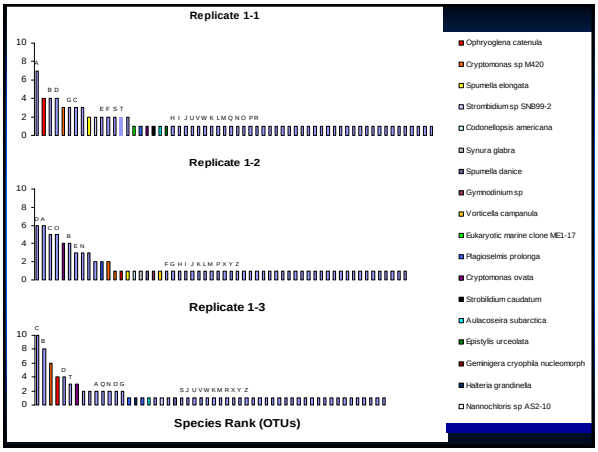
fx source
1
0.077
0.029

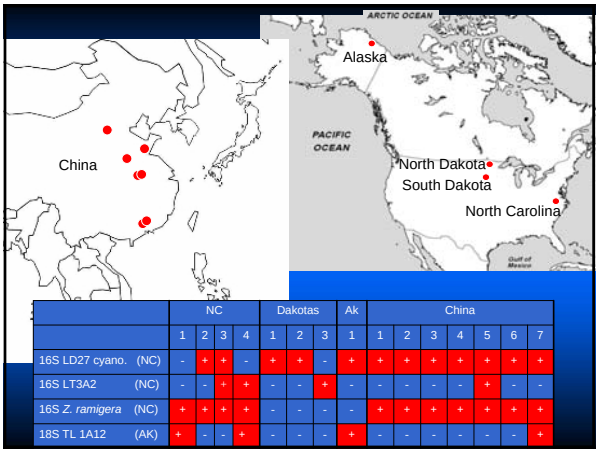
Zoogloea ramigera

fx source
1
0.024
0.0045

Asterionella formosa

fx source
1
0.034
0.018





		NC				Dakotas			Ak	China						
		1	2	3	4	1	2	3	1	1	2	3	4	5	6	7
16S LD27 cyano.	(NC)	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+
16S LT3A2	(NC)	-	-	+	+	-	-	+	-	-	-	-	-	+	-	-
16S *Z. ramigera*	(NC)	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
18S TL 1A12	(AK)	+	-	-	+	-	-	-	+	-	-	-	-	-	-	+

Conclusions

- Natural microbial populations are characterized by some common and many "rare" OTUs (species)
- The common taxa vary in abundance across lakes, but even modest replicate samples generally show the same taxa
- Not all taxa have to be known to find similarities (or differences) among aquatic systems since abundance of "key" taxa varies over time and space.
- The "metagenomic" microbial bioindicator approach for characterizing aquatic ecosystems and risk assessment based on microarrays shows promise.
- Important questions remain, including:
  - adaptation to local environment
  - mechanism and magnitude of microbial dispersal
  - how can this approach be made "user-friendly?"

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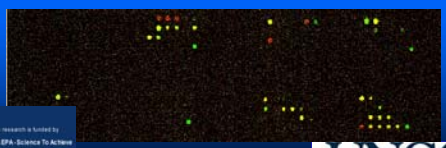
- John Clamp – NC Central Univ.
- Gao Shan – Protozoology Lab, Ocean Univ. of China
- Funding: EPA, UNCG GMAP



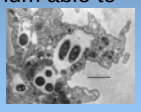
This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # RD-83162701

Characterization of Naturally Occurring Amoeba-Resistant Bacteria

Farone, A. L.[1], M. B. Farone[1], J. H. Gunderson[2], and S. G. Berk[2]
[1]Middle Tennessee State University
[2]Tennessee Technological University

Legionella-Like Amoebal Pathogens (LLAPs)

- 1956—description of an obligate intracellular parasite of free-living amoebae that lysed amoeba
- 1986—T. Rowbotham in England reported the isolation of a Legionella-like bacterium able to induce amoebal lysis
- 1998—First description of LLAP isolated from U.S. soil
- Present—over 30 groups or species of pathogens capable of infecting free-living amoebae



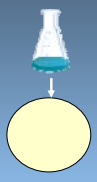
Occurrence of Infected Amoebae in Cooling Towers Compared with Natural Aquatic Environments

- 40 natural aquatic environments were compared to 40 cooling tower samples
- 22 cooling towers showed infected amoebae
- 3 natural samples had infected amoebae
- 16 times more likely to encounter infected amoebae in cooling towers

Screening of Samples for Infected Amoebae

1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

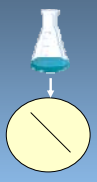
Screening of Samples for Infected Amoebae



1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

Nonnutrient agar cross-streaked with UV-inactivated *E. coli*

Screening of Samples for Infected Amoebae



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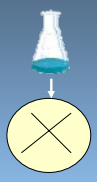
Screening of Samples for Infected Amoebae



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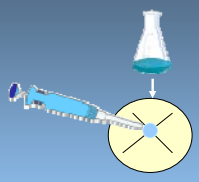
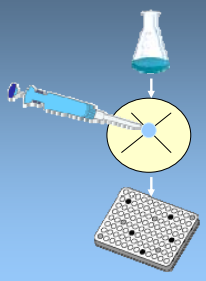
Screening of Samples for Infected Amoebae



1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

Nonnutrient agar cross-streaked with UV-inactivated *E. coli* + 50 µl concentrated sample

Screening of Samples for Infected Amoebae

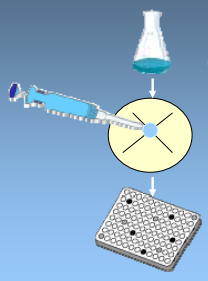


1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

Nonnutrient agar cross-streaked with UV-inactivated *E. coli* + 50 µl concentrated sample

- Determine presence of amoebae
- Wash plates
- Transfer aliquots to 96-well plates

Screening of Samples for Infected Amoebae



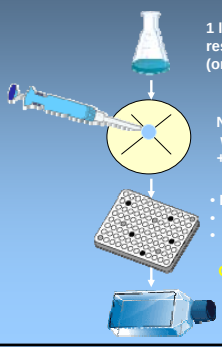
1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

Nonnutrient agar cross-streaked with UV-inactivated *E. coli* + 50 µl concentrated sample

- Determine presence of amoebae
- Wash plates
- Transfer aliquots to 96-well plates

Observe for infected native amoeba

Screening of Samples for Infected Amoebae



1 liter water sample centrifuged and resuspended in 0.5 ml Tris buffer (or biofilm sample)

Nonnutrient agar cross-streaked with UV-inactivated *E. coli* + 50 µl concentrated sample

- Determine presence of amoebae
- Wash plates
- Transfer aliquots to 96-well plates

Observe for infected native amoeba

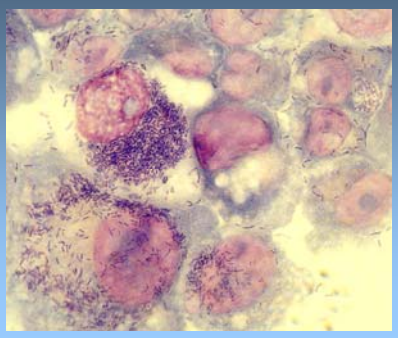
Transfer positive samples to monolayers of *A. polyphaga*

Isolation of the Unculturable Amoebal Pathogens

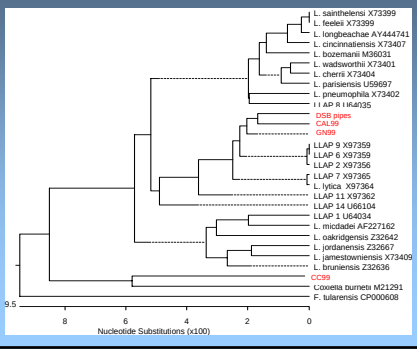
Acanthamoeba polyphaga
+
amoebal pathogen + contaminants

- Physical methods
- Biological methods
- Chemical methods

Cooling Towers

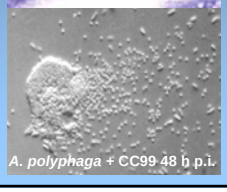


Phylogenetic Tree of Unculturable Cooling Tower Isolates

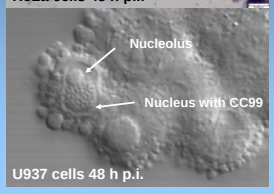


Bacterium CC99 + *A. polyphaga*

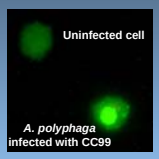
- **Unculturable**
- **Coccoid, < 0.5 µ**
- **Motile**
- **Infects host nucleus**
- **Lysis within 48 h**

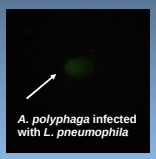


- **Infectious for human cell lines, U937 macrophage-like cells and HeLa cells**
- **Infects nucleus**
- **Lyse cells in 72 h**



Fluorescent *In Situ* Hybridization with 16S rDNA-specific Probes

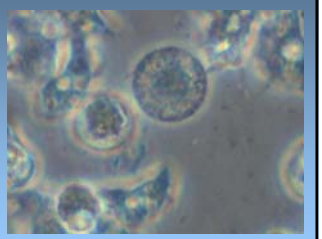




- Use molecular methods (FISH and real time PCR) to detect distribution of novel AAMs in aquatic systems

Current Studies: Water Distribution Systems

- Unculturable AAMs from infected amoebae found in fire safety sprinkler system and fire hydrant samples



Significance

- Infected amoebae can be found in water distribution systems
- The AAM infecting the amoebae can be novel and unculturable and therefore undetectable
- Bacteria pathogenic for humans are thought to have evolved in association with amoebal hosts

Acknowledgements

- This research was funded by U.S. EPA Science to Achieve Results (STAR) Program Grants
- Drs. Anthony Newsome and Nizam Uddin
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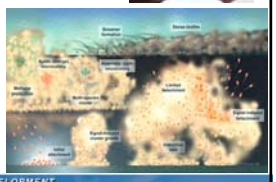
Nicholas J Ashbolt

Biofilm Sampling and Screening Techniques for Amoeba-Related Biofilm Pathogens

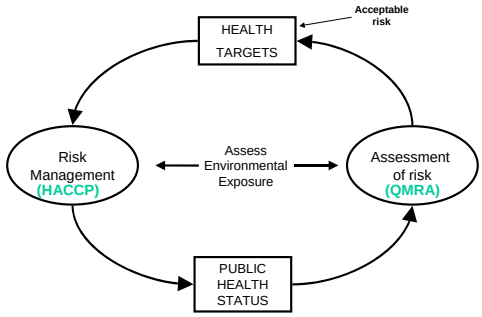
The U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms in Water
Cincinnati, June 18-20, 2007

Points covered

1. Risk management and that 'Pathogen' events can be short-term - how to sample?
2. Pathogen concerns from biofilms
3. Biofilm-amoeba research aims



WHO Risk management approach



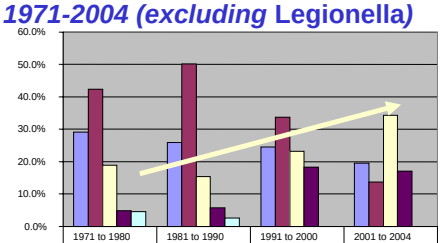
Fewtrell & Bartram (2003) IWA Publishing

Highest uncertainty with DS risks

- DS information gaps include:
  - Fecal pathogen 'intrusions' into DS
  - **Non-fecal pathogen growth in biofilms**
  - Sequestration, inactivation and sloughing of pathogens from biofilms
- Current high uncertainty when modeling infection risks due to unknown DS biofilm effects

So what is going on in distribution systems?

Trends in outbreaks from U.S. public water systems 1971-2004 (excluding Legionella*)*

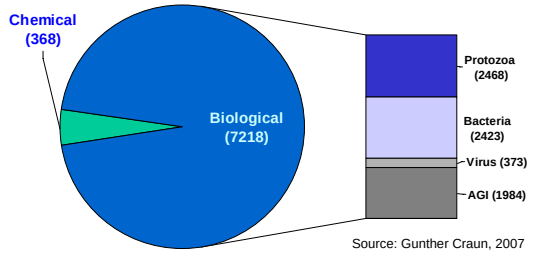


Source: Gunther Craun, 2007

	1971 to 1980 (n=285)	1981 to 1990 (n=227)	1991 to 2000 (N=142)	2001 to 2004 (n=35)
untreated groundwater	29.1%	26.0%	24.6%	19.6%
water treatment deficiency	42.5%	50.2%	33.8%	13.7%
distribution system deficiency	18.9%	15.4%	23.2%	34.3%
miscellaneous or unknown	4.9%	5.7%	18.3%	17.1%
untreated surface water	4.6%	2.6%	0.0%	0.0%

Time Period

Chemical vs. Biological causes of distribution system illness cases, from outbreaks 1981-2002



Source: Gunther Craun, 2007

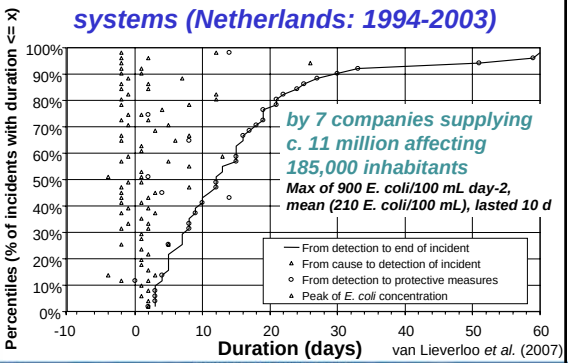
Note: does not include *Legionella*

Number and percentage of samples with positive E. coli detection (in first samples) in finished water and in distribution systems 2000-2003

Country	Samples with +ve *E. coli* detection	
	Finished water	Distribution system
France	369/54,560 (0.7%)*	903/114,138 (0.8%)
Netherlands	17/39,545 (0.04%)	99/107,593 (0.09%)
Germany	1/20,737 (0.005%)	15/12,530 (0.1%)
Total	387/114,842 (0.34%)	1017/264,261 (0.38%)

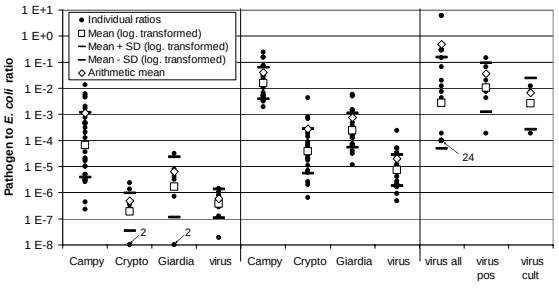
from Van Lieverloo et al. (2006) Chapter 5, MICRORISK Final Report, EU, Brussels [*disinfected systems 100%, 6%, 38% resp`y]

Duration of 50 'fecal' events in distribution systems (Netherlands: 1994-2003)



van Lieverloo *et al.* (2007)

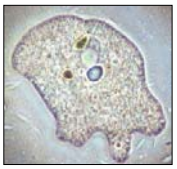
Estimated pathogen:E. coli ratios

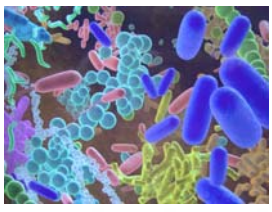


van Lieverloo *et al.* (2007)

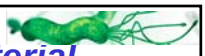
2. Concerns that make biofilms a problem

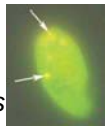
- Biofilms sequester fecal pathogens and allow the growth of opportunistic pathogens





Water-based bacterial pathogens





- Various *Legionella* strains
- *Mycobacterium avium, M. ulcerans*
- *Burkholderia pseudomallei*
- *Helicobacter pylori*
- *Aeromonas* & *Vibrio* spp.
- *Campylobacter* spp.

- All grow associated with amoeba in biofilms & may be active but non-culturable

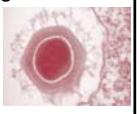
Pathogens also protected in biofilm ecosystems

- Biofilm slime 'mops-up' chlorine disinfectants & pathogens
- Acanthamoebae cysts remained viable
  - after treatment with 100 mg/L chlorine (free and combined) for 10 min, as well as
  - 80°C for 10 min – containing viable legionellae
- Implying that conventional hyper-disinfection or 80°C heating may be insufficient for long-term control of Acanthamoebae-bound Legionellae in water distribution systems

Storey *et al.* (2005) Scand. J. Infect. Dis. 36(9):656-662

And it may get worse!

- *Acanthamoeba polyphaga* Mimivirus largest known DNA virus
- The word "girus" used to recognize its intermediate status
  - genome complexity which is closer to small parasitic prokaryotes than to regular viruses[1]
- Possibly > legionellae in causing community & nosocomal pneumonia[2]; Mouse model possible[3]



750 nm dia

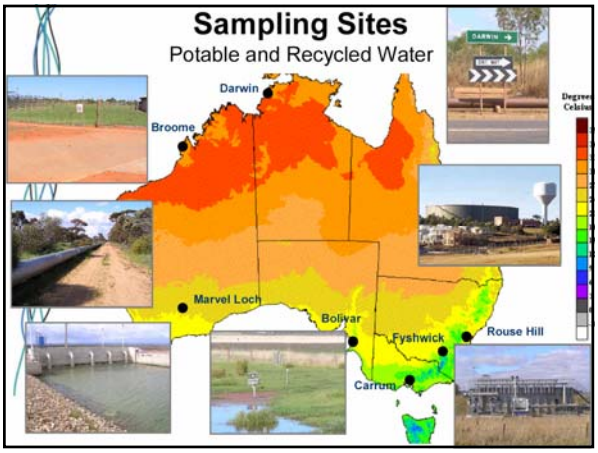
[1]Claverie *et al.* (2006) Virus Res.117(1):133-44
[2]La Scola *et al.* (2005) Emerg. Inf. Dis. 11(3):499-52
[2]Berger *et al.* (2006) Emerg. Inf. Dis. 12(2):248-55
[3]Khan *et al.* (2007) Microb. Pathog. 42(2-3):56-61

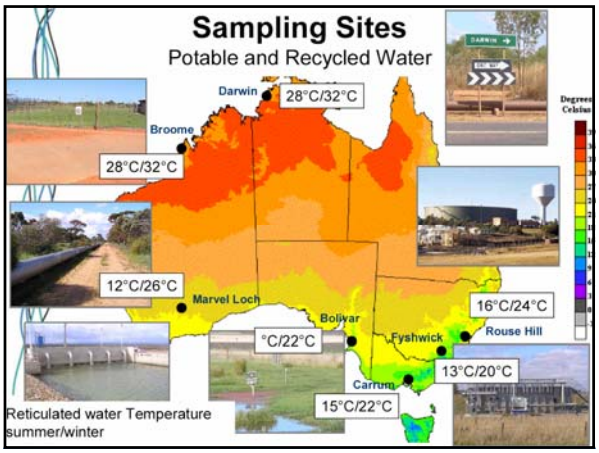
However, biofilms provide a history of contamination

- Because of their sequestering nature, biofilms are a good integrator of passed contamination
- Hence, biofilms may provide a preferable target to monitor than water – more representative, particularly for small systems with infrequent sampling & for short duration events

So how to sample biofilms?

CRC-WQT (Australia) project: Understanding the Growth of Opportunistic Pathogens in mains





Biofilm sampling
Pipe excavation

Biofilm sampling

Biofilm harvesting

Biofilm flushings

Post Doc-1: 'Biofilm' pathogen sampling device

- This project, in conjunction with NHSRC/NRMRL T&E facility, will focus on a sampler that:
  - Is in the main flow but with properties that sorb chemical & microbial analytes
  - High surface area that encourages biofilm
  - Easily retracted and removed from full-pressure water main

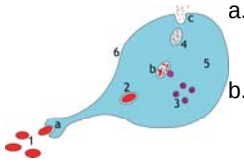
3. Is there a biofilm-amoeba 'virulence' marker

- *Legionella* story has illustrated the 'Trojan horse' analogy
  - And the 'microbial gym' where the amoeba is the training ground to also enable infection of human macrophages

Post Doc-2: Genetics of the biofilm amoeba-bacterial-mimivirus environment

- MAC, Legionellae etc. are readily found in pipe biofilms, but the question is their significance?
- Rather than chasing strains one-by-one, is there a common ('virulence') factor involved (mimivirus)?
- Using *Legionella-Acanthamoeba-Mimivirus* as a model

Steps in a macrophage ingesting a pathogen



a. Pathogen (1) ingestion through phagocytosis, a phagosome (2) is formed
b. The fusion of lysosomes (3) with the phagosome creates a phagolysosome; the pathogen is broken down by enzymes
c. Waste material is expelled or assimilated

http://en.wikipedia.org/wiki/Macrophage

Possible general 'virulence' factor for amoeba-based pathogens

e.g. Inhibition of phagosome maturation is an important mechanism for virulence in *Mycobacterium tuberculosis*

- novel gene, *pmiA*, which is involved in production of a specific cell wall glycolipid, which, in turn, plays a role in preventing phagosome maturation

Robinson *et al*. (2007). Infect. Immun. 75:581–591.

We are what we eat!

- Giant viruses are nucleocytoplasmic large DNA viruses (NCLDVs) that infect algae (phycodnaviruses) and amoebae (Mimivirus)
- Islands of bacterial-type genes, including apparently intact prokaryotic mobile genetic elements occur within these viruses
- Hypothesize that NCLDV genomes undergo successive accretions of bacterial genes, acquire within their bacteria-feeding eukaryotic hosts
- Such acquisition may be driven by the intimate coupling of recombination and replication in NCLDVs. Filée *et al*. (2007) Trends in Genetics 23(1):10-15

Associated PhD student on Mimivirus occurrence

- Commenced March 2007, UNSW
- First year in Sydney to screen material collected from the CRC-WQT project on opportunistic pathogens in distribution systems
  - Using q-PCR for mimiviruses
  - Annular reactors for pathogen sorption/desorption studies & risk assessment

Hence, long-term biofilm research goals

- See if phagosome maturation is a general mechanism (target) for bacterial virulence
- Describe the role of mimiviruses in lateral 'virulence' gene transfer in biofilms
- Investigate the role of ABNC cells in animal dose-response models and efficacy of chlorine disinfection

This presentation does not necessarily reflect official U.S. EPA policy

Overview of NCER & STAR

Barbara Klieforth, MSPH
National Center for Environmental Research
June 18, 2007

EPA's Mission

- Protect human health and safeguard the natural environment — air, water, land — upon which life depends.

ORD's Customers

- **EPA Program Offices and Regions**
- **Other Agencies and Policy Partners**
- **Place-Based Customers (states, tribes, local communities)**
- **Academic Research Community**
- **Environmental Technology Providers**

ORD

- Our mission is to conduct research to inform Agency decisions with sound scientific information
- EPA's Program and Regional Offices are our principal clients
- Maintaining the quality of our scientific workforce is crucial
- Communicating our results, and why they matter, is an important new emphasis
- **STAR program fills a unique niche** by supporting research not conducted or funded by other agencies and is directly relevant to the mission of EPA

ORD research ⟶ better decisions ⟶ positive environmental outcomes

EPA STAR Program Summary

- **Mission: include this country's universities and nonprofit groups in EPA's research program and ensure the best possible quality of science in areas of highest risk and greatest importance to the Agency**
- **Issue approximately 20-25 RFAs each year**
- **Each year: receive 2500-3200 grant applications**
- **Award about 300-400 new STAR grants, fellowships & SBIR contracts per year**
- **Manage about 1000 active research grants and fellowships**

STAR Program Excels

*The National Research Council said**

- EPA requires a strong and balanced research program to fulfill its mission and the *STAR program is an important part of the overall EPA research program*
- *STAR program fills a unique niche* by supporting important research that is not conducted or funded by other agencies and is directly relevant to the mission of EPA
- *STAR processes compare favorably and in many cases substantially exceed* those in other research-supporting organizations
- *STAR research results have already improved the scientific foundation for decision making* even though the program is young and many of the projects have not yet been completed

* National Research Council, *The Measure of STAR: Review of the U.S. Environmental Protection Agency's Science to Achieve Results (STAR) Research Grants Program, National Academy Press, 2003.*

A Decade of Accomplishments

- **NCER has accomplished a lot:**
  - Total number of RFAs Issued: 233
  - Total number of grants awarded: 2,281
  - Total number of fellowships awarded: 1372
  - Total number of journal articles published: >6,500
  - Total number of institutions awarded grants: >900
  - Total grant dollars awarded: >$970M
  - NCER contributed 50% of papers to 2005 BOSC reviews

NCER's Programs

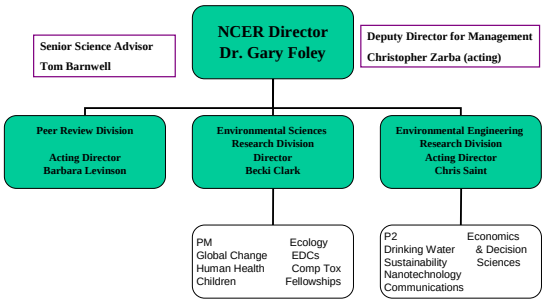
How we do our funding

- **Science To Achieve Results (STAR) Grants**
- **STAR and GRO Fellowships**
- **EPSCoR**
- **Small Business Innovation Research (SBIR)**
- **Earmarks**

NCER's People Make the Difference

- 22 PhD Degrees
  - Agronomy; Engineering (Civil, Mechanical, Environmental and Mechanical), Geology, Atmospheric Chemistry, Marine Biology, Toxicology, Epidemiology
- 13 Masters of Science
  - Environmental Health, Environmental Science, Environmental Engineering, Civil Engineering, Public Health, Environmental Management, Library Science, Business, Geology
- 1 Juris Doctor

Organizational Structure



EPA STAR Research Program

- **Goal-directed solicitation planning**
- **Significant cross-agency and interagency involvement with solicitation planning, writing, and review**
- **Competitive solicitations: award about $60 million dollars annually**
- **Joint Solicitations with other Agencies**
- **External peer review**
- **Internal relevancy review:** program office and regional input
- **Fund highest priority projects**
- **Communicate research results through website, ORD laboratories, program office and regional meetings, and publications** ***(www.epa.gov/ncer)***

STAR Research Partners

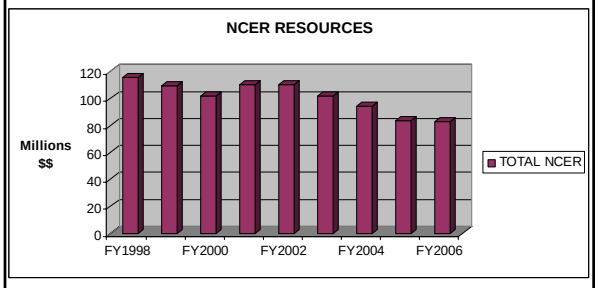
- Current Partners
  - American Chemistry Council
  - Association of California Water Associations (ACWA)
  - American Water Works Association Research Foundation (AWWARF)
  - Department of Energy
  - Department of Homeland Security
  - NASA
  - NIEHS
  - NIOSH
  - NOAA
  - NSF
  - Office of Naval Research
  - Strategic Environmental Research and Development Program
  - USDA
- Potential Future Partners
  - European Union

Research Priorities

Focus areas for research supported by funding through grants, fellowships, and contracts

- **Drinking Water**
- Particulate Matter
- Global Change
- Ecological Services
- Human Health Research
  - Children's Health
  - Tribal Centers
- Endocrine Disrupting Chemicals
- Computational Toxicology
- Economics and Decision Sciences
- Pollution Prevention
- Sustainability
- Nanotechnology
- Exploratory Research

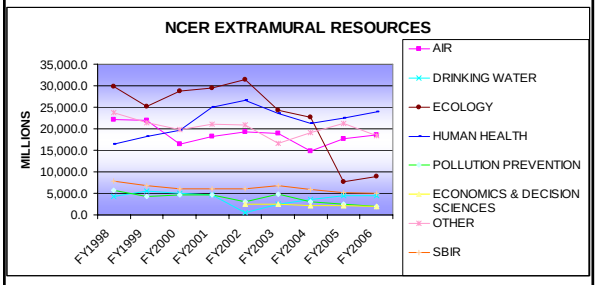
Science To Achieve Results (STAR) Program



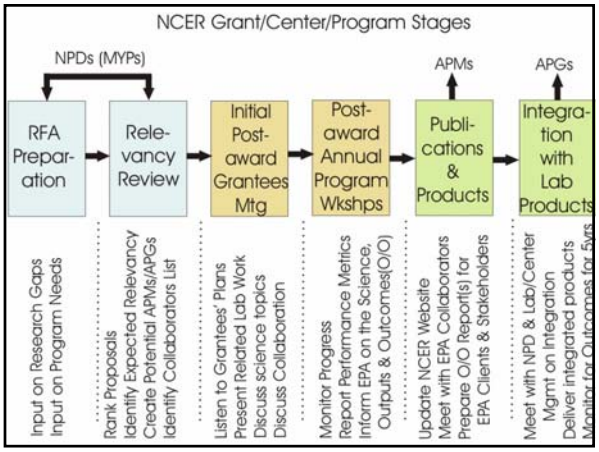
RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions

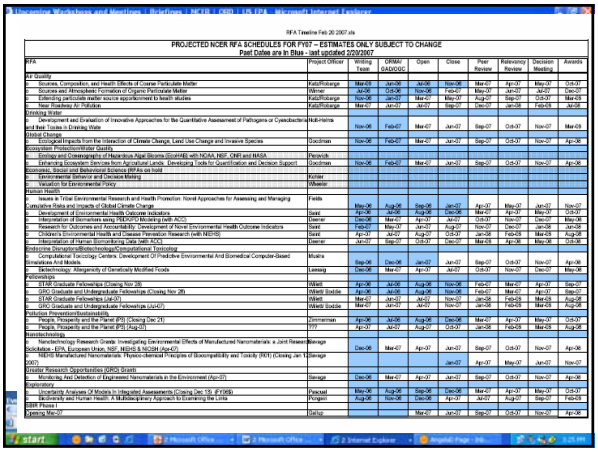
Science To Achieve Results (STAR) Program

Funding by Program Area



RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions




PROJECTED NCER RFA SCHEDULES FOR FY07 – ESTIMATES ONLY SUBJECT TO CHANGE
Past Dates are in Blue - last updated 2/20/2007

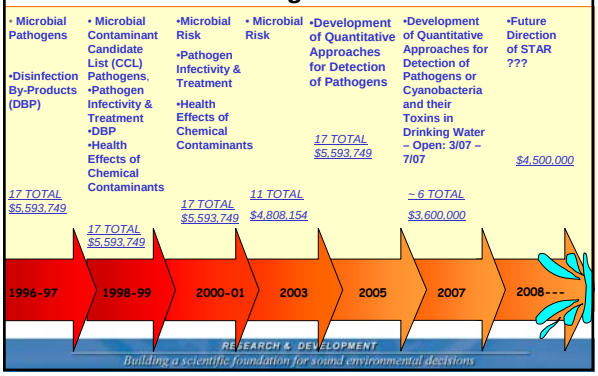
Science To Achieve Results (STAR) Program

NCER's Drinking Water Program

- **Program began in FY 1996**
- **Funding levels between $2.5-5.0 M/yr**
- **Since inception NCER had funded research in a wide variety of areas**
- **Research completed 3-4 years after award**
- **Solicitation preparation and Programmatic Reviews have extensive participation from OW, ORD, and Regional Offices**

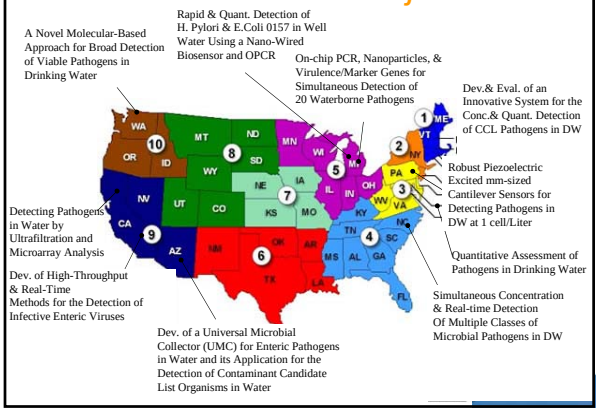
RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions

Evolution of Research for the Drinking Water Program

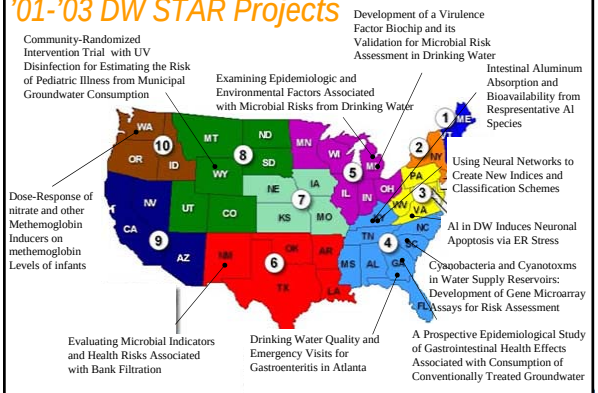


RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions

2005 DW STAR Projects



'01-'03 DW STAR Projects



Science To Achieve Results (STAR) Program

All-Investigators Drinking Water Science Progress Review Workshops

- U.S. EPA Microorganisms in Drinking Water – August, 2003
- The U.S. EPA/U.S. Geological Survey Meeting on Cryptosporidium Removal by Bank Filtration – September, 2003
- U.S. EPA Workshop on Pharmaceuticals in the Environment – August, 2005
- **U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms in Drinking Water - June 18-20, 2007**

Science To Achieve Results (STAR) Program

NCER Web Site:

http:www.epa.gov/ncer

Science To Achieve Results (STAR) Program

Communicating NCER Activities

- Online Access and Resources
  - Solicitations (RFAs)
  - Abstracts, Progress Reports, Final Reports, Bibliographies
  - Topical and Regional Research Summaries and Research Capsules
  - Powerful search window
- Publications
  - Synthesis Reports and Individual Summary Reports
  - State-of-Science Reports (SOSs)
  - SBIR abstracts and summaries

Science To Achieve Results (STAR) Program

Communicating NCER Activities

- Annual Science Progress Review Workshops
  - Workshop Proceedings
- Scientific Conferences
  - Participation in various sessions/symposia

Science To Achieve Results (STAR) Program

NCER Solicitations

- **Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens or Cyanobacteria and their Toxins in Drinking Water - *Closes: July 10, 2007***
- Greater Research Opportunities: Detection and Monitoring of Engineered Nanomaterials - Closes September 13, 2007
- Ecological Impacts from the Interaction of Climate Change, Land Use Change and Invasive Species - Closes: June 26, 2007
- Enhancing Ecosystem Services from Agricultural Lands: Developing Tools for Quantification and Decision Support - Opens: July 2007
- Interpretation of Biomarkers Using Physiologically Based Pharmacokinetic Modeling – Closes: September 18, 2007
- Research for Outcomes and Accountability: Development of Novel Environmental Health Outcome Indicators - Opens: June 2007
- Accountability: Development of Novel Environmental Health Outcome Indicators - Opens: June 2007
- Ecology and Oceanography of Hazardous Algal Blooms (EcoHAB) with NOAA, NSF, ONR and NASA - Opens: July 2007
- Exploratory Investigations in Food Allergy (R21): through NIH-NIAID - Opens July 2007
- 5th Annual P3 Awards: People Prosperity and the Planet - Opens: August 2007
- Children's Environmental Health and Disease Prevention Research (with NIEHS) - Opens: August 2007

http://epa.gov/ncer

How to Navigate through your STAR Experience

- Communicating with your Project Officer
- Interactions with EPA staff
  - Grant vs Cooperative Agreement
- Post Award Monitoring
  - Reporting
    - Annual and Final
  - Site Visits
- Presentations/Publications
  - All-Investigator's Mtgs and EPA Seminars
  - STAR logo

How to Navigate through your STAR Experience (Cont'd)

- International Travel
- No-cost Extensions
- Supplemental Funding
  - < 15K
  - > 15K

Overview of Methods for Detection of Pathogens in Water

and

Introduction to a Highly Multiplexed Nucleic Acid-Based Pathogen Identification Assay

R. Paul Schaudies Ph.D.
703-298-3720

GnArraytion Inc
Genomic Cliff Notes

Analytical Consulting Services
Inspired by Technology, Driven by Innovation

Challenges for Identification of Waterborne Pathogens

- Processing large volumes of water
- Concentration of inhibitors
- Low numbers of target organisms
- Multiple classes of target organisms
- Detection of live organisms
- Simultaneous identification of multiple organisms

Sample Collection

Sampling Methods

- Filtration
- Centrifugation
- Affinity isolation
  - Whole organism capture
  - Nucleic acid extraction

Sampling is the ultimate driver of system sensitivity

Viability

- Culture
- ATP
- NADH
- RNA
  - Total RNA
  - Induced genes

Detection without Amplification

- Capture by structural recognition
- Inherent enzymatic activity
- Secondary enzymatic activity
  - Photons
  - Electrons
- Spectral methods
- Cantilevers
- Flow cytometry
- Protein microarrays

Detection with Amplification

- PCR, qPCR, NASBA, RAM
  - Ribosomal targets for abundance
- Loop-mediated isothermal amplification
- Multiplexed PCR with microarray
- Whole sample amplification with microarray
- Fluorescence vs. electrochemical

Detection Systems without Amplification

- Biodetection Enabling Analyte Delivery System (BEADS)
- Fiber optic microarrays
- Immuno arrays
- Antibodies on tapered optical platforms
- Luminex LabMAP
- Automated Water Analyser Computer Supported System (AWACSS)

Detection Systems with Amplification

- Lab on a chip design
- PCR amplification
- Small sample size
- Requires exceptional sample concentration
- Military systems
  - Large
  - Expensive

GnArraytion Inc
Genomic Cliff Notes

- Early stage R&D company **developing molecular infectious disease diagnostics**
- Spinout from SAIC**, a Fortune 300 technology company**
- Products & Services
  - **Microbial genotyping microarrays**
    - CDC Category A pathogen array – under evaluation by CDC
    - HIV genotyping array – under evaluation by FDA
    - Food and water-borne pathogens undergoing laboratory validation
  - **Library of unique & functional biomarkers for human pathogens**
  - **Bioinformatic analysis**
  - **Diagnostics testing services**
  - **Contract R&D services**

Broad spectrum identification and characterization of waterborne pathogens

Approach: apply bioinformatics and laboratory methods to simultaneously identify and characterize a broad spectrum of infectious agents

Applicability: can be integrated with a variety of systems and platforms, from bench-top laboratory instruments to field portable devices

GnArraytion Inc
Genomic Cliff Notes

Molecular Radar

- **High fidelity signatures for human & animal pathogens**
  - Simultaneous high confidence identification of multiple pathogens
  - Functionally complete characterization in hours
  - Can detect a broad range of infectious disease agents
  - Customizable resolution
- **"Platform Agnostic"**
  - Bioinformatics provides bulk sequence
  - Biomarkers can be derived for microarrays, PCR or alternative platforms
  - Modularity allows integration with different platforms for different applications
- **Customizable suite of analysis methodologies for**
  - Epidemiological monitoring
  - Forensics
  - Environmental monitoring

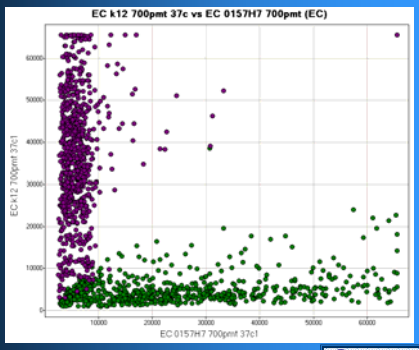
GnArraytion Inc
Genomic Cliff Notes

Food and Water-Borne Pathogen Microarray Design

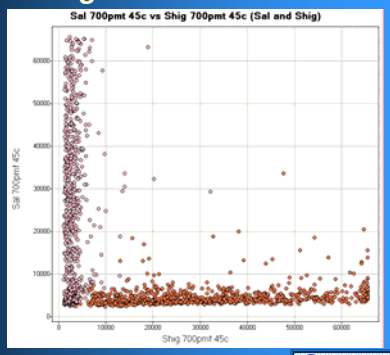
Food/Water Testing Array Content	# oligos
Aeromonas hydrophila	560
Aeromonas punctata plasmid pFBAOT6	467
Brucella abortus	125
Brucella melitensis	500
Burkholderia mallei	750
Burkholderia pseudomallei	750
Camphylobacter jejuni	750
Clostridium botulinum	750
Coxiella burnetii	750
E. coli O157:H7	750
Helicobater pylori	750
Hepatitis D	25
Listeria monocytogenes	750
Norwalk Virus	40
Pseudomonas aeruginosa	850
Rickettsia conorii	850
Salmonella enterica	850
Shigella flexneri	850

GnArraytion Inc
Genomic Cliff Notes

E. coli K12 vs *E. coli* O157:H7



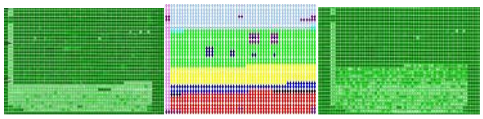
Shigella vs *Salmonella*



GenArraytion Pathogen Array

- Sequences selected following initial screening arrays
- Organisms arrayed in groups to aid rapid visual analysis
- Bioinformatics required for detailed strain level analysis

Bacillus anthracis Ames vs Sterne on VER 1 Array



B anthracis Sterne

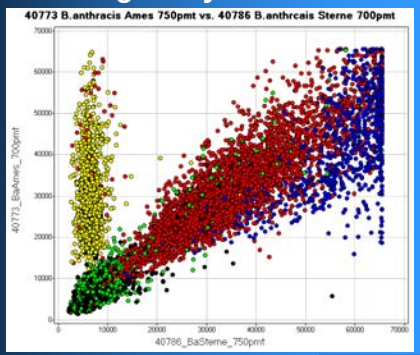
B anthracis Ames

ID	Spot Color
pXO1	Blue
pXO2	Yellow
Genomic	Red
Controls	Purple

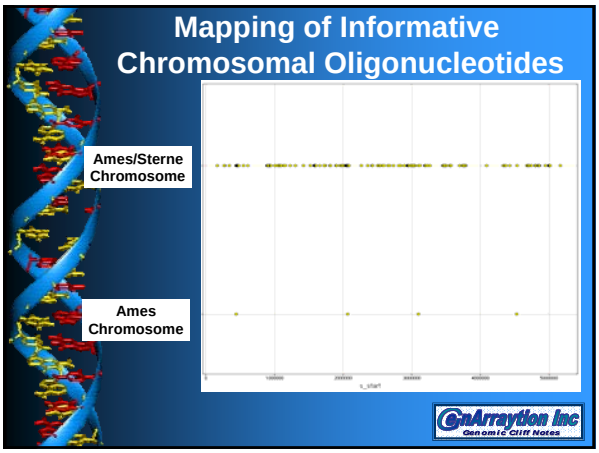
Bacillus Screening Array

- 2000 *B. anthracis* chromosomal and plasmid unique sequences
- 2000 *B. cereus* chromosomal unique sequences
- 2000 *B. thuringiensis* chromosomal and plasmid unique sequences
- Oligonucleotides to 29 different *Bacillus*-specific virulence/toxin genes

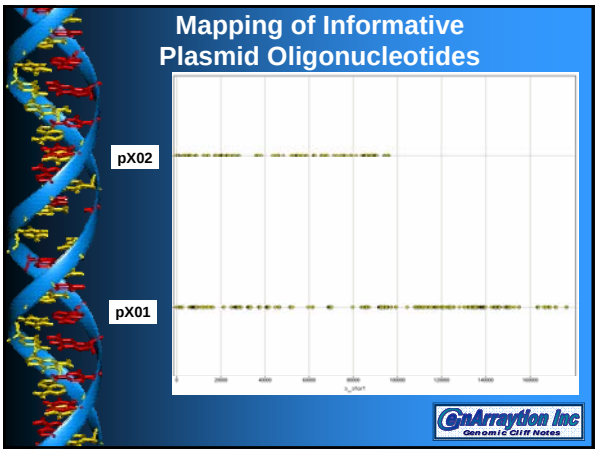
Screening Array Ames vs Sterne



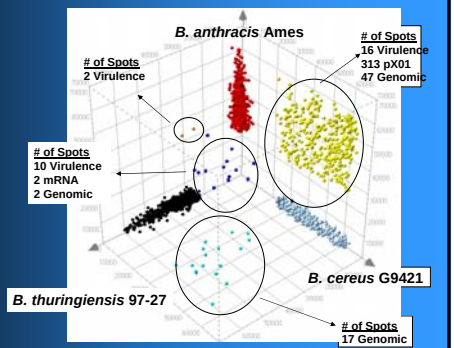
Mapping of Informative Chromosomal Oligonucleotides



Mapping of Informative Plasmid Oligonucleotides



Virulence Factors for *Bacillus*



Customized Level of Specificity

- Strain level sequences
- Species level sequences
- Genus level sequences
- Bacterial sequences
- Viral sequences
- Protozoan sequences

Summary

- GenArraytion's Molecular Radar provides high fidelity identification and characterization of microorganisms
- We have achieved resolution down to the level of strain for pathogens and near-neighbor organisms
- We can design and validate arrays for any DNA or RNA containing organism at desired level of resolution

Robust PEMC Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter

Raj Mutharasan
Frank A. Fletcher Professor
Department of Chemical and Biological Engineering

Drexel UNIVERSITY

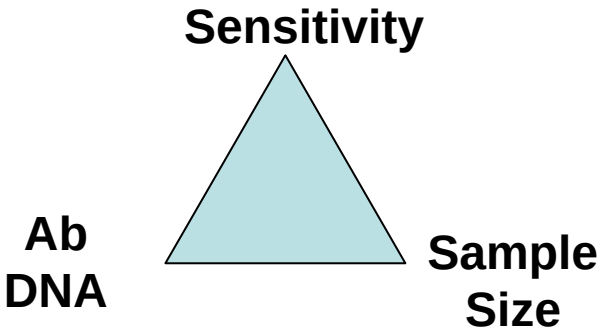
Workshop on Innovative Approaches for Detecting Microorganisms in Water
Cincinnati, Ohio, June 19th, 2007

Objectives

1. **Explore and establish experimentally piezoelectric-actuated millimeter-sized cantilever sensors suitable for detecting one pathogen in one liter of water using new cantilever oscillation and measurement modalities**

2. **Develop flow cell-PEMC sensor detection assembly for large sample volume**

3. **PEMC sensor for confirming pathogen identity by DNA signature**



Model pathogen*: Cryptosporidium parvum* oocysts
Surrogate: E. coli O157:H7

Progress

1. **Sensitive mode established; model experiments with E. coli O157:H7 and Crypto**

2. **Successful 1 liter samples completed using modified flow cell; 1 cell/mL completed**

3. **Preliminary results for DNA-based detection of E. coli O157:H7 successful**

Model pathogen*: Cryptosporidium parvum* oocysts

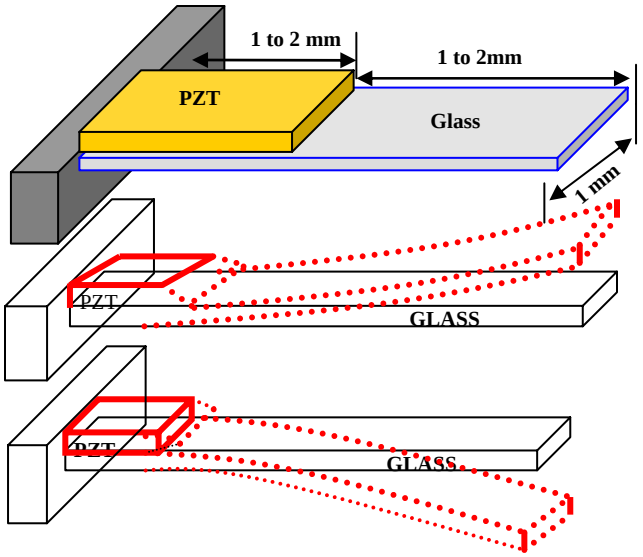
Content

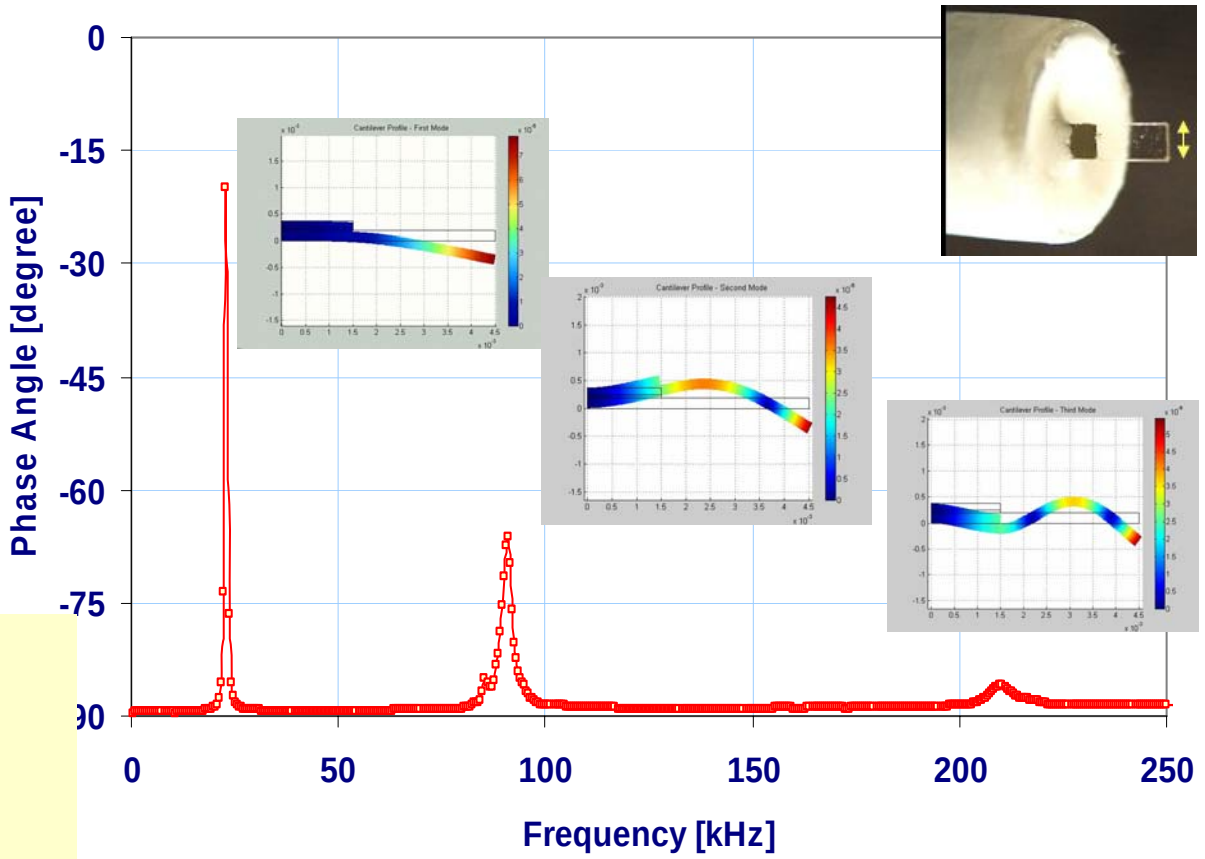
- Cantilever sensor (mass change sensitivity)
- *E. coli* in buffer
- *E. coli* in proteinous matrix
- Crypto in buffer
- Stx2-gene based detection - buffer and beef wash
- DNA-detection in buffer and in serum

Team

USDA-ARS

Cantilever Sensor measures mass





Sensing Principle

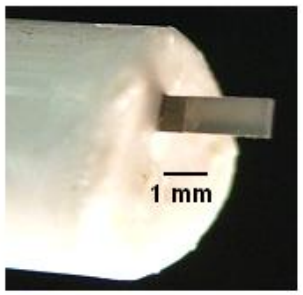
- Resonant frequency depends on cantilever's mass.
- Surface is immobilized with a recognition molecule (eg. Antibody; ssDNA)
- When target attaches to the cantilever, mass changes, and resonant frequency changes

A pathogen is 1 μm in size and weighs 10^{-12} g (1 pg)

A 60 kD protein is a few nm and weighs10^{-19} g (0.1 ag)

Higher modes

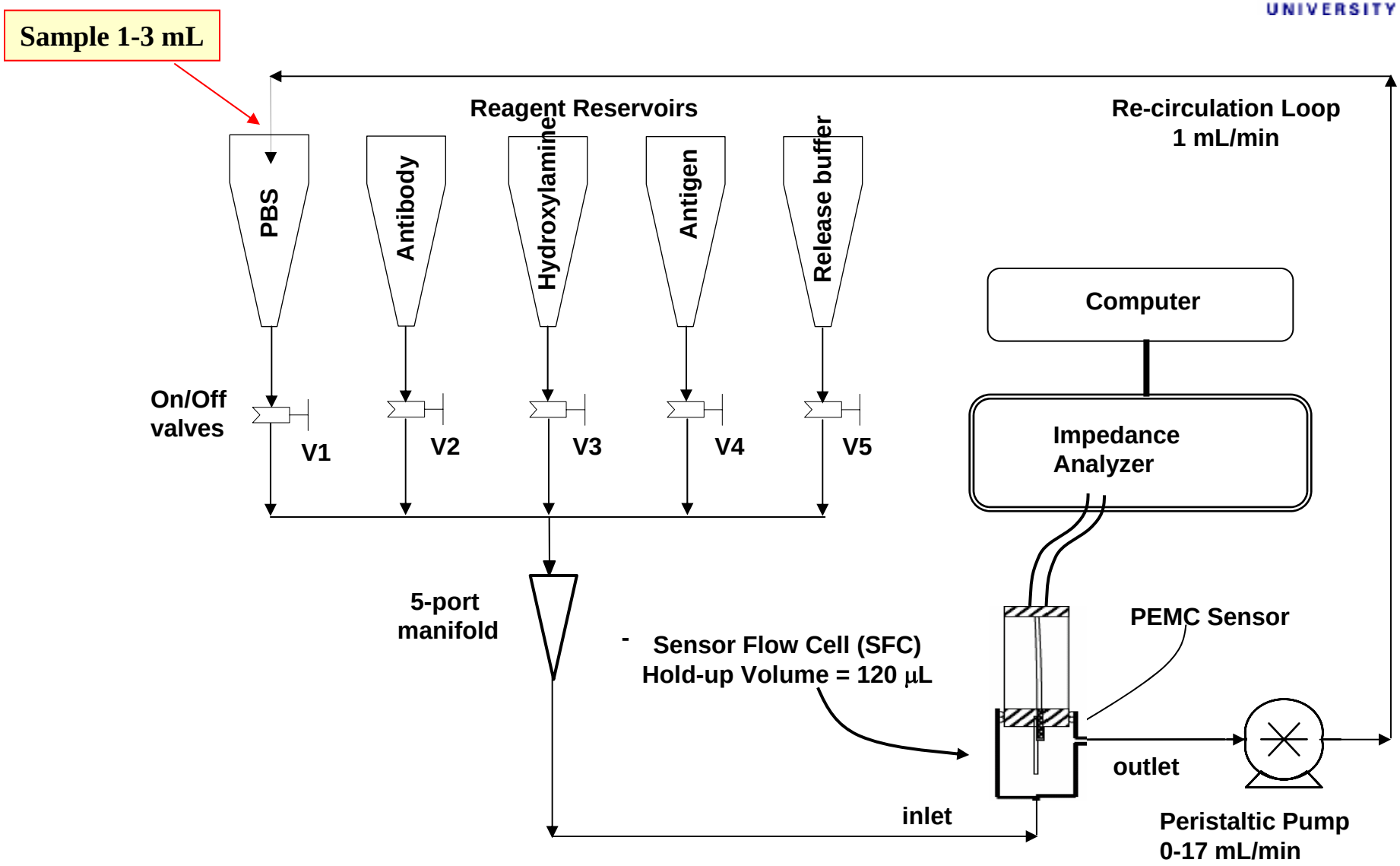
Higher frequency modes are more sensitive



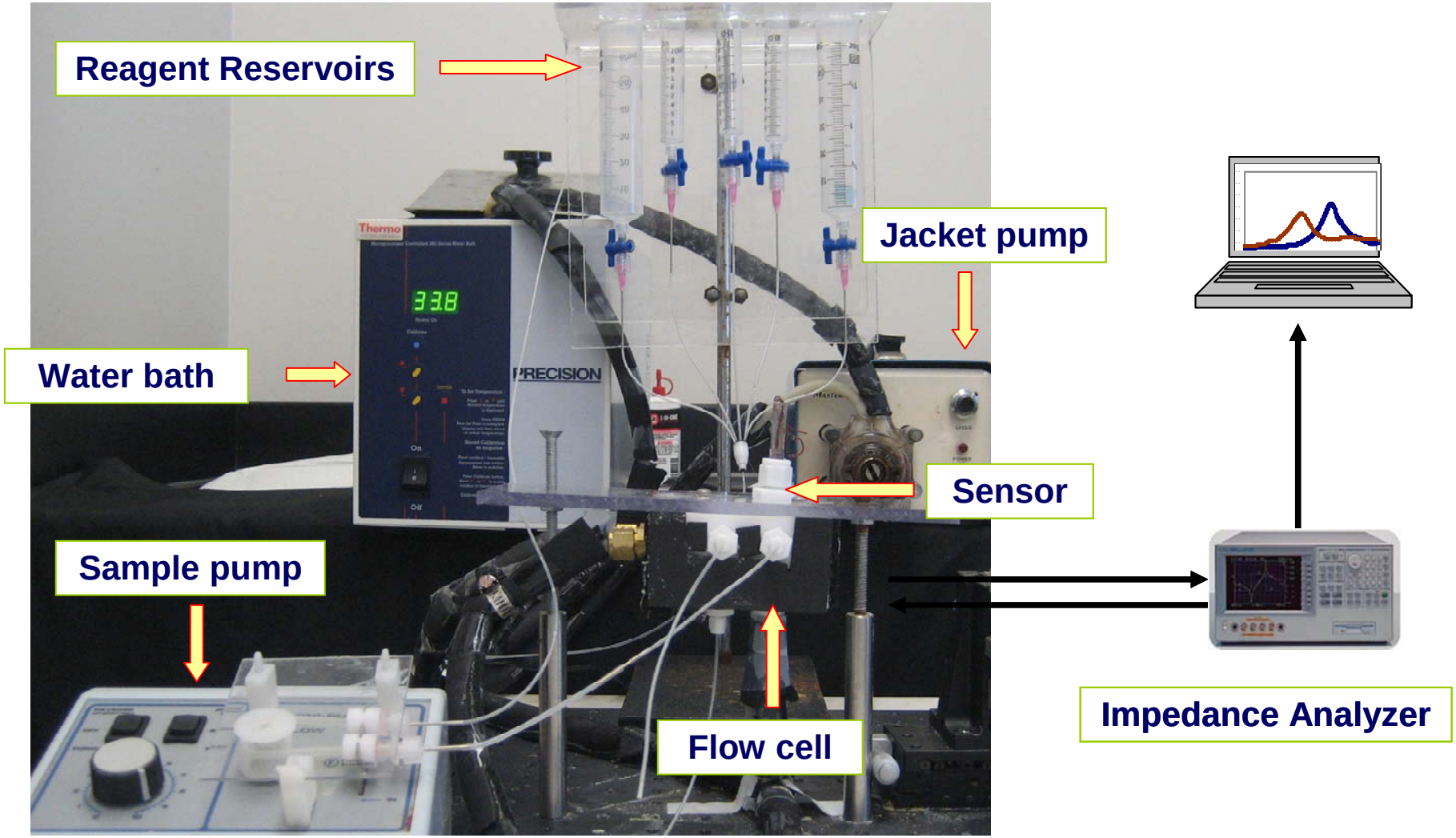
$$f_n = \frac{\nu_n^2}{2\pi}\sqrt{\frac{EI/L^3}{\rho AL}} \Rightarrow \frac{\nu_n^2}{2\pi}\sqrt{\frac{k}{M_e}}$$

$$\text{Sensitivity} = \frac{\Delta m}{\Delta f_n} = \frac{k\nu_n^4}{2\pi^2}\frac{1}{f_n^3}$$

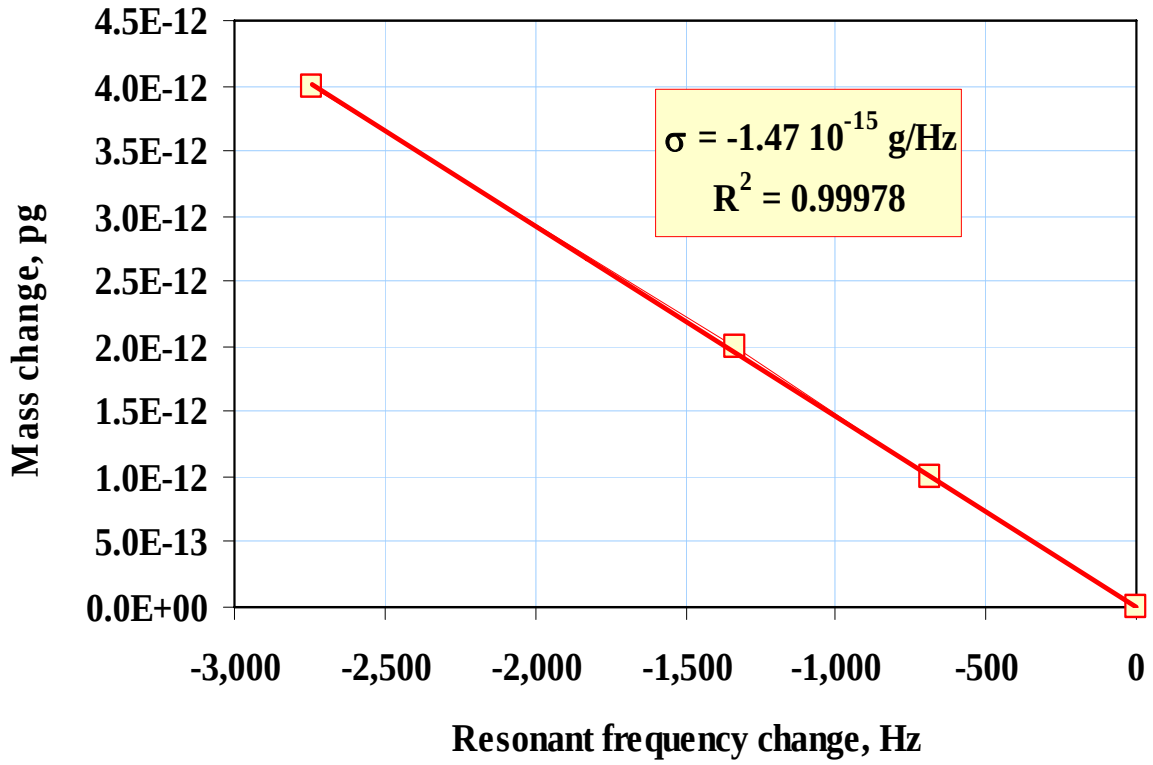
Experimental Apparatus



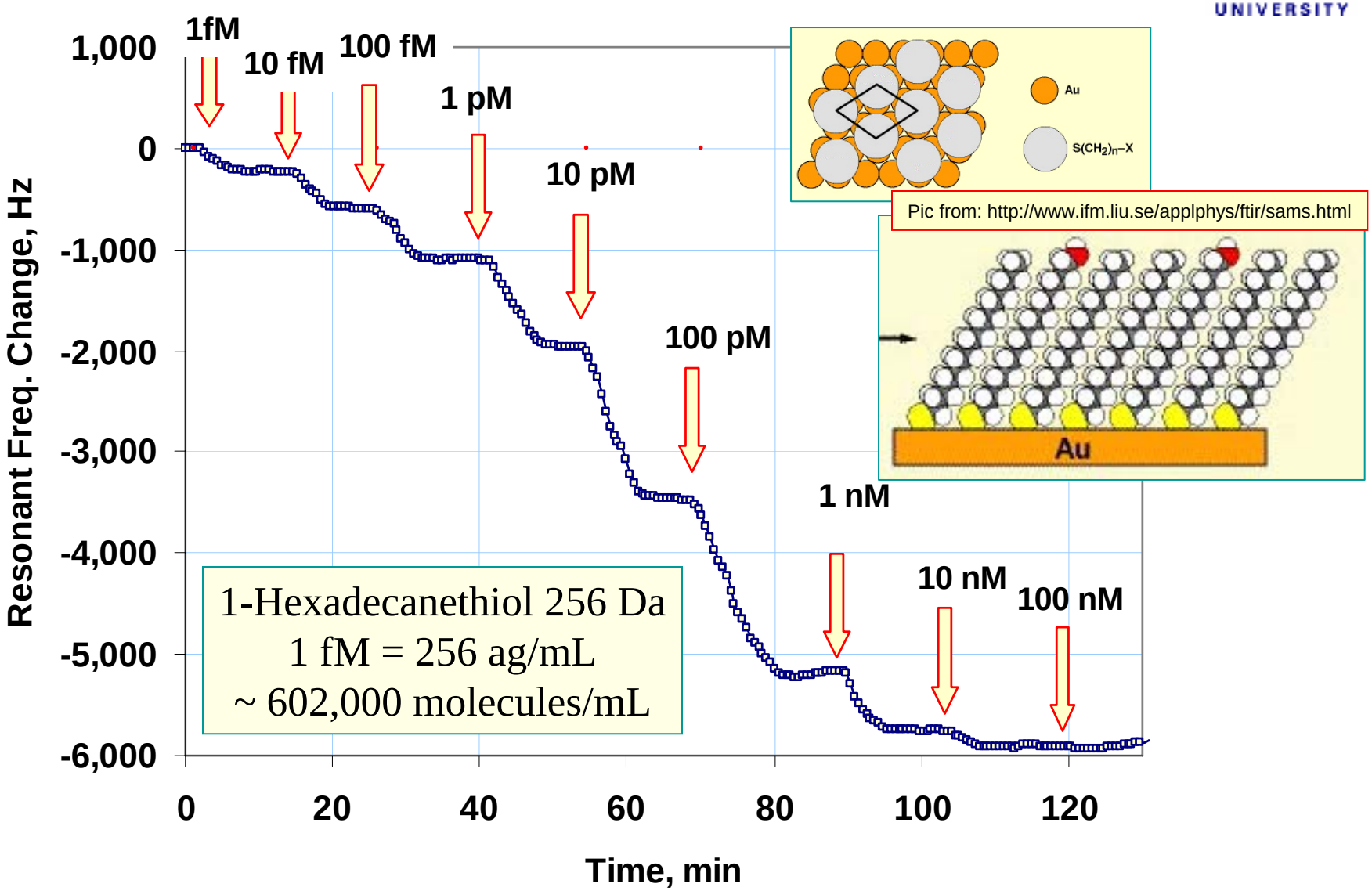
Experimental Arrangement



Mass change sensitivity

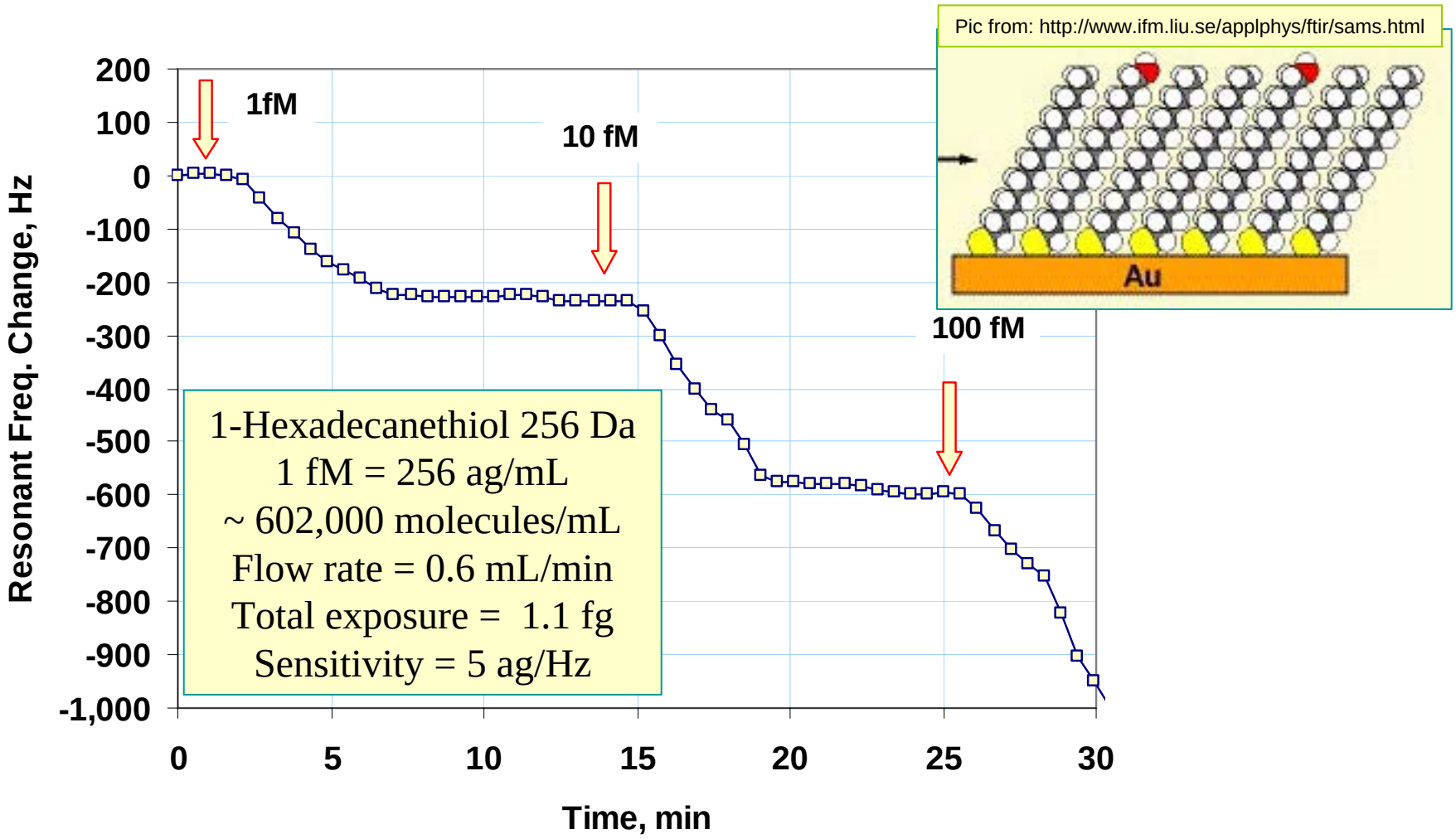


Mass change sensitivity



Rijal, K.,Mutharasan, R., Method for Measuring the Self-Assembly of Alkanethiols on Gold at Femtomolar Concentrations. *Langmuir* **2007,** 23, (12), 6856-6863.

Self-Assembly of 1-Hexadecanethiol



Rijal, K.,Mutharasan, R., Method for Measuring the Self-Assembly of Alkanethiols on Gold at Femtomolar Concentrations. *Langmuir* **2007,** 23, (12), 6856-6863.

Interface Chemistry

Borosilicate Glass (160 μm)

APTES
(silane)

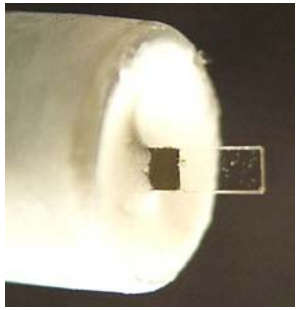
$-NH_2$ functional group

EDC/SulfoNHS

$NaIO_4/NaCNBH_3$

Carboxylic Group

Glycosidic Group



Quartz ~160 μm

Sputter Cr

Gold <111>

Protein G, C or A

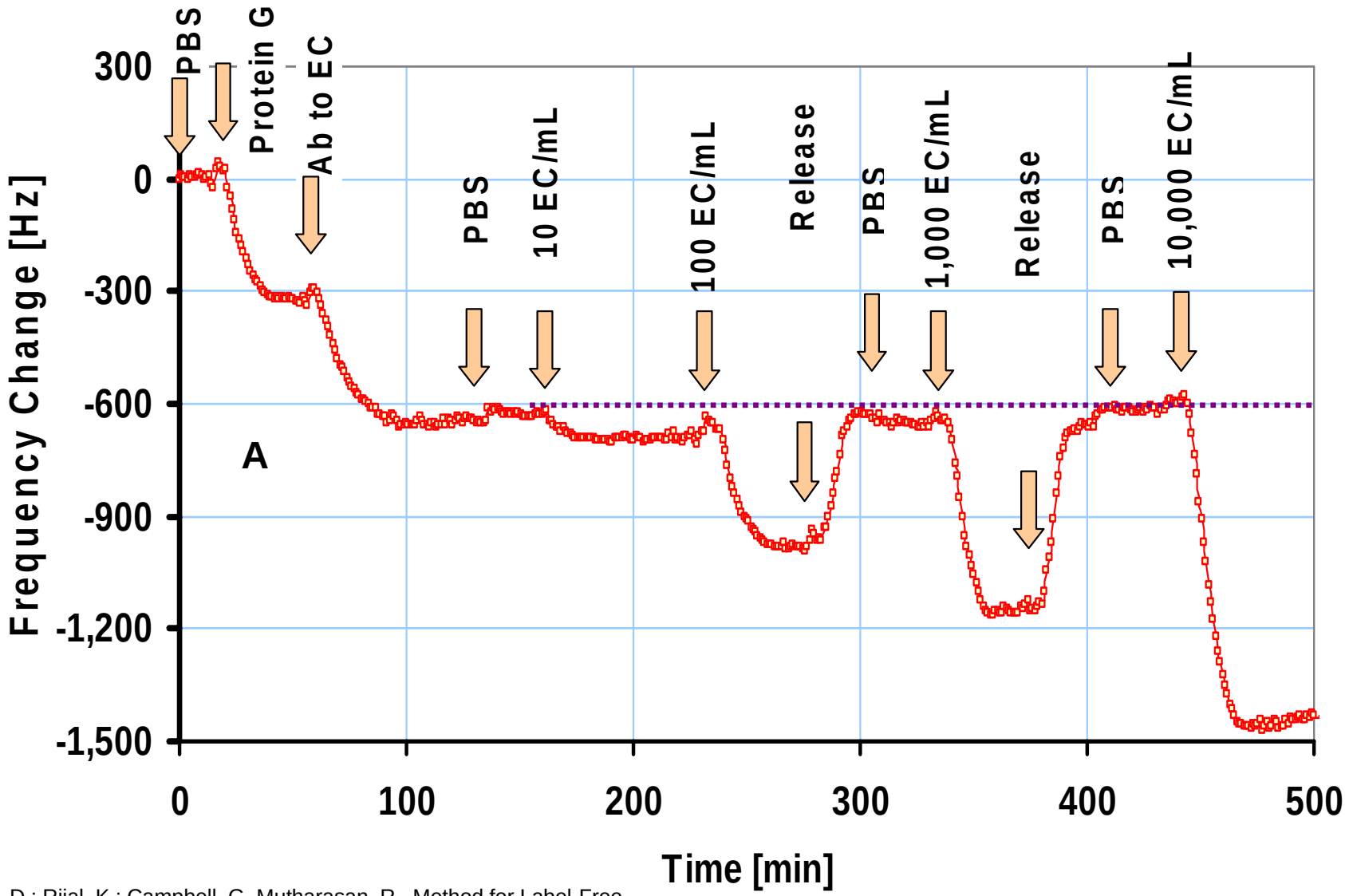
Immobilize Ab

Thiolic Acid(s)

EDC/SulfoNHS

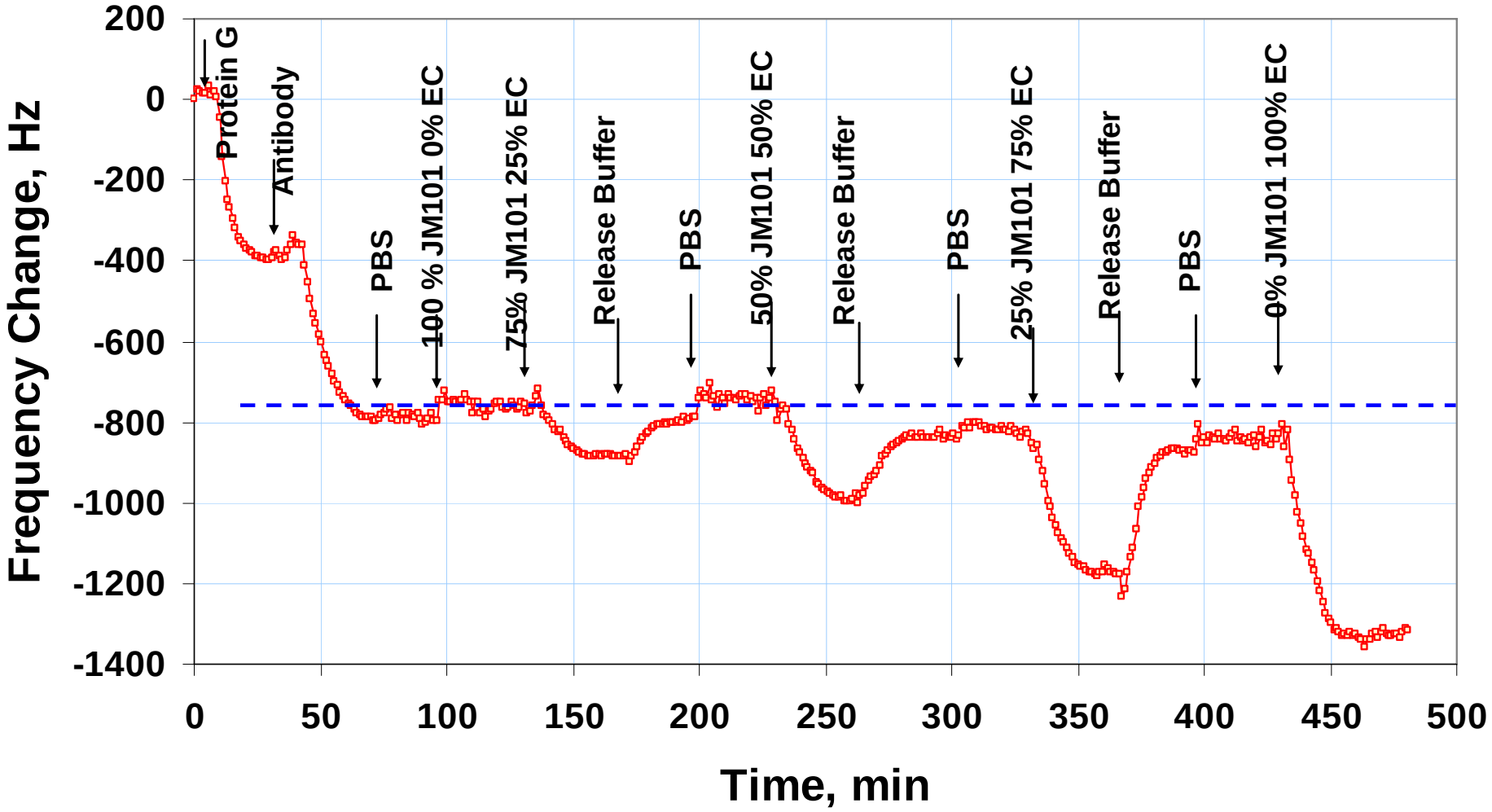
Immobilize Ab

E. Coli O157:H7 in buffer



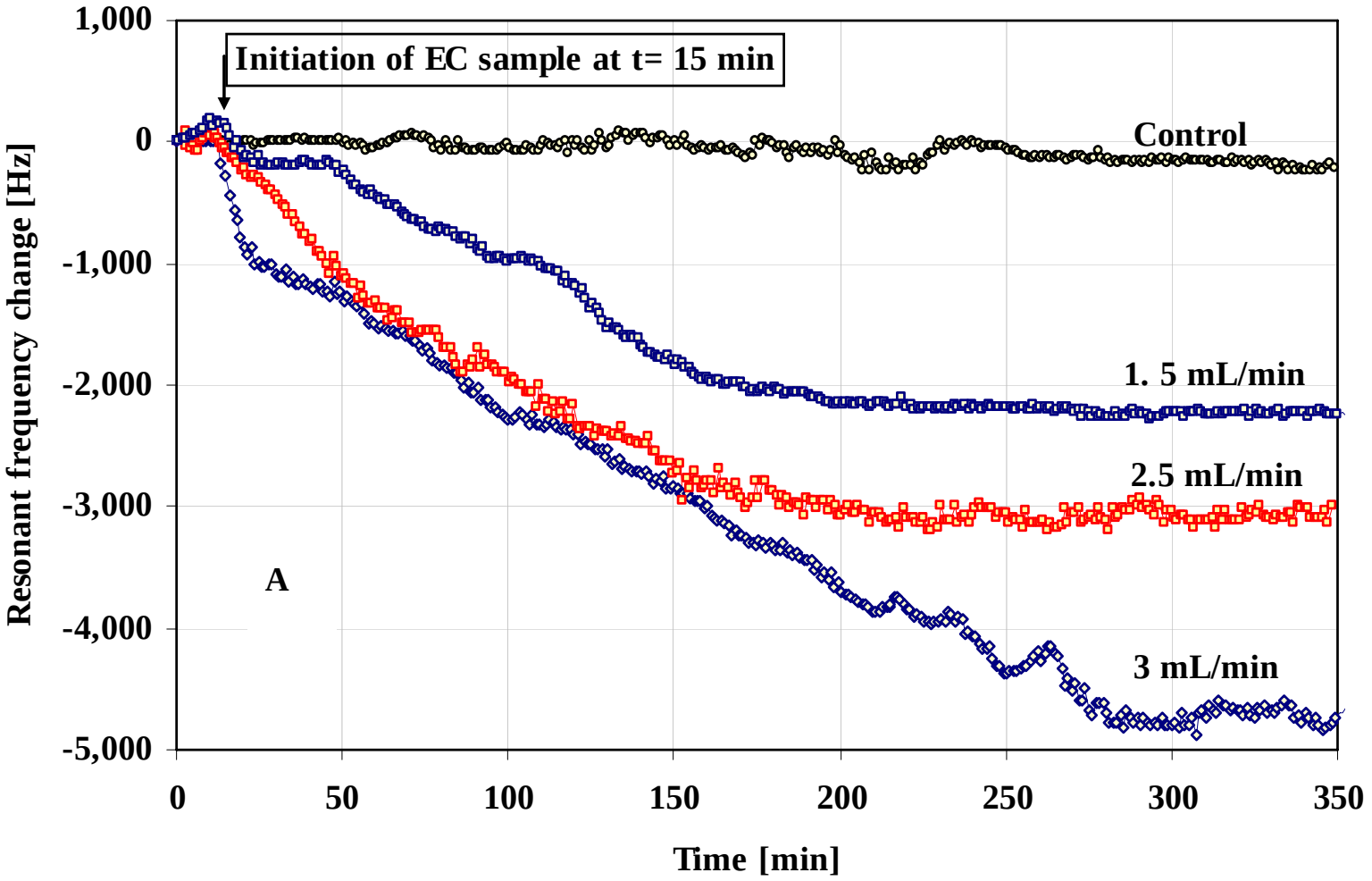
Maraldo, D.; Rijal, K.; Campbell, G.,Mutharasan, R., Method for Label-Free Detection of Femtogram Quantities of Biologics in Flowing Liquid Samples. *Analytical Chemistry* **2007,** 79, (7), 2762-2770

E. Coli O157:H7 in buffer



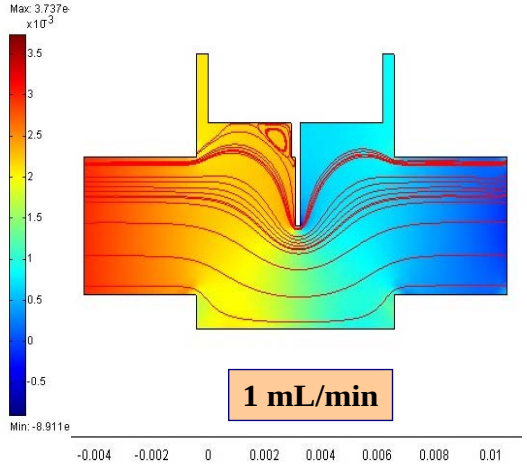
Maraldo, D.; Rijal, K.; Campbell, G.,Mutharasan, R., Method for Label-Free Detection of Femtogram Q uantities of Biologics in Flowing Liquid Samples. *Analytical Chemistry* **2007,** 79, (7), 2762-2770

One liter *E. coli* O157:H7 sample in buffer

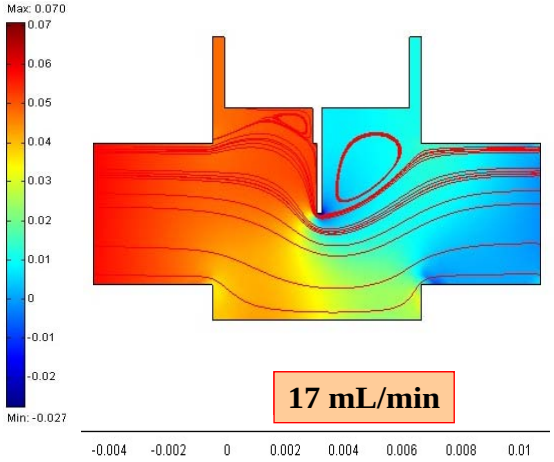


Campbell, G. A.,Mutharasan, R., A method of measuring *Escherichia coli* O157:H7 at 1 cell/mL in 1 liter sample using antibody functionalized piezoelectric-excited millimeter-sized cantilever sensor. *Environmental Science Technology* **2007,** 41, (5), 1668-1674.

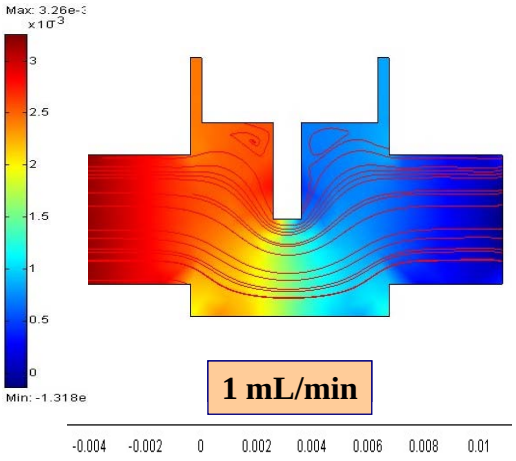
Flow cell geometry and flow field (Obj 2)



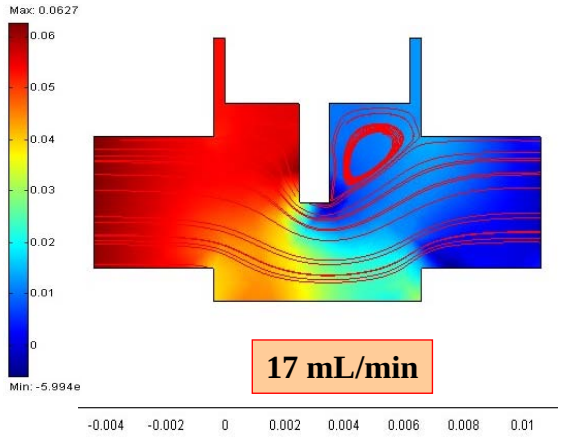
1 mL/min



17 mL/min

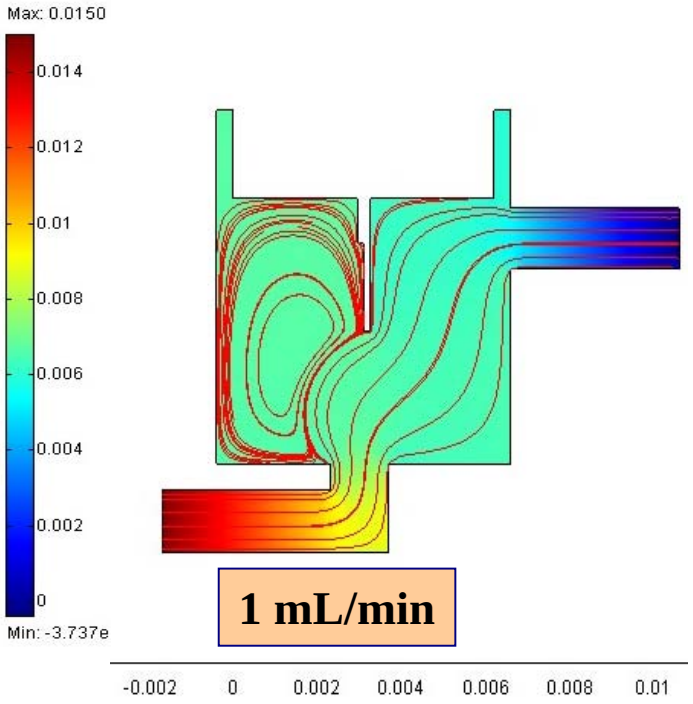


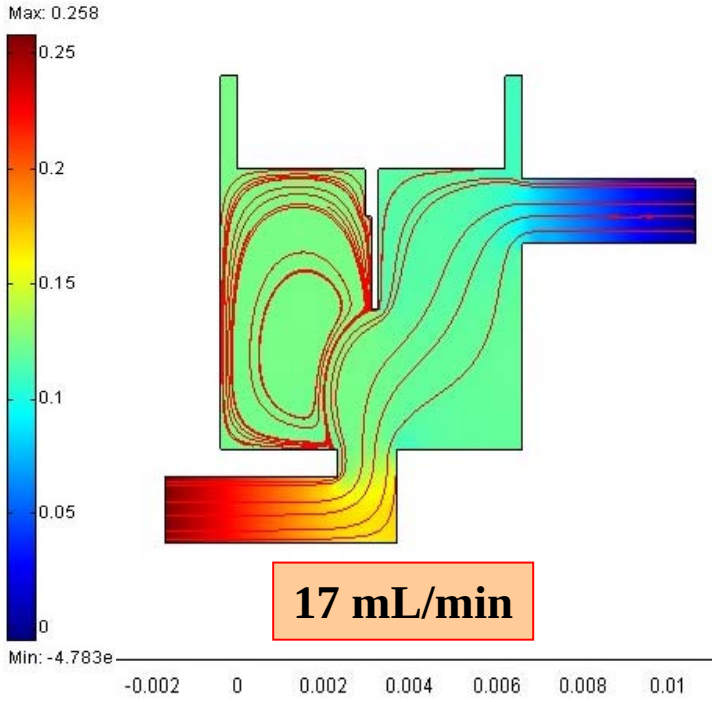
1 mL/min



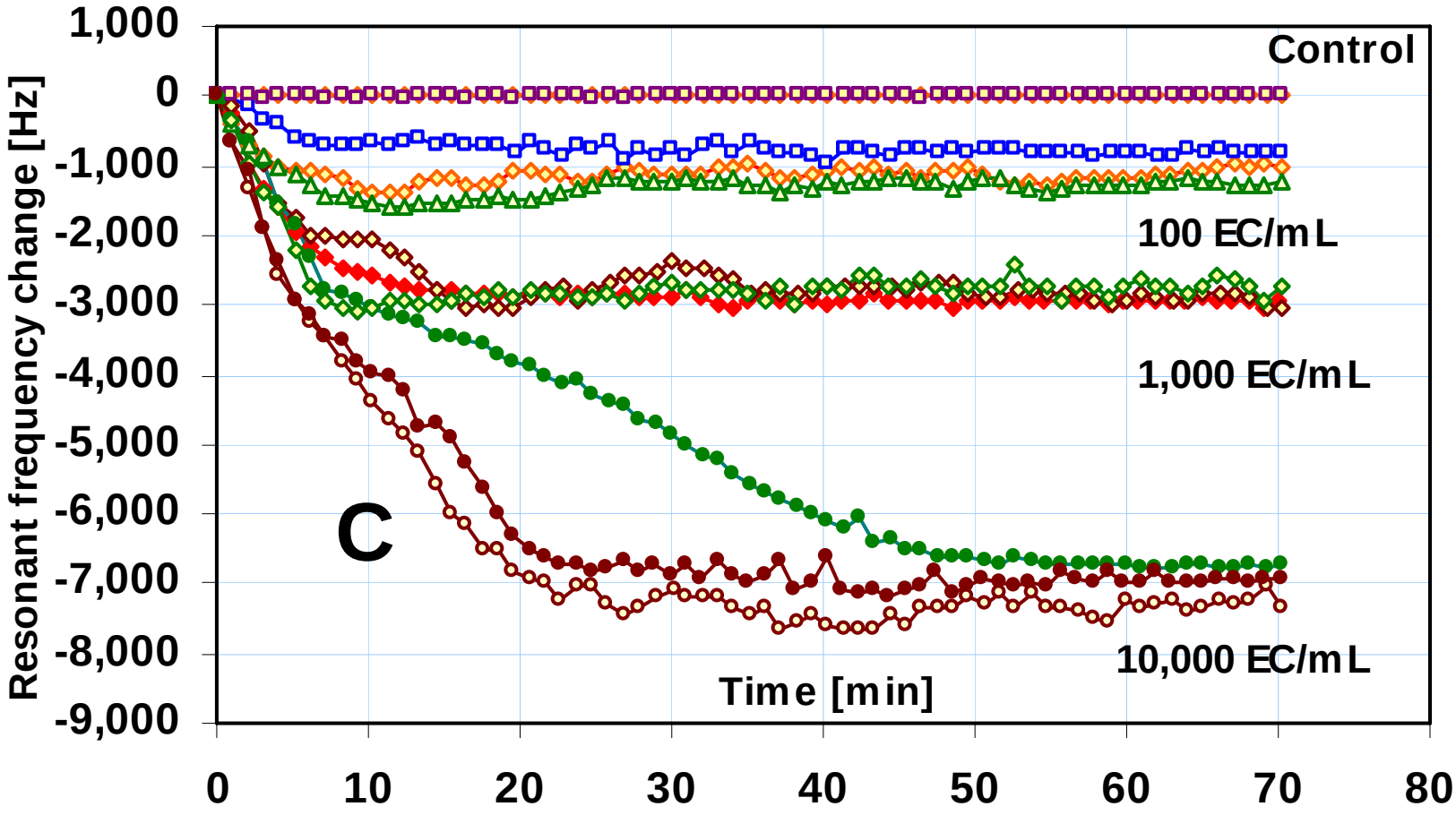
17 mL/min

Flow cell geometry and flow field (Obj 2)



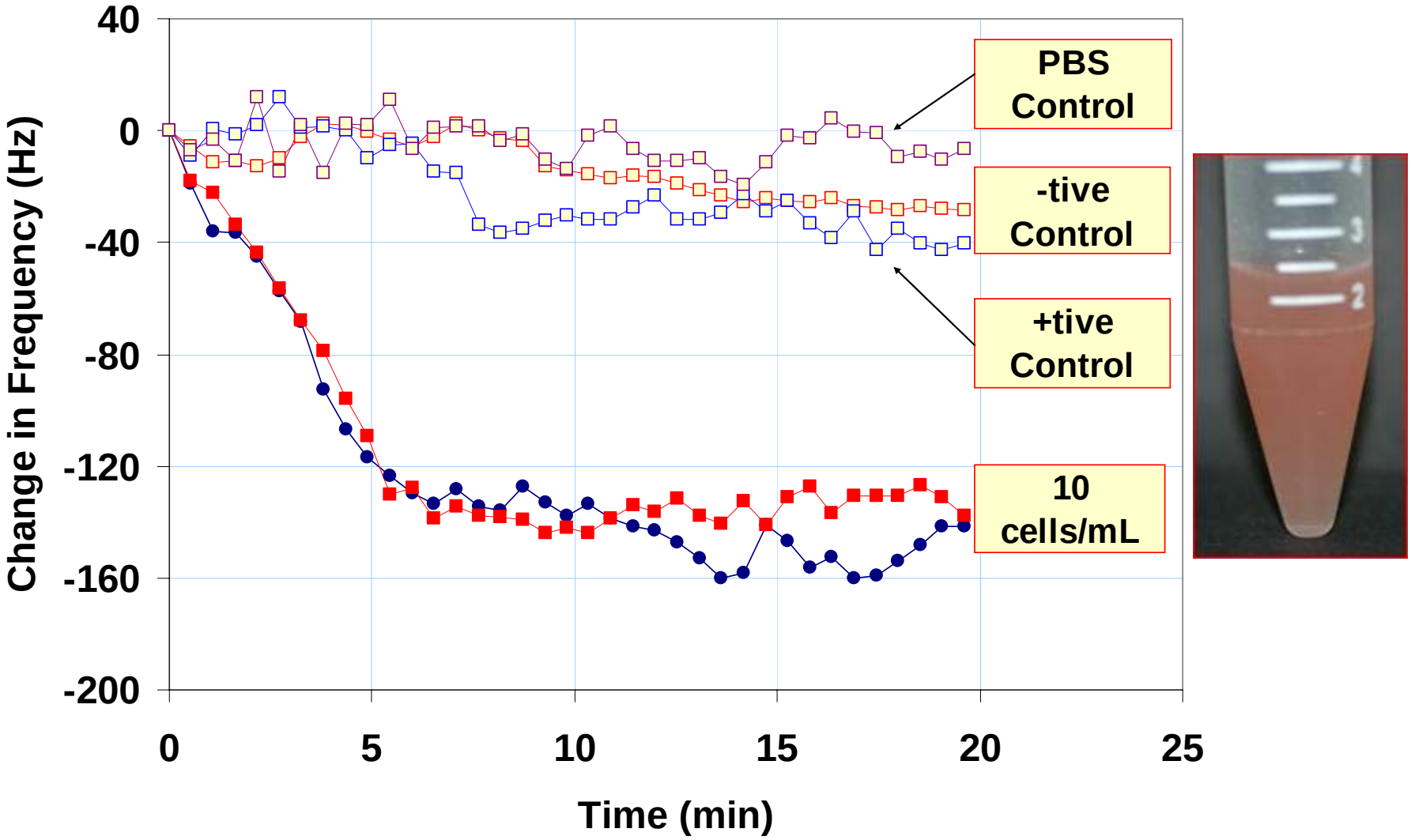


Repeatability - E. coli O157:H7 in buffer



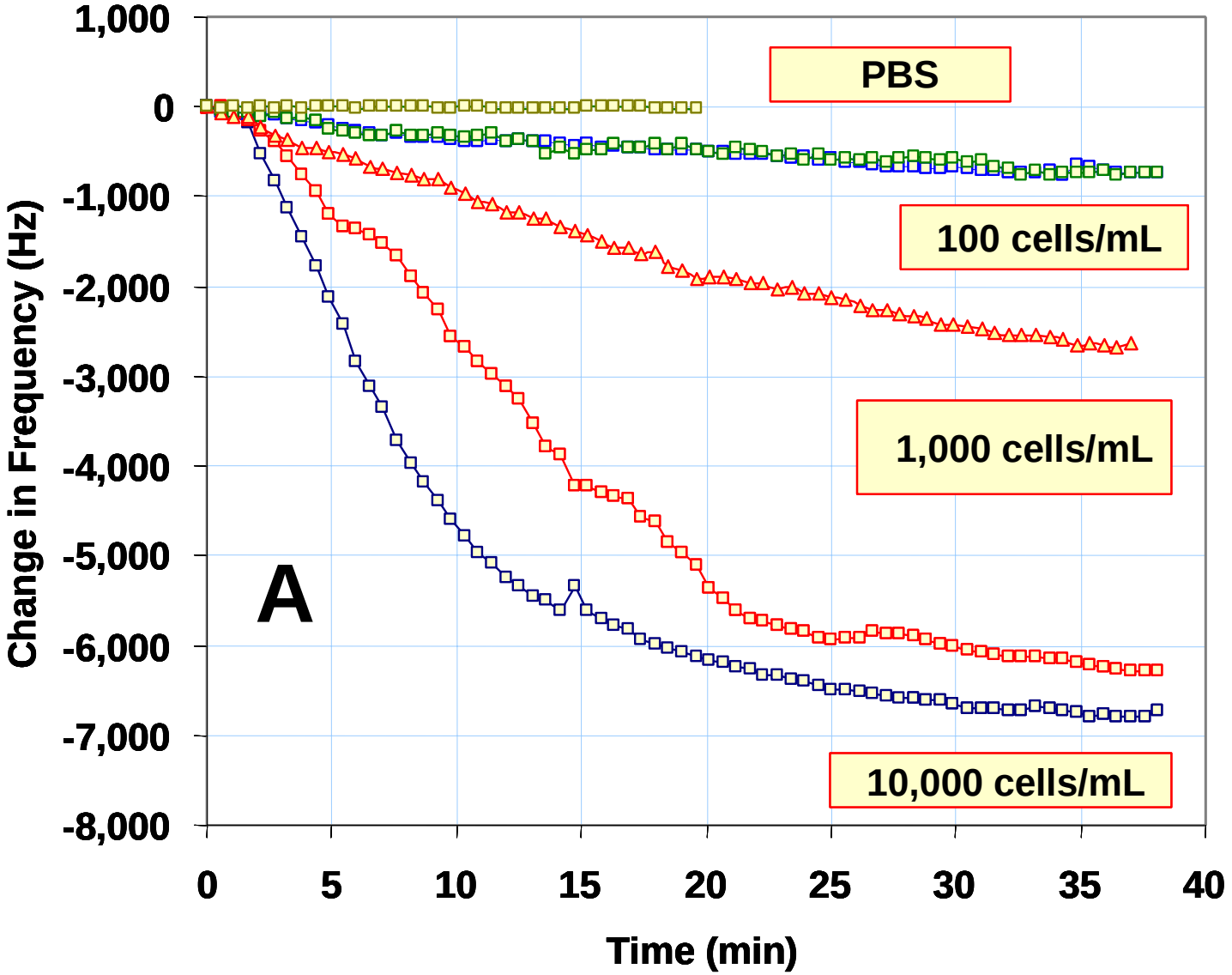
Maraldo, D.; Rijal, K.; Campbell, G.,Mutharasan, R., Method for Label-Free Detection of Femtogram Quantities of Biologics in Flowing Liquid Samples. *Analytical Chemistry* **2007,** 79, (7), 2762-2770

E. coli O157:H7 in ground beef wash



Maraldo, D.,Mutharasan, R., 10-Minute Assay for Detecting *Escherichia coli* O157:H7 in Ground Beef Samples using Piezoelectric-Excited Millimeter-Sized Cantilever (PEMC) Sensors. *Journal of Food Protection* **2007,** in press.

E. coli O157:H7 in ground beef wash



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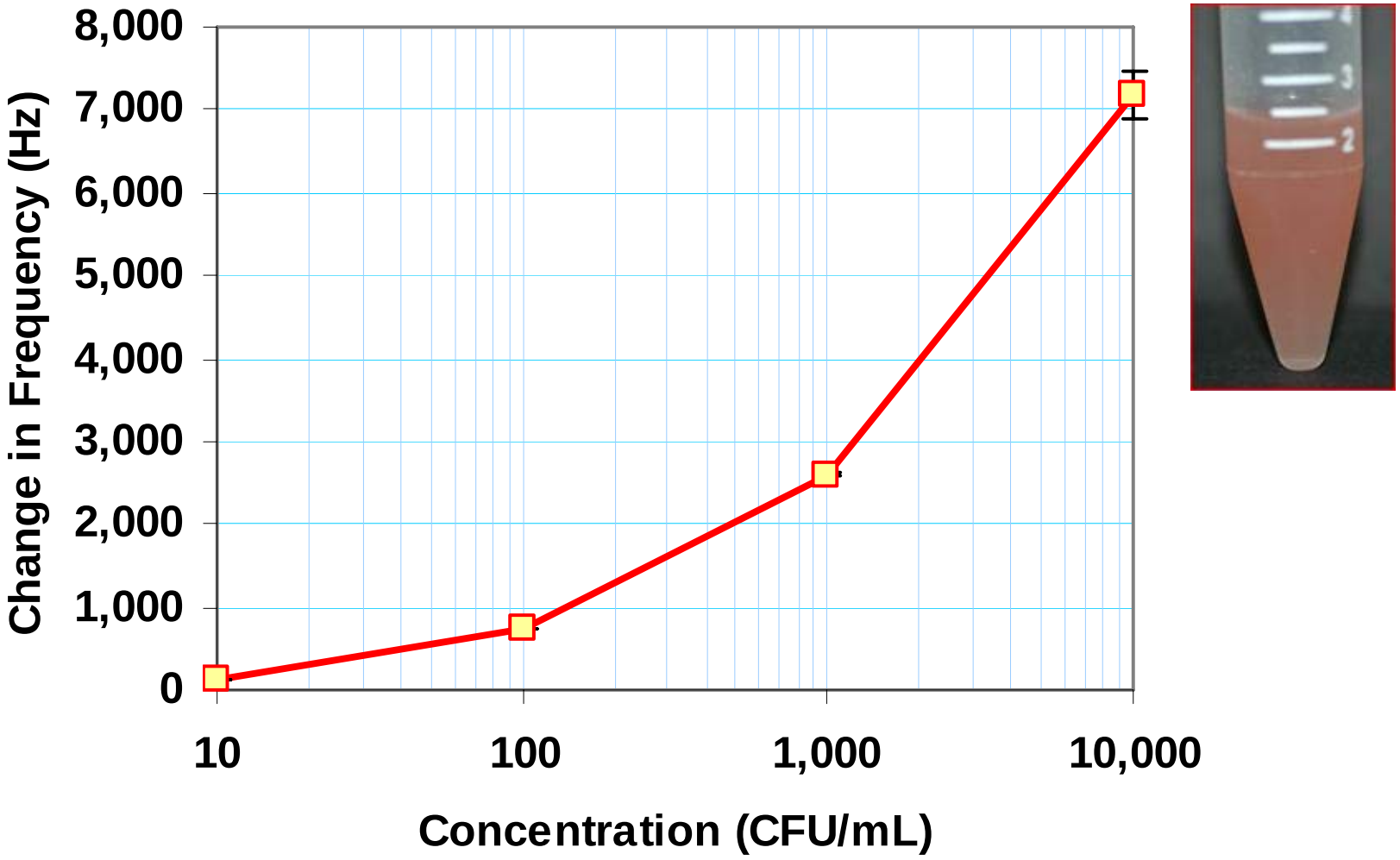
E. coli O157:H7 in ground beef wash



Maraldo, D.,Mutharasan, R., 10-Minute Assay for Detecting *Escherichia coli* O157:H7 in Ground Beef Samples using Piezoelectric-Excited Millimeter-Sized Cantilever (PEMC) Sensors. *Journal of Food Protection* **2007,** in press.

Conclusions

- Cantilever sensor mass change sensitivity = 1 ag/Hz
- *E. coli* in buffer – Detection limit – 10/mL (in theory one cell)
- *E. coli* in proteinous environment – Detection limit 10/mL (in theory one cell)
- Crypto in buffer – done 100/mL
- DNA-detection in buffer and in serum – Feasible without a sample prep step
- Stx2-gene based detection - buffer and beef wash – done 4450 cells; 100 appears to be feasible

Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water

Udi Zuckerman, Ph.D.

Saul Tzipori, DVM, Ph.D., DSc

Tufts University Cummings School of Veterinary Medicine
Grafton, Massachusetts

EPA 6/19/07

Tufts Cummings School of Veterinary Medicine
The Division of Infectious Diseases
North – Grafton, MA

An efficient concentration method is a key to a successful detection of waterborne pathogens

Overview

- Milestones of the Continuous Flow Centrifugation methodology (CFC) developed at Tufts
- Accomplishments of previous EPA STAR award 1999 - 2003
- Objectives of current STAR award 2006 – 2009
- Progress: new automated method/equipment for multiple waterborne pathogens
- Future tasks
- Acknowledgements

Milestones of the Continuous Flow Centrifugation (CFC) methodology developed at Tufts

1994 - Initial testing of a Haemonetics blood separator – 1st prototype

1996 – Field Testing at Tel Aviv University (Jordan River) 2nd prototype

1999 – EPA STAR 1st award

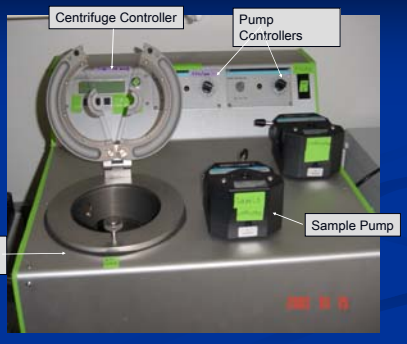
2003 – Method 1623 – 3rd prototype

1st prototype

2nd prototype

3rd prototype

Portable Continuous Flow Centrifuge (PCFC) used for Tier 2 validation



PCFC Protocol (Cont.)

Start Concentration

- Insert the inlet tubing into the pump's head
- Press the PCFC start button
- Push the pump's start button

PCFC Protocol (Cont.)

Stop Concentration

- Disconnect the tubing
- Unscrew the lid's lock, open the lid & pull out the bowl
- Inject 5 ml of elution buffer through the inlet port

PCFC Protocol (Cont.)

Elution

- Assemble the wrist shaker clamps aligned in horizontal position
- Clamp the bowl in an upright position
- Set the speed to 600 rpm & agitate for 10 min
- Rotate the bowl to 9 o'clock position, agitate for 5 min
- Rotate to 3 o'clock position, agitate for 5 min

PCFC Protocol (Cont.)

Detection

- Remove caps from the bowl's ports
- Invert the outlet port & decant the bowl's contents into a 250 ml conical centrifuge tube
- Rinse the bowl by adding 2ml DI water into the inlet port
- Tilt the bowl gently, make sure the residual is located on the bottom
- Invert the outlet port & decant the residual into the centrifuge tube
- Proceed as described in section 13, USEPA Method 1623

Tier 2 mean results of the PCFC compared to Method 1623 criteria

Matrix/Organism	Method 1623 Acceptable Range of Mean Recovery (%)	PCFC Study Mean Recovery (%)
Reagent Water *Cryptosporidium*	21-100	42.5
Giardia	17-100	47.2
Source Water *Cryptosporidium*	13-111	37.4
Giardia	15-118	32.6

PCFC Approved by EPA as a Standard Concentration Method

EPA United States Environmental Protection Agency

Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA December 2005

Changes in the December 2005 Version of the Method
The method was revised again in 2005 to support promulgation of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the June 2003 version include:
Nationwide approval of the use of portable continuous-flow centrifugation as a modified version of the method. The product met all method acceptance criteria for *Cryptosporidium* using 50-L source water samples.

Cont..

CFC 200

2005 - the CFC 200 and 625B bowl became commercially available

2006 – the second EPA STAR was award

1st automated CFC prototype

Current STAR award 2006 – 2009 objectives

- Simultaneous concentration of representative microorganisms from each group of the CCL list
- Validation of the concentration methodology through EPA programs
- Detection and quantitative identification of the CCL list using multiplex miniaturized fiber optic bead microarrays coupled with a compact scanner
- Side by side comparison of this detection methodology with EPA standard methods

Expending the CFC methodology beyond protozoa concentration

- Design of a new multiple pathogens bowl
- Design of a portable computerized concentration/elution equipment
- Design of a disposable tubing kit
- Choosing the programming software
- Testing variable operating protocols

How does it work?

- **Filtration components are based on size exclusion which is prone to clogging and the overall procedure is labor intensive and expensive**
- **The new automated CFC methodology employs centrifugal force to sediment the protozoa and bacteria inside the bowl with minimal clogging problems.**
- **The modified bowl allows the "particle-free sample" to flow through the positive charged component in the core and the viruses are adsorbed by the positive electrostatic forces**
- **Elution buffers are injected sequentially where the trapped protozoa/bacteria first, then the viruses second, are dislodged and the concentrates are delivered to two separate sterile bags.**

Constructing a new bowl – modified core for virus capturing

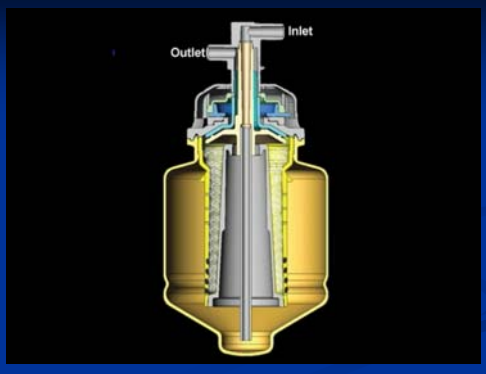
Virus component

High separation core

Testing the modified core component

Filtering large volumes of tap/source water spiked with MS2 bacteriophages

New bowl for simultaneous pathogen concentration



Automated CFC protocol

Water Sample 10 – 100 L

Concentration

Assemble the modified bowl/tubing harness
Turn on the CFC, select operation mode
Protozoa, bacteria and viruses are concentrated inside the bowl

Elution

Protozoa/bacteria buffer is injected, the bowl goes through shaking cycles, the concentrate (~200ml) is delivered to a sterile infusion bag. Virus buffer with a neutral charge is then injected, the bowl goes through rinsing cycles, the concentrate (~20ml) is delivered to a 2nd bag

Detection

The first concentrate is divided into bacteria and protozoa aliquots, and together with the virus concentrate are then processed for detection using standard or rapid methods

Spiking experiments using the automated CFC

Portable automated continuous flow centrifuge

Disposable Kit

Automated concentration and elution

Testing the recovery efficiency of the automated CFC with 10 L tap water samples spiked with multiple microorganisms

- *C. parvum* (EasySeed) were spiked and oocysts were detected from the concentrate using method 1623
- MS2 bacteriophages (ATCC 15597-B1) were spiked and detected from the concentrate using the agar overlay method (the host was E. coli 1559).
- B. anthracis spores (kanamycin resistant strain, sterne) from Tufts stock reference were spiked and detected by MF

Recovered concentrates and detection methods

Virus concentrate

Agar overlay procedure for MS2 phage

Bacteria/protozoa concentrate

EPA Method 1623 for *C. Parvum*

Vacuum filtration and media growth for *B. anthracis*

Recovery (%) of *C. parvum* oocysts, *B. anthracis*, and MS2 bacteriophages from 10L (N=7) tap water samples using an automated CFC and a modified bowl (9,000rpm & 0.5 liter/min)

C. Parvum spike (mean +/- SD)	*C. parvum* recovery (mean +/- SD)	*B. anthracis* spike (CFU mean +/- SD)	*B. anthracis* recovery (mean +/- SD)	MS2 spike (mean +/- SD)	MS2 recovery (mean +/- SD)
100 +/- 2.5	40 +/- 12.2	23.3 +/- 4.6	43.6 +/- 16.4	$2.6*10^7$ +/- 1.3 $*10^7$	48.1+/-28.2

We currently test the system with 100L of tap and source water samples

Summary of the advantages of the automated concentrator

- Portable, compact and automatic device that can operate from 220, 110, and 12 DC
- Simultaneously concentrates bacteria, protozoa algae and viruses
- Integrated elution
- Process large volumes (100L) without clogging (possibly >1000L)
- Safe handling, self contained and rapid procedure
- Disposable kit eliminates the need to disinfect the equipment
- The concentrate could be tested in the field or transported to the lab
- Cost effective and efficient
- Ideal for continuous monitoring

The next phase

- Our collaborator on the EPASTAR project, also from Tufts, is currently working on the bioinformatix of the CCL list for the microarray detection: this will be presented at next meeting
- Once the detection platform is complete, the automated PCFC concentrates will be tested using this technology
- The detection will be compared with currently approved standard methods

Acknowledgements

- EPASTAR program for the current financial support (Grant 2005-STAR-K1) and the previous (Grant 99 – STAR – C1)
- Haemonetics for the substantial financial and technical support over the past 10 years
- Staff of the Division of Infectious Diseases for technical support (Greg Rei, Curtis Rich)

UC RIVERSIDE UNIVERSITY OF CALIFORNIA

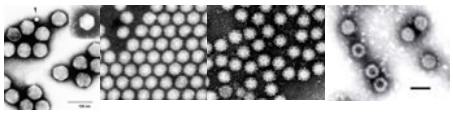
Development of High-Throughput and Real-Time Methods for the Detection of Infectious Enteric Viruses

Yu-Chen Hwang[1], Hsiao-yun Yeh[2], Marylynn V. Yates[1], Ashok Mulchandani[2], and Wilfred Chen[2]

[1]Environmental Sciences Department
[2]Chemical & Environmental Engineering Department
University of California, Riverside

Human Enteric Viruses

- Common infectious viral agents
  - waterborne diseases
  - fecal-oral transmission
  - stable in aquatic environments
- enteric *adenoviruses, enteroviruses, noroviruses, rotaviruses*
  - coxsackievirus, hepatitis A virus & poliovirus

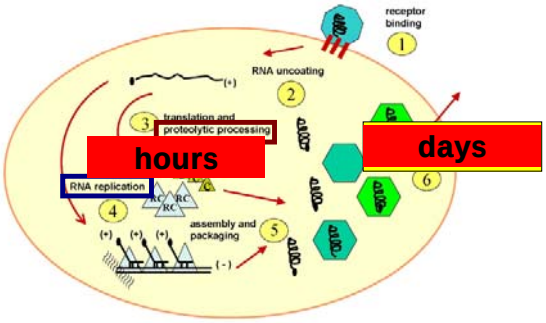


Methods for the Detection of Viruses

Principle	Infectivity	Detection limit (particles/ml)	Time
Electron Microscopy	No	1.E+05	< 24 h
ELISA			
viral antigen	No	1.E+05	< 2 h
antiviral antibody	Yes	1.E+05	< 2 h
Quantitative PCR	No	1.E+00	< 4 h
Plaque Assay	Yes	1.E+00	< 8 days

Koopmans & Duizer. 2004. Int J Food Microbiol

Viral Replication Cycle

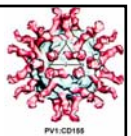


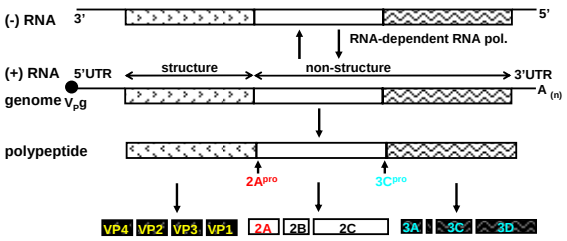
www.molbio.uni-luebeck.de

Outline

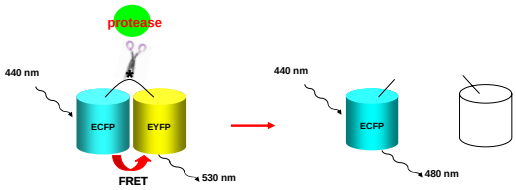
- Reporter cell targeting viral protease activity for infectious PV1
- Fluorescent probe to monitor viral genome replication
  - *in situ* detection of infectious HAV
  - real-time fluorescent assay of viral RNA

Poliovirus



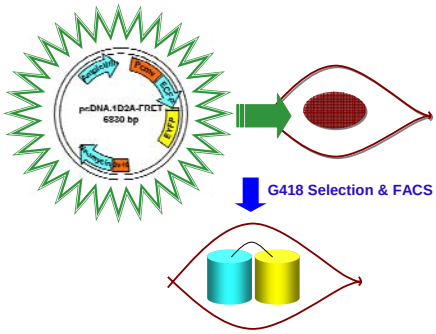


Fluorescence Resonance Energy Transfer

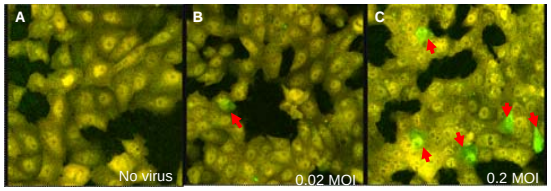


* protease cleavage sequence derived from poliovirus VP1-2A junction
Ser-Thr-Lys-Asp-Leu-Thr-Thr-**Tyr**-**Gly**-Phe-Gly-His-Gln-Asn-Lys-Ala

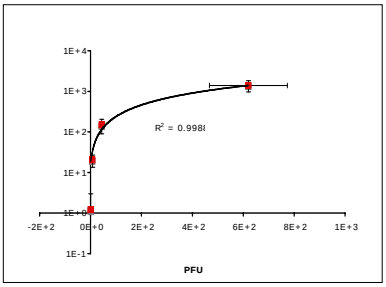
Reporter Cell Line



Reporter Cells Challenged w/ PV1



Reporter System vs. Plaque Assay



Conclusions & Pitfalls

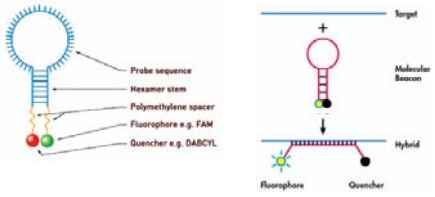
- Stable & strong expression of the fluorescent substrate
- Achieved detection limit of 1 PFU within 8h pi while plaque assay takes 48h pi

- Image-based microscopy limits processing capacity
- HTS using flow cytometer under investigation

Molecular beacon (MB)

Single-stranded hairpin oligonucleotide probe

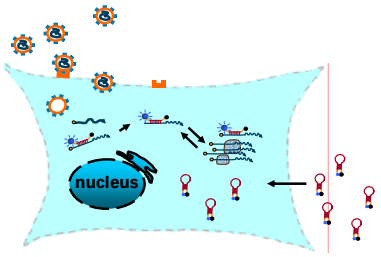
- probe sequence: 10-50 nt
- GC stem: 6-8 nt
- fluorophore & quencher
- high S/N ratio -> high sensitivity



Tyagi et al. 1996. *Nature Biotech.*

Monitor viral replication *in situ*

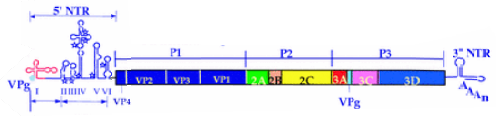
- fixation
- permeablization
- hybridization & washing
- microscopy



MB to detect infectious viruses

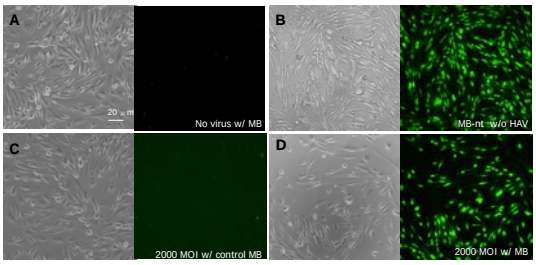
- Previous study (Wang et al., 2005. AEM)
  - detected 1 PFU of CVB6 using MB at 6 h pi
- Challenges for HAV detection
  - long incubation time to produce visible plaques (8 days)
  - some HAV infections result in no obvious CPE
  - *in situ* fluorescent assay using MB for early detection

HAV MB



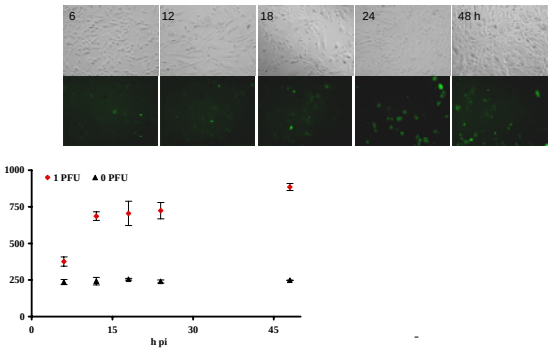
- Probe sequence
  - conserved region at 5'NTR among 26 HAV strains
- MB sequence
  - 5' FAM – CTTGGGCCGCCGCTGTTACCCTATCCCCCAAG – DABCYL 3'
- Hepatitis A virus
  - ATCC strain VR 2089

in situ Detection of HAV

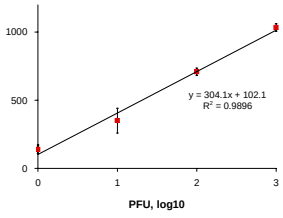


*cells were fixed, permeabilized, and hybridized with MB 6 h pi

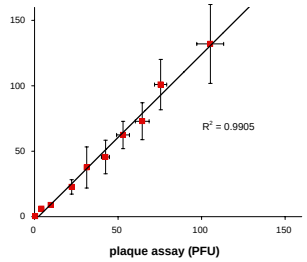
FrhK-4 infected w/1 PFU HAV



Standard Curve



Fluorescence Assay vs. Plaque assay

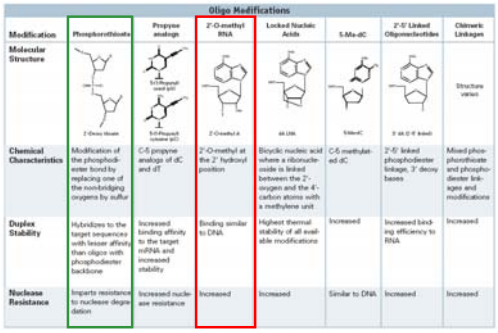


Conclusions & Pitfalls

- Achieved detection limit of 1 PFU HAV after 6 h pi
- Comparable to plaque assay

- Required extensive post-treatment of the sample
- MB are prone to degradation & photobleaching

Nuclease-resistant MB

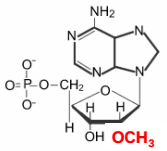


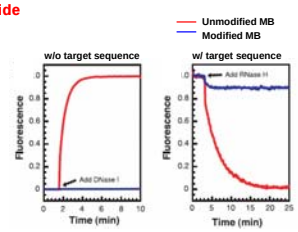
www.genelink.com

Nucleotide Modification I

- replace 2' hydrogen with methoxide

2'-O-Methyl oligoribonucleotide



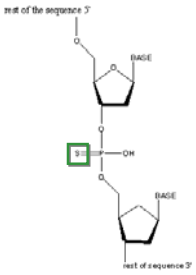


Bratu et al. 2003. *PNAS*.

Nucleotide Modification II

- modify phosphodieaster bond

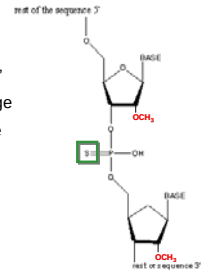
- Replace the non-bridging oxygen with sulfur at the phosphate group



TIB MOLBIOL, LLC

Nuclease–resistant MB

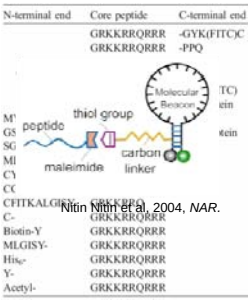
- Combination of methoxy group at 2' position with phosphorothioate linkage provides greater nuclease resistance



TIB MOLBIOL, LLC

Cellular Delivery

- HIV Tat-derived peptide containing arginine and lysine facilitates cellular delivery of MB
- Short incubation time (<30 min) with high efficiency
- No interference with the target or the fluorescence of MB

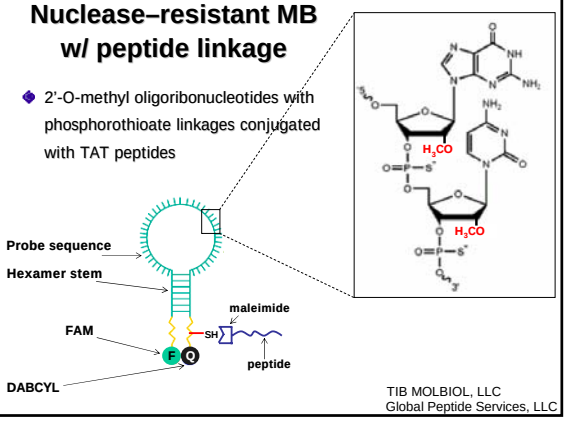


Nitin Nitin et al, 2004, *NAR.*

Brooks et al, 2005. *Adv. Drug Deliv. Rev.*

Nuclease–resistant MB w/ peptide linkage

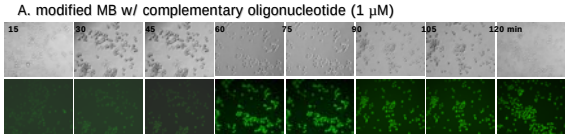
- 2'-O-methyl oligoribonucleotides with phosphorothioate linkages conjugated with TAT peptides



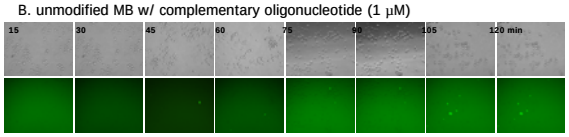
TIB MOLBIOL, LLC
Global Peptide Services, LLC

Cellular Delivery of MB in Real time

A. modified MB w/ complementary oligonucleotide (1 μM)



B. unmodified MB w/ complementary oligonucleotide (1 μM)



Summary & Future Work

- The modified probe facilitated cellular delivery of the nuclease-resistant MB, providing cellular detection of infectious viruses in real time
- Real-time monitoring of viral replication in progress
- HTS for viral pathogens will be assessed

Acknowledgements

- Yates' Lab
  - Dr. J. Jason L. Cantera
- Mulchandani & Chen's Lab
  - U Loi Lao, Shailendra Singh
- Dr. D. Carter & Ms. B. Walter, UCR Genomic Center
- Dr. R. Tsien, UCSD (CFP)
- Dr. A. Miyawaki, Brain Science Institute, Japan (YFP)
- EPA STAR & EPA GRO graduate fellowship for Yu-Chen Hwang

Timely multi-threat biological, chemical, and nuclide detection in large volume water samples.

Presented by
Paul Galambos, Sandia National Labs Dept 17492,
pcgalam@sandia.gov
Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000

Introduction

Handheld, robust, multi-agent detector

- Problem: Need to detect multiple dangerous agents (CBNE – Chem/Bio/Nuclear/Explosive) in various dirty samples at high levels of sensitivity and specificity (low false negatives and low false positives). Needed by soldier on the battlefield.
- Solution under development: Bead based multiplexed detection of many agents in the same solution with raw sample handling and cleanup.

- **Can we apply this solution to detection of dangerous microorganisms in water?**

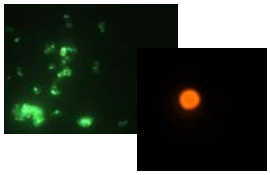
Outline

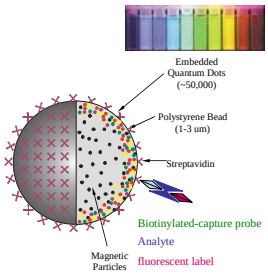
- Two key enablers – bead and concentrator
- Milk – problem and modeled system solution
- Milk system testing and future developments
- Strawman water system discussion

Magnetic Bead Chaperone

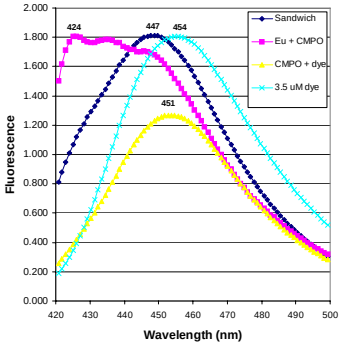
Chaperone characteristics:

- Magnetic particles for preconcentration of target analytes
  - Highly selective, strong forces
- Surface chemistry for target capture
  - Chem, bio, nuclear, explosives
- QD Bar-code for target ID
- Sandwich Assay

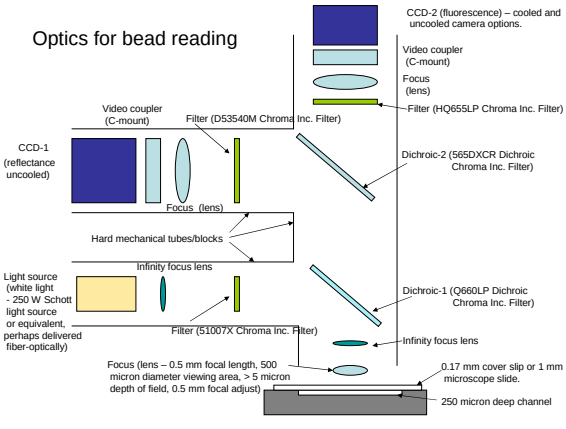




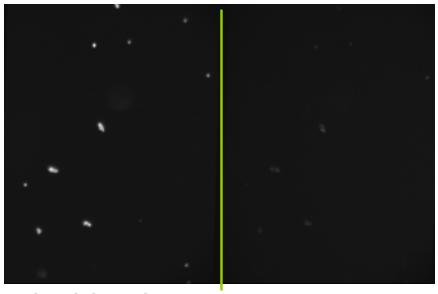
Radionuclide detection



Optics for bead reading



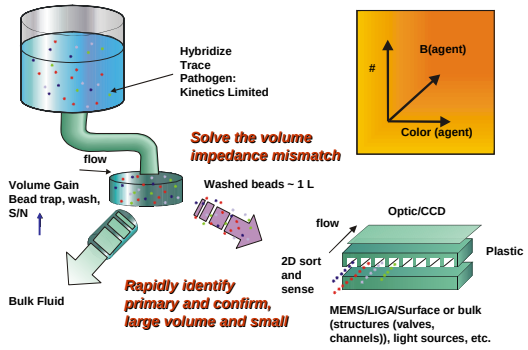
Biodetection – Botulinum toxin substitute in milk



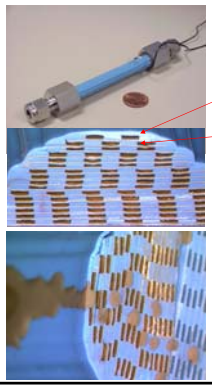
Two-signal detection.

Bead identification on left; captured antibody with fluorescent label on right. Software uses information from both images to identify bead location, type and capture of target antibody.

Key Enabler: High Volume Trace Sampling - Concentrator



Meso-scale Trap Prototype using LTCC technology



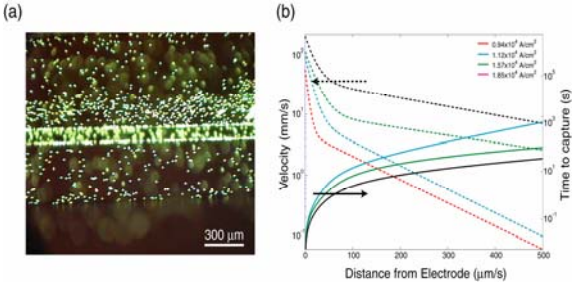
Each row has electrodes in parallel within each fluid channel (top row has two channels, with two electrodes in parallel)

Rows of electrodes are connected to each other in series (the top row, which has two electrodes in parallel, has its electrodes connected in series to electrodes in row 2, which has three channels and three electrodes in parallel)

Thus if we pass a total of 1 A of current through the entire device (total resistance of 20 ohms, which will require 20V), the top row will have 0.5 A going through each of the two conductors in the two channels). The rows in the center of the device (where there are 6 channels and 6 electrodes) will have 0.17 A passing through each wire.

Testing will involve passing a sample of magnetic beads through the trap, and observing the signal intensity measured downstream of the trap with a spectrometer.

Experiments with single wire demonstrate electromagnetic capture of magnetic beads for sample cleanup.



Model description*

- TTI (Time-To-Identify) = $t_{collection} + t_{mixing} + t_{trapping} + t_{sensing} + t_{transport}$

$t_{collection}$ = f(flowrate, target size, pipe size, viscosity)

t_{mixing}= f(Volume, # beads, #targets, target size, probability of capture)

$t_{trapping}$ = f(flowrate, bead size, pipe size, viscosity)

$t_{sensing}$ = f(SNR,Quantum eff, dark current, signal stgth, wavelength)

$t_{transport}$ = f(flowrate, scale)

* Accepted for publication, International Journal of Nanofabrication, September 2007

Milk problem

- Milk supply is vulnerable to contamination (perhaps on purpose) between the cow and the grocery shelf (reference Wien, PNAS 2006 paper on milk vulnerability)

Calculations pertaining to milk from model

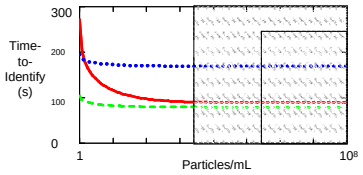
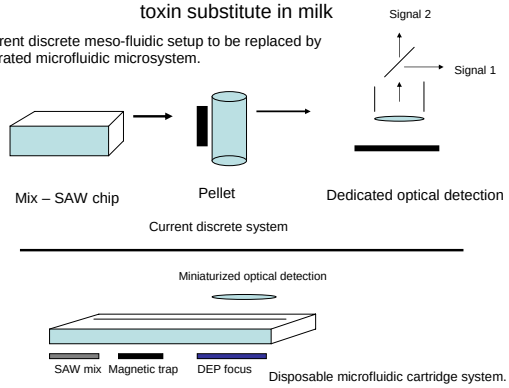


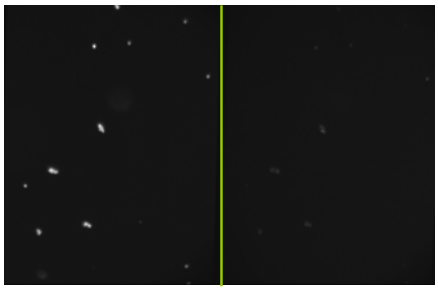
Figure 6. Variation in system performance for small analytes (e.g. *botulinum* toxin) with respect to the number of beads and bead count rate in 1ml samples. This figure represents system performance envelopes expected in raw milk samples. The dashed curve, solid curve, and dotted curve curves are for ($2x10^4$ beads, 10^3/s count rate), (10^6 beads,10^4/s count rate) and (10^6 beads,10^5/s count rate), respectively.

Rapid and accurate bead-based identification botulinum toxin substitute in milk

- Current discrete meso-fluidic setup to be replaced by integrated microfluidic microsystem.



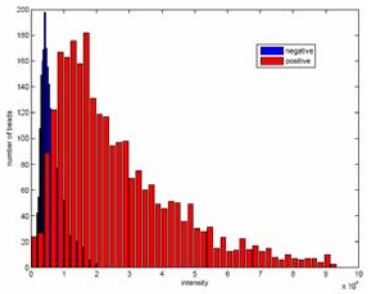
Biodetection – Botulinum toxin substitute in milk



Two-signal detection.

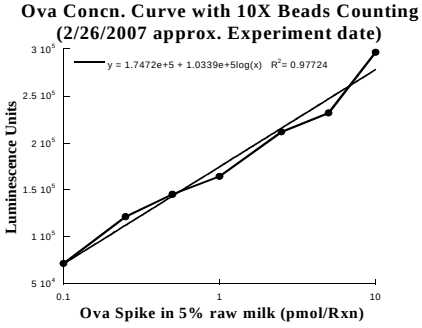
Bead identification on left; captured antibody with fluorescent label on right. Software uses information from both images to identify bead location, type and capture of target antibody.

Test Results: Identification of Botulinum substitute (Ovalbumin) in Milk



Shift in number of beads at higher intensity at wavelength of antibody label indicates positive capture of target (Ovalbumin – Botox substitute).

Sensitivity curve – Ova in milk



Time-to-Identify (TTI) Experimental

- **14 samples identified correctly in one day.**

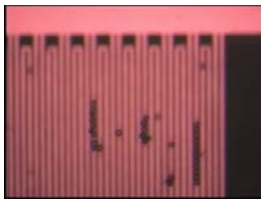
Sample Timeline

Add all reagents to tube- 1 minute
Mix (on SAW chip)- 15 minutes
Clean SAW chip and pipette mixture into clean tube- 2 minutes
Pellet beads- 10 minutes
Clean pellet and re-suspend pellet in buffer- 2 minutes
Centrifuge pellet and pipette sample onto glass slide- 3 minutes
Move sample to scope and check focus on sample- 3 minutes
Data collection (180 frames)- 15 minutes
Save data and start code: 1 minute
Code analysis-10 minutes
Data output with positive or negative ID- 3 minutes
Time Sum: 65 minutes

***Note*:** Because the next sample can be prepared and mixed while the previous sample is being optically analyzed, we are obtaining positive or negative ID's approximately every 30-35 minutes.

- **Modifications to hardware and procedure to reduce time and increase sensitivity are on-going in preparation for field test in July.**

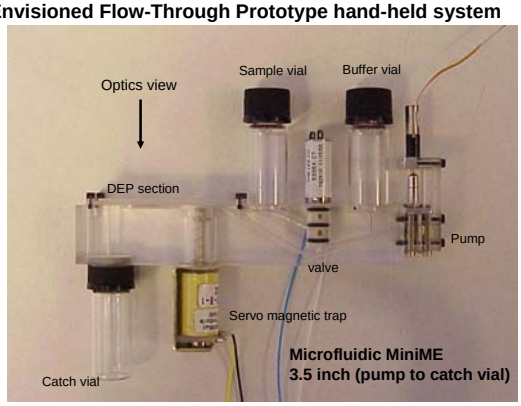
DEP (dielectrophoretic) focus will allow flow-through continuous bead reading.



9 micron diameter Spherotech Nile Red Fluorescent Magnetic beads at 5V, 1MHz (5 V and 0V signals). Switched to 15 MHz at the end of the video- disperses the beads

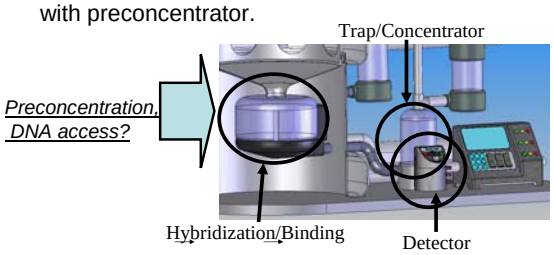
Bottom Line: we can create 2D null points where the latex magnetic beads will migrate. By modifying the dimensions + spacings of the electrodes, we can ensure that single beads are on an axis (above right movie). Questions to be answered are the frequency effects (which freq to use?) and interparticle interactions that will cause beads to pearl-chain as in the above right movie. We could design the electrodes to define 3D nulls that would separate individual beads, but translational motion would be difficult (would need shifting traps to get translation of the trapped beads).

Envisioned Flow-Through Prototype hand-held system



Strawman system – water

- Replace 1 mL milk sample collection with large volume of water to small volume of bead solution with preconcentrator.



I invite your input.

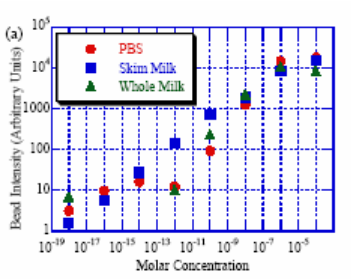
Conclusions

- Bead based detection of botulinum substitute in milk indicates utility of concept for liquid based dangerous agent detection in dirty liquids.
- System concept adaptable to many problem scales – spanning macro to nano scales.
- Opportunity to adapt partially developed bead-based detection systems to high throughput sensors for micro-organism detection in water.

Acknowledgements

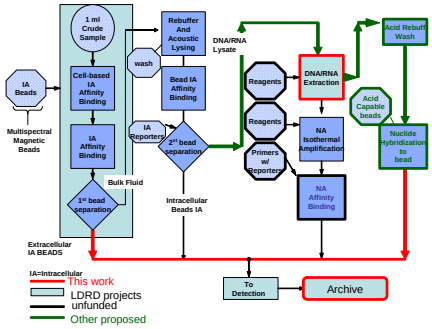
- Sandia LDRD office and UC Davis (Jim Cullor) funded this work.
- Beady project team: Mark Derzon, Achyuthan Komandoor, Jaime Mcclain, Chris Bourdon, Conrad James, Kamyar Rahimian, Mike Kaminski
- USAMRIID – George Ludwig

Extras



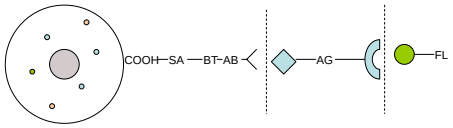
- Measurements with confocal microscope show sensitivity advantage
- Raw milk causes problems – being worked
- We need this kind of curve in the new MiniME

  Note: Use later sensitivity curve from Achyuthan



- DEP video, trapping video

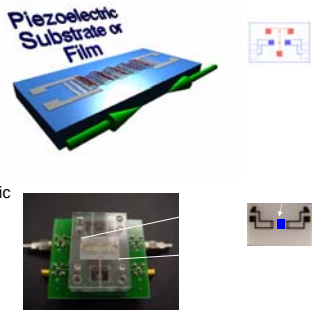
FL – Fluorescent label
AB – Surrogate antibody
AG– Surrogate antigen
SA - Streptavidin
BT - Biotin



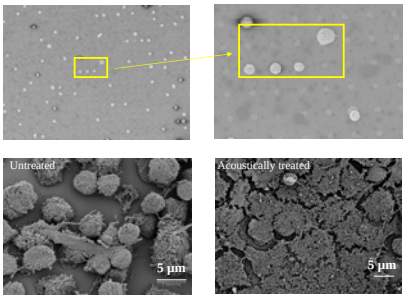
Surrogate Sandwich Assay

Summary: Acoustic Technology

- Robust & Simple = Reliable & Low cost
- Small active region (<1 mm
- No mechanical failure
- Rapid (seconds to minute
- Thermal Shift less than 1°
- Low power Surface Acoustic Waves (SAW) (<20mW)
- Can be incorporated into existing MEMS



Problem: Specific Cell Capture & Lysis



On-chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens

U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms in Water

Cincinnati, OH

June 19, 2007
1:00 PM

MICHIGAN STATE UNIVERSITY

Syed A. Hashsham

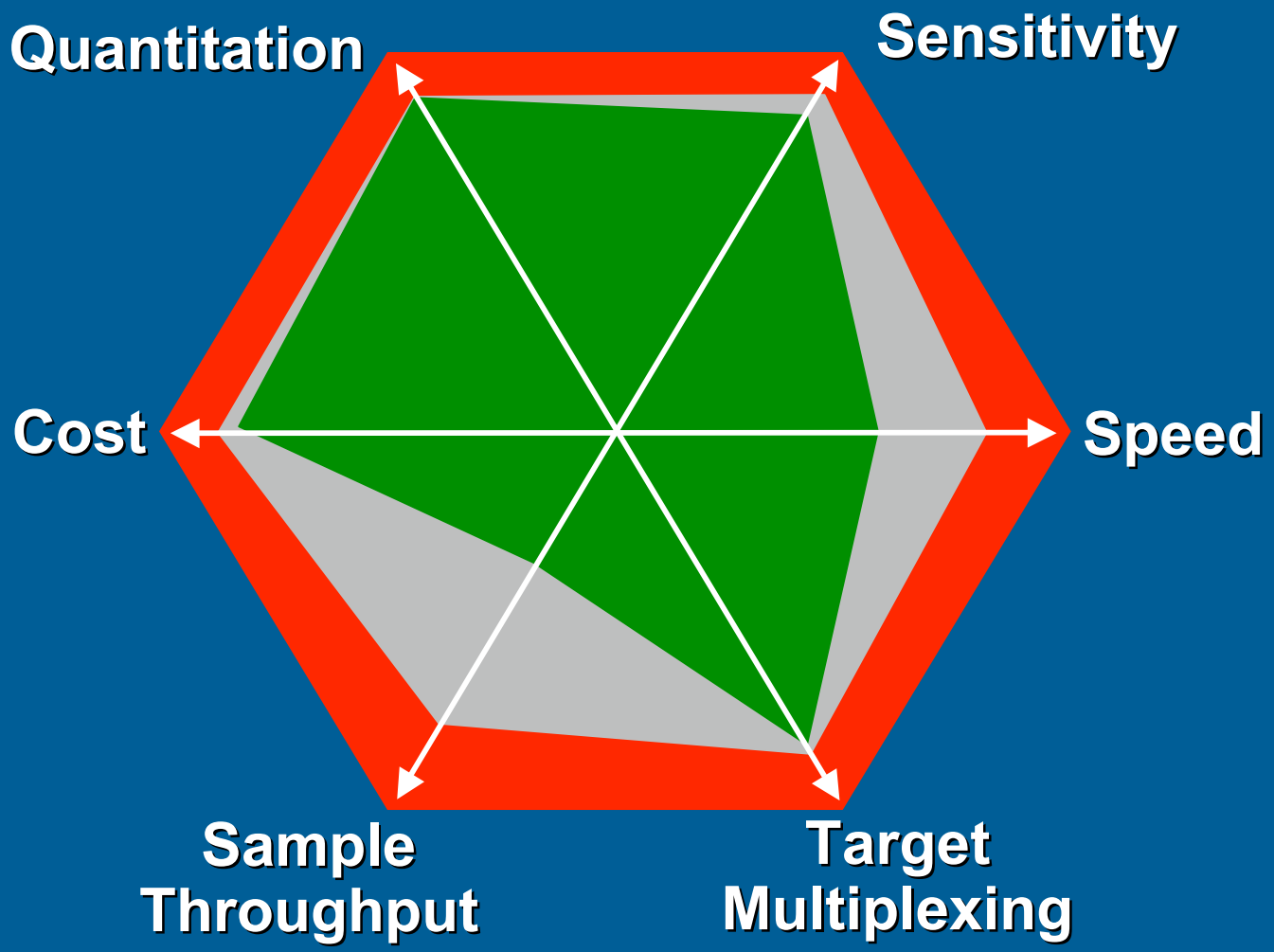
Edwin Willits Associate Professor

Department of Civil and Environmental Engineering and Center for Microbial Ecology
Michigan State University

Outline

1. Microfluidic Biochip-based Parallel Detection of Pathogens
2. High Throughput Screening for Genetic Markers
3. Reducing the Time and Cost to Detect using Nano-particles
4. Challenges and Outlook for Diagnostics/Screening of Genetic Markers

Pushing the Diagnostic/Screening Envelope: Multiple Directions!



Microfluidic DNA Biochip

Target is labeled with dye

Cy3 or Cy5

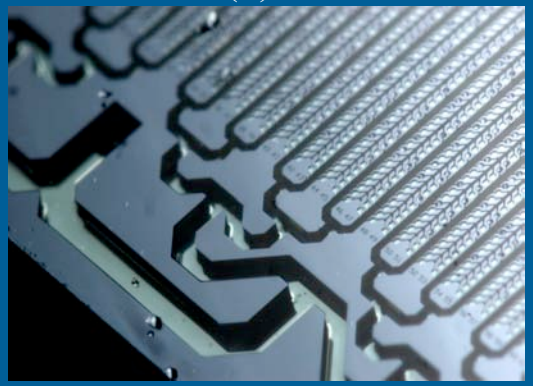
AGCTAAAGCTGACCGGTGCGGAGTGCGTA

ACTGGCCACGCCT

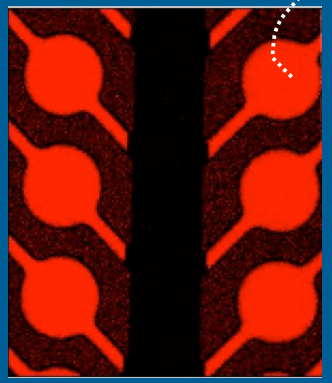
Probe attached to chip surface at 3' end

(a)

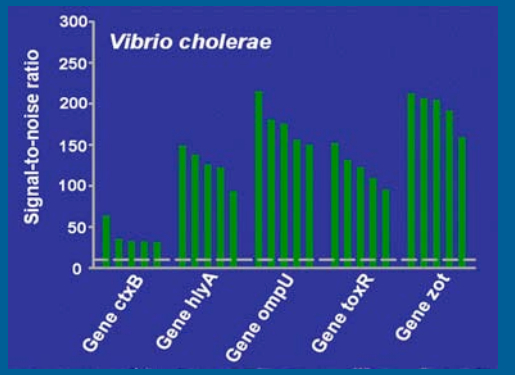
(b)

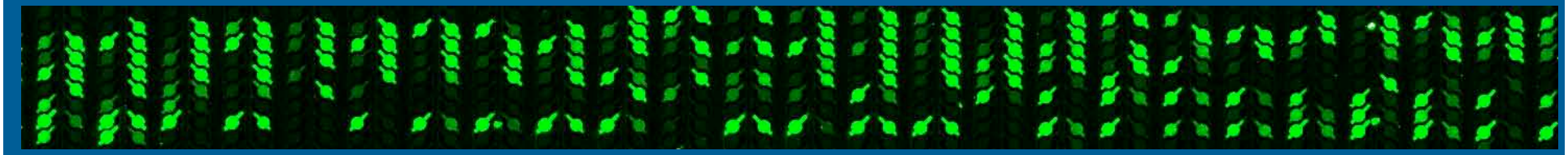


(c)

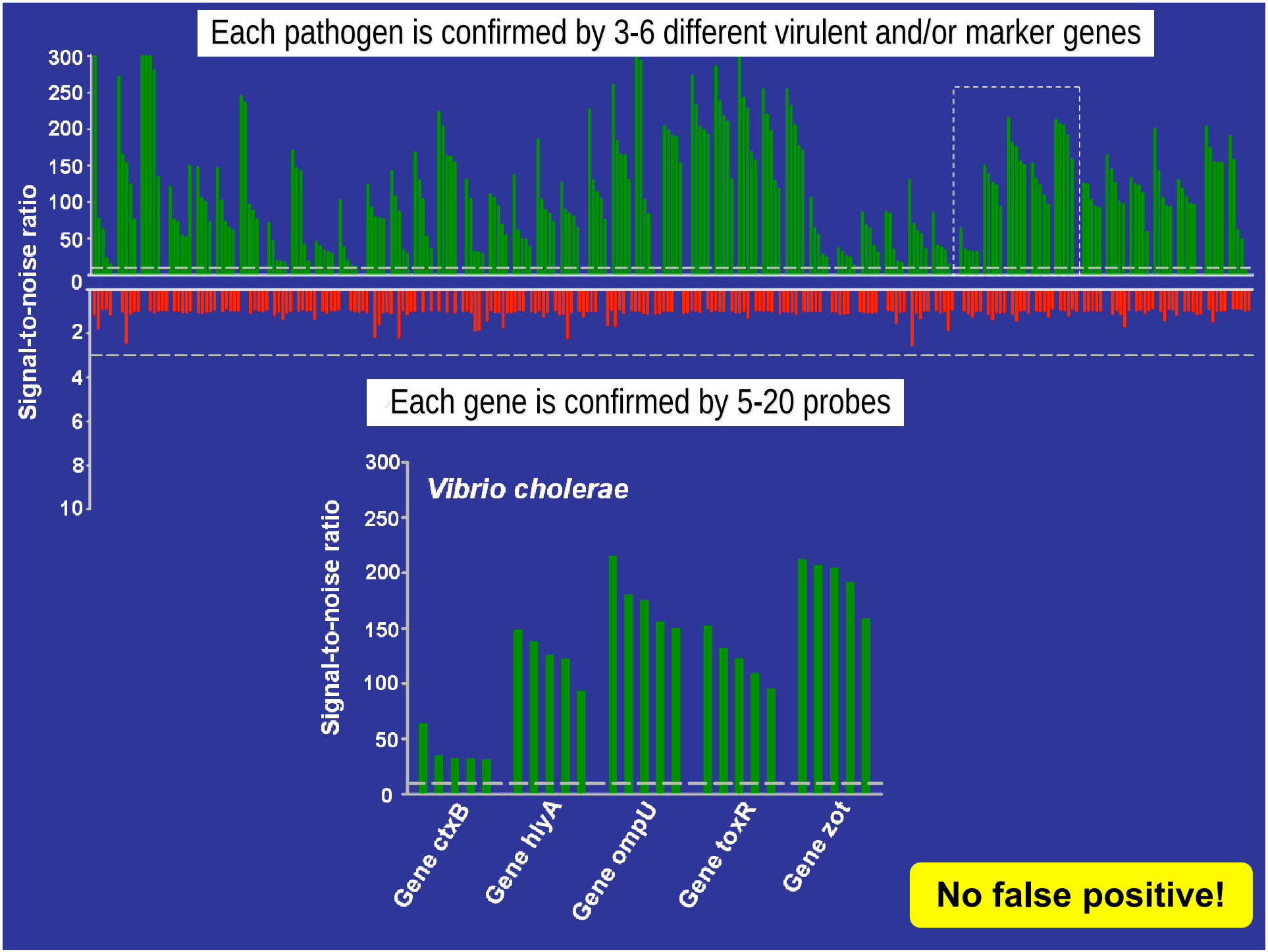


(d)





(e)


Each pathogen is confirmed by 3-6 different virulent and/or marker genes
Signal-to-noise ratio
300
250
200
150
100
50
0
2
4
6
8
10
Each gene is confirmed by 5-20 probes
Vibrio cholerae
Signal-to-noise ratio
300
250
200
150
100
50
0
Gene ctxB
Gene hlyA
Gene ompU
Gene toxR
Gene zot
No false positive!

List of 20 Waterborne Pathogens

1. *Aeromonas hydrophila*
2. *Burkholderia pseudomallei, mallei*
3. *Campylobacter jejuni*
4. *Clostridium perfringens*
5. *Enterococcus faecalis, faecium*
6. *Escherichia coli, Shigella*
7. *Helicobacter pylori*
8. *Klebsiella pneumoniae*
9. *Legionella pneumophila*
10. *Leptospira interrogans*
11. *Listeria monocytogenes*
12. *Mycobacterium avium, paratuberculosis, tuberculosis, leprae*
13. *Pseudomonas aeruginosa*
14. *Salmonella typhimurium* DT104
15. *Staphylococcus aureus*
16. *Vibrio cholerae, mimicus, vulnificus*
17. *Vibrio parahaemolyticus*
18. *Yersinia enterocolitica, pestis, pseudotuberculosis*
19. *Cryptosporidium parvum, hominis*
20. *Giardia lamblia, intestinalis*

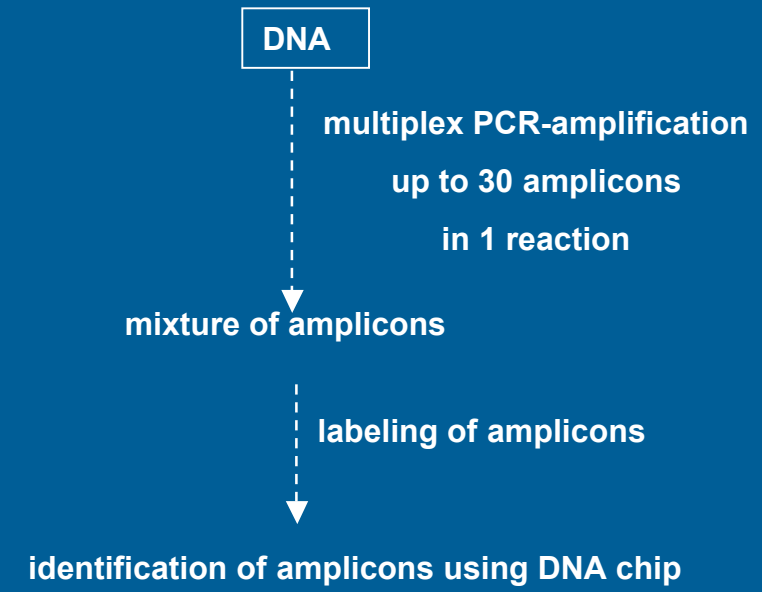
Enhancing Sensitivity

Multiplex PCR-amplification followed by **DNAchip-based amplicon identification**

Without Multiplex Amplification

~1 % of the population

With Multiplex Amplification



Overall Screening Approach

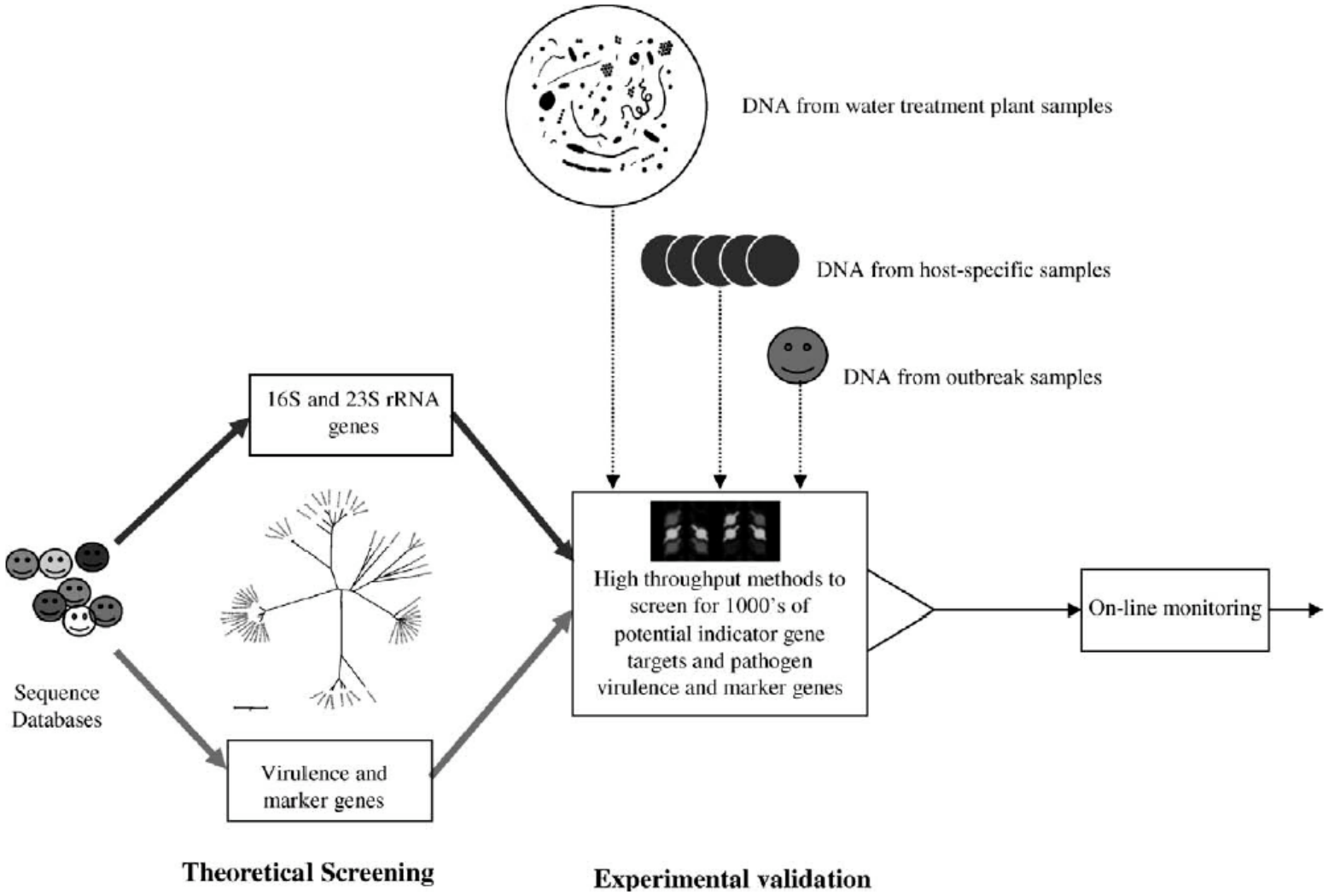


Figure 1—The overall process of screening for new indicators and developing focused methods to monitor the targets consists of two major tasks: (1) comprehensive design of probes/primers/target markers to evaluate the potential indicator groups, and (2) centralized high throughput screening of a large number of samples to find and validate new indicators. Validated markers can then be used to develop methods for online monitoring.

(Stedtfeld et al., Water Environment Research, 2007)

Automated sequence collection: Functional Gene Pipeline Repository (FGPR)

- Harvests Functional Genes from GenBank using **Hidden Markov Model (HMM)**
- Training sequences chosen by experts is input
- Matching sequences are output

Updated monthly

nning with tters F-K	beginning with letters L-Q	beginning with letters R-T
feoA	lam	racR
feoB	lasA	rtxD
FGLN	lasB	scl
fhaB	lbpA	scpB
fhaC	lbpB	seA
filA	lef	seC
fimA	lepA	serA
fimD	lepB	sfaA
flaA	lgtA	sipB
flaB	lgtB	sipC
flhA	lic2A	ska

Select	Score	New Hit	PID	NID	Definition					
☐	1075.8	✪	AAF14359	AF121135	glycosyltransferase [Neisseria gonorrhoeae]	Neisseria gonorrhoeae	1	100	346	Harvey,H.A.
☐	1074.7	✪	AAA92074	U15992	glycosyltransferase	Neisseria gonorrhoeae	1	100	362	Danaher,R.J.
☐	1073.0	✪	AAA68009	U14554	glycosyl transferase	Neisseria gonorrhoeae	1	100	348	Gotschlich,E.C.
☐	1070.6	✪	CAB83816	AL162753	lacto-N-neotetraose biosynthesis glycosyl tranferase [Neisseria meningitidis Z2491]	Neisseria meningitidis Z2491	1	100	346	Parkhill,J.
☐	1070.4	✪	AAK70338	AF313394	glycosyl transferase LgtA [Neisseria gonorrhoeae]	Neisseria gonorrhoeae	1	100	346	Tong,Y.
☐	1044.3	✪	AAM33861	AF470659	LgtA [Neisseria meningitidis]	Neisseria meningitidis	1	100	362	Zhu,P.
☐	1043.4	✪	AAM33875	AF470665	LgtA [Neisseria meningitidis]	Neisseria meningitidis	1	100	362	Zhu,P.
☐	1033.2	✪	AAM33869	AF470662	LgtA [Neisseria meningitidis]	Neisseria meningitidis	1	96	348	Zhu,P.
☐	1031.5	✪	AAM33855	AF470657	LgtA [Neisseria meningitidis]	Neisseria meningitidis	1	96	348	Zhu,P.
☐	1027.8	✪	AAN08510	AY134876	LgtA [Neisseria lactamica]	Neisseria lactamica	1	96	348	Zhu,P.

Developed by James R. Cole at MSU

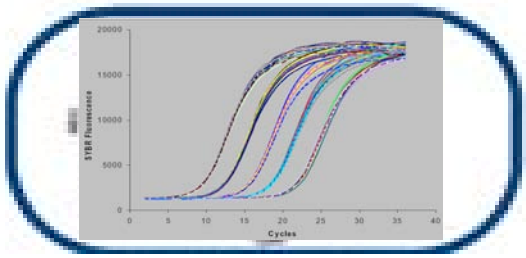
Survey/Screening of Multiple Samples

1. presence of antibiotic resistance, virulence, and indicator genes from various reclamations systems and treatment facilities,
2. correlation between pathogens and indicators with different hosts,
3. release into surface waters and maybe persistence,
4. finding host specific markers.

Table 1. Preliminary Sampling

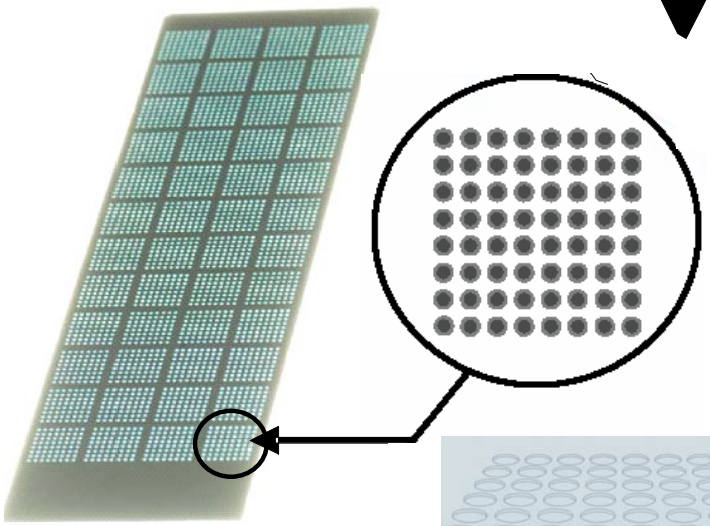
Examined	Sample	Temporal, Host, and Geographic Variation
1, 2, 4	Waste Water Treatment Plant	Monthly (Jan-Dec 2006) sampling of raw waste water at East Lansing WWTP
1, 2, 4	Host Specific Fecal Samples	Horse, Cow, Human, Sheep, Chicken, Cow Manure Treatment, Facility Influent, Cow Manure Treatment Facility Effluent
1,2,3	Agricultural/River Water	Temporal and Geograhpic sampling (results not shown).

High-throughput Assay Screening Tool: BioTrove OpenArray™

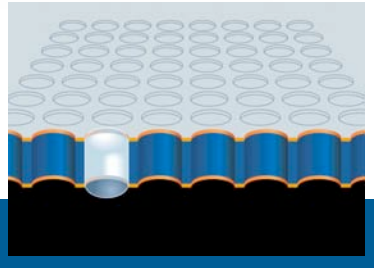


Sensitivity and Specificity of QPCR

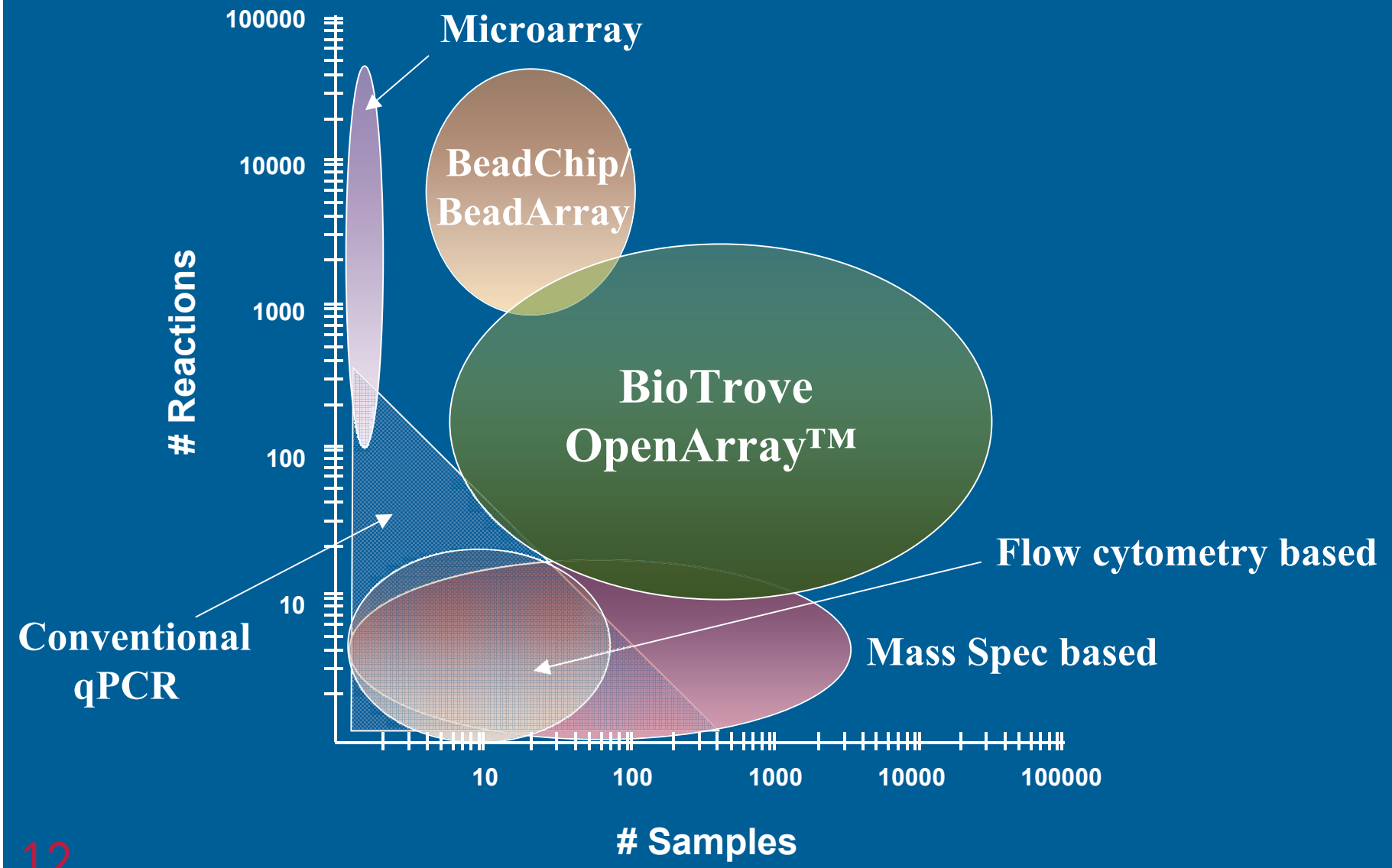
Throughput of Microarray



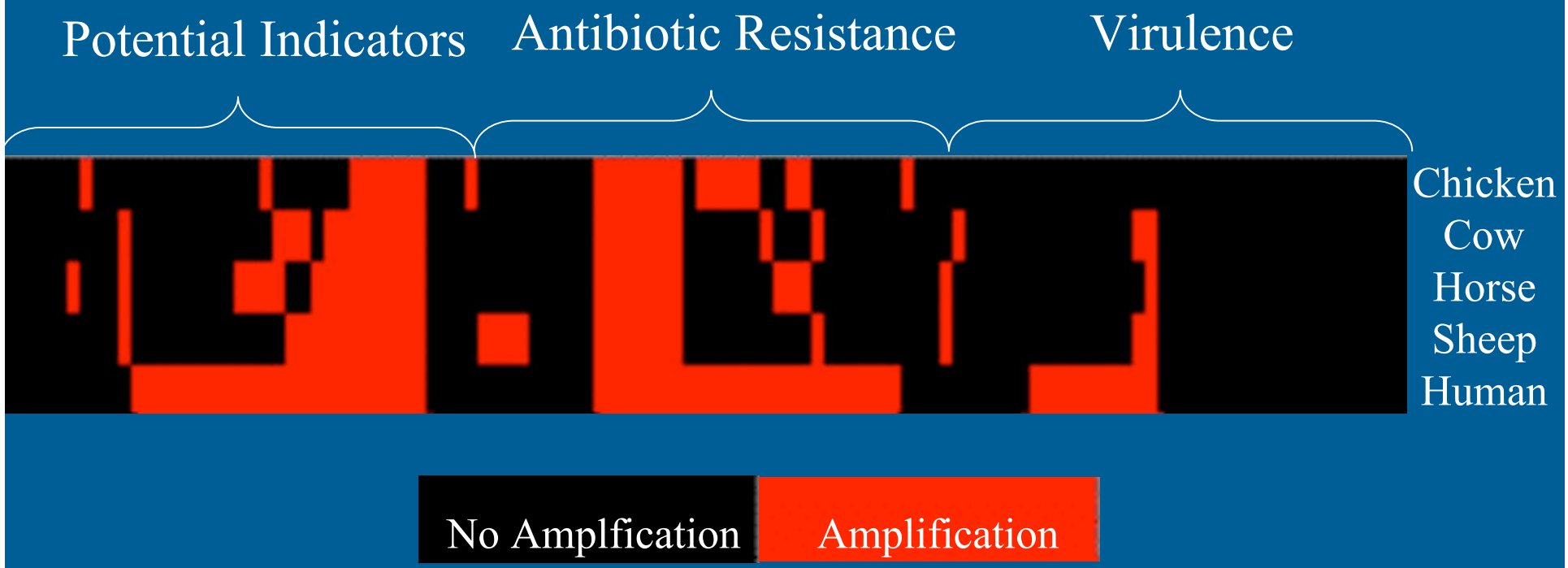
OpenArray™



NT cycler and computer with Analysis Software


Microarray
BeadChip/
BeadArray
BioTrove
OpenArray™
Flow cytometry based
Mass Spec based
Conventional
qPCR
100000
10000
1000
100
10
Reactions
10
100
1000
10000
100000
Samples

Indicator Screening Results: Fingerprint of Fecal Samples from various Hosts



Dye-doped nanoparticle-based detection

Zhao et al., 2006 PNAS

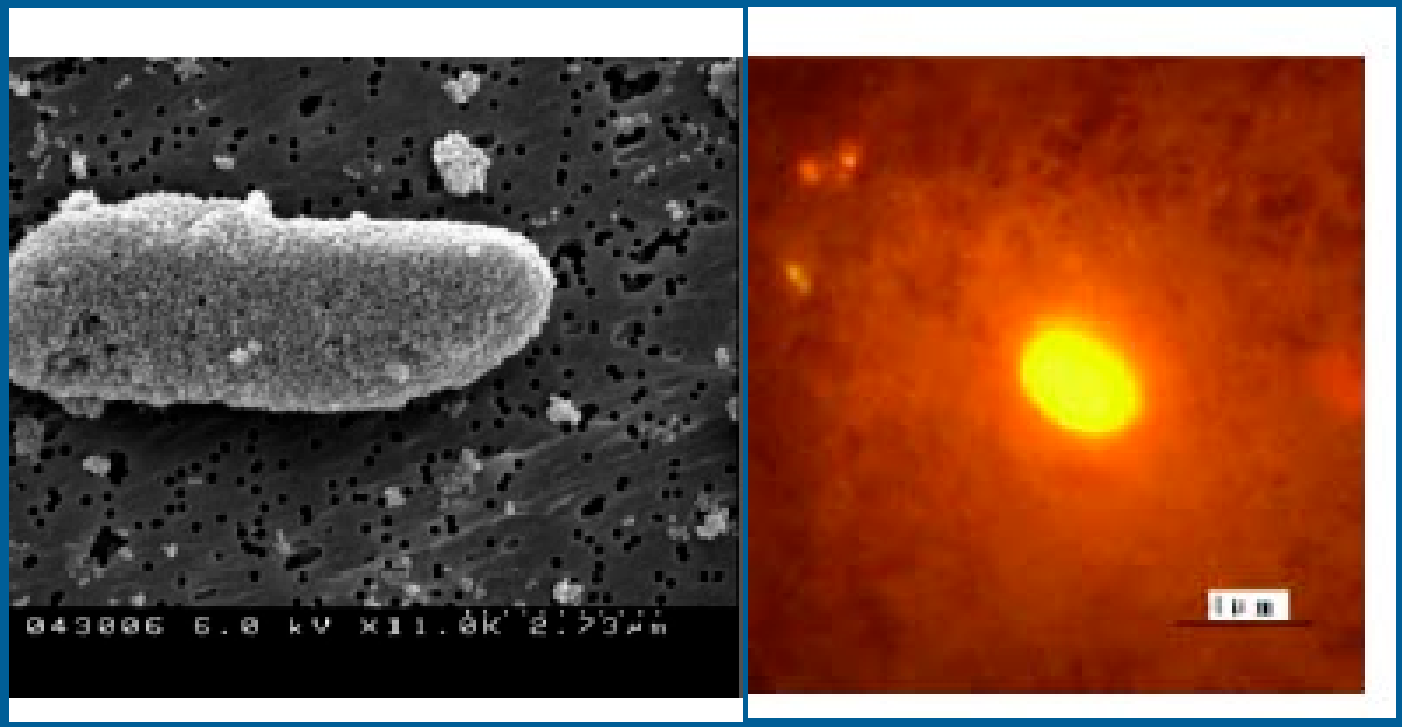
PNAS

A rapid bioassay for single bacterial cell quantitation using bioconjugated nanoparticles

Xiaojun Zhao*, Lisa R. Hilliard*, Shelly John Mechery*, Yanping Wang†, Rahul P. Bagwe*, Shouguang Jin†, and Weihong Tan*‡

*Center for Research at the Bio/Nano Interface, Department of Chemistry, and The Shands Cancer Center, University of Florida, Gainesville, FL 32611; and †Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL 32610

Edited by Nicholas J. Turro, Columbia University, New York, NY, and approved September 9, 2004 (received for review July 5, 2004)



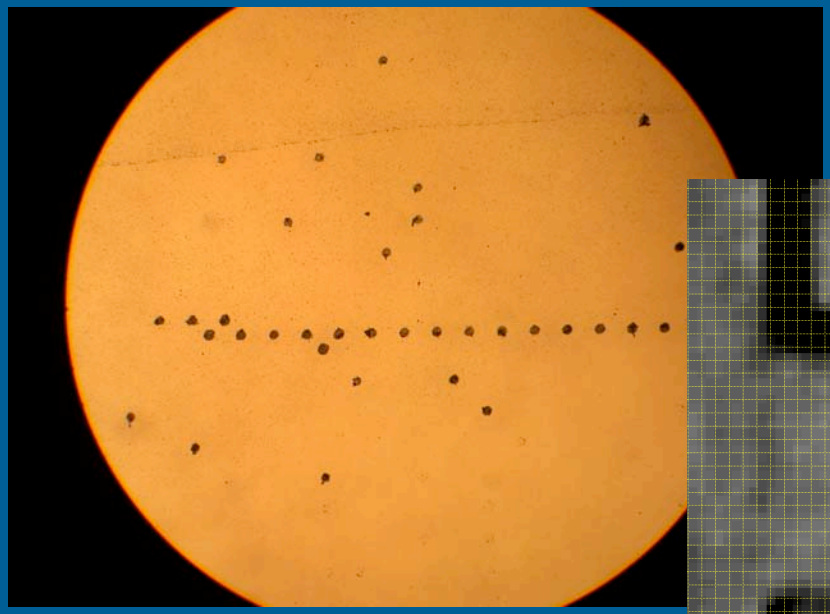
SEM and Fluorescence image of *E. coli* 0157:H7 incubated with antibody conjugated dye doped nano-particles

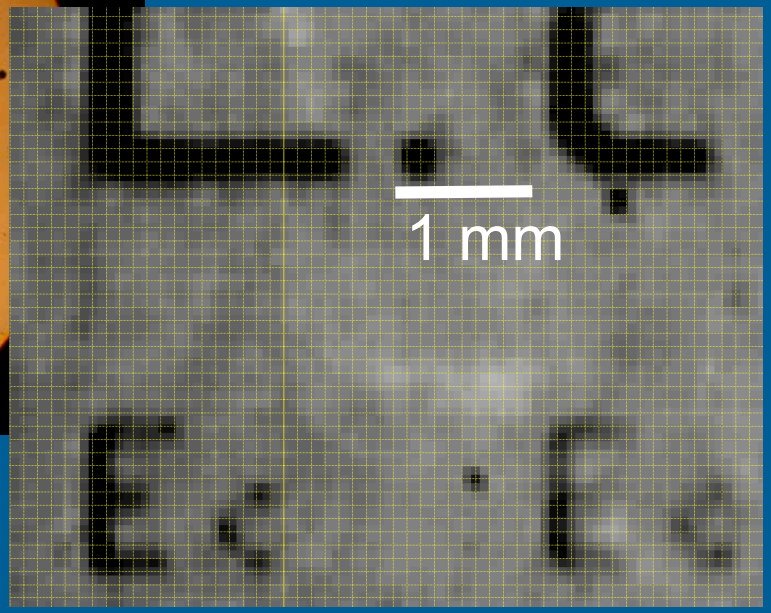
Can we Reduce the Cost of Visualization?

$50,000

$500

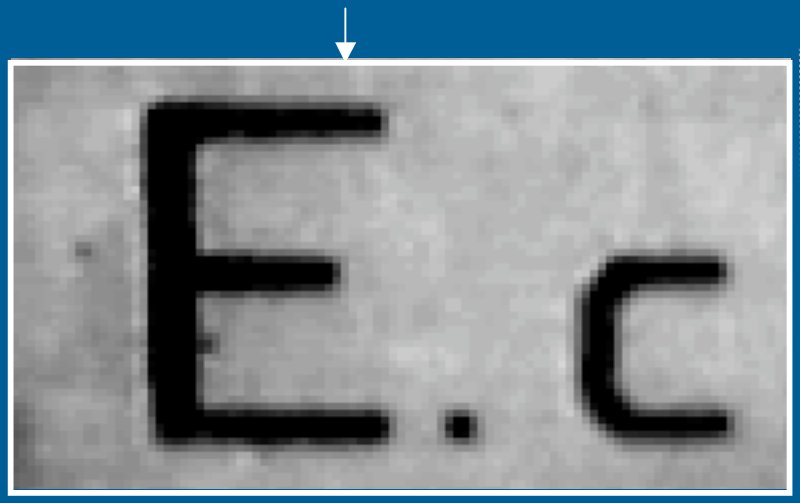
Free





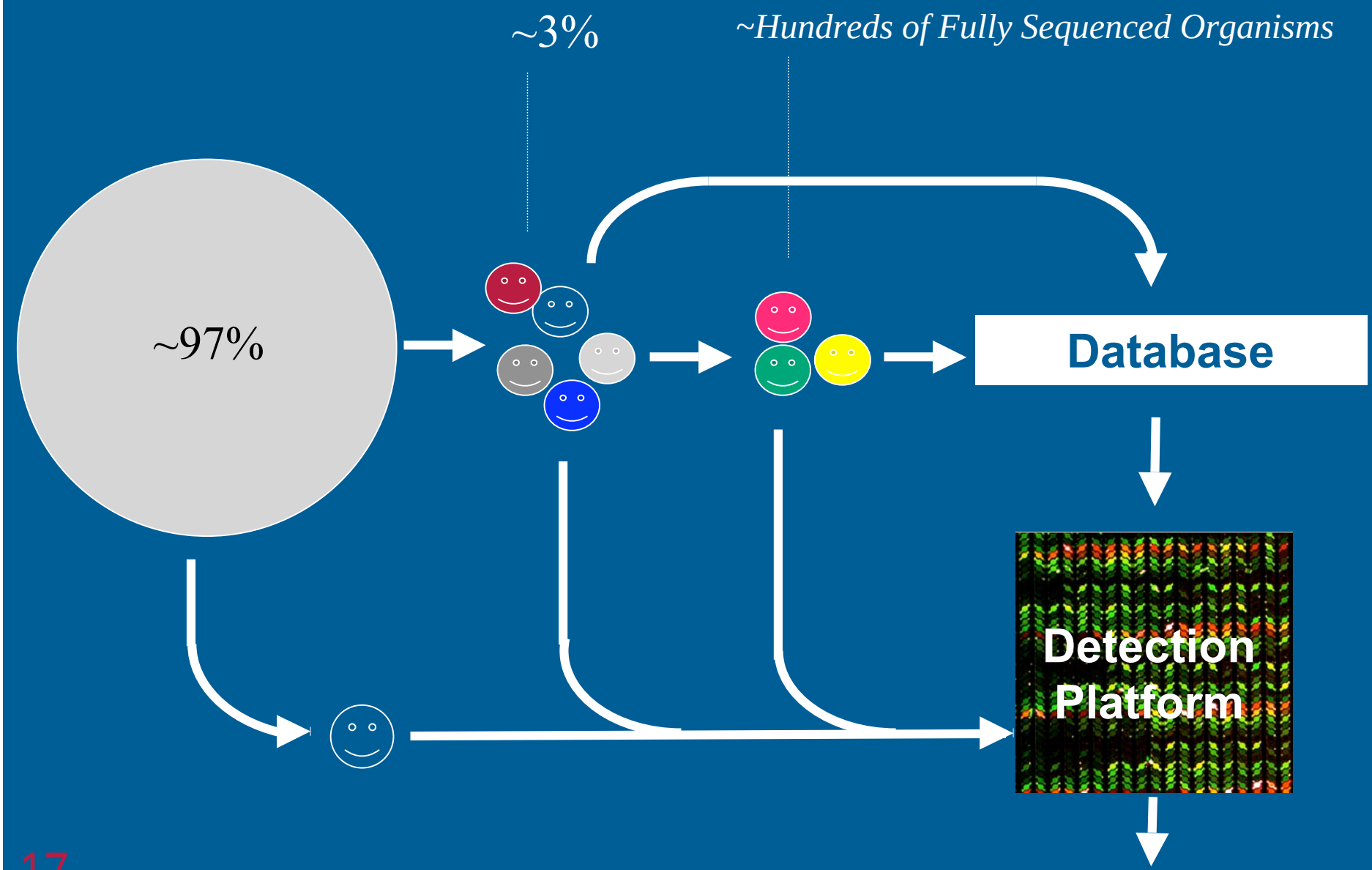
Messy but Beautiful!

One of the patterns after hybridization, and labeling with gold and silver nanoparticles



Glass slide with in situ synthesized probes printed in a pattern “E.c” of various dimensions

The Universal Challenge



Variable Virulence Factors- Variable Effects

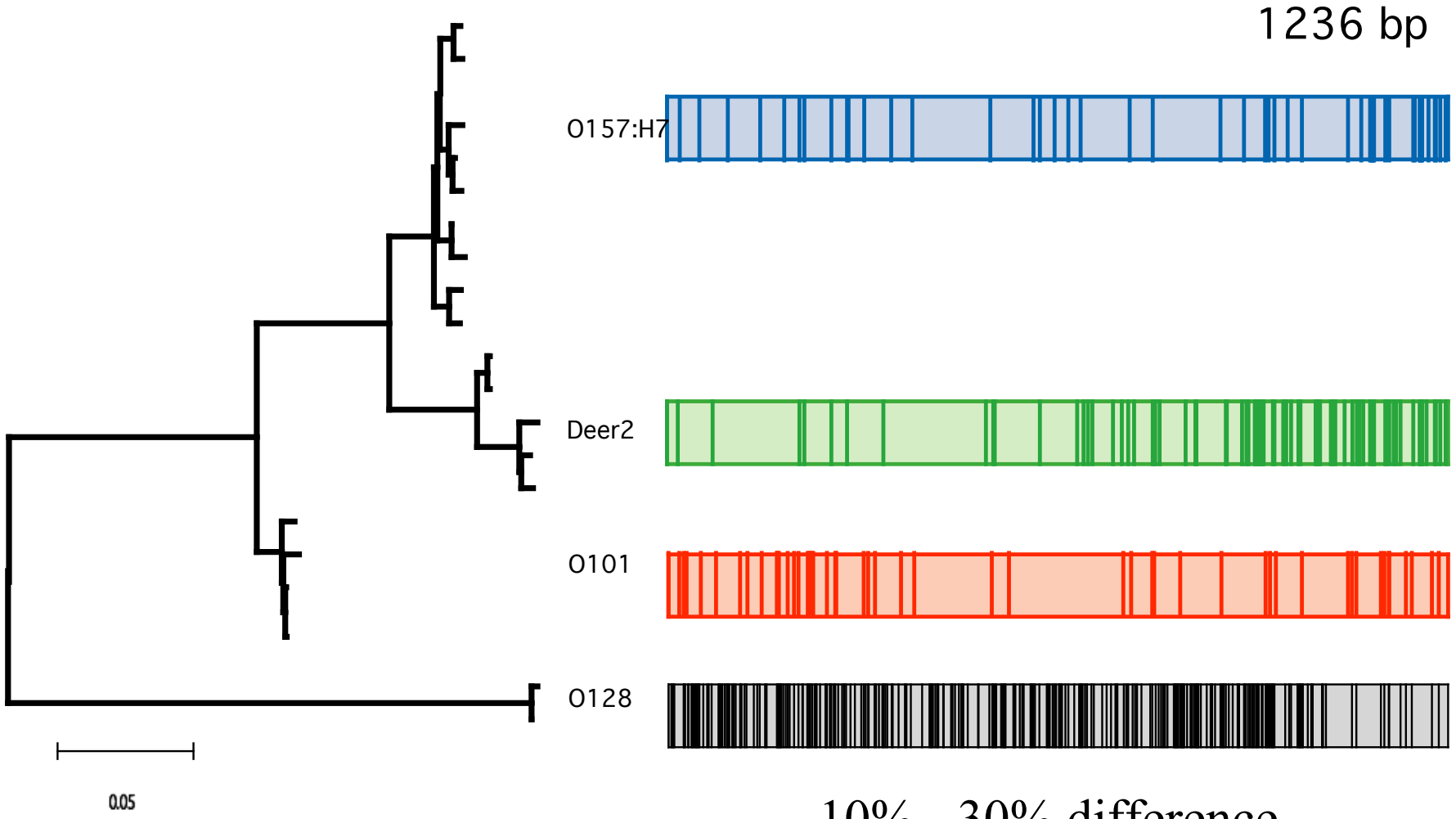
Strain	Pathotype	*eae*	*stx1*	*stx2*	*setIA*	*SenA*	*ehxA*	*hylA*	*espP*	*sat*	*iucC*	*shu/chu*	*ipaH*
K12	Not pathogenic	-	-	-	-	-	-	-	-	-	-	-	-
W3110	Not pathogenic	-	-	-	-	-	-	-	-	-	-	-	-
EDL 933	EHEC	●	●	●	-	-	●	-	●	-	-	●	-
Sakai	EHEC	●	●	●	-	-	●	-	●	-	-	●	-
CFT073	UPEC	-	-	-	●	-	-	●	-	●	●	●	-
S. flexneri 2a str. 301	EIEC	-	-	-	●	●	-	-	-	-	●	-	●
S. sonnei Ss046	EIEC	-	-	-	-	●	-	-	-	-	●	-	●
S. dysenteriae 1 str. 197	EIEC	-	●	-	-	●	-	-	-	-	-	●	●
S. boydii 4 str. 227	EIEC	-	-	-	-	●	-	-	-	-	●	-	●

EHEC: Enterohemorrhagic *E. coli*; UPEC: Uropathogenic *E. coli*; EIEC: Enteroinvasive *E. coli*

Tourlousse et al., *Water Environment Research*, 2007

Variation in gene sequence

Shiga toxin 2 (*stx2*) variants

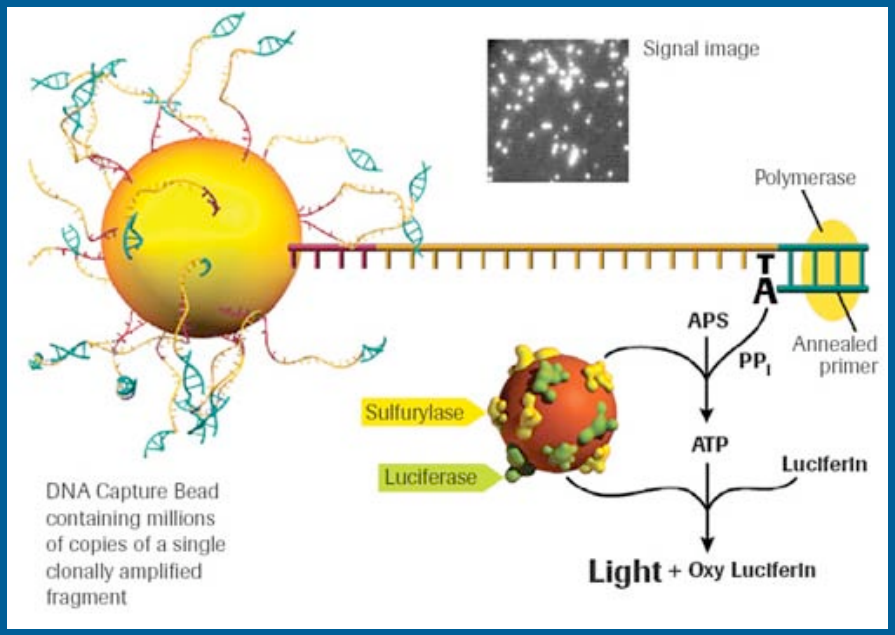


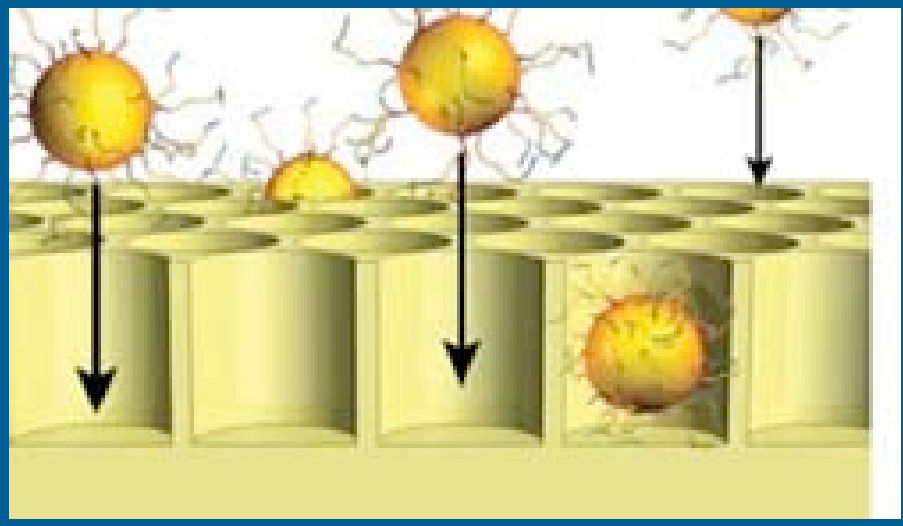
10% - 30% difference

Can we screen for most populations (including the unknowns) and genetic markers in parallel?

(Sample Throughput, Target Multiplexing)

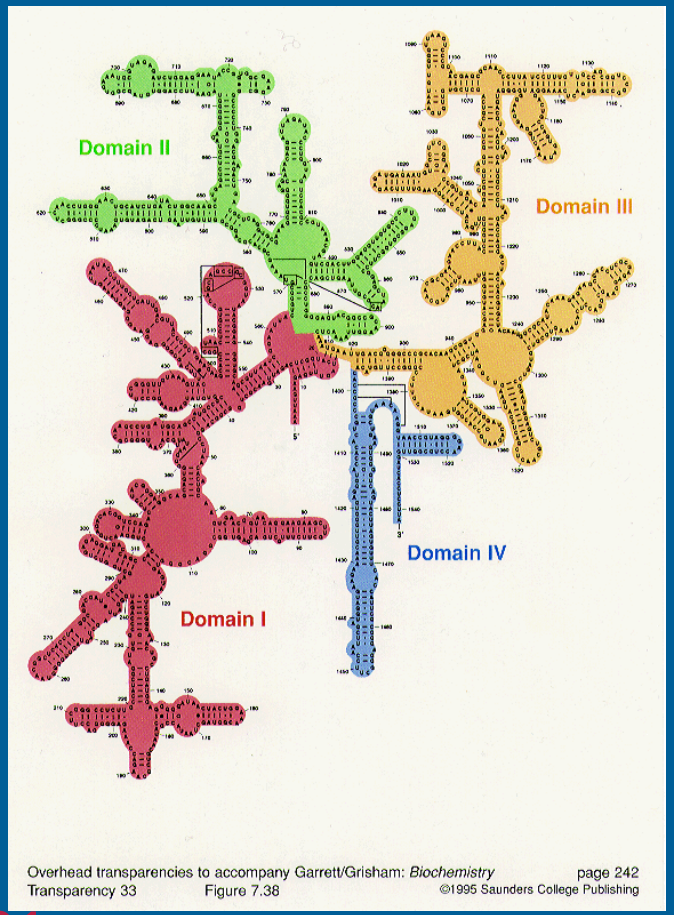
454 Sequencing



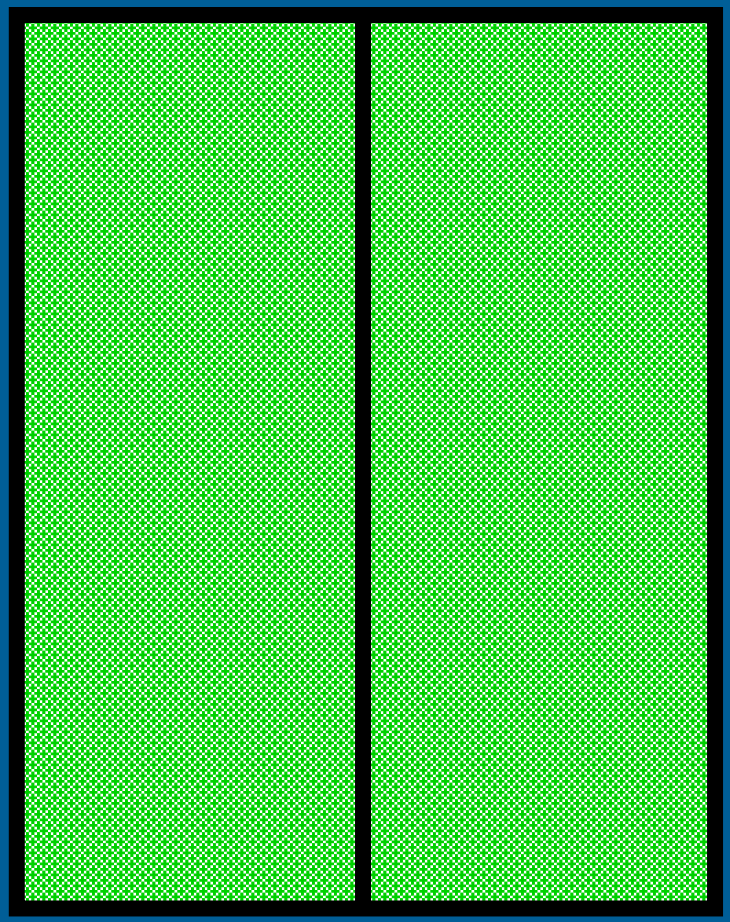


200 bp long 16S rRNA sequences of the whole community

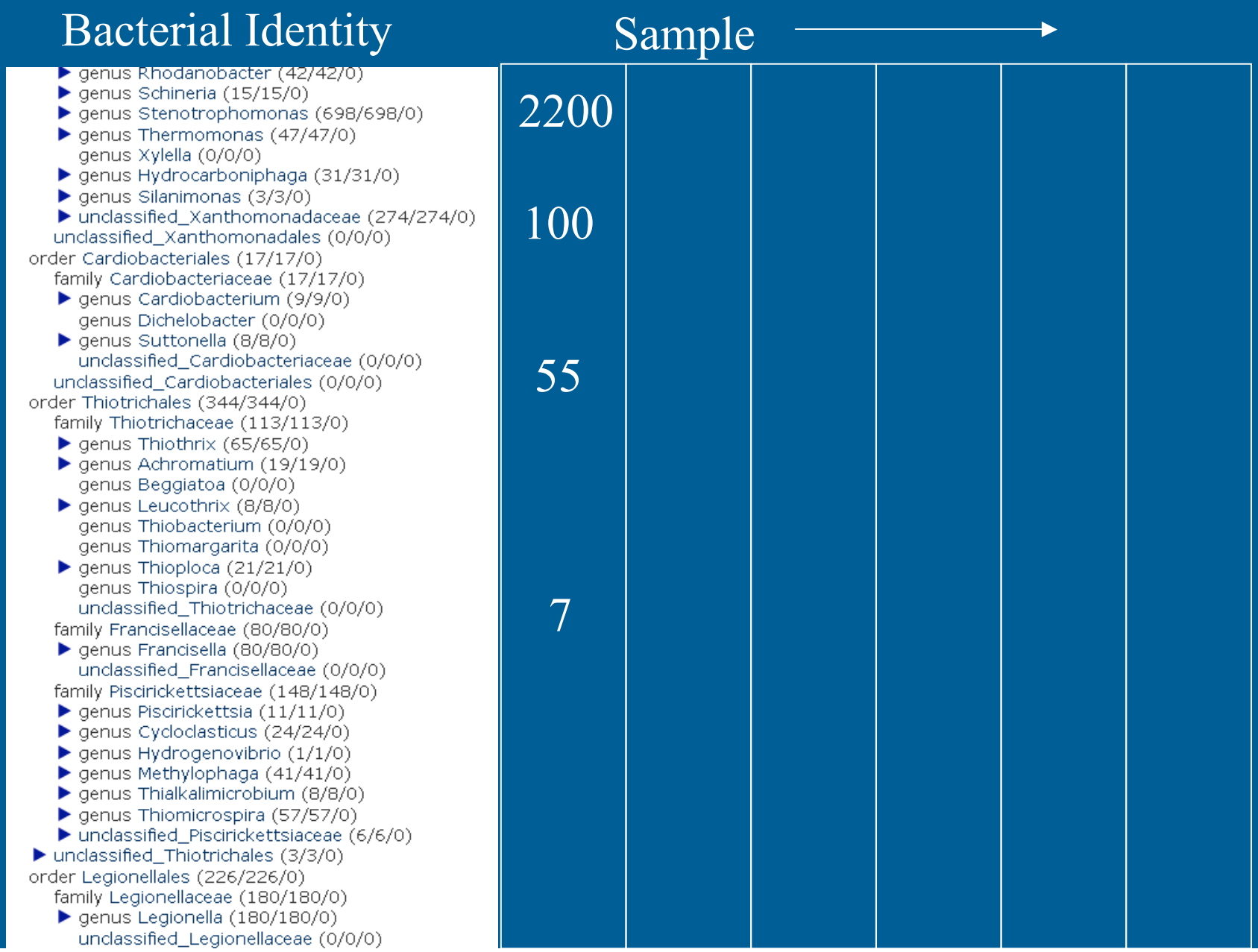
22 Tags x 2 Chambers
= 44 samples each with 10,000 to 15,000 sequences
$10,000 per plate ($230 per sample; 7 cents per 1000 bases)



Tags for
V4 Region
amplifies 200 bp



What to Expect from 454 Sequencing



Potential Indicator Screening

Strategic Samples

- Outbreaks
- Samples suitable for source tracking
- Focused on specific systems
- Temporal

Integrated with Currently Used Standard Methods

Acknowledgements

Environmental Protection Agency (Grant No. **R833010**)
Michigan Economic Development Corporation
21st Century Jobs Fund

Group

Doctoral candidates:

Robert Stedtfeld	Design/Sealing/Overall
Tiffany Stedtfeld	Validation
Ahsan Muneer	Software/Modeling/Communication
Dieter Tourlousse	Sample Processing/DNA Biochip
Farhan Ahmad	micro-PCR Analysis
Gregoire Seyrig	PCR Reagent Stability
Yu Yang, Vidya Srinivasan	Nanoparticles

Post-doctoral Associates

Jean Marie Rouillard	Probe Design
Onnop S.	Chip synthesis

PIs: Syed Hashsham, Volodymyr Tarabara, Erdogan Gulari, and James Tiedje

Novel Molecular-Based Approach for Broad Detection of Viable Pathogens in Drinking Water

John Scott Meschke[1] and Gerard Cangelosi[2]
[1]Department of Environmental and Occupation Health Sciences, University of Washington
[2]Seattle Biomedical Research Institute

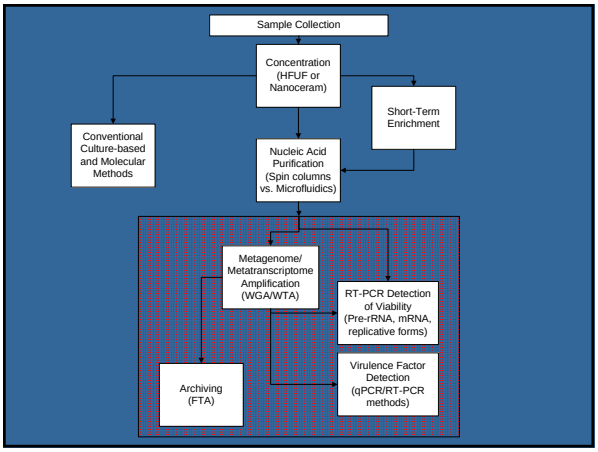
This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R833011

Statement of the Problem

- Current methods for direct detection of pathogens in drinking water are limited
  - Sensitivity
  - Breadth of detection
  - Speed
  - Viability
  - Ability to quantify
- **Must consider method as a whole**!

Our Approach

- Four CCL2 Microbes
  - Echovirus, Adenovirus, MAC, Aeromonas
- Sample Prep
  - Concentration
  - Nucleic Acid Extraction/Purification
- Novel Approach to Detection
  - WGA/WTA
  - Pre-rRNA
- Archiving



Concentration Approaches

- HFUF (Fresenius)
- Nanoceram Fastflow (alumina nanofibers)
- Capsule filter from Scientific Methods, inc.

FTA

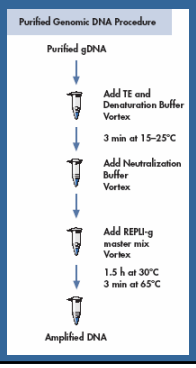
- Developed by Professor Leigh Burgoyne at Flinders University; now commercially marketed by Whatman
- FTA Cards contain chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidation and UV damage.
- Prevents the growth of bacteria and other microorganisms
- Nucleic acids collected on FTA Cards are stable for years at room temperature
- FTA Cards are stored at room temperature before and after sample application, reducing the need for laboratory freezers

WGA Approaches

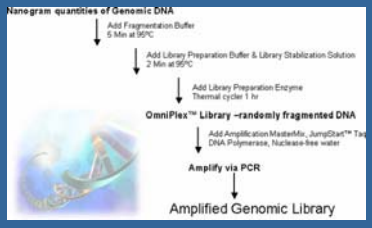
- Whole Genome Amplification
- Multiple Displacement Amplification (MDA) Technology
- Omniplex Library Approach

Multiple Strand Displacement (MDA)

- E.g. Qiagen Repli-g Ultrafast
- Isothermal genome amplification
- DNA products of up to 100 kb
- Avoids the high sequence bias of PCR-based amplification methods
- Alkaline denaturation buffer for gentle denaturation of genomic DNA
- Avoids fragmentation of template DNA caused by heat denaturation methods



Omniplex Library Approach



- Developed by Rubicon Genomics; now licensed to Sigma under name Genomeplex

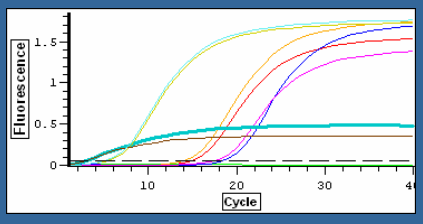
Preliminary Results: WGA

- Significantly improves sensitivity for *E. coli*, *MAC*, Adenovirus types 2 and 41
- Minimum 3-4 CT units (~1log10) improvement in detection in clean system; factoring in volume differences up to 2000* fold increase in detection achievable.
- In dirty system, similar results (3-4 CT units) for 100ml mock extract; for 500ml mock extract (~2 CT units)

WTA

- Whole Transcriptome Amplification
- Transplex from Sigma; also developed by Rubicon Genomics.
- RNA is incubated with a reverse transcriptase and non-self-complementary primers comprised of a quasi-random 3' end and a universal 5' end
- Annealed primers are extended by polymerase, displacing single strands which become new templates for primer annealing and extension
- Creating an OmniPlex® library

Preliminary Results: WTA



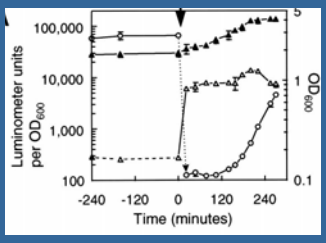
- Echovirus as detected by pan-entero primers

Pre-rRNA

- Design of RT-PCR methods targeting the region bridging mature rRNA and 5' leader region
- Short-term enrichment

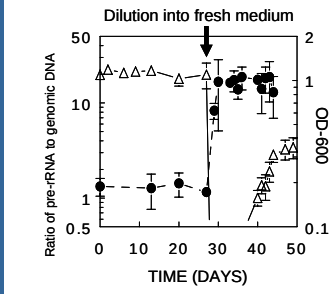
http://www.web.virginia.edu/Heidi/chapter12/chp12.htm

Pre-16S rRNA and total SSU rRNA pools during outgrowth from stationary phase on LB broth



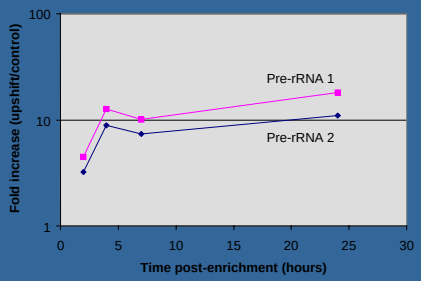
- Overnight cultures were diluted 20-fold into fresh broth at time zero (arrow).
- Symbols: open circles, culture OD600 (right axis); open triangles, pre-16S rRNA per OD600 (left axis); filled triangles, total SSU rRNA per OD600 (left axis). Datum points on the pre-16S rRNA curve connected by dashed lines were below background level.

(Cangelosi and Brabant, 1997).



Ratio of pre-SSU rRNA to genomic DNA upon nutritional shift-up of *Mycobacterium tuberculosis*. Stationary phase cells were diluted into fresh 7H10 broth at the time indicated by the arrow. Closed circles, pre-rRNA to genomic DNA ratio. Open triangles, OD-600 of culture (cell density).

Pre-rRNA up-shift in *M. avium*



- MAC is a slow grower (doubling time 24 hours)
- Cells were stored in tap water at room temp for 14 days, then enriched by dilution into culture broth at time 0 hours. Results were obtained with 2 different primer sets targeting the 5' leader region.

Aeromonas Pre-rRNA assay

- Two primer sets target the region bridging the mature rRNA and 5' leader
- 50 fold increase in pre-RNA within 15 minutes when water starved cells are enriched

Summary

- Sensitivity
  - Concentration by HFUF or Nanoceram
  - WGA/WTA
- Breadth of detection
  - WGA/WTA
  - FTA archiving
- Speed
  - All rapid methods; limited by concentration/purification
- Viability
  - Pre-rRNA for Bacteria; Nascent strand for Viruses
- Ability to quantify (????)
  - qPCR methods

Acknowledgements

Funding:
US EPA Star Grant R826828

Collaborators:
Gerard Cangelosi, SBRI
Kris Weigel, SBRI

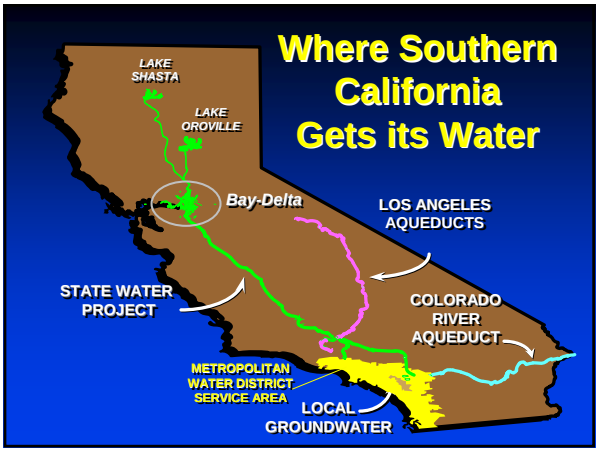
EOHML Staff/Students:
Jennifer Parker
Nicky Beck

Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis

Anthea K. Lee
Metropolitan Water District of Southern California

Metropolitan Water District of Southern California (MWD)

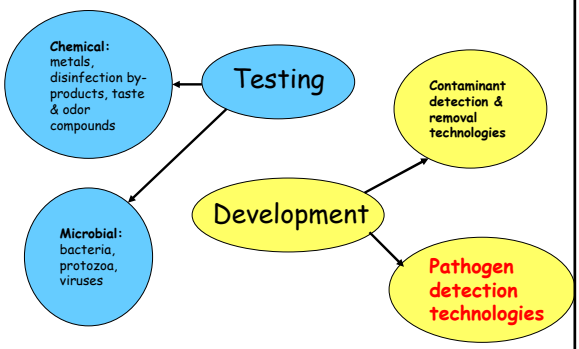
- Consortium of 26 cities and water districts
- Provide water for >18 million people in Southern California
- Delivers an average of 1.7 billion gallons of water daily



Distant & Local Water Reservoirs

5 Treatment Plants

Water Quality Laboratory

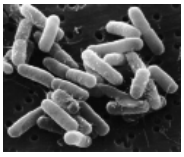


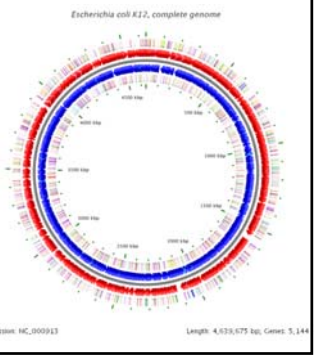
Purpose

- **Detect very few pathogens in very large volumes of water**
- **Use and optimize molecular tools**

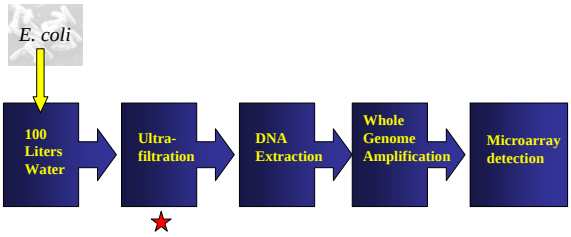
Proof of Principle

- ***E .coli* K12**
- **Sequence available**

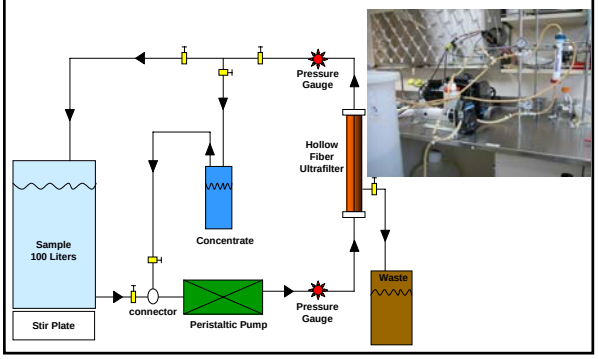




Experimental Design



Ultrafiltration



Ultrafiltration Recovery Efficiency

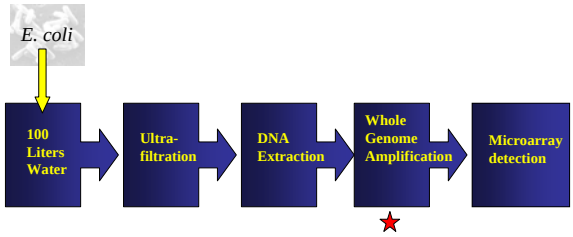
Organism	Mean	Range	N	Breakthrough (%)
Bacteriophage MS2	67	32 – 85	30	0.04
Echovirus 1	76	65 – 87	3	0.02
Bacillus subtilis	64	38 – 89	26	0
Salmonella typhimurium	57	41 – 73	10	0
Cryptosporidium parvum	86	80 – 96	6	0

* 100 L of treated drinking water was spiked with organisms at concentrations ranging from 6 per L to 1 × 10^7 per L and concentrated to 100 mL using a 65kDa MWCO filter (HPH1400, Minntech).

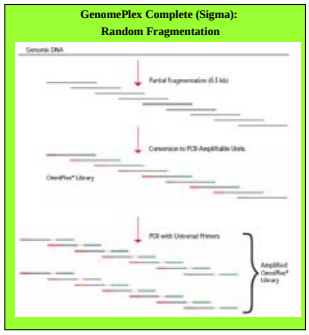
Improvements for Ultrafiltration

- **Increase Efficiency to >80%**
  - **Blocking**
  - **Elution**
- **Additional concentration step?**

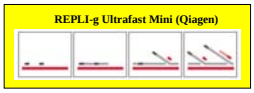
Experimental Design



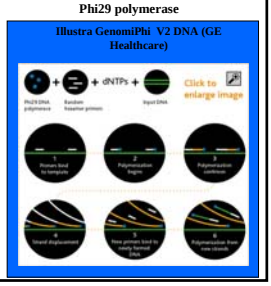
Whole Genome Amplification Techniques



DOP-PCR (Roche):
Random priming & Taq polymerase



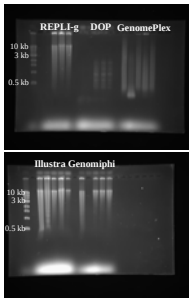
Multiple Strand Displacement:
Phi29 polymerase



Comparison of WGA Kits

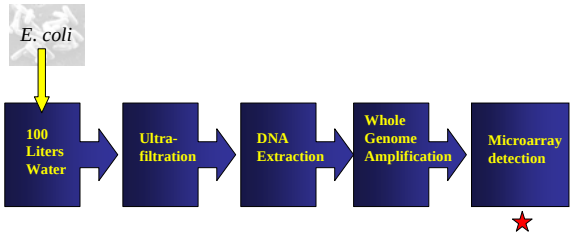
Kit	expected yield* (ug/mL)	actual yield** (ug/mL)
REPLI-g Ultrafast Mini (Qiagen)	350-500	357 644
Illustra Genomiphi V2 (GE Healthcare)	200-350	317 214
GenomePlex Complete (Sigma)	40-93	30 none detected
DOP-PCR (Roche)	not specified	9 5

*starting material 10 ng genomic DNA
**results from 2 independent experiments



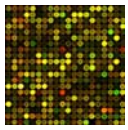
agarose gels loaded with equal amounts of WGA product

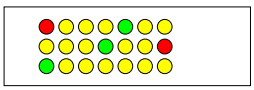
Experimental Design



What is a Microarray?

- Ordered array of polynucleotides affixed to a solid surface
- Glass microscope slide is a common surface
- Each probe is a unique sequence (100->40,000 spots)
- Hybridize with fluorescently- labeled target nucleotide



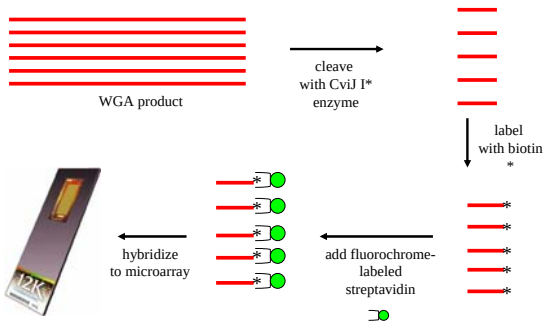


E. coli K12 microarray

- 40 bp probes
- every 800 bp
- ~5800 probes
- cognate mismatch for each probe
- factory standard positive and negative controls

Combimatrix Custom Array

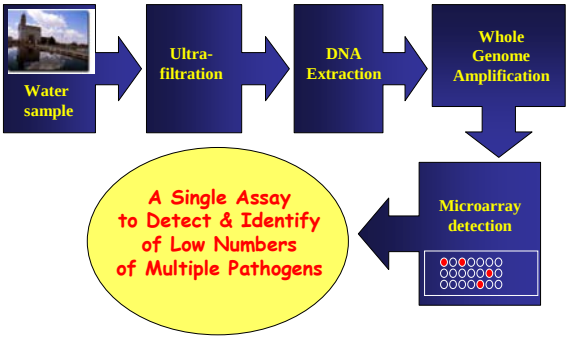
Target Preparation



Beyond Proof of Principle

- **Apply techniques to model pathogens: *Cryptosporidium parvum*, human adenovirus 2, *Salmonella typhimurium*.**
- **Confirm infectivity of concentrated pathogens**
- **Design custom microarray:**
  - **waterborne pathogens including CCL organisms, toxigenic *E. coli*, *Legionella*, *Campylobacter*, *Salmonella*, *Shigella*, *Yersinia*, *fecal indicators*, *Cryptosporidium*, *Giardia*,**

Summary of Pathogen Detection



Acknowledgments

Paul Rochelle
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colleagues at MWD
EPA STAR grant

Identification of Bacterial DNA Markers for the Detection of Human and Cattle Fecal Pollution

Orin C. Shanks

Office of Research and Development
National Risk Management Research Laboratory, Water Supply and Water Research Division

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FECAL BACTERIA are the most common biological contaminant.

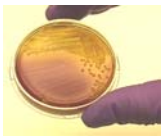
Sample Area:
39% rivers/streams (269K miles)
45% lakes/ponds (7.7 million acres)
51% estuaries (15K square miles)

1

Current Recommended Water Quality Monitoring Criteria for Fecal Pollution

Microbial "Fecal Indicators"

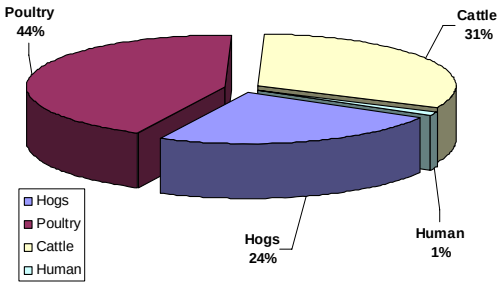
- *E. coli* and enterococci
- Represents fecal pollution event
- Measures fecal bacteria from multiple animal sources



2

Fecal Production in U.S.

Based on USDA data statistics



RL Kellogg, CH Lander, DC Moffitt, N Gollehon - NRCS and ERS GSA Publ. No. NPS00-0579. Washington, DC: USDA, 2000

$1x10^9$ tons/year

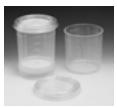
3

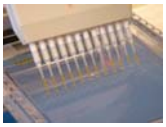
Wildlife Contributions

4

Microbial Source Tracking

CONCEPT… Match microbe from a polluted site and an animal source to suggest the origin of fecal pollution





5

Research Goals

1. **Identify human- and cow-specific bacterial DNA markers**
2. **Develop PCR-based assays with potential for Microbial Source Tracking applications**

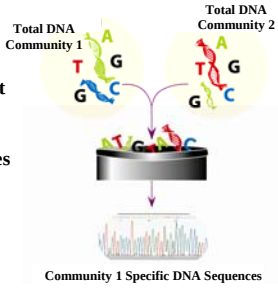
Goal 1: Identify Host-Specific Bacterial DNA Markers

Metagenomic Approach... Characterize differences in total microbial DNA obtained from animal fecal specimens.

Advantages:	**Limitations:**
▪ Survey entire community	▪ Complexity
▪ No cultivation	▪ Expense
▪ No genetic information	

Enrichment for Host-Specific Bacterial DNA Markers

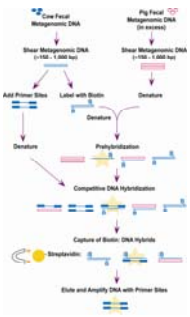
- **Genome Fragment Enrichment**
- **Simultaneously compare two complex microbial communities**
- **Directed sequencing of variable genetic regions**



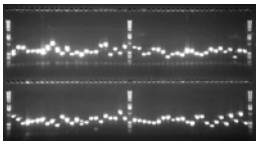
Shanks et al. (2006) Journal of Microbiological Methods. 66:321-330

Method Overview

GFE



Generate Enriched DNA Library



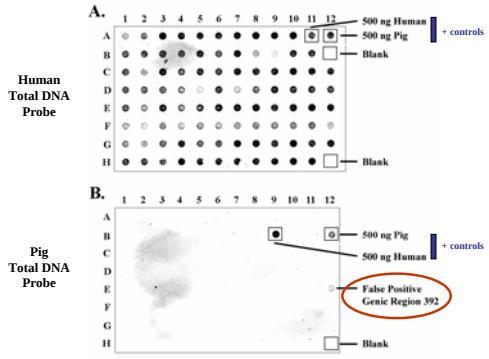
DNA Sequencing

Summary of Sequenced DNA Clones obtained by GFE

	Human	Cow	Total
Sequenced Clones	351	468	819
Non-Redundant Sequences	54	88	142
False Positives	6	10	16

- **17% Redundant Sequences**
- **2.3% False Positive**

Confirm Specificity with Dot Blots



Goal 2: Develop PCR-Based Methods

- **DNA Target Selection:**
  - Sequence similarity search (n = 677)
    - Blastx with refseq database
    - Only *Bacteroidales*-like (n = 279)
  - Function annotation
    - Only predicted membrane associated function
    - No antibiotic or multidrug resistant genes
    - No mobile or extrachromosomal elements
- **PCR Assay Design:**
  - Design primer sets (n = 29)
  - Optimization
- **Establish Specificity for Fecal Microbial Community:**
  - All detect either human or cow
  - None detect pig

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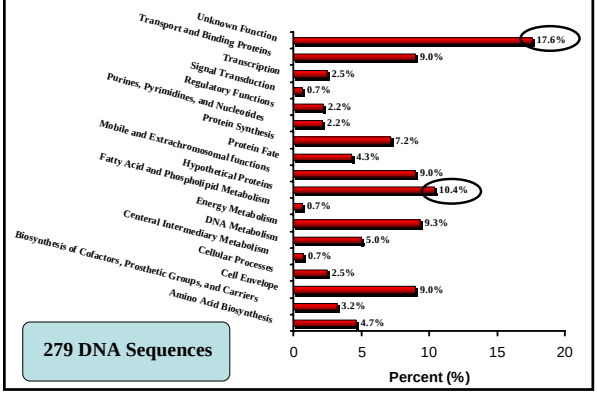
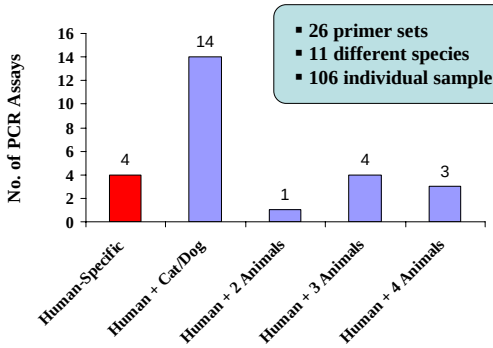
Functional Annotation



279 DNA Sequences

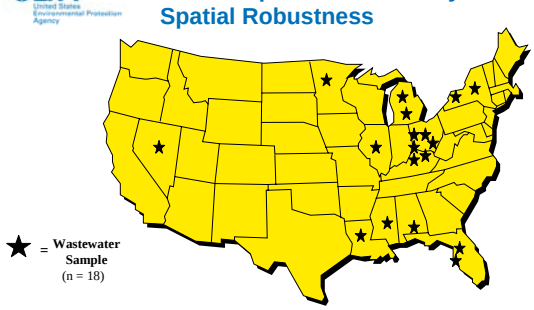
Human PCR Assay Specificity



- **26 primer sets**
- **11 different species**
- **106 individual samples**

14

Human-specific PCR Assay Spatial Robustness



Assay 19	Assay 22	Assay 30	Assay 7
100%	100%	100%	61%

15

Cow PCR Assay Specificity

		% of Samples Positive by:		
Animal Group	No. of Samples	Assay 1	Assay 2	Assay 3
Birds	32	0	0	0
Human	25	0	0	0
Agriculture	64	0	0	**2**
Wildlife	99	0	0	0
Pets	25	0	0	0

- **3 primer sets**
- **27 different species**
- **245 individual samples**

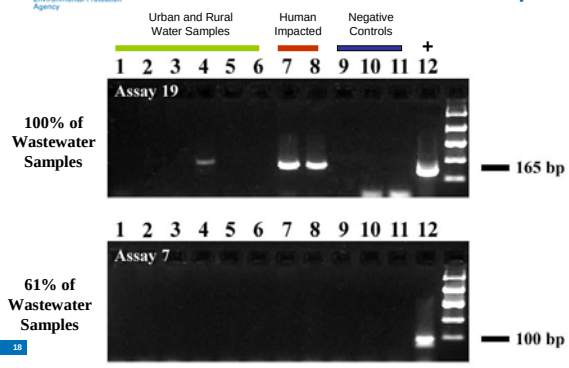
16

Cow-specific PCR Assay Spatial Robustness

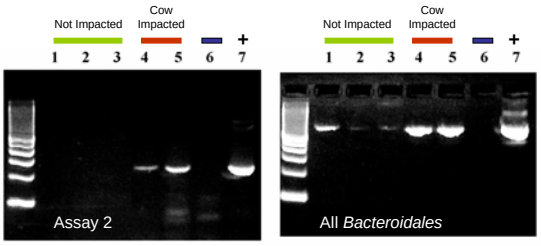
		% of Samples Positive By:		
Locality	No.	Assay 1	Assay 2	Assay 3
Nebraska	101	70.93	70.3	87.1
West Virginia	26	69.2	69.2	100
Georgia	10	100	100	100
Texas	1	100	100	100
Delaware	11	63.6	100	100
	148	**72**	**80**	**91**

17

Detection of Human-Specific Fecal Pollution from Environmental Samples



Detection of Cow-Specific Fecal Pollution from Environmental Samples



80% of Individual Cow Samples

General Fecal Indicator
Bernard & Field (2000) 66: 1587-1594

Conclusions

- **Genome Fragment Enrichment identifies differences between fecal microbial communities**
  - Low false positive rate (2.6 %)
  - Alternative to large scale sequencing
- **Host-specific PCR assays**
  - Specific for target animal groups
  - Broad distribution among target samples
  - Detection of fecal pollution from environment
- **Future Microbial Source Tracking application**
  - Abundance of target in fecal samples?
  - Survival of target DNA in environment?
  - Relevance to current culture-based methods?
  - Link to public health risks?

Acknowledgements

Contributing Authors:
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Michelle Bonkosky

*Shanks et al. (*2006) Applied and Environmental Microbiology. 72:4054-4060
Shanks et al. (2007) Applied and Environmental Microbiology. 73:2416-2422

Detection of waterborne pathogens using Real Time qPCR and Biosensor methods

Sangeetha Srinivasan,
Evangelyn Alocilja, Joan Rose, and Erin Dreelin
Michigan State University
Project No. RD83300501
Grant awarded in 2006

MICHIGAN STATE UNIVERSITY

RESEARCH NEEDS

- There are numerous potential waterborne pathogens for which methods are needed (eg. Microbes on the Contaminant Candidate List, occurrence is needed for regulatory purposes)
- Rapid assessment is needed for during and after disasters, as well as during weather related events
- Specificity and Sensitivity need to be evaluated in real-world samples for new methods.
- Molecular methods have seen great advances in clinical applications but have limited assessment for water.

Groundwater Risks Lessons Learned from emerging bacterium *E.coli* 0157:H7

Walkerton, Ontario Outbreak (occured In small community Using Ground water).

Source: Application of Animal Waste/Manure

Monitoring and Disinfection not addressed.

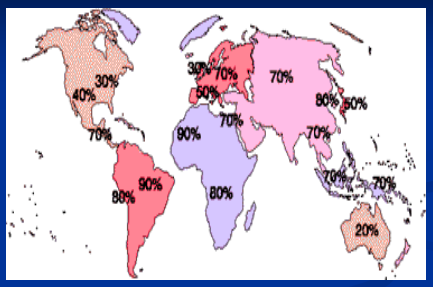
2300 CASES
7 DEATHS
27 CASES of HUS

5 years later community still suffering.

Walkerton — 5 years later

Tragedy Could Have Been Prevented

Another emerging potential waterborne pathogen is *Helicobacter pylori* (contaminant in CCL)



30-50% of the world's population are colonized with it

Source: Helicobacter *Foundation website:* www.helico.com

The WHO has classified *H. pylori* as a Class I carcinogen because of the association of *H. pylori* and gastric malignancies.

Rolle-Kampczyk *et al*. (2004) found a significant correlation between well water contaminated with *H. pylori* detected by PCR and colonization status in humans using that water. (*Int J Hyg Environ Health. 2004 Sep;207(4):363-8)*

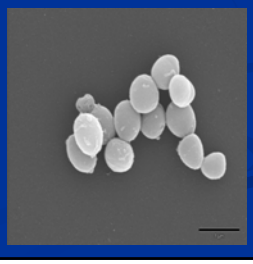
Water supplies contaminated with fecal material may be a potential source of *H. pylori* transmission (Hulten *et al., Gastroenterology. 1996 Apr;110(4):1031-5 1996*).

Detection of *H.pylori* and other pathogens in water

- Difficult to cultivate and identify via conventional techniques
- Small numbers
- Slow growth rate
- Transform to VBNC state
- Rapid and sensitive detection of waterborne pathogens is necessary

Transformation of *H.pylori* into VBNC state

Cultivable state



VBNC state

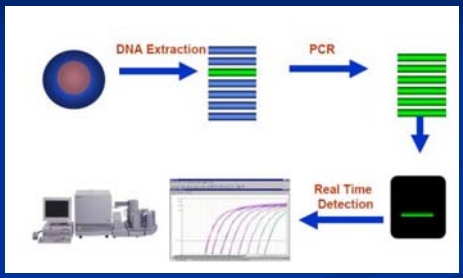
Objectives of QPCR/Biosensor Research Project

- Contrast two rapid molecular methods (one antibody based and one DNA based) for detection of pathogens in water.
  - Develop a real-time qPCR assay
  - Develop a nano-wire enabled antibody-based conductometric biosensor

Target:

- Pathogens such as *H.pylori* and *E.coli* O157: H7.

Real time qPCR: Basic Principle



Advantages

- **Reliable and exact quantification of bacteria**
  high reproducibility in the beginning of exponential phase
- **High specifity**
  Usage of an additional third probe
- **High sensitivity**
  lower detection limit potentially = 1 bacteria
- **High objectivity**
  fully automated process

Target :

vacA gene fragment

Primers used:

- VACA.txt-103F
  Sequence: GCAATAGCAATCAAGTGGCTTTG
- VACA.txt-182R
  Sequence: GCGCGCTTCCACATTAGC

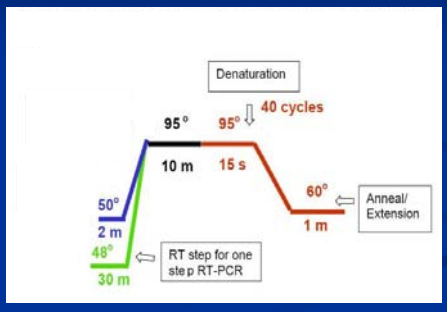
Software used:

Primer Express (ABI)

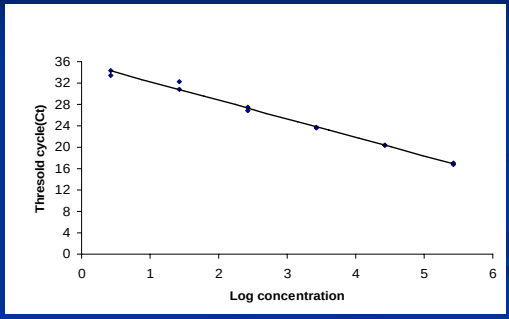
Master mix:

SYBR green (ABI)

Real time PCR cycle conditions



Standard Curve of log concentration Vs ct, with r^2=0.991



Journal of Applied microbiology (In press)

Efficiency:

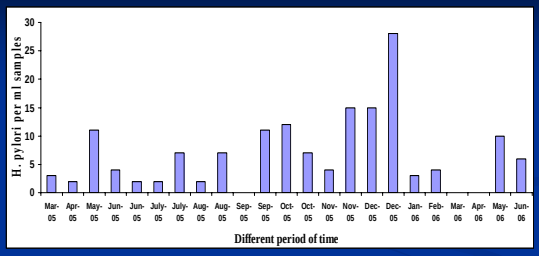
- 90 % of *H.pylori* was recovered from the pure culture.
- 15% was recovered from wastewater.

Specificity:

- Negative for *E.coli*, *E.faecium* and *C.jejuni*

Journal of Applied microbiology (In press)

One Year Survey of waste water

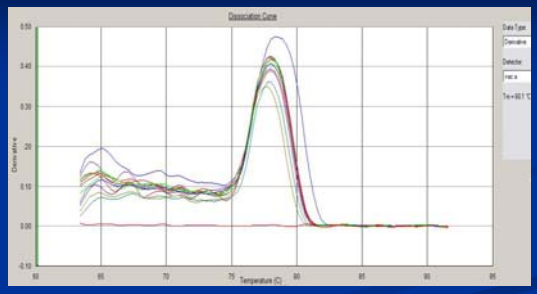


Quantitative real –time PCR analysis by absolute quantification and expressed in amounts of *H. pylori* per ml of water sample. Data are shown as mean value of three replicate. Standard deviation is less then 0.05

- **86% of sewage samples were positive.**
- **Numbers ranged from 2 to 28 cells/ml.**

Journal of Applied microbiology (In press)

Melting curve



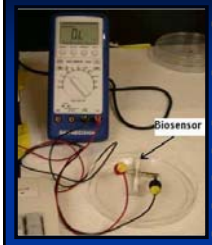
Showed good reproducibility and consistent Tm value in each run.

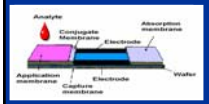
Journal of Applied microbiology (In press)

Significance

- Highly sensitive.
- Higher specificity achieved inspite of using SYBR-green method.
- 100 times more sensitive than conventional PCR.
- Sample processing to data analysis finished within 5-6 hours

Polyaniline Nanowire Biosensor

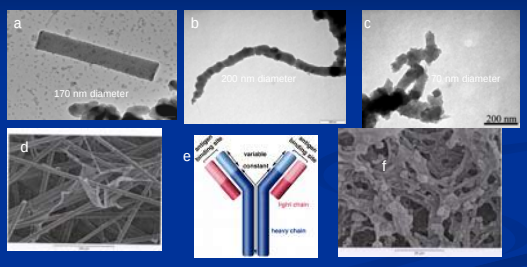




Schematic of the biosensor

- Polyaniline is utilized as a molecular nanowire to form a circuit
- Reports binding event between antigen and antibody
- Suitable for field based detection
- Rapid – detection in 6 min
- Sensitive – 10^1 – 10^2 cfu/ml
- Highly specific
- Reagentless
- Disposable
- Inexpensive
- Targets: *Helicobacter pylori* and *E. coli* O157:H7

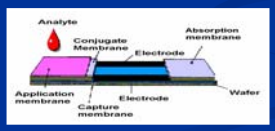
Nanowire/Antibody Based Biosensors



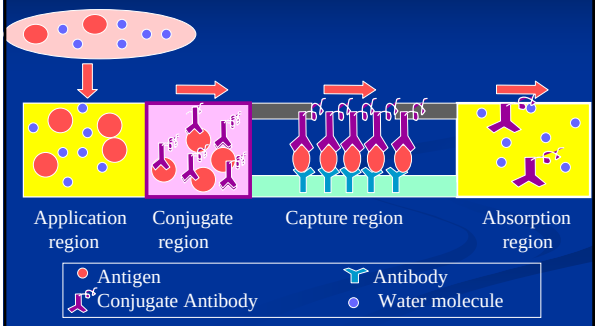
(a, b, c) Synthesized polyaniline nanowires, (d) nanowires conjugated with antibodies, (e) antibody schematic, (f) capture zone of biosensor with bacterial cells

Biosensor components

- Sample Application pad → Cellulose membrane
- Conjugate pad → Fiber Glass membrane
- Capture pad → Nitrocellulose membrane
- Absorption pad → Cellulose membrane



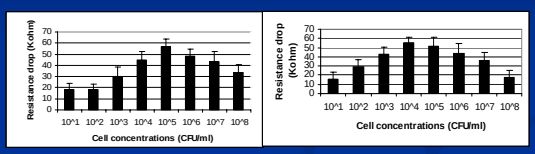
Detection Mechanism



Detection and Data Analysis

- Drop 0.1 ml sample (control) on the application pad
- Measure signal 6 min after sample application
- Calculate drop in resistance value:
  - Control – sample
- Resistance drop > 30%: positive

Previous Data on Biosensor Response

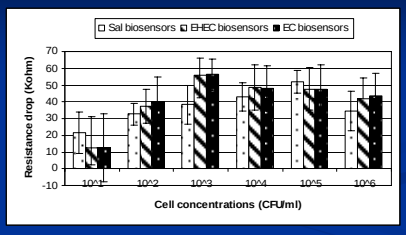


Biosensors were functionalized with antibodies specific to *E. coli* O157:H7 (EHEC) and used to test for *E. coli* O157:H7 (ATCC #43895) in pure culture.

Biosensors were functionalized with antibodies specific to *Salmonella spp.* (Sal) and used to test for *Salmonella typhimurium* in pure culture.

Biosensors and Bioelectronics, 2003:18, 813-819

Previous Data on Biosensor Response



Biosensors were functionalized with antibodies specific to *Salmonella* (Sal), *E. coli* O157:H7 (EHEC), and non-pathogenic *E. coli* (EC) and used in strawberry samples inoculated with a mixture of *E. coli* O157: H7, *E. coli* K-12, and *Salmonella* Typhimurium cells.

Biosensors and Bioelectronics, 2003:18, 813-819

Goals

- Design qPCR experiment with new primers for detection of *E.coli O157* targeting virulence genes.
- Examine manure, sewage, other surface and ground waters in seeded experiments for sensitivity and interferenes, as well as the need for pre-processing of samples for both biosensor and qPCR
- Compare qPCR and biosensor techniques for naturally occurring detection in the environmental samples.

Acknowledgment

Environmental Protection Agency (EPA) for funding this project (Michigan State University contract number RD83300501)

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Microarray Detection of Human Viruses from Community Wastewater Systems

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Syed A. Hashsham[1], Erdogan Gulari[2], Joan B. Rose[1].
[1]Michigan State University
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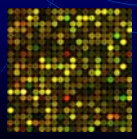
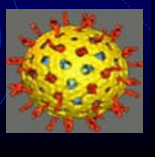
MICHIGAN STATE UNIVERSITY





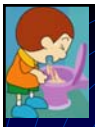
Acknowledgements

- Dr Hashsham, Bob, Dieter, Sarah for the use of microarray equipment
- Dr Gulari, Jean-Marie Roulliard for technical advice
- Joan Rose lab, MSU

This project is funded in part by an EPA Star Grant (2003-STAR-H1) **"DEVELOPMENT OF A VIRULENCE FACTOR BIOCHIP AND ITS VALIDATION FOR MICROBIAL RISK ASSESSMENT IN DRINKING WATER"**

Goals of (2003-STAR-H1)

1. Selection of gene targets microorganisms of interest to water safety;
2. Designing probes
3. Synthesize microfluidic biochips
4. Validate and field-test the synthesized biochips
5. Undertake a pilot risk analysis
6. Viral and bacterial indicator targets



Viral outbreaks

- 65% of groundwater outbreaks
  - (Yates et al, 1985)
- 25% - 50% of recreational freshwater outbreaks
  - (MMWR, 2004)
- Current drinking and recreational standards are bacterial indicator based

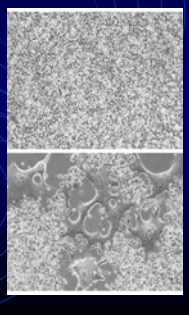
Table 1 Human enteric viruses that may be waterborne transmitted

Genus	Popular name	Disease caused
Enterovirus	Poliovirus	Paralysis, meningitis, fever
	Coxsackievirus, A, B	Herpangina, meningitis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, heart anomalies, rush, pleurodynia, diabetes?
	Echovirus	Meningitis, fever, respiratory disease, rush, gastroenteritis
Hepatovirus	Hepatitis A	Hepatitis
Reovirus	Human reovirus	Unknown
Rotavirus	Human rotavirus	Gastroenteritis
Mastadenovirus	Human adenovirus	Gastroenteritis, respiratory disease, conjunctivitis
Calicivirus	Human calicivirus	Gastroenteritis
	Norwalk virus	Gastroenteritis, fever
	SRSV	Gastroenteritis
	Hepatitis E	Hepatitis
Astrovirus	Human astrovirus	Gastroenteritis
Parvovirus	Human parvovirus	Gastroenteritis
Coronavirus	Human coronavirus	Gastroenteritis, respiratory disease
Torovirus	Human torovirus	Gastroenteritis

(Bosch, 1985)

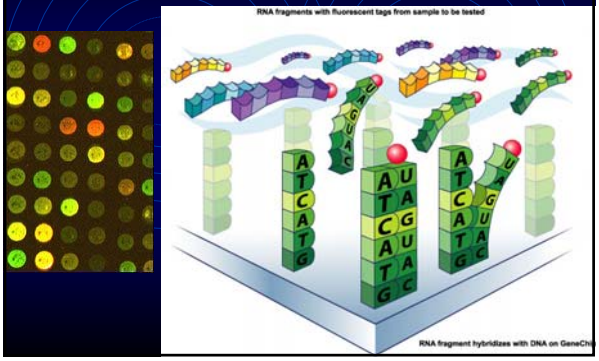
Detection methods

- Culture
- Direct observation
- Antigen detection
  - EIA, LA, ICG
- Molecular detection
  - RT PCR, ICC PCR, microarray/probe detection

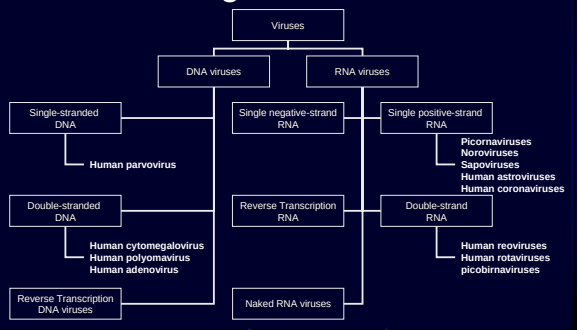


Microarrays

- Multiple pathogen detection
- High throughput
- No target bias
- Culture independent
- Results can be read off computer



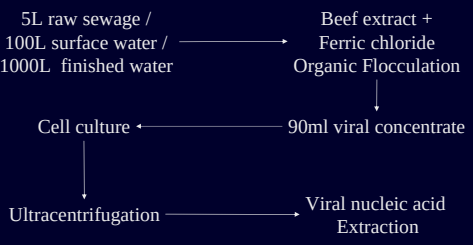
Target viruses



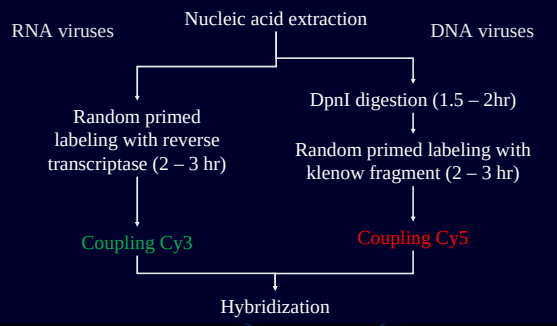
Chip layout

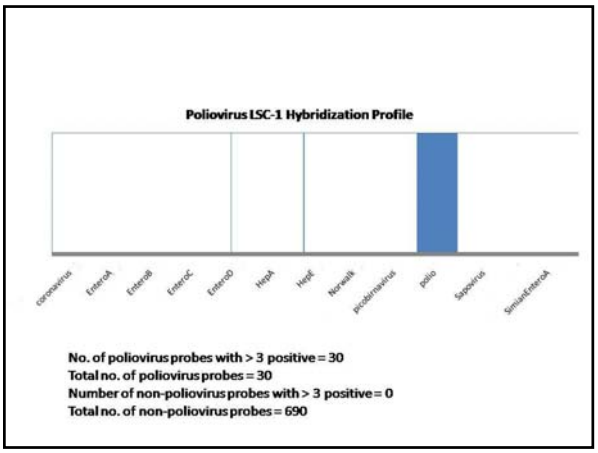
- ~ 30 probes per target group
- 5 copies per probe
- Version 1: 24 viral targets
- Version 2: 26 viral targets
- Version 3: 27 viral targets
  - 4050 spots on chip
  - < 40% chip utilised

Sample Preparation

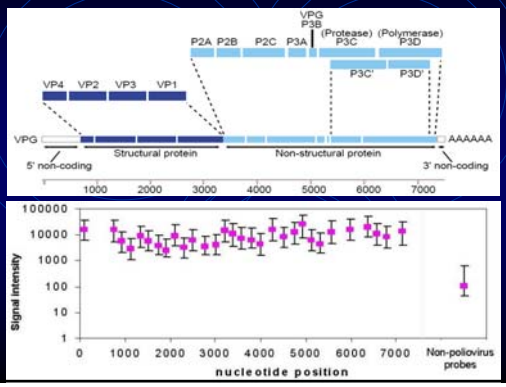


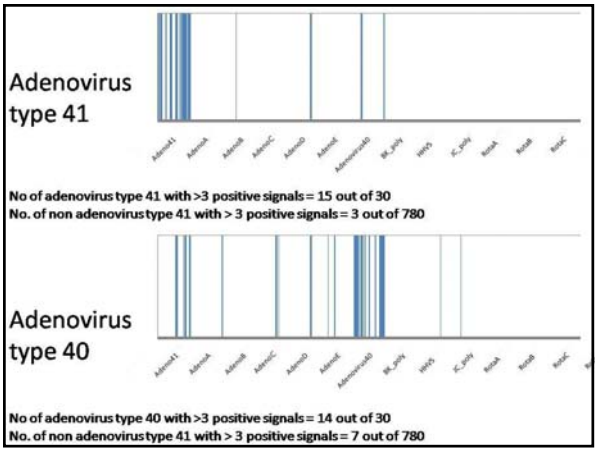
Labeling and Hybridization

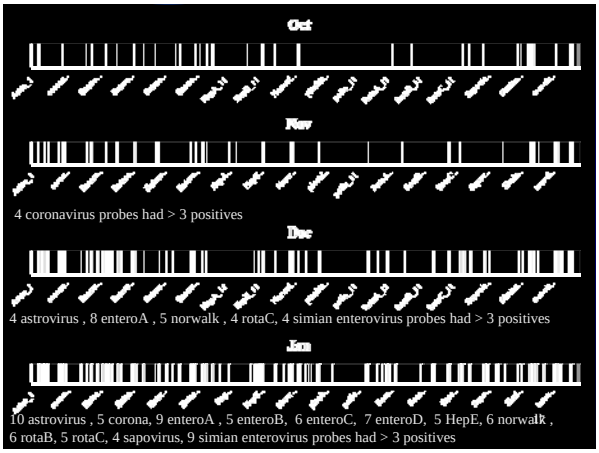




Signal Distribution Across Viral Genome

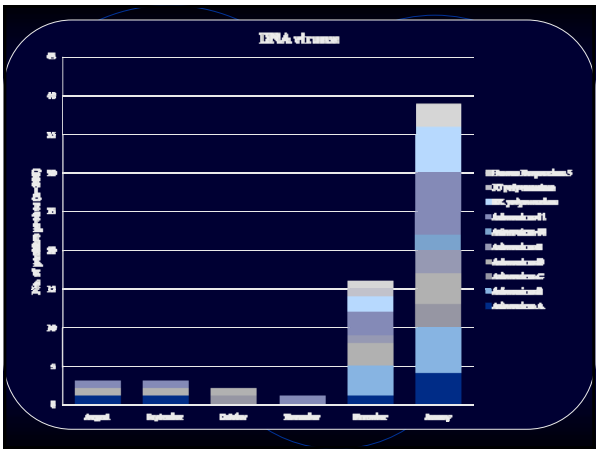






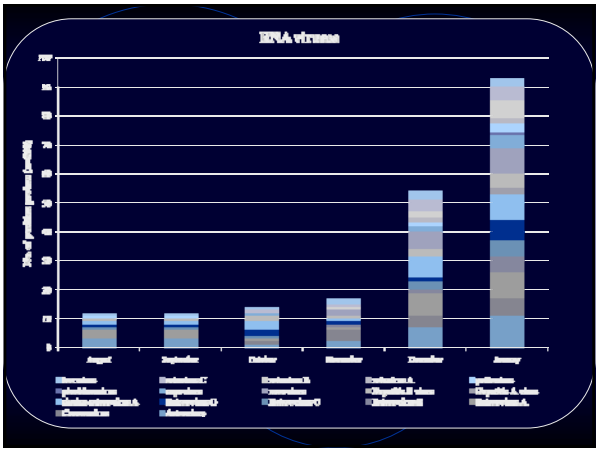
When is a spot positive

- Each virus family has 30 unique probes × 5 copies
- Probe is +ve if 4 or 5 of 5 copies has Signal to Noise Ratio (SNR) > 2
- SNR Threshold is arbitrary, higher is better
- A putative hit is recorded if > 5 probes are +ve.



Observations

- Human polyomaviruses detected
  - JC » BK
- Multiple adenovirus types present
  - Especially Adenovirus 41, B, D
- No one group virus ubiquitous



Observations

- ↑ in virus detects from Aug to Jan
- Torovirus, hepatitis E virus, enterovirus A, enterovirus D, astrovirus present in every sample taken
- Astrovirus most frequently detected and most no. of positive probes

Replicability

DNA viruses

% distribution positive probes (Aug – Jan)

- Adenovirus 41 (21.9%)
- Adenovirus B (15.6%)
- Adenovirus D (15.6%)
- BK polyomavirus (12.5%)
- Adenovirus A (10.9%)
- Adenovirus C (6.3%)
- Adenovirus E (6.3%)
- Human cytomegalovirus (6.3%)
- Adenovirus 40 (3.1%)
- JC polyomavirus (1.6%)

RNA virus

- Single stranded + RNA
- Astorvirus (13.4%)
- Enterovirus A (12.4%)
- Other enteroviruses (10.9%)
- Noroviruses (8.4%)
- Coronaviruses (7.4%)
- Enterovirus D (6.4%)
- Hepatitis E virus (6.4%)
- Enterovirus C (5.9%)
- Torovirus (5.4%)
- Enterovirus B (3.5%)
- Sapovirus (3.5%)
- Poliovirus (3.0%)
- Hepatitis A virus (1.0%)

- Double stranded RNA
- Rotavirus C (5.4%)
- Rotavirus B (4.5%)
- Rotavirus A (2.0%)
- Picobirnavirus (0.5%)

Achievements

- Probes for major waterborne viruses designed
- Microarrays tested against 1 RNA and 2 DNA virus
- Virus profiling of sewage
  - Seasonality
  - Relative proportions

Future Work

- Additional sewage samples
  - Seasonal trend continue?
- Method sensitivity using spiked sewage samples
- Corroborate signals using other methods
  - Toroviruses
  - Astroviruses
- Analyzing other matrices

This research is funded by
U.S. EPA - Science To Achieve Results (STAR) Program
Grant # 2003-STAR-H1

Thank you

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Quantitative Assessment of Pathogens in Drinking Water

Kellogg J. Schwab Ph.D.
Johns Hopkins University
Bloomberg School of Public Health
Department of Environmental Health Sciences

Characteristics of Enteric Bacteria

- Generally have moderate to low infectious dose
  - Ingesting 10s to 1000s infectious bacteria can cause illness
- Persistence
  - Potential degradation of vegetative bacteria
  - Moderate to low resistance to environmental conditions and chemical inactivation
- Size
  - Intermittent in size (0.5-2 µm)
- Adsorption
  - Bacteria are frequently particle associated under ambient conditions

Characteristics of Enteric Protozoa

- Generally have low infectious doses
  - Ingesting 10s of infectious protozoa can cause illness
- Persistence
  - Significant resistance to environmental degradation and chemical inactivation
- Size
  - Relatively large in size (2-10 µm)
- Adsorption
  - Protozoa can be freely suspended or associated with biofilms

Characteristics of Enteric Viruses

- Generally have a very low infectious dose
  - Ingesting 10 – 100 infectious virions can cause illness
- Persistence
  - Non-enveloped
  - Resistant to environmental degradation and chemical inactivation
- Size
  - Very small (20-100 nm)
- Adsorption
  - Isoelectric point
    - Virions are negatively charged at ambient pH

Sample Processing – Low Microbial Titers

- Although high levels of pathogens can be shed from ill individuals or animals, typically microbial concentrations in surface or groundwater are quite low
- We do not know how many pathogens are present in an environmental sample
  - Assume that there are only a few
  - The goal is to be able to detect one
- Concentrate and purify large volumes of water
  - Starting volumes
    - 100's to 1,000's of L of water

Virus Recovery from Water

- 100s to 1000s of liters of water are filtered
  - 1MDS 0.2 µM positively charged cartridge filter

Viral Elution Using 3% Beef Extract pH 9.5

Viral Eluate

- 1-2 L of eluate (beef extract, amino acids, urea) is further concentrated
  - Acid precipitation
  - Polyethylene glycol precipitation
- Suspend pellet in 10-30 mls PBS
  - For cell culture, multiple small volumes (0.2 - 1 ml) are inoculated onto cells
  - For molecular assays further concentration and purification is needed

Viral Eluate

- Working sample volume for molecular detection is approximately 100 µl
  - 100 L to 100 µl = 10^6 fold concentration
  - Sample volume in a RT-PCR reaction = 1 to 20 µl
    - Thus 1 to 20 L of starting volume assayed
- 1MDS filtration/elution recovery efficiency ranges from 30-60%

Microbial Recovery from Water

- Ultrafiltration
  - 30 to 65 kD ultrafilter
  - Potentially filter 100s of liters depending on turbidity levels
  - In most instances, a secondary concentration step is required
  - Improved recovery efficiency (50 – 80%)
    - Elution of trapped microbes can be challenging
- Polyethylene Glycol precipitation
  - Phase separation of proteins from water
  - Limited to ≤ 1 L
  - Recovery (60-90%)

Co-concentration of Inhibitors

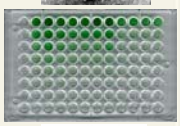
- Samples are comprised of complex matrixes
- Irrespective of the concentration and elution technique, in most instances inhibitors are also concentrated
  - From the water
    - Humic and fulvic acids, organics, etc
  - From the eluate
    - Beef extract, salts
- Multiple inhibitors are common
- Inhibitors must be removed, inactivated or diluted for successful detection

Sample purification

- Most filtration methods recover intact, viable microorganisms
- Subsequent sample purification depends on the detection method
  - Intact infectious microbes
    - Maintain membrane / protein integrity while limiting toxicity
  - Nucleic acid
    - Liberate nucleic acid while preventing degradation

Classical Viral Detection Methods

- Electron microscopy (10^5 - 10^6)
  - Direct EM
  - Immune EM
- Immunoassays (10^4)
  - RIA
  - ELISA
  - Immunofluorescence
- Cell Culture (1-10)
  - CPE
  - Plaque assay

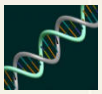


Cell Culture – Part Science Part Art

- Viruses amenable to cell culture can reflect infectivity
- There is no universal cell line
- Many human viruses of interest do not replicate or replicate very poorly in tissue culture
  - Norovirus, HAV,
- It can take days to weeks to obtain results
- Systems have integrated cell culture with molecular detection

Molecular Detection Techniques

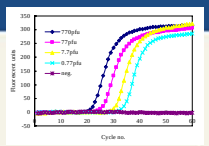
- Molecular detection methods
  - Nucleic Acid
    - Polymerase Chain Reaction (PCR)
    - Reverse Transcription –PCR (RT-PCR)



Molecular Detection of RNA

- RT-PCR and PCR are very sensitive
  - Always the potential for contamination
    - Amplicon contamination
    - Positive control contamination
      - Laboratory viral stocks
      - Clones, RNA constructs
    - Sample cross-contamination
- It is critical to monitor for false negatives and false positives while maintaining sensitivity and specificity
- Multiplexing can result in masking low levels of one target by the presence of high levels of a second target
- Gel electrophoresis is not a definitive detection end-point for single-round PCR

Quantitative PCR



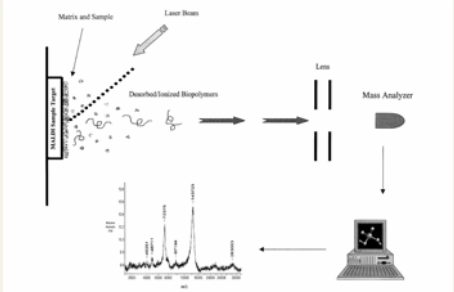
- Quantitative Reverse Transcription –PCR (qRT-PCR)
  - Based on cycle threshold (Ct) values
  - Critical that appropriate standards are developed and used
- Integrated probe confirmation improves specificity and can reduce cross-contamination
- Use of a single tube Ct (i.e. no dilution series) to quantify levels of nucleic acid can be problematic if there is partial inhibition

	no inhibition	inhibition
– Undiluted	30	37
– 10^{-1}	33.3	33.3
– 10^{-2}	36.6	36.6

Advanced Detection Techniques - Proteomics

- Molecular detection methods
  - Proteins
    - Proteomics
    - Matrix assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) with peptide sequence confirmation by MS/MS analysis of peptide fragments
  - Of interest is determining the infectious nature of the detected microorganisms
    - Specific protein fragments present in intact, infectious virions

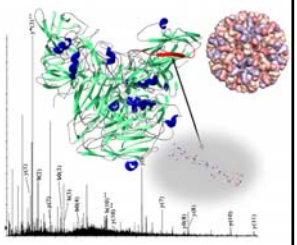
MALDI Sample Analysis



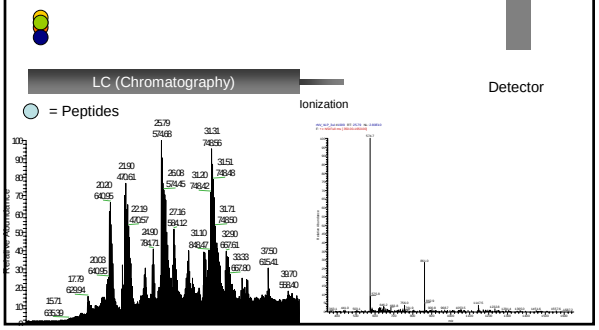
Bakhtiar and Tse, 2000. Mutagenesis **15:**415-30

Detection of VLPs using Mass Spectrometry (MS)

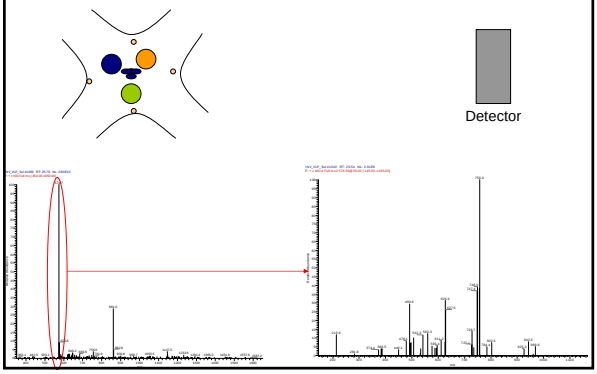
- Detect capsid protein
  - Multi-copy
  - Uniquely identifiable
- Purify protein
- Digest with protease (trypsin)
- Chromatographically separate peptides and then determine amino acid sequence (LC-MS/MS)
- Search masses against genome databases (e.g., NCBI)



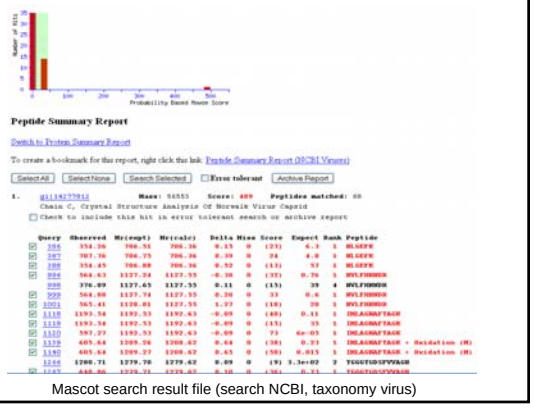
LC-ESI-MS



Peptide Sequencing



Sequence → Search



Mascot search result file (search NCBI, taxonomy virus)

Biological Mass Spectrometry Research Directions

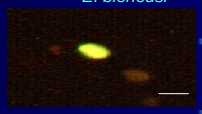
- Quantitation
  - Stable isotope internal standards
  - Assess:
    1. Recovery during preparation
    2. Absolute concentration of target analyte in sample
- Speed
  - Simplify sample preparation methods and processes
  - Maintain robustness and reproducibility
- Accuracy
  - Develop suite of specific peptide targets
  - Target only desired protein/ peptide biomarkers

The Graczyk's Lab: **Multiplexed Fluorescent *In Situ* Hybridization (FISH) Microsporidia**

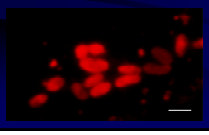
E. hellem Hester et al. (2000) *J Eukaryot Microbiol* **47:**299-308.
Graczyk et al. (2007) *J Clin Microbiol* 45:1255-60

Encephalitozoon hellem

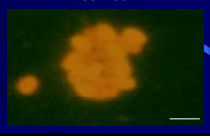
E. bieneusi



E. intestinalis



E. cuniculi



Why use ribosomal RNA as the target?

- Denaturation not required (rRNA is partly single stranded)
- Ribosomes present in multiple copies
- Regions of rRNA range from highly conserved to highly variable (up to strain specific)

Summary

- Low levels of microorganisms in environmental samples require concentration and subsequent recovery
  - Existing concentration procedures have advantages and disadvantages
    - No method provides 100% recovery
- Cell culture (or integrated cell culture-PCR) analysis is one of the few existing methods that addresses infectivity
  - Limited to microbes amenable to replication in the cell lines being used
  - Can be technically challenging and requires maintenance of tissue culture facility

Summary

- Molecular assays can be rapid and automated
- Limited by lack of information regarding infectivity
- Care must be taken to prevent inhibition and contamination
- PCR amplicons must be verified for specificity
- qRT-PCR can quantify target levels but the use of appropriate standards and critical analysis of Ct values are essential
- Proteomics has great potential for detection of multiple targets if sensitivity can be improved

Acknowledgements

- Collaborators
  - Drs Rolf Halden, Thaddeus Graczyk
  - Students – Kristen Gibson, David Colquhoun
- Funding – EPA STAR

An Overview of Pathogen Research in the Microbiological and Chemical Exposure Assessment Research Division

Ann Grimm, Ph.D., US EPA
Innovative Approaches for Detecting Microorganisms in Water, June 20, 2007

Agenda

- Risk Assessment and the EPA's Office of Research and Development
- An overview of the Microbiological and Chemical Exposure Assessment Research Division
- Research emphasis of MCEARD

Microbial Risk Assessment

Step 1: Problem Formulation

Step 2: Analysis
- **Characterization of Exposure**
- **Characterization of Health Effects**

Step 3: Risk Characterization

Step 4: Management of Risk

EPA's Office of Research and Development

Step 1: Problem Formulation — National Center for Environmental Assessment

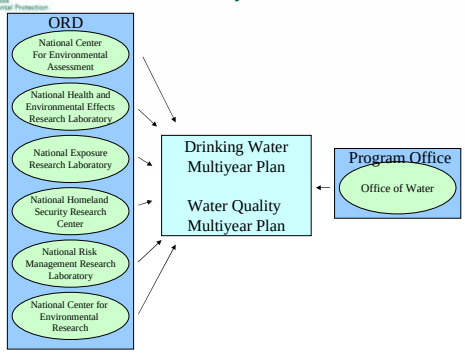
Step 2: Analysis
- **Characterization of Exposure**
- **Characterization of Health Effects**

National Exposure Research Laboratory
National Health and Environmental Effects Research Laboratory
National Homeland Security Research Center

Step 3: Risk Characterization — National Center for Environmental Assessment

Step 4: Management of Risk — National Risk Management Research Laboratory

Multiyear Plans



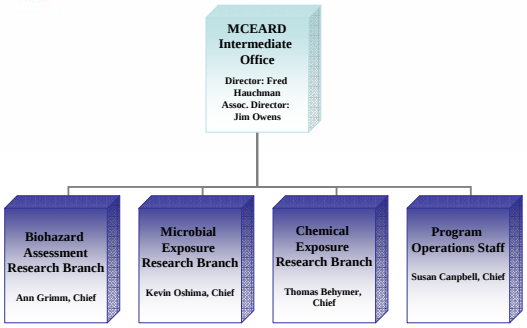
Contaminant Candidate List

- Adenoviruses
- *Aeromonas hydrophila*
- Caliciviruses
- Coxsackieviruses
- Cyanobacteria (blue-green algae), other
- freshwater algae, and their toxins
- Echoviruses
- *Helicobacter pylori*
- Microsporidia (Enterocytozoon & Septata)
- *Mycobacterium avium intracellulare* (MAC)

National Exposure Research Laboratory (NERL)

Focus: NERL studies the impact of stressors on people and/or the environment. MCEARD research focuses on pathogen or chemical stressors that impact public health.

MCEARD-Overview



MCEARD Microbiology

In total, the Division currently has 35+ scientists conducting or supporting research in environmental microbiology.

Pathogens of Interest:

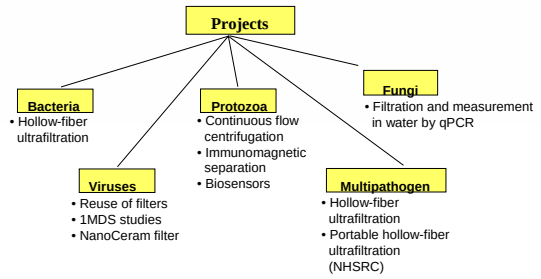
Parasites	Bacteria	Viruses	Fungi
•*Cryptosporidium*	•Cyanobacteria (endotoxins)	•Enteroviruses	•*Aspergillus* sp.
•*Giardia*	•*Helicobacter pylori*	•Adenovirus	•*Fusarium* sp.
•*Toxoplasma gondii*	•*Mycobacterium avium*	•Reovirus	
•Microsporidia	•*Mycobacterium paratuberculosis*	•Rotavirus	
•*Cyclospora*	•*Aeromonas*	•HAV	
	•Indicator bacteria	•HEV	
		•Caliciviruses	
		•Astrovirus	

Project Areas

Occurrence
- Sample Collection and Concentration
- Pathogen Detection and Typing Assays
  - •Molecular
  - •Cultural

Exposure
- New ways of detecting exposure
- Dose response studies using animal models
- Study of cellular response to infection

Sample Collection and Concentration



Molecular Detection

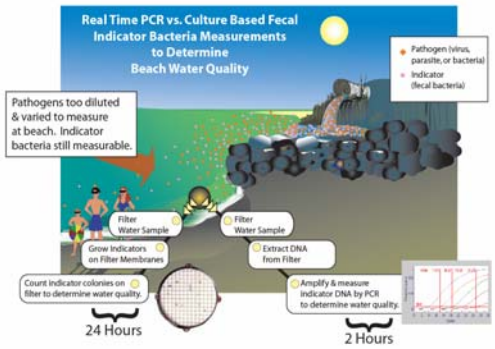
•**PCR/qPCR:** PCR-based amplification detection assays are a key focus of the Division. Assays for a wide variety of organisms are in various stages of development, evaluation or validation.

•**NASBA:** Nucleic acid sequence based amplification is being evaluated as an alternative to PCR for some organisms.

•**Microarrays:** Microarrays are being investigated for typing and for multiple pathogen detection.

•**Proteomics:** MALDI-TOF analysis of microorganisms is being investigated for typing and characterization.

Comparison of a qPCR and Culture Method for *Enterococcus*



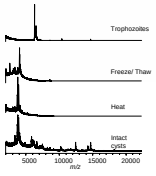
Method Concordance

Agreement between EPA-ORD QPCR method results and reference laboratory culture method results with respect to being above or below the 104 Enterococci/100 ml marine beach water posting criterion

Agreement among all samples	Agreement among samples below posting criterion by culture	Agreement among samples above posting criterion by culture
84%	39%	100%

Proteomics

Mass spectrometry is used to identify protein signatures unique to specific organisms



Giardia lamblia mass spectra for intact, heat and freeze/thaw treated oocysts, and purified trophozoites

- Rapid identification
- Used to rapidly speciate
- In some cases, may be able to differentiate live from dead

Culture/Viability

- **Culture Assays:** Bacterial culture methods have been and are being developed to improve detection of microorganisms.
- **Cell Culture Assays:** Cell culture based methods are being optimized and developed to better detect viable viruses and parasites.
- **Integrated Cell Culture/PCR and enrichments:** Several methods are under development that involve enriching for viable organisms, either by cell culture or other methods such as pretreatments that limit PCR amplification by non-viable organisms.

Integrated Cell Culture/RT-PCR Method

Sample Collection & Elution
↓
Concentration
↓
Tissue Culture
↓
Detection

Exposure

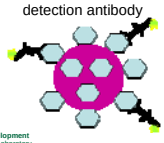
- **Novel Methods:** New approaches to detecting human exposure to pathogens are being investigated and developed.
- **Dose Response Models:** Animal models are being used to help estimate the risk posed by exposure to various organisms.
- **Cellular Response to Infection:** Microarray analysis is being used to determine the effect of infection on cells. The goal is to identify markers of infection.

Microbead immunoassay

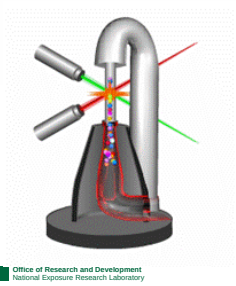
1. Microscopic beads are coated with one specific protein (*Cryptosporidium*, norovirus or rotavirus)

2. Saliva samples are incubated with beads in microplate wells; salivary antibodies react with the protein

3. Samples are incubated with labeled anti-human detection antibody



Luminex dual laser flow cytometer



Red laser is used for bead classification. The discriminator signal depends of the bead type.

Green laser is used for assay quantification. The reporter signal is proportional to the number of detection antibodies attached to the bead.

At least 100 beads of each type are measured; the median reporter signal for each type is reported.

Issues and Future Directions

- Microbial Source Tracking
- Prions
- Better Indicators
- Viability
- Relative Risks of Animal-borne Pathogens
- Method Validation
- Emerging Pathogens
- Better Occurrence Methods
- More Exposure Methods

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MCEARD Microbiologists

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Debbie Flannigan
Shay Fout
Susan Glassmeyer
Richard Haugland
Mohammad Karim
Dawn King
Lu Li
Dennis Lye
Audrey McDaniels
Brian McMinn
Kevin Oshima
Stacy Pfaller
Cliff Rankin
Eric Rhodes
Shawn Siefring
Gerard Stelma
Gretchen Sullivan
Manju Varma
Eunice Varughese
Steve Vesper
Eric Villegas
Mike Ware
Larry Wymer

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms in Water

U.S. Environmental Protection Agency
Andrew W. Breidenbach Environmental Research Center
26 W. Martin Luther King Drive
Cincinnati, OH
June 18–20, 2007

JUNE 18, 2007

INTRODUCTION AND OVERVIEW

The U.S. Environmental Protection Agency (EPA) Progress Review Workshop on Innovative Approaches for Detecting Microorganisms in Water was held on June 18-20, 2007, in Cincinnati, Ohio. The workshop, co-sponsored by EPA's Office of Research and Development's (ORD) and the Office of Water (OW), brought together approximately 78 researchers and regulatory personnel from academia, industry, and government to discuss ongoing research on innovative approaches to detect microorganisms in water in a real-time manner. The workshop served as a stimulus for increased collaborations among the various researchers and included information on various national and international efforts and initiatives.

SESSION 1
Moderator: Barbara Klieforth, U.S. EPA, Office of Research and Development (ORD)

Barbara Klieforth welcomed participants to the two and a half day workshop designed to be an interactive meeting between researchers in academia, industry, and the government. The workshop is intended to be a forum for researchers to share information and, more importantly, to stimulate progress and collaboration on furthering the development of cost-effective, timely and innovative solutions in assessing and managing microbial contaminants in water. Another primary function of this workshop is to officially kick-off the start of the twelve recently funded Science To Achieve Results (STAR) grants from the 2005 solicitation "*Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens in Drinking Water*" by ORD's National Center for Environmental Research's (NCER). The STAR program awards competitive research grants to leading researchers from the academic and nonprofit communities.

Innovative Pathogen Detection in the Context of the National Program for Drinking Water Research
Audrey Levine, U.S. EPA, ORD, National Program Director (NPD) for Drinking Water

Dr. Levine explained that the rationale for the workshop was a result of the immense interest in monitoring pathogens in a real-time manner. There is an increasing demand for the real-time assessment of pathogens to ensure the safety and security of drinking water, recreational water, water used for irrigation, food processing and production, and reclaimed and recycled water. There are many specific research needs, including the investigation of potential interferences to detection methods such as particulates, salts, and metals. It is important to examine the relationship between pathogens and chemical and microbial indicators.

EPA's strategic directions are used to develop science questions that in turn are used to develop research questions. The research programs are designed around core research areas (e.g., human health and ecology)

and problem-driven research areas (e.g., drinking water, air quality); the core research develops tools to approach problem-driven research. ORD research is outcome-oriented and provides support for regulatory decisions. EPA's National Research Programs relate to each of EPA's five strategic goals. Microbial issues are investigated by the Drinking Water and Water Quality Research Programs (Goal 2) and the Homeland Security, Human Health, and Ecosystems Research Programs (Goal 4). Each National Research Program is lead by an NPD. Research programs within ORD are developed by a Research Coordination Team and are guided by Multi-Year Plans (MYPs), which include specific Long-Term Goals (LTGs). Research is evaluated by Annual Performance Goals and Annual Performance Measures.

The legislative authorities for water include the Safe Drinking Water Act (SDWA) and the Clean Water Act (CWA). The SDWA requires EPA to set maximum levels for contaminants in water delivered to users of public water systems based on sound science and risk-based standard setting. Under the SDWA, EPA examines source water in lakes, streams, and oceans to ensure source water protection; assesses pathogens in its Underground Injection Control Program; and determines the microbial safety of the water system via its Total Coliform Rule (TCR). The components of the SDWA help keep drinking water safe and manage risks. The CWA sets water quality criteria and guidelines and technology-based standards for ambient water, including providing effluent guidelines for the regulation of point sources, the Combined Animal Feeding Operations Rule, and human health and aquatic life water quality criteria.

The Drinking Water Research Program's (DWRP) two LTGs are to: (1) characterize risks, and (2) manage risks. Research needs of these LTGs being addressed by STAR researchers include the development of assessment tools (e.g., monitoring tools) and methods for quantifying pathogens. Ongoing research also involves source water and water resources, including investigations of: the prevalence and persistence of pathogens in surface and ground water; the effectiveness of management practices for controlling pathogens; and source tracking. The Water Quality Research Program's LTGs are to: (1) improve water quality on a watershed basis, and (2) improve coastal and ocean water quality.

Dr. Levine reiterated that the goals of this workshop are to: (1) learn about research efforts pertaining to innovative pathogen detection, (2) identify potential research collaborations, and (3) identify research gaps and needs.

Regulatory Perspective From the U.S. EPA's Office of Water
Phil Oshida, U.S. EPA, Office of Water (OW), Office of Ground Water and Drinking Water (OGWDW)

Dr. Oshida explained that EPA maintains headquarters in Washington, DC, and 10 regional offices throughout the United States. The regional offices carry out most of the regulations working with state and local governments. The OGWDW develops groundwater drinking regulations and is divided into two groups, those that work with the SDWA and those that work with the CWA. The results of the innovative researched performed by the DWRP are used by regulatory personnel for regulatory decisionmaking. The three divisions within the OGWDW are the Standards and Risk Management Division, Drinking Water Protection Division, and Water Security Division.

The SDWA regulatory process is not a linear process and all phases occur simultaneously. One phase is the determination of priority contaminants, another phase is the development of the Contaminant Candidate List (CCL), and the third phase is the Six Year Review, which examines existing regulations to determine if revisions or updates are needed. Statutory requirements for the drinking water regulatory processes include the CCL, unregulated contaminant monitoring, regulatory determination for the CCL, regulation development, and the Six Year Review. In classifying the CCL3, first the CCL universe was identified; this was followed by a screening process involving ORD, the Office of Science and Technology, and the Office of Pesticide Programs that narrowed the number of possible contaminants and resulted in a preliminary CCL (PCCL). The PCCL then was evaluated and reviewed. The proposed CCL3 will be available in 2008.

Per the SDWA, the contaminants on the CCL must meet all three of the following criteria: (1) The contaminant may have an adverse effect on the health of persons. (2) The contaminant is known to occur or there is substantial likelihood that the contaminant will occur in public water systems with a frequency and at levels of public health concern. (3) Regulation of such contaminant presents a meaningful opportunity for health risk reduction for persons served by public water systems. This standard-setting process includes five steps: (1) occurrence assessment; (2) analysis of test methods, availability, monitoring requirements, and compliance reporting; (3) risk assessment; (4) technology performance assessment; and (5) cost-benefit analysis.

Outbreaks in drinking water occur because indicator and treatment systems, although good, are not always adequate. Treatment failures and disinfection-resistant pathogens also contribute to outbreaks. Existing tests may not allow timely response to indicator or treatment failures; therefore, there is a need for a rapid test that allows for strain-level identification.

OW programs evolve to be responsive to new challenges to drinking water safety by developing innovative methods. These innovative methods may offer advantages over current methods as a result of their speed, specificity, and ease as compared to microscopic methods. Also, these methods may allow response to contamination events before it is too late and detect contaminants that do not co-occur with indicators. These innovative methods may not replace indicators but could supplement this approach. Possible applications include field-portable devices; online monitoring; a faster, less-expensive screen for *Cryptosporidium* and *Giardia*; tools and methods to control airline water quality; and the use of "omics" for the screening of known and emerging pathogens.

Use of Innovative Detection Methods for Detecting Contaminant Candidate List Pathogens
James Sinclair, U.S. EPA, OW, OGWDW

EPA's approach for controlling pathogens in drinking water is to use water treatment and monitor coliform indicators. Some emerging pathogens, however, cannot be controlled by treatment and/or coliform indicator monitoring and require individual regulations. The mechanism for regulating currently unregulated contaminants is the CCL. Contaminants to be considered for regulation are placed on the Drinking Water CCL, and CCL contaminants needing more information become research priorities. The three criteria for contaminants to be put on the CCL are that they: (1) are not currently regulated or anticipated to be regulated in drinking water by a future regulation, (2) may occur in drinking water, and (3) cause adverse health effects in drinking water consumers. To make regulatory decisions regarding microorganisms, information on pathogen health effects, treatment, and occurrence in water are needed; analytical methods also are needed.

Occurrence information for regulatory determination is gathered via the Unregulated Contaminant Monitoring Rule (UCMR), which is EPA's regulation for receiving occurrence information for drinking water, and other research surveys as deemed appropriate. The UCMR is a 1-year survey of selected drinking water systems with three monitoring options (e.g., assessment monitoring, screening survey, and prescreen survey) based on method availability. The survey is usually, but not always, conducted on CCL contaminants. Methods used for surveys ideally: (1) determine occurrence in drinking water at levels of concern; (2) detect viable, infective organisms; and (3) detect disease-causing species or strains. EPA has traditionally relied on culture-based methods for the detection of emerging pathogens. These methods may not distinguish between pathogenic and nonpathogenic forms and may be expensive, slow, and labor-intensive. Additionally, not all pathogens can be cultured. These deficiencies may limit collection of occurrence information.

The National Research Council (NRC) was tasked with reviewing EPA's CCL process and noted a "bottleneck" in evaluating drinking water pathogens. The NRC recommended using molecular methods of all types, especially polymerase chain reaction (PCR)-based methods, to overcome problems encountered

with traditional methods. Genetic methods, including microarrays, should be used to detect: (1) RNA to distinguish between live and dead organisms, and (2) detect virulence factor genes (e.g., Virulence Factor Activity Relations [VFAR]) to identify virulence in microorganisms. Another recommendation was the need to define method objectives and use molecular methods if they meet those objectives for a particular UCMR survey option. One NRC reviewer suggested that molecular methods be used for the prescreen survey. EPA also could combine culture methods with genetic methods so that the overall method would have the benefits of both.

EPA has taken several steps to implement these recommendations. Workshops focusing on PCR quality assurance (QA) guidance, VFARs, and microarrays have been held, and a guidance document on PCR QA has been released as a result of one of the workshops. Additionally, microarray, virulence factor, and molecular method detection projects are underway. In 2004, EPA also created the Environmental Technology Council (ETC) to consider the use of innovative methods for solving environmental problems. This program identifies areas in which technology is a critical factor in providing a cost-effective solution and leverages existing resources to promote innovative technologies. The ETC Program includes members from ORD and program and regional offices. One of 11 action teams focuses on detection of microbial contaminants in drinking water. Those interested in participating in the program can contact Drs. Keya Sen (OW) and Sam Hayes (ORD) or visit http://www.epa.gov/etop/forum/problem/microarray_technology.html for more information.

Discussion

Dr. Mark Sobsey asked if there was any effort to develop a suite of indicators that would be useful in identifying pathogens, including those not covered by the TCR (e.g., viruses). Dr. Sinclair responded that he was not aware of any, but this might be considered under upcoming TCR revisions. There is much discussion about using other indicators, but as he is not a member of the action team, he cannot speak to the team's specific plans.

A participant asked how many pathogens are listed on the current CCL. Dr. Sinclair responded that there are nine pathogens on the CCL2, which are listed on the CCL Web Site (http://www.epa.gov/safewater/ccl/ccl2.html).

LATE-PCR: Maximizing Detection Information From a Single Tube

Kenneth Pierce, Brandeis University

Linear-After-the-Exponential (LATE)-PCR is an improved method of asymmetric PCR. The method is similar to symmetric PCR in terms of reagents and cycling profiles, the main difference being that one of the primers is present in a lower concentration. Asymmetric PCR is inefficient because the lower concentration of the primer reduces its melting temperature, and the reaction is not being run at optimal conditions. Lowering the annealing temperature to increase efficiency allows nonspecific amplification from the excess primer. To correct this, the laboratory has designed limiting primers that have melting temperatures at least as high as those of the excess primer. LATE-PCR also allows the uncoupling of the primer from probe design and detection. LATE-PCR modifies the limiting primer so that the limiting primer melting temperature is higher than the excess primer melting temperature.

The laboratory utilized this improved PCR process to detect hepatitis C viral RNA. The hepatitis C virus genome is comprised of positive-strand RNA, and proteins are synthesized from viral RNA with no DNA intermediate. The laboratory used Armored RNA® (Asuragen, Inc.) as a surrogate, with the sequence corresponding to the 5' untranslated region. The laboratory achieved real-time detection of hepatitis C virus amplification, and the post-PCR melting profiles reveal a tremendous amount of information. The researchers found that targets with the same melting temperatures can have distinct fluorescence signatures;

minor sequence variations also show distinct signatures. Additionally, molecular beacons can be used with LATE-PCR to identify specific pathogens when multiple pathogens are present.

Low-temperature probes have many advantages. Mismatch-tolerant probes provide strong signals, have the potential to detect an extremely high number of sequence variations, and the signal ratios (i.e., fluorescence signatures) can distinguish most nucleotide variations within the region hybridized by the probes. Sequence-specific probes show strong signals with LATE-PCR products, can detect a high number of unique targets, and can identify multiple target types in the same sample. Additionally, when using multiple probes, colors, sequence variants, mismatch-tolerant probes, and amplicons, it is possible to achieve more than 1 million possible variants in a single closed tube. A whole system approach that includes LATE-PCR, "Dilute-'N-Go" sequencing, multiplexing, qualitative end-point analysis, uncoupled annealing and detection, and low temperature probes can have a broad range of diagnostic applications, including pathogen testing, genetic testing, and cancer testing. The laboratory is working with Smiths Detection to develop a field-portable device that is designed to be immersible in disinfectant, thereby avoiding potential cross-contamination.

Discussion

Dr. Keya Sen asked if the method can be used to determine copy number. Dr. Pierce explained that it does ascertain the copy number for a particular target. Quantification of the endpoint can be determined by the intensity of the signal because the signal stays proportional to the initial target throughout the reaction.

Dr. Sen asked if the method was effective on closely related sequences as well as widely different targets. Dr. Pierce responded that the probes must be sequence specific, but they can be widely different. The limit occurs in the multiplexing, which is a limitation for any type of PCR. The laboratory has accomplished 15 different targets in one reaction and is working to improve this.

A participant asked if a highly differentiated response is seen with a single mismatch. Dr. Pierce replied that in most cases, this is seen.

A participant asked how an error in the TAQ polymerase would impact results. Dr. Pierce responded that an error in incorporation would have to occur in the first round of the PCR. Errors such as this are extremely rare, occurring at a rate of 1 per 1 million reactions.

A participant asked if the samples were from clean buffers or serum samples. Dr. Pierce replied that they were the Armored RNA® samples, which are viral particles that have been placed in a standard buffer. The participant asked what the results might be using serum samples. Dr. Pierce responded that there are inhibitors in serum so this would need to be countered in the preparation, or the targets would need to be extracted from the samples. Smiths Detection is working on a method to perfect this.

A participant asked how well the software is developed and if it is available. Dr. Pierce replied that the program the laboratory uses is expensive DNA stockware that costs $3,000 per year to license one computer but is extremely useful.

Advanced Oxidation Technologies and Nanotechnologies for Water Treatment: Fundamentals, Development, and Application in the Destruction of Microcystin LR
Dionysios D. Dionysiou Dionysius, University of Cincinnati

The eutrophication of water favors the formation of cyanobacteria harmful algal blooms (cyano-HABs), which cause the production and release of more than 50 genera of bioactive compounds that are harmful to humans and the ecosystem. The most commonly found cyanotoxin in cyano-HABs is microcystin LR, a hepatotoxin. Microcystin LR has a highly stable cyclic structure with functional groups that are highly

water soluble; it is toxic in the *trans* configuration. The World Health Organization (WHO) has placed a provisional limit of 1 μg/L of microcystin LR in drinking water. Although it is on the Drinking Water CCL, the EPA has not regulated it in terms of the best available technology or maximum contaminant level. Cyanobacterial contamination is reported worldwide, including in the United States, Australia, China, and Mexico, and a fatal outbreak occurred in Brazil in 1996.

Titanium dioxide (TiO_2) photocatalysis is being investigated as a water purification and detoxification technology. It allows for the complete mineralization and disinfection of water without the addition of other chemicals or the production of hazardous wastes. TiO_2 nanoparticles in slurry have been used successfully for the degradation of microcystin LR. When using TiO_2 photocatalysts, the TiO_2 must be removed, which is a problem for water treatment systems. This research focuses on a green approach to remove TiO_2 while increasing the photocatalytic activity. The approach uses surfactant templates that include a crystal phase that changes the concentration of the Ti in the surfactant, which changes the poststructure of the final material. It is possible to create a TiO_2 membrane with an outer layer porosity that can control selectivity. Using nano-based methods and catalytic materials with increased surface area can increase catalytic activity.

High-performance liquid chromatography-tandem mass spectrometry is used to destroy toxicity and to understand the effects that intermediate products have on the environment and human health. The laboratory determined that the chemical mechanism of TiO_2 photocatalysis allows the formation of 22 radical intermediates, most not previously reported. Hydroxyl radicals attack microcystin LR at four different sites. Additionally, TiO_2 has high catalytic activity under visible and ultraviolet (UV) radiation; this allows the use of sustainable and renewable solar energy in the technology. This is promising for the use of solar light in the remediation of water resources contaminated with biological toxins and other chemicals.

Discussion

A participant asked if the cost of TiO_2 is comparable to that of powdered activated carbon. Dr. Dionysius responded that he was unsure of the cost comparison, but TiO_2 that the laboratory used was made in mass quantities and was not expensive. A participant commented that part of the cost is not associated with the material itself but in collateral costs in attempting to control the material. Dr. Dionysius agreed that this was an engineering challenge.

A participant stated that cyanotoxins are a small percentage of HAB organic matter and asked about the efficiency of the method when this is considered. Dr. Dionysius responded that the efficiency will decrease, and more tests need to be run in the presence of natural organic matter. Although there will be lower rates, the toxin still will degrade.

Cyanobacteria and Cyanotoxins in Water Supply Reservoirs: Development of Gene Microarray Assays for Risk Assessment
Parke Rublee, University of North Carolina (UNC) at Greensboro

This cyanobacteria research project focuses on drinking water reservoirs in North Carolina, a state in which there are no natural lakes. The researchers routinely sample 12 reservoirs and randomly sample others for microcystin levels and are attempting to develop gene microarray assays to determine risk. The approach has been to generate small subunit ribosomal DNA (rDNA) libraries from genomic DNA extracted from the summer samples of the reservoirs. Probes to known cyanotoxin genes are generated and spotted on microarray slides for rapid cyanobacteria assessment. To date, sampling has not included blooms that have demonstrated significant toxin production because such blooms have not occurred. Currently the laboratory is evaluating 100 to 200 clones derived from each sample of 10 reservoirs distributed across North Carolina with the goal of encompassing the entire diversity of cyanobacteria in North Carolina. Another goal is to

correlate abundances of cyanobacteria with physical and chemical parameters from the lake samples so that factors that promote bacterial growth and toxin production can be determined. This effort is part of a larger project to develop a more comprehensive metagenomic ecosystem assessment in a single microarray test.

Bioindicator organisms have limited environmental tolerances; the presence of overlapping species indicate where parameters are restricted. The characteristics of the environment can be described depending on which organisms are present. Each organism has its own characteristic tolerances and when added to a system, the more details and complexity involved provide more information about the system. Currently, evaluation of water quality occurs after treatment. If water could be evaluated before treatment in a real-time fashion, the treatment process could be improved and risks to human health could be decreased. In addition to detection of pathogens, a microarray method could be used for water quality assessment for municipal and industrial systems, environmental assessments of aquatic ecosystems, bioremediation monitoring, and synoptic detection of pollutants and toxins, including biological or chemical weapons (i.e., dual use). This dual-use application is desirable for utilities. To develop such a method, there are many fundamental questions regarding targets, spatial and temporal variability, replication and sample size, biogeography, and sensitivity. The best approach to determining microbial bioindicator markers is via empirical field testing, which involves the discovery of unique signatures from well-defined sites and includes unknown species.

The laboratory used small 16S (prokaryotic) and 18S (eukaryotic) rDNA libraries of clones to compare lakes in North Carolina, Alaska, the Dakotas, and China to determine biogeographical differences. The results indicated that natural microbial populations are characterized by some common and many rare species (i.e., operational taxonomic units). Common taxa vary in abundance across lakes, but even modest replicate samples generally show the same taxa. Not all of the taxa have to be known to find similarities or differences among aquatic systems because abundance of key taxa varies over time and space. The metagenomic microbial bioindicator approach for characterizing aquatic ecosystems and risk assessment based on microarrays shows promise, but important questions remain.

Discussion

A participant asked how the researchers confirmed that the 16S primers were identifying everything present in the sample. Dr. Rublee explained that this was not done because it does not matter if everything is identified. The goal is not to census everything but to identify meaningful sequences for the array. Sequences that were easy to amplify but not informative also were discarded.

A participant asked how the researchers know that chimeras are not present. Dr. Rublee responded that a chimera check was completed. Although chimeras occur at a low rate, the researchers were cognizant of the possibility.

A participant asked why the researchers did not sequence everything and then design an algorithm to determine important sequences. Dr. Rublee replied that the laboratory hopes to have 454 Sequencing, but the resources at the university are limited. If the technologies evolve beyond glass microarray slides, the method will become more user friendly.

Characterization of Naturally Occurring Amoeba-Resistant Bacteria
Mary Farone, Middle Tennessee State University

Legionella-like amoebal pathogens (LLAP) were first described in 1956 as obligate intracellular parasites of free-living amoebae with the ability to lyse their hosts. The first description of LLAP in U.S. soil occurred in 1998, and currently there are more than 30 groups or species of pathogens capable of infecting free-living amoebae. The difference between LLAP and *Legionella* bacteria is that LLAP cannot be cultured. Researchers compared the occurrence of infected amoebae in cooling towers versus natural

aquatic environments and found that infected amoebae are 16 times more likely to occur in cooling towers than in natural aquatic environments. Samples were screened, and the presence of amoebae was determined. The aliquots containing the amoebae were added to 96-well plates and observed for infected native amoebae. Positive samples then were transferred to monolayers of *Acanthamoeba polyphaga*. Physical, biological, and chemical methods were used to separate the infecting LLAP from the mixture.

Using these methods, the researchers were able to identify typical LLAP bacteria in cooling towers and create a phylogenetic tree. Most of the unculturable cooling tower isolates are closely related to LLAP, but one isolate is much more closely related to *Coxiella burnetii*. This bacterium is unculturable, coccoid, motile, infects the host nucleus, and lyses cells within 48 hours of infection. It can infect human cell lines, including U937 macrophage-like cells and HeLa cells. The researchers used fluorescent *in situ* hybridization (FISH) with 16S rDNA-specific probes to detect distribution of novel amoeba-associated microorganisms (AAMs) in aquatic systems and discovered that unculturable AAMs from infected amoebae are present in fire safety sprinkler system and fire hydrant samples. This is significant because: (1) infected amoebae can be found in water distribution systems, (2) the AAM infecting the amoebae can be novel and unculturable and therefore undetectable, and (3) bacteria pathogenic for humans are thought to have evolved in association with amoebal hosts.

Discussion

A participant asked Dr. Farone to briefly describe the sampling method used in the fire hydrant and sprinkler system experiments. Dr. Farone responded that the enrichment process was the same as the one described for the cooling tower experiments. Water was collected from sprinklers, and pipes were swabbed for biofilms.

A participant asked if the pathogens are found inside the amoebal cyst or in the active amoeba. Dr. Farone responded that they were found in the active amoebae; the laboratory has not studied the cysts very much.

A participant asked how the pathogens were undetectable but then detected. Dr. Farone responded that for purposes of her talk undetectable meant undetectable by traditional culture methods.

Biofilm Sampling Techniques and Screening Techniques for Amoeba-Related Biofilm Pathogens
Nick Ashbolt, U.S. EPA, ORD, National Exposure Research Laboratory (NERL)

More information is needed about fecal pathogen intrusions into distribution systems; nonfecal pathogen growth in biofilms; and sequestration, inactivation, and sloughing of pathogens from biofilms. Currently, there is high uncertainty when modeling infection risks as a result of unknown distribution system biofilm effects. Trends in outbreaks from U.S. public water from 1971 to 2004 indicate that distribution system deficiency is increasing. Ninety-five percent of all illnesses caused by distribution system deficiencies from 1981 to 2002 are biological (vs. chemical) in nature.

Biofilms are a concern to human health because biofilms sequester fecal pathogens and allow the growth of opportunistic pathogens. Water-based bacterial pathogens include various *Legionella* strains; *Mycobacterium avium* and *M. ulcerans*; *Burkholderia pseudomallei*; *Helicobacter pylori*; and *Aeromona*s, *Vibrio*, and *Campylobacter* species. All of these organisms grow associated with amoeba in biofilms and may be active but nonculturable. Another concern regarding biofilms is that they protect pathogens because the biofilm slime does not allow chlorine to act effectively. One study showed that *Acanthamoeba* cysts containing viable *Legionella* were not killed by either chlorine or heat treatment, indicating that conventional hyperdisinfection or 80°C heating may be insufficient for long-term control of *Acanthamoebae*-bound *Legionella* bacteria in water distribution systems. Additionally, the *A. polyphaga*-associated virus, mimivirus, is the largest known DNA virus and may be a more common cause of community and nosocomial infections than *Legionella*. Biofilms do, however, provide a history of contam-

ination. Because of their sequestering nature, biofilms are a good indicator of past contamination, and therefore may provide a preferable target to monitor when compared to water. Biofilms are more representative, particularly for small systems with infrequent sampling and for short-duration events.

An Australian project aims to obtain an understanding of the growth of opportunistic pathogens in water mains. One project, in conjunction with EPA's National Homeland Security Research Center (NHSRC) and National Risk Management Research Laboratory (NRMRL), will focus on a biofilm pathogen sampling device that: (1) resides in the main flow but with properties that sorb chemical and microbial analytes; (2) possesses a high surface area that encourages biofilm formation; and (3) is easily retracted and removed from the full-pressure water main. A second project will investigate the genetics of the biofilm amoeba-bacteria-mimivirus environment. The significance of pathogens found in pipe biofilms will be determined. Is there a common virulence factor (e.g., mimivirus) involved? A third project is investigating mimivirus occurrence. Long-term biofilm research goals are to: (1) determine if phagosome maturation is a general mechanism (i.e., target) for bacterial virulence; (2) describe the role of mimiviruses in lateral virulence gene transfer in biofilms; and (3) investigate the role of alive-but-not-culturable cells in animal dose-response models and efficacy of chlorine disinfection.

Discussion

A participant asked if it is possible to perform a bottle brush and use water pressure to perform the sampling (e.g., pipeline "pigging"). Dr. Ashbolt responded that this is one possible method, but weaknesses also are being catalogued for purposes of homeland security. The method also must be easy and inexpensive. There also are other technologies that researchers will investigate.

A participant asked what type of disinfectant was used and if a change in the biofilm was noticed as a result of a change in disinfectant. Dr. Ashbolt responded that he had not examined this specifically, but the structure of biofilms are different; chlorine selects for more virulent strains.

A participant asked if other water quality parameters are being investigated. Dr. Ashbolt responded that he had investigated other parameters and would be interested in partnering with another laboratory to examine others.

A participant asked what triggers biofilm formation in pipes. Dr. Ashbolt replied that the surface contains nutrients so biofilm formation cannot be stopped. Biofilms have the ability to grow in stainless steel pipes in outer space. The participant clarified that he meant factors other than nutrients. Dr. Ashbolt responded that phosphorus-limited or carbon-limited situations may be factors in certain distribution systems depending on the nature of source water. Breakdown of organic matter also leads to more biofilm formation. Treatment methods must be carefully considered so that the treatment does not inadvertently increase biofilm growth and formation.

Overview of the U.S. EPA's Office of Research and Development and the Science To Achieve Results (STAR) Program

Barbara Klieforth, U.S. EPA, ORD, National Center for Environmental Research (NCER); and Angela Page, U.S. EPA, ORD, NCER

Ms. Klieforth explained that EPA's mission is to protect human health and safeguard the natural environment—air, water, and land—on which life depends. ORD's mission is to conduct research to inform Agency decisions with sound scientific information; program and regional offices are ORD's principal clients. The STAR Program fills a unique niche by by supporting extramural research that is: directly relevant to the mission of EPA; complementary to existing intramural research; not currently conducted or funded by other ORD Labs and Centers and is. STAR involves U.S. universities and nonprofit groups in EPA's research program and ensures the best possible quality of science in areas of highest risk and

greatest importance to the Agency. EPA issues 20 to 25 Requests for Applications each year and receives 2,500 to 3,000 grant applications in response. The Agency funds 300 to 400 new STAR grants and fellowships each year, managing approximately 1,000 active grants and fellowships. The National Research Council (NRC) reviewed the STAR Program, The Measure of STAR, and found that it exceeds expectations and is an important part of the overall EPA research program. STAR research results have improved the scientific foundation for decision-making. Since its inception in 1995, the STAR, and found that it exceeds expectations and is an important part of the overall EPA research program. STAR research results have improved the scientific foundation for decisionmaking. Since its inception, the STAR Program has awarded more than $970 million to more than 900 institutions that has resulted in the publication of more than 6,500 peer-reviewed journal articles.

In addition to STAR grants and fellowships, NCER funds research projects via the Experimental Program to Stimulate Competitive Research (known as EPSCoR), the Small Business Innovation Research Program, and earmarks. NCER personnel hold advanced degrees and expertise in a wide range of fields. STAR is an integral part of NCER's responsiveness to Agency needs. The competitive solicitations are designed with cross-Agency and interagency involvement, and the highest priority projects are funded following extensive internal and external peer review with program office and regional input. Research results are communicated via the NCER Web Site (http://www.epa.gov/ncer), ORD laboratories, program offices, regional meetings, and publications. A new emphasis is being placed on communicating via listservs. Although NCER's budget has steadily decreased, funding for drinking water research has remained steady.

NCER's Drinking Water Program was established in 1996 with funding levels between $2.5 and $5 million per year. Solicitation preparation and programmatic reviews have extensive participation by OW, ORD, and regional offices. The STAR Drinking Water Research Program has evolved from focusing on chemical contaminants in drinking water to assessing microbial risk. The current focus is on the development of quantitative approaches for detection of pathogens and cyanobacteria and their toxins.

For the benefit of new and returning STAR grantees, Ms. Angela Page described STAR grants procedures. Although STAR grants are awarded to institutions, the Principal Investigator (PI) is the grant representative for the institution; the Project Officer (PO) is the EPA representative. Communication between the PO and the PI is important, and e-mail communication is preferred so that there is a record of all correspondence. As ORD labs become interested in entering into a cooperative agreement with a 2005 grantee, all official requests must be sent from the PI to the PO. EPA researchers are not authorized to direct research detailed in a cooperative agreement. All grantees must submit annual and final reports to their PO and a shorter Web version of these reports to EPA's contractor. Grantees also should include the STAR logo with their grant number in all presentations. International travel must be requested by the PI and approved by EPA prior to making travel arrangements. No-cost extensions may be requested via e-mail to the PO; the first year is automatic, but requests for a second year require justification. A no-cost extension cannot be used as a means to exhaust remaining funds. Supplemental funding may be requested. Justifications for supplemental funding requests less than $15,000 are not quite as extensive as those greater than this amount. Request in excess of $15,000 must meet three criteria and adequately justify that the funding is within the scope of the original grant and is intrinsically necessary for the completion of the research (i.e., results will be jeopardized without the supplemental funding).

JUNE 19, 2007

SESSION 2

Moderators: James Owens, U.S. EPA, ORD, NERL; and Sandhya Parshionikar, U.S. EPA, OW, OGWDW

Overview of Methods for Simultaneous Detections of Pathogens and Introduction to a Highly Multiplexed Nucleic Acid-Based Assay

R. Paul Schaudies, GenArraytion, Inc.

Waterborne pathogen identification has many challenges, including the large volumes of water involved, the concentration of inhibitors, the low numbers and multiple classes of target organisms, the detection of live organisms, and the simultaneous identification of multiple organisms. Sampling is the ultimate driver of system sensitivity. Viability of organisms may be determined by culture, examining ATP or NADH levels, or by analyzing RNA. Methods by which detection can occur without amplification include capture by structural recognition, spectral methods, cantilevers, flow cytometry, and protein microarrays. PCR, loop-mediated isothermal amplification, multiplexed PCR with microarray, and whole-sample amplification with microarray are examples of methods of detection that include amplification.

GenArraytion, Inc. is an early-stage research and development (R&D) company developing molecular infectious disease diagnostics that provides microbial genotyping microarrays, a library of unique and functional biomarkers for human pathogens, bioinformatic analysis, diagnostics testing services, and contract R&D services. The approach to broad-spectrum identification and characterization of waterborne pathogens is to apply bioinformatics and laboratory methods to simultaneously identify and characterize a wide variety of infectious agents. The Molecular Radar method that the company has developed provides high fidelity signatures for human and animal pathogens and is a customizable suite of analysis methodologies for epidemiological and environmental monitoring and forensics. The method is "platform agnostic" in that it is compatible with a range of platforms. The food- and waterborne pathogen microarray design includes hundreds of oligonucleotide sequences from each of nearly 20 common food- and waterborne pathogens. Sequences are selected following initial screening arrays, and organisms are arrayed in groups to aid rapid visual analysis. Additional bioinformatics are required for detailed strain-level analysis. A *Bacillus* screening assay was developed using 2,000 chromosomal and plasmid unique sequences from both *Bacillus anthracis* and *B. thuringiensis*; 2,000 chromosomal unique sequences from *B. cereus*; and oligonucleotides to 29 different *Bacillus*-specific virulence and/or toxin genes. Informative chromosomal and plasmid oligonucleotides were mapped. Fourteen virulence factors were shared by the three *Bacillus* organisms.

The GenArraytion arrays provide customized levels of specificity, including strain-level, species-level, genus-level, bacterial, viral, and protozoan sequences. GenArraytion's Molecular Radar provides high-fidelity identification and characterization of microorganisms. Resolution has been achieved to the strain-level for pathogens and near-neighbor organisms. The company has the ability to design and validate arrays for any DNA- or RNA-containing organism at the desired level of resolution.

Discussion

A participant asked what was the size of the oligonucleotide. Dr. Schaudies responded that the oligonucleotides were comprised of approximately 35-50 nucleotides, with a maximum size of 50.

A participant commented that the method uses bioinformatics to make the oligonucleotides specific and asked how the array would perform in a dirty matrix where sequences were unknown. Dr. Schaudies

replied that the array had been tested in this manner and some, but not all, of the sequences could be identified.

Dr. Ashbolt asked what was the efficiency of recovery. Dr. Schaudies answered that the array had not been tested on large volume samples yet; all work has been geared toward demonstrating the specificity of the sample.

Dr. Ashbolt asked if the food- and waterborne pathogen array included oligonucleotide sequences for hepatitis D virus as stated on the slide or if it was a mistype and hepatitis E virus was meant. Dr. Schaudies stated that he would investigate that.

A participant asked what the desired platform with which to use the technology would be. Dr. Schaudies responded that any platform that was extremely sensitive and fast would be desirable.

Robust Piezoelectric-Excited Millimeter-Sized Cantilever Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter
Raj Mutharasan, Drexel University

The objectives of this research project are to: (1) experimentally explore and establish piezoelectric-actuated millimeter-sized cantilever (PEMC) sensors suitable for detecting one pathogen in 1 L of water using new cantilever oscillation and measurement modalities, (2) develop a flow cell-PEMC sensor detection assembly for large sample volumes, and (3) develop a PEMC sensor for confirming pathogen identity by DNA signature. Thus far, project researchers have: established the sensitive mode via model experiments with *Escherichia coli* O157:H7 and *Cryptosporidium*; successfully completed 1 L samples using modified flow cell (1 cell/mL); and obtained preliminary results for DNA-based detection of *E. coli* O157:H7. The principles of cantilever sensing are that resonant frequency depends on cantilever's mass, the surface is immobilized with a recognition molecule (e.g., antibody or single-stranded DNA), and when the target attaches to the cantilever, the mass and resonant frequency change. High frequency modes are more sensitive. The platform of the experimental apparatus is a liquid chromatograph. The mass change sensitivity is very high and can measure very small mass changes. Calibration curves of 1-hexadecanethiol self assembly, thiolated 15-mercury, and *E. coli* O157:H7 in buffer illustrate the successful correlation of changes and the reproducibility of results. The system also was tested with *E. coli* O157:H7 in ground beef wash and Cryptosporidium oocysts in tap water.

Following verification of the reproducibility of the method, the Shiga toxin 2 (*Stx2*) virulence factor gene sequence was chosen as a probe. There is one copy of the *Stx2* gene per cell, and *Stx2* and *Stx1* share approximately 56 percent homology. The *Stx2* gene also codes for the Stx2A and Stx2B anti-terminator Q protein. Three different sample preparation methods were used and compared. When comparing probe used in buffer versus ground beef wash, there was a 30 percent reduction in efficiency. A 16S ribosomal RNA (rRNA) probe was used in human serum, and researchers found that the probe worked in a dirty environment. The researchers concluded that the cantilever sensor mass change sensitivity is 1 ag/Hz, and the detection limit of *E. coli* in both buffer and in a proteinaceous (dirty) environment is 10/mL (i.e., in theory, one cell). DNA detection in buffer and serum is feasible without a sample preparation step. In terms of *Stx2* gene-based detection in buffer and beef wash, 4,500 cell detection was demonstrated; but, 100 cells appears to be feasible.

Discussion

A participant asked what the sensitivity would be when there are many pathogens present in the matrix. Dr. Mutharasan replied that the beef wash is a dirty matrix with a significant number of different bacteria. When many non-antigenic bacteria are present, the kinetics of sensor reduces.

A participant asked how dissolved compounds in water (i.e., more complex environments) will affect the technology. Dr. Mutharasan responded that the technology was tested on clinical samples and sensitivity decreased by approximately 10 to 20 percent compared to buffer.

A participant asked if the one-cantilever system can use multiple antibodies for the initial screen. Dr. Mutharasan answered that it can, and this is the desired approach. Commercial applications of the technology will include the simultaneous identification of multiple pathogens.

Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water
Udi Zuckerman and Saul Tzipori, Tufts University

An efficient concentration method is a key to a successful detection of waterborne pathogens. During the last 13 years, researchers at Tufts University have developed a continuous flow centrifugation (CFC) method to accomplish this. The third prototype, the Portable Continuous Flow Centrifuge (PCFC), was developed in 2003. The system was validated for *Cryptosporidium* in EPA-approved test laboratories. Tier 2 validation with *Cryptosporidium* and *Giardia* in reagent and source water indicated that the PCFC had recovery rates from 32.6 to 47.2 percent, well within the Method 1623 acceptable range of mean recovery. The PCFC was approved by EPA in 2004 as a standard concentration method.

The objectives for the current STAR grant are to: (1) simultaneously concentrate representative micro-organisms from each group of the CCL list, (2) validate the concentration methodology through EPA programs, (3) detect and quantitatively identify microorganisms on the CCL list using multiplex miniaturized fiber optic bead microarrays coupled with a compact scanner, and (4) perform side-by-side comparisons of this detection methodology with EPA standard methods. During Year 1 of the grant, researchers focused on expanding the CFC methodology beyond protozoa concentration via the design of a new multiple pathogens bowl; portable, computerized concentration/elution equipment; and a disposable tubing kit. Additionally, programming software was chosen and variable operating protocols were tested. In this method, in contrast to filtration which is based on size exclusion, centrifugal force is applied, and the modified bowl traps the protozoa, bacteria, bacterial spores and algae due to their sedimentation. Then, it allows the "particle-free sample" to flow through the positive-charged component in the core; the viruses are adsorbed by the positive electrostatic forces. Buffers then are injected to release bacteria and viruses. The automated CFC protocol includes concentration, elution, and the concentrate is ready for detection. The recovery efficiency of the automated CFC was tested with 10-L tap water samples spiked with multiple microorganisms, including *Cryptosporidium parvum*, MS2 bacteriophages, and kanamycin-resistant *B. anthracis* spores. The mean recovery rates were 40 (±12.2), 48.1 (±28.2), and 43.6 (±16.4) percent, respectively.

The automated concentrator is a portable, compact, and automatic device that simultaneously concentrates bacteria, protozoa, algae, and viruses and allows for integrated elution. It can process large volumes of water without clogging in a safe-handling, self-contained, and rapid procedure. The disposable kit eliminates the need to disinfect the equipment. The device is cost effective, efficient, and ideal for continuous monitoring. A collaborator currently is working on the bioinformatics of the CCL for microarray detection. Once the detection platform is complete, the automated PCFC concentrates will be tested using this technology, and detection will be compared with currently approved standard methods.

Discussion

A participant asked if the actual time for operating the test was 6 hours. Dr. Zuckerman replied that the system processes at a rate of 1 L per minute. The concentration step takes 3 hours, and the detection step takes an additional 3 hours.

A participant asked if PM was collected via the centrifugation and the bacteria and viruses collected through the filtration matrix. Dr. Zuckerman responded that bacteria, bacterial spores, algae, and particulates are compacted in the bowl of the device, and viruses enter the inner core and attach to positively charged material inside the core. The participant asked if viruses are associated with PM if the viruses would end up in the PM fraction. Dr. Zuckerman answered that if the viruses are bound to large particles, then at least a portion of them would end up in the sediment in the bowl, which is why 100 percent recovery efficiency is not achieved.

A participant asked what amounts of microorganisms were spiked during testing. Dr. Zuckerman responded that 100 *Cryptosporidium*, 22 *Bacillus* spores, and 10^5 to 10^7 MS2 bacteriophages.

A participant asked if the researchers molecularly tested the environmentally spiked water to determine if organic matter could be removed to increase recovery rates. Dr. Zuckerman answered that the system was tested with *E. coli* O157:H7 and a standard microarray system, and up to 20 colony-forming units of *E. coli* could be detected in 10 L of water spiked with 20 to 50 *E. coli* bacteria.

Development of High-Throughput and Real-Time Methods for the Detection of Infective Enteric Viruses
Yu-Chen Hwang, University of California at Riverside

Human enteric viruses are common infectious agents that remain stable in aquatic environments and cause waterborne diseases via fecal-oral transmission. Enteric viruses include adenoviruses, enteroviruses, noroviruses, and rotaviruses. Electron microscopy, enzyme-linked immunosorbent assay (ELISA) for viral antigen, and quantitative PCR (qPCR) do not determine the infectivity of viruses present in water; plaque assays are the gold standard in viral detection and determine infectivity but take several days to complete. Therefore, there is a need to develop a rapid detection method for aquatic environments.

Poliovirus is a nonenveloped, positive-sense, single-stranded RNA virus. Examination of protease activity may be a possible method for rapid detection. Fluorescence resonance energy transfer (FRET) was employed to target protease activity. A cell line was genetically engineered to express a substrate that serves as a cleavage site and expresses protease early in the viral replication cycle. A reporter cell line was chosen that stably expressed the FRET fluorescent substrate. The reporter cells were challenged with poliovirus, and results showed that the reporting system performed comparably to the plaque assay method. Therefore, the researchers showed stable and strong expression of the fluorescent substrate while achieving a detection limit of 1 plaque-forming unit (PFU) within 8 hours postinfection.

A molecular beacon is a single-stranded hairpin oligonucleotide probe with a probe sequence of 10 to 50 nucleotides and a GC stem of 6 to 8 nucleotides. It acts as a fluorophore and quencher and possesses high sensitivity; therefore, it is a highly sensitive method to monitor viral replication *in situ*. Hepatitis A virus (HAV) is a major bottleneck for the industry because there is a long incubation time before it produces visible plaques and some HAV infections do not result in obvious cytopathic effects (CPEs). Using a molecular beacon, the laboratory achieved a detection limit of 1 PFU of HAV after 6 hours postinfection, which is comparable to plaque assay results. This method, however, required extensive post-treatment of the sample, and molecular beacons are prone to degradation and photobleaching.

To address the limitations of molecular beacons, the laboratory modified the molecular beacon to improve its resistance to nuclease activity by replacing the 2' hydrogen with methoxide and modifying the phosphodiester bond. The combination of the methoxy group at the 2' position with phosphorothioate linkage provides greater nuclease resistance. The laboratory determined that the modified probe facilitated cellular delivery of the nuclease-resistant molecular beacon, providing cellular detection of infectious viruses in real time. The future focuses of the laboratory will be real-time monitoring of viral replication in progress and assessing a high-throughput method for viral pathogens.

Discussion

A participant asked if the system actually allows detection within 6 hours postinfection. Ms. Hwang responded that 8 hours is more accurate.

A participant asked how much labor is involved before the start of the incubation period. Ms. Hwang replied that the 8 hours reflects the post-sample processing and not just the incubation period.

A participant asked if the researchers had considered the extent to which the fluorescent signal may give a false positive. Ms. Hwang responded that this had been considered and natural fluorescence patterns are easily distinguished from others.

Dr. Ashbolt asked if the researchers had examined HAV strains that do not form plaques. Ms. Hwang answered that this needed to be addressed.

A participant asked if there had been a direct comparison between the nuclease-resistant and the standard molecular beacons. Ms. Hwang responded that these tests were in progress and the results would be available within the next few weeks.

A participant asked if the researchers had explored quantum dots or gold nanoparticles. Ms. Hwang answered that this was being investigated.

Timely Multi-Threat Biological, Chemical, and Nuclide Detection in Large Volume Water Samples
Paul Galambos, Sandia National Laboratories

There is a need to detect multiple dangerous agents (e.g., chemical, biological, nuclear, and explosive) in dirty samples with high levels of sensitivity and specificity. A bead-based, multiplexed detection method is under development. Can this method be applied to the detection of dangerous microorganisms in water? The magnetic bead chaperone is the key enabling reagent and does not photobleach. Radionuclide detection must be highly acidic for success and the sample must be split. Optical bead reading is used to capture images of two signals, and appropriate software uses information from both images to identify bead location, type, and capture of target antibody. Another key need is the development of a concentrator that will solve the volume impedance mismatch and rapidly identify and confirm large and small volumes.

Testing of a mesoscale trap prototype will involve passing a sample of magnetic beads through the trap and observing the signal intensity measured downstream of the trap with a spectrometer. This technology quantitatively measures trapping time. In the proposed model, time-to-identify (TTI), a new performance metric, is the sum of the collection, mixing, trapping, sensing, and transport times. This TTI model can be applied to the real-life problem of detecting botulism toxin in milk because the milk supply is vulnerable to contamination, perhaps on purpose, between the cow and the grocery shelf. The toxin is smaller and is therefore easier to detect. Per the model, a reasonable amount of sensitivity can be achieved in a short amount of time. Test results indicate that this method allows for rapid and accurate bead-based identification of botulinum toxin substitute in milk. The process took 65 minutes to complete, but the researchers hope to decrease this to 10 to 15 minutes. Modifications to the hardware and procedure so that time is reduced and sensitivity is increased are ongoing in preparation for a field test in July 2007. A dielectrophoretic focus will allow flow-through continuous bead reading. An hand-held system that can analyze large volumes of water is envisioned; input from participants at this workshop is sought in this endeavor.

In conclusion, bead-based detection of botulinum substitute in milk indicates the utility of the concept for liquid-based dangerous agent detection in dirty liquids. The system concept is adaptable to many problem

scales, from the macroscale to the nanoscale. Additionally, there is an opportunity to adapt partially developed bead-based detection systems to high-throughput sensors for microorganism detection in water.

Discussion

A participant asked what was the upper limit of multiplexing. Dr. Galambos responded that it can be millions with 10 different colors at 10 different levels. The upper limit is not restrained by the number of barcodes. With quantum dots, the number is practically unlimited.

A participant asked what volume the researchers speculate will be used in the final product. Dr. Galambos replied that currently it is working at the milliliter level and could be expanded to 1 L without much difficulty.

A participant commented that water often contains magnetic particles and asked how this would affect the method in terms of detection. Dr. Galambos answered that the researchers built in several stages to eliminate false positives. A participant asked if researchers tested the system with magnetic particles. Dr. Galambos replied that it had not been tested intentionally but had been tested with raw milk, urine, and blood. A participant commented that the method is based on capture by quantum dots and identification by fluorescent signal, and magnetic particles should not be a problem.

A participant asked how this method was similar to the Luminex system. Dr. Galambos responded that he had not run a direct comparison with the Luminex system and added that this method was different than the Luminex system in a variety of ways.

SESSION 3

Moderators: Sam Hayes, U.S. EPA, ORD, NRMRL; and Shay Fout, U.S. EPA, ORD, NERL

On-Chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens

Syed Hashsham, Michigan State University

Many parameters are involved in developing detection methods: quantitation, sensitivity, cost, speed, sample throughput, and target multiplexing. Currently, it is not possible to incorporate all parameters into every detection method. One method to detect pathogens is a microfluidics DNA biochip. There are no false positives because each pathogen is confirmed by three to six different virulent and/or marker genes and each gene is confirmed by five to 20 probes. Multiplex PCR amplification followed by DNA chip-based amplicon identification can enhance sensitivity. The Functional Gene Pipeline Repository (FGPR) harvests functional genes from GenBank using a Hidden Markov Model; training sequences chosen by experts are put into the system, and matching sequences are the result.

The laboratory designed assays and chips to include genes for antibiotic resistance, indicators, and virulence to examine all of these in environmental samples. Multiple samples were surveyed and screened for the presence of antibiotic resistance, virulence, and indicator genes from various reclamations systems and treatment facilities; correlation between pathogens and indicators with different hosts; release into surface waters and possible persistence; and host-specific markers. BioTrove OpenArray™, a high-throughput assay screening tool, also was used by the laboratory. Indicator screening results showed temporal changes in raw wastewater and provided a fingerprint of fecal samples from various hosts.

The researchers asked if quantification is possible without standard curves. An experimental cycle threshold (Ct) value was obtained using the genome size of the target organism, the GC content of the target organism's genome, the theoretical melt temperature of the last seven bases of the primer's 3' end,

and the amplicon length. The resulting curve is used to determine the amount without a standard curve. The predicted Ct versus theoretical Ct is based on an empirical equation.

The next direction the laboratory will take is dye-doped nanoparticle-based detection. Tests indicate that nanoparticle-based technology results correlate with standard growth curves. The nanoparticle-based technology also is faster than cell-based reverse transcription (RT)-PCR, DNA-based RT-PCR, absorbance, and plate counting. Silver and gold nanoparticle-based technologies also may decrease the cost of visualization. The detection platform for the technology is the universal challenge. Another research question is whether populations, including the unknowns, and genetic markers can be screened in parallel. It is possible that 1-minute micro-PCR will be available in the future, but it currently is a long way off.

Discussion

Dr. Sobsey asked how screening for genes in water merely based on the extraction of nucleic acids can inform about the risks from infectious pathogens. The organism is the important factor; detection of genes must still be groundtruthed. Dr. Hashsham responded that this is a very broad and very fast screening tool that is the first step in identifying infectious organisms. Dr. Sobsey commented that it is important to link these nucleic acid-based analytical tools to traditional culture methods.

A Novel Molecular-Based Approach for Broad Detection of Viable Pathogens in Drinking Water
John Scott Meschke, University of Washington

Current methods for direct detection of pathogens in drinking water are limited by sensitivity, breadth of detection, speed, viability, and their ability to quantify. It is necessary to consider the method as a whole. The laboratory's approach includes four CCL2 microbes (echovirus, adenovirus, *M. avium*, and *Aeromonas*). Steps include concentration and nucleic acid extraction and purification followed by a novel approach to detection that includes whole-genome amplification (WGA), whole-transcriptome amplification (WTA), or a pre-rRNA approach. Archiving is the last step of the process. The method is being used in parallel with benchmarks to determine its accuracy. Hollow-fiber ultrafiltration (HFUF) is the best method for concentration but requires a grab sample. FTA® cards are used for archiving because they contain chemicals that lyse cells, denature proteins, and protect nucleic acids from nucleases, oxidation, and UV damage. Growth of bacteria and other microorganisms is also inhibited on the cards. Nucleic acids collected on FTA® cards are stable for years at room temperature, and because they are stored at room temperature before and after sample application, there is a reduced need for laboratory freezers.

Two WGA approaches include multiple displacement amplification (MDA) and the OmniPlex® library. Qiagen's REPLI-g UltraFast Mini Kit was used to perform the MDA. This method avoids the high sequence bias of PCR-based amplification methods as well as the fragmentation of template DNA caused by heat denaturation methods. The OmniPlex® library provides an amplified genomic library of randomly fragmented DNA. Preliminary results indicate that WGA approaches significantly improve the sensitivity for *E. coli*, *M. avium*, and adenovirus types 2 and 41. There is an improvement of a minimum of 3 to 4 Ct units in detection in a clean system; factoring in volume differences up to a 2,000-fold increase in detection is achievable. In a dirty system (lake water), similar results (3 to 4 Ct units) were obtained for 100 mL of mock extract. When the volume was increased to 500 mL mock extract, the results achieved approximately 2 Ct units improvement. Sigma's TransPlex™ kit was used for the WTA approach. Preliminary results were achieved with echovirus. The pre-rRNA approach involves the design of RT-PCR methods that target the region bridging the mature rRNA and the 5' leader region, providing short-term enrichment. Preliminary results have been obtained with *M. avium* and *Aeromonas*.

In summary, sensitive methods include HFUF concentration, WGA, and WTA. Methods that increased breadth of detection include WGA, WTA, and FTA® archiving. In terms of speed, all discussed methods were rapid but limited by concentration and purification. The pre-rRNA approach for bacteria and a nascent

strand approach for viruses are good methods when considering viability, and qPCR has the ability to quantify.

Discussion

A participant commented that presumably there is one copy of pre-rRNA per ribosome and asked if this was the leader sequence for the ribosome. Dr. Meschke responded that there is much more mature rRNA than pre-rRNA because the pre-rRNA degrades more quickly and is more ephemeral.

A participant commented that FTA® cards originally were designed to store blood and asked for confirmation that the researchers were using them to store water samples. Dr. Meschke confirmed this and added that they are suitable for storing nucleic acid extractions.

A participant asked if any pre-rRNA induction studies have been completed that investigate how the response differs in a resting cell. Dr. Meschke answered that some cells were given a dose of nutrients and some cells were starved.

A participant asked whether induction is needed for the pre-rRNA. Dr. Meschke replied that there is a short enrichment step of nutrients in broth.

A participant commented that to induce different pathogens different media is needed and asked if this had been pursued. Dr. Meschke agreed that this is true and plans to investigate this.

Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis
Anthea K. Lee, Metro Water District of Southern California (MWDSC)

The MWDSC is a consortium of 26 cities and water districts that provides water for more than 18 million Southern Californians, delivering an average of 1.7 billion gallons of water daily. Southern California receives 42 percent of its water from local sources, including the Los Angeles Aqueduct, and the remainder is imported; the MWDSC mostly manages imported water. There are many storage locations across a wide region, and water quality may be quite different across locations. The MWDSC manages five different treatment plants around Southern California, each with different percentages of local and imported water. The MWDSC Water Quality Laboratory monitors influent, effluent, and distribution waters from all treatment plants and makes treatment recommendations as necessary. Its two main functions are to: (1) test (EPA and non-EPA-regulated) chemical and microbial components; and (2) develop new pathogen detection technologies, including improvements and modifications to existing methods. The purpose of this research is to detect very few pathogens in very large volumes of water using and optimizing molecular tools.

The laboratory used standard *E. coli* K12 for its proof-of-principal experiments. The experimental design included comparison of ultrafiltration, DNA extraction, WGA approaches, and microarray detection of *E. coli* in 100 L of water. Previous data indicated that the mean recovery efficiency of ultrafiltration was 57 to 86 percent, depending on the organism, with little to no breakthrough. Ultrafiltration efficiencies can be improved to greater than 80 percent with blocking, elution, and possibly an additional concentration step. The laboratory used four brands of kits to test three different WGA techniques: random fragmentation (one kit), degenerate oligonucleotide primer-PCR (one kit), and multiple strand displacement (MSD; two kits). The two MSD kits, Qiagen's REPLI-g UltraFast Mini Kit and G.E. Healthcare's Illustra Genomiphi V2, performed well. According to the results, the best kit was the REPLI-g UltraFast Mini Kit. A microarray is an ordered array of oligonucleotides affixed to a solid surface with unique sequences that act as probes to hybridize with the fluorescently labeled target nucleotide. The microarray allows the bias and completeness of WGA approaches to be tested, and cognate mismatches allow for background subtraction.

With the new STAR grant, the laboratory will apply the described techniques to model pathogens (e.g., human adenovirus 2, *C. parvum*, *Salmonella typhimurium*), confirm infectivity of concentrated pathogens, and design a custom microarray for waterborne pathogens that includes CCL organisms, toxigenic *E. coli*, *Legionella*, *Campylobacter*, *Salmonella*, *Shigella*, *Yersinia*, fecal indicators, *Cryptosporidium*, and *Giardia*. The ultimate goal is to develop a single assay to detect and identify low numbers of multiple pathogens.

Discussion

A participant suggested that the researchers ensure that there are no restriction sites within the 40 mers that are on the array. Dr. Lee replied that this was going to be completed.

A participant asked if the researchers had considered examining the effect of the time that the *E. coli* is in the water because the presence of chlorine could affect the results. Dr. Lee responded that sodium thiosulfate is added to neutralize the chlorine. The researchers have performed viability experiments with *E. coli* K12 and have determined that there is little loss of viability over time.

A participant asked what the researchers plan to do regarding infectivity. Dr. Lee answered that she has an interest in researching infectivity sometime in the future to confirm that the infectious agent that is used at the beginning is what is present at the end.

Simultaneous Concentration and Real-Time Detection of Multiple Classes of Microbial Pathogens From Drinking Water
Mark Sobsey, UNC–Chapel Hill

The objectives of the research project are to: (1) develop and field evaluate new and improved methods to concentrate viruses (and possibly other classes of pathogens) in water of different qualities with regards to particulate and dissolved organic matter; (2) develop and evaluate secondary concentration for post-filtration sample processing; (3) optimize viral nucleic acid amplification by real-time PCR for rapid detection of low virus concentrations in drinking water samples; and (4) provide the developed methods to selected water virology laboratories for collaborative testing of the methods to validate their performance. The researchers are supplementing the development and testing of positively charged glass wool filter material by testing commercially available Argonide NanoCeram® positively charged alumina fiber filters, which have many advantages.

The initial focus of the laboratory was HFUF, using two in a series to improve throughput. Experiments compared microbe recoveries with and without overnight pretreatment of HFUFs using eluting solution and found no significant differences in microbial recoveries. Experiments with no pretreatment, however, had significantly shorter processing times than those with pretreated HFUFs. Three eluting solutions were tested for enhanced microbial recovery, and no statistically significant differences for recoveries were achieved by any of the eluting solutions tested. Multiple classes of relatively low concentrations of microbial indicators and pathogens were recovered from dechlorinated drinking (tap) water. Adsorption efficiencies of different adenoviruses to MDS filters from surface and tap water were compared. The recovery rates for surface water were low, so impurities in water that decrease efficiencies must be addressed. Researchers investigated the adsorption and elution of adenovirus 2 and 41 using NanoCeram® flat filters and determined that absorption rates were good, but virus recovery was low. Adenovirus recovery following adsorption to and elution from NanoCeram® electropositive microporous filter medium was good with the exception of surface water.

The researchers have experienced variable adenovirus and norovirus recovery by polyethylene glycol (PEG) precipitation, so they are exploring modifications of the method and investigating centrifugal ultrafiltration, HFUF, and immunoaffinity and cell receptor-mediated virus capture. The researchers also

will further evaluate real-time PCR with adenovirus types 2, 5, 8, 40, and 41. Standard curves for different adenovirus concentrations will be developed, and lower detection limit and amplification efficiency will be evaluated. The researchers also plan to examine the relationship between Ct value in real-time PCR and infectivity in cell culture.

Thus far, the laboratory has demonstrated good recovery of viruses by HFUF, variable virus recovery by MDS and Argonide electropositive adsorbent filters, and variable recovery by PEG precipitation. A potentially improved elution medium, protein hydrolysate, is being examined. Real-time PCR for adenoviruses and noroviruses is being optimized and calibrated.

Discussion

Dr. Ashbolt asked if the researchers are examining synthesizing their own targets as a positive control or if they are investigating commercially available products. Dr. Sobsey responded that the researchers are interested in both. The goal for norovirus and adenovirus is to develop a suitable product to add to commercial product lines.

A participant asked why some recoveries were 7 percent and some were 40 percent. Dr. Sobsey explained that he did not have a good answer for this and that it possibly was laboratory error.

A participant asked if the elution rates had been verified. Dr. Sobsey replied that this work was forthcoming.

A participant asked if direct microscope counting was used. Dr. Sobsey answered that direct microscopy or other approaches, including hybrid approaches, were used when culture is not the best approach.

A participant asked if it was possible that their method damaged the cells, possibly explaining the loss of recovery. Dr. Sobsey replied that the filters used are for blood filtering, but it is possible that some damage is occurring. It may be feasible to stabilize and protect the cells so that this does not happen.

POSTER SESSION

The participants took a 1-hour break to view the project posters in the main atrium of the Andrew W. Breidenbach Environmental Research Center.

SESSION 4

Moderators: Nichole Brinkman, U.S. EPA, ORD, NERL; and Hiba Ernst, U.S. EPA, OW, OGWDW

Identification of Bacterial DNA Markers for the Detection of Human and Cattle Fecal Pollution

Orin Shanks, U.S. EPA, ORD, NRMRL

According to the National Water Quality Inventory 2000 Report, fecal bacteria are the most common biological contaminants. Current recommended water quality monitoring criteria for fecal pollution include the use of microbial fecal indicators such as E. coli and enterococci that represent fecal pollution events and measure fecal bacteria from multiple animal sources. In the United States, it is estimated that 1 billion tons of fecal matter are produced each year; less than 1 percent is attributed to humans. The main source are poultry (44%), cattle (31%), and hogs (24%). These estimates do not reflect additional wildlife fecal production.

Microbial source tracking matches microbes from a polluted site with animal sources to suggest the origin of fecal pollution. The research goals of this project are to: (1) identify human- and cow-specific bacterial

DNA markers, and (2) develop PCR-based assays with the potential for microbial source tracking applications. To accomplish the first objective, researchers have adopted a metagenomic approach that characterizes differences in total microbial DNA obtained from animal fecal specimens. The literature suggests that false positives occur approximately 50 percent of the time, with some estimates at 80 to 90 percent. To improve these rates, genome fragment enrichment (GFE) was used to simultaneously compare two complex microbial communities. GFE allows the generation of an enriched DNA library from which DNA sequencing can occur. Results show that sequenced DNA clones obtained by GFE have 17 percent redundant sequences and 2.3 percent false positives, much lower than traditional methods; this specificity was confirmed via dot blots.

To complete the second objective, now that a list of DNA sequences with a low rate of false positives has been generated, target selection must occur. Bacteroidales-like DNA sequences were chosen as targets because Bacteroidales bacteria are abundant in feces, obligate anaerobes, and increasingly used in microbial source tracking. Functional annotation reinforced the original goal of examining novel genes. Host-specific (e.g., human and cow) PCR assays were tested for specificity and spatial robustness. Pilot studies of human- and cow-specific fecal pollution from environmental samples have begun. Results indicate that host-specific PCR assays are specific for target animal groups, show a broad distribution among target samples, and can detection fecal pollution from the environment. In the future, microbial source tracking may be applied to determining target abundance in fecal samples, examining the survival of the target DNA in the environment, identifying its relevance to current culture-based methods, and linking the presence of target DNA to public health risks.

Discussion

A participant asked if the researchers knew the identities of the discovered sequences. Dr. Shanks responded that it had been found via functional annotation and one marker has extracytoplasmic polymerase function.

A participant asked if the number of targets will be sufficient given the large number of animal species. Dr. Shanks answered that currently the researchers can only work with the reference collection that is available. Efforts are being made to increase the size of the reference collection, but the targets do not have to be totally specific. For example, shorebirds (e.g., ducks, geese, seagulls) share a certain amount of homology.

Dr. Ashbolt asked if the researchers had examined juvenile fecal matter; *Cryptosporidium* is an important organism in this regard. Dr. Shanks replied that this had not been examined currently, but researchers are developing a tool to address these questions more thoroughly because these are important questions.

Dr. Sen asked if the PCR was single or multiplexed. Dr. Shanks answered that it was single PCR with 29 different primers.

Detection of Waterborne Pathogens Using Real-Time PCR and Biosensor Methods
Sangeetha Srinivasan, Michigan State University

There are numerous potential waterborne pathogens for which detection methods are needed, and rapid assessment is crucial during and after disasters and weather-related events. Additionally, specificity and sensitivity need to be evaluated in real-world samples. Although molecular methods have seen great advances in clinical applications, there has been limited assessment for water applications. The researchers are using *E. coli* O157:H7 as a result of lessons learned in previous outbreaks; Another emerging potential waterborne pathogen is *H. pylori*. The WHO has classified *H. pylori* as a Class I carcinogen, and research indicates that there is a significant correlation between well water contaminated with *H. pylori* and colonization status in humans using that water. The researchers have chosen to focus on *H. pylori* in

addition to *E. coli* O157:H7 because it is difficult to cultivate and identify via conventional techniques, it exists in small numbers and has a slow growth rate, and it can transform into a viable-but-non-culturable (VBNC) state. The objectives of this research project are to: (1) contrast two rapid molecular methods for the detection of pathogens in water, (2) develop a real-time qPCR assay, and (3) develop a nanowire-enabled antibody-based conductometric biosensor.

The advantages of qPCR are that it allows for the reliable and exact quantification of bacteria and has high specificity, sensitivity, and objectivity. The *H. pylori* target sequence was the *vacA* gene fragment, and SYBR® green was used. When the efficiency of the method was tested, results indicated that 90 percent of the *H. pylori* was recovered from the pure culture and 15 percent from wastewater. The results were negative for *E. coli*, *E. faecium*, and *Campylobacter jejuni*, indicating the method's specificity. In a 1-year qPCR survey of wastewater, 86 percent of sewage samples were positive for *H. pylori*. The melting curve showed good reproducibility and a consistent melting temperature value in each run. Results show that the method is highly sensitive (100 times more so than conventional PCR) and specific. The complete protocol, from sample processing to data analysis, is completed within 5 to 6 hours.

A polyaniline nanowire biosensor has been developed that reports the binding event between antigen and antibody and is suitable for field based detection because it is rapid, sensitive, highly specific, reagentless, disposable, and inexpensive. The microbial targets for this technology are *H. pylori* and *E. coli* O157:H7. The biosensor is comprised of cellulose, fiberglass, and nitrocellulose membranes. The detection mechanism includes application of the antigen followed by conjugation, capture, and absorption. Previous data on biosensor response are promising, and further investigation will be conducted.

Future projects for the laboratory are to: (1) design a qPCR experiment with new primers for the detection of virulence genes in *E. coli* O157:H7; (2) examine manure, sewage, and surface and groundwater in seeded experiments for sensitivity and interferences; (3) examine the need for preprocessing of samples for both biosensor and qPCR; and (4) compare qPCR and biosensor techniques for naturally occurring detection in the environmental samples.

Discussion

A participant asked what the measurement was of the gap between the cathodes. Ms. Srinivasan replied that she would ask the PI, Dr. Evangelyn Alocilja, and forward the information to the participant.

Dr. Schwab asked if a cost analysis, including additional equipment, had been completed and if SYBR® green truly was less expensive than Taqman®. Ms. Srinivasan responded that this had been completed and SYBR® green was less expensive. A participant commented that the cost of the various components have very little to do with the overall cost of the method; licensing, patent, and royalty costs are significant.

Microarray Detection of Human Viruses From Community Wastewater Systems
Mark Wong, Michigan State University

The objectives of this research project are to: (1) select gene targets for microorganisms of interest to water safety, (2) design probes, (3) synthesize microfluidic biochips, (4) validate and field test the synthesized biochips, (5) undertake a pilot risk analysis, and (6) investigate viral and bacterial indicator targets. Because 65 percent of groundwater outbreaks and 25 to 50 percent of recreational freshwater outbreaks are caused by viruses, the laboratory has decided to focus on viral pathogens. Viral detection methods currently available include culture, direct observation, and antigen and molecular detection. Microarray is used because it is a high throughput method that allows for multiple pathogen detection with no target bias. The method is culture independent, and the results can be read off of a computer. The chip layout includes approximately 30 probes per target group with five copies per probe. The latest version has 27 viral targets. RNA and DNA viruses are labeled separately and display two different signals.

Tests of the technology indicate that some probes are cross reactive (adenoviruses 40 and 41) but closely related. When this method was used to analyze raw sewage in Michigan from October through January, many viral probes showed positive results. This is explained by the enhanced survival of viruses in cold weather or the increased secretion of seasonal viruses. There was a significant number of astroviruses, which were not thought to be a significant viral threat. Both DNA and RNA viruses displayed a seasonal trend. Human polyomaviruses were detected, and multiple adenovirus types were present, especially adenoviruses 41, B, and D. No single virus group, however, was ubiquitous. Torovirus, hepatitis E virus, enterovirus A, enterovirus D, and astroviruses were present in every sample taken. Astroviruses were the most frequently detected viruses in the raw sewage. Adenovirus 41 was the most frequent DNA virus seen in the experiment.

Thus far, the laboratory has designed probes for major waterborne viruses, tested microarrays tested against one RNA and two DNA viruses, and profiled sewage for viral seasonality and relative proportions. Future work will include analyzing additional sewage samples to determine if the seasonal trend continues, determining the method's sensitivity using spiked sewage samples, corroborating signals using other methods, and analyzing further matrices.

Discussion

A participant commented that the enterovirus detection that the researcher found was the opposite of what is seen clinically (i.e., increased enterovirus disease incidence in summer, decreased disease incidence in winter) and asked how this could be explained. Mr. Wong responded that the cell culture method used preferentially selects for enterovirus, so its presence may be overestimated. Another possibility is that more viruses are excreted than previously realized or that the cold conditions protect the virus in sewage.

Dr. Sobsey asked how the researchers explain the capability of certain viruses to grow when using cell culture as a method. Mr. Wong replied that this is a good question, and carryover is one possibility. Some viruses cannot be cultured because it is not recognized that infection is taking place as a result of the lack of CPEs.

Dr. Sobsey recommended that the researchers investigate pig, poultry, and cow sewage and perform the same research process. Mr. Wong answered that the researchers had plans to this.

Dr. Schwab asked if the results indicated any agricultural impact. Mr. Wong responded that if there were impacts, they are limited.

Dr. Schwab asked whether the researchers expected to see hepatitis E virus. Mr. Wong answered that they were surprised but did determine that it is human-specific hepatitis E virus.

A participant asked about the ability of microarrays to quantify. Mr. Wong replied that microarrays will not generate quantitative data except in the roughest sense (e.g., random sampling). It will not tell how many viruses are in the sample.

Quantitative Assessment of Pathogens in Drinking Water
Kellogg Schwab, Johns Hopkins University

Enteric bacteria generally have a moderate-to-low infectious dose, have moderate-to-low resistance to environmental conditions and chemical inactivation, and are intermediate in size (0.5-2 µm). The bacteria are frequently particle associated under ambient conditions. Enteric protozoa generally have low infectious doses, significant resistance to environmental degradation and chemical inactivation, and are relatively large in size (2–10 µm). Protozoa can be freely suspended or associated with biofilms. Enteric viruses generally have a very low infectious dose, are resistant to environmental degradation and chemical

inactivation, and are very small (20–100 nm). Virions are negatively charged at an ambient pH. Although large numbers of pathogens can be shed from ill individuals or animals, microbial concentrations in surface or groundwater typically are quite low. It is unknown how many pathogens are present in an environmental sample, but it is assumed that there are not many; the goal is to be able to detect one while concentrating and purifying large volumes of water.

The researchers are performing viral elution using 3 percent beef extract at a pH of 9.5. The working sample volume for molecular detection is approximately 100 µl, and MDS filtration/elution recovery efficiency ranges from 30 to 60 percent. Two methods of microbial recovery from water include ultrafiltration and PEG precipitation. In concentrating inhibitors, environmental samples are more complex than laboratory samples and are comprised of complex matrices. No matter which concentration and elution techniques are used, inhibitors are also concentrated in most instances, and multiple inhibitors are common. Inhibitors must be removed, inactivated, or diluted for successful microbial detection. Most filtration methods recover intact, viable microorganisms, and the subsequent sample purification depends on the detection method (e.g., intact infectious microbes, nucleic acids).

Classic viral detection methods include electron microscopy, immunoassays, and cell culture. Cell culture is part science and part art. Viruses amenable to cell culture can reflect infectivity, but there is no universal cell line. Because many human viruses of interest do not replicate or replicate poorly in tissue culture, it can take days to weeks to obtain results. Many systems have integrated cell culture with molecular detection methods, including PCR. PCR is very sensitive, and performing multiplex PCR can result in masking low levels of one target by the presence of high levels of a second target. qRT-PCR is becoming a standard molecular technique for detection. Proteomics techniques, including matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS), also can be used as pathogen detection methods. These methods are useful in determining the infectious nature of the detected microorganisms using specific protein fragments present in intact, infectious virions. MALDI-TOF-MS has the ability to detect the capsid of virus-like particles. Future research directions using MS will focus on quantitation, speed, and accuracy, and multiplexed FISH as a method of detection also will be investigated. rRNA will be used as a target because denaturation is not required, ribosomes are present in multiple copies, and regions of rRNA range from highly conserved to highly variable.

In summary, low levels of microorganisms in environmental samples require concentration and subsequent recovery, and cell culture is one of the few existing methods that addresses infectivity. Molecular assays can be rapid and automated but are limited by the lack of information regarding infectivity. qRT-PCR can quantify target levels, but the use of appropriate standards and critical analysis of Ct values are essential. Proteomics has great potential for detection of multiple targets if the sensitivity can be improved.

Discussion

A participant asked how reproducible is the laser desorption in this study. Dr. Schwab responded that it is highly reproducible if care is taken to ensure that the sample is included in the matrix and the volumes are accurate. The volumes used in these experiments were in the range of microliters, and the concentrations are in the clinical range.

Dr. Meschke commented that it is impossible to achieve 100 percent recovery. Each step of a process has its own recovery rates, and it is not possible to additively determine a percent recovery or percent detection at the end of a method. He suggested using distributional data and probabilistic approaches to determine what the results really mean.

Dr. Sen asked how a baseline is set to compare the PCR results. Dr. Schwab responded that each template used has an algorithm that determines the baseline fluorescence. It is necessary to be cognizant about the capabilities of the methods, especially because these methods were developed for clinical and not environ-

mental uses. It is important to normalize to the standards and examine the standards for accuracy before performing the experiment.

Dr. Hashsham asked what is being done to improve the limit of detection. Dr. Schwab responded that the samples are concentrated at the beginning of the process so that they can be decreased to a small volume by decreasing the buffer.

JUNE 20, 2007

Recap and Overview of Pathogen Research in the Microbiological and Chemical Exposure Assessment Research Division of NERL

Ann Grimm, U.S. EPA, ORD, NERL

Dr. Grimm summarized the previous 2 days' talks, stating that several common themes ran through the presentations, all of which related to the need for better methods. Some of these themes included the need for better sample preparation procedures; for more rapid real-time monitoring methods; for detection methods that could specifically quantitiate and speciate microoganisms; and for detection assays that specifically detected viable and infectious pathogens. In addition, there was the need for the development of technologies that would lead to high throughput, inexpensive, methods that could identify multiple pathogens.

Dr. Grimm then gave a short presention on the work done in MCEARD on innovative methods. To first understand MCEARD's role in the USEPA, particularly in the Office of Research and Development, she described how the Laboratories and Centers of ORD were organized around the risk assessment paradigm. This paradigm includes four steps: (1) problem formulation, (2) analysis, (3) risk characterization, and (4) risk management. In terms of analysis, the two important items to characterize are pathogen exposure as well as the resulting health effects. The Laboratories and Centers in ORD are tasked with these different research activities. Specifically, the National Center for Environmental Assessment is responsible for problem formulation and risk characterization. Analysis is performed by NERL (exposure research) and the National Health and Environmental Effects Research Laboratory (health effects); it was also noted that much of NHSRC's research is related to analysis, although they also conduct risk assessment and risk management activities. Finally, NRMRL carries out risk management research. The Drinking Water and Water Quality MYPs guide ORD's drinking water research.

As indicated in the paradigm, NERL studies the impact of stressors on people and/or the environment, and MCEARD research specifically focuses on pathogen or chemical stressors that impact public health. MCEARD is organized into three technical branches (Biohazard Assessment Research, Microbial Exposure Research, and Chemical Exposure Research) as well as one administrative branch. MCEARD has more than 35 scientists that conduct or support research in environmental microbiology, making it one of the largest single groups focused on this topic in the world. Pathogens of interest include parasites, bacteria, viruses, and fungi. Projects are divided into the areas of occurrence and exposure and for the most part focus on method development. Occurrence projects include ones on improving sample collection and concentration procedures, and others related to pathogen detection and typing assays (e.g., PCR/qPCR, nucleic acid sequence-based amplification, microarrays, proteomics and culture assays). Exposure projects include the development of new methods to detect exposure, dose-response studies using animal models, and studies of cellular responses to infection. Potential future research directions for the Division include the study of microbial source tracking, prions, indicators, viability determination, animal-borne pathogens, method validation and emerging pathogens. This would be in addition to continued efforts to improve occurrence methods and develop new exposure methods.

Panel Discussion

Co-Leads: Keya Sen, U.S. EPA, OW, OGWDW; and Ann Grimm, U.S. EPA, ORD, NERL
Panel Members: Parke Rublee, UNC–Greensboro; Mark Sobsey, UNC–Chapel Hill; J. Scott Meschke, University of Washington; and Syed Hashsham, Michigan State University

Dr. Sen began the discussion by asking the panelists if true real-time monitoring is possible. Dr. Meschke responded that he did not believe so given the current state-of-the-science. Even with the best detection methods, exposures occur; treatment options may be a better area to explore. Dr. Sobsey added that the answer is dependent on what is being measured. If the goal is to measure a predictive item instead of the microbe itself, then it is possible. Microbes cannot be measured immediately, easily, or reliably in all cases, but measurement of chemical indicators is a possibility. Additionally, it should be determined if researchers are exploring this avenue adequately, including examining analytes that are predictive of risk and incorporating them into future monitoring methods.

Dr. Rublee commented that many real-time monitoring methods utilize physical and chemical parameters (e.g., chlorophylls associated with cyanobacteria). Real-time monitoring is partly an engineering problem, and he encourages the interaction and coordination of engineers and biologists. Combining molecular techniques with the emerging nanoparticles field may result in progress toward real-time monitoring. The field of microfluidics also is a source of optimism. Although real-time monitoring is not available now, he is optimistic that it will be forthcoming.

Dr. Jerry Stelma commented that distribution systems to important buildings in the United States have real-time monitoring in case of terrorist activity. Immunoassays can measure large numbers of specific pathogens, but in dealing with everyday drinking water hazards (e.g., cross-connection, treatment failure) there are significantly fewer organisms present for detection. Concentration and viability are the issues that researchers must manage.

Dr. Hashsham commented that the barriers are not the tools but the decisions. PCR can be performed in 6 minutes, but the water industry has no impetus to perform this type of monitoring. They ask, "Why should we perform this type of monitoring and provide it quickly? What would we do if we found something?" There is no legal requirement for them to monitor their water supply in real time. True real-time monitoring would occur before water is consumed, but monitoring before a person is at risk also can be considered real-time monitoring. Therefore, this type of real-time monitoring is possible. Dr. Stelma added that the two issues to influencing the water industry to use this type of monitoring are expense and body count. It is expensive to perform these tests, and the number of people who become sick as a result of contaminated drinking water is relatively low.

Dr. Sobsey stated that if the goal of monitoring is to reduce risks, then the system and the water source must be known to inform measurement decisions and allow better risk assessment. Designing analytical systems is based on the supporting framework. Epidemiological information is necessary to this framework, so more effort should be placed in surveillance. He described the Virus Watch Program and routine measurements of viruses in sewage that occurred more than 40 years ago. He recommended that researchers read "Viruses in Families" (Fox JP, Hall CE. *Lancet* 1971;1(7711):1240-1241). Community risks must be known, and examining sewage and determining what is present will allow this community-level knowledge. Analytical capabilities and the ability to increase the concentration of microorganisms have made real-time monitoring a possibility. Opportunities are being missed; there are many things that can be done now, but more analysis is necessary.

Dr. Schaudies commented that it is necessary to determine the worth of real-time monitoring and asked what EPA could do to help this. Another participant commented that it would be useful to know what concentrations are needed to detect microorganisms and at what level testing needs to be done.

Dr. Meschke added that it is important to determine if the goal is to detect an exposure that has already occurred or if the goal is to prevent an outbreak. Real-time monitoring implies that prevention is the goal. Researchers cannot think of indicator methods in a vacuum and should determine what the data needs are before proceeding.

Mr. Wong stated that thresholds tend to be probabilistic, so it is difficult to attain a firm "line" above which one can say exposure to levels indicate sickness and those below do not. Additionally, some microbes have long-term sequelae (e.g., diabetes from adenovirus) where acute illness is not seen, and chronic illness is not seen until many years following exposure. A standard target dose concentration may not be obtainable. One possibility is to address what dose indicates chronic disease versus what dose indicates acute illness. Many microbes may cause illness at any dose.

Dr. Sobsey commented that food-borne outbreaks happen constantly and are not being managed well. Levels of pathogens in food are not known, and there is no mechanism in place to immediately collect contaminated food and analyze it. Opportunities for receiving better data from outbreaks are being missed. Currently, beach studies are following human swimmers to measure pathogens in water and determine illness from these exposures. A clinical diagnostic component should be added to these studies. It may be possible to mitigate illness if this information is obtained, but without the clinical data, there is a large gap in the knowledge. Dr. Meschke added that water may not be the only route of infection for many pathogens. The cost versus risks and benefits to any study, including the beach studies, must be calculated, especially as food-borne illness is much more common than waterborne illness; the hot dog cart may be a confounder to the beach study.

Dr. Sen asked what the current opinion was regarding animal model studies. Dr. Sobsey indicated that he was in favor of such studies. Some pathogens will never be approved for human exposure studies (e.g., hepatitis A virus and hepatitis E virus), so data must be acquired in another manner. Primate models or natural exposures are two methods by which to determine dose response rates. The caveat is that it must be confirmed that the animal models mimic the disease etiology in humans. Dr. Stelma added that rat models are overused and models more closely resembling humans must be developed. Dr. Sobsey agreed and stated that animal use in experimental toxicology is not fully appropriate but necessary because carcinogens cannot be given to humans. The data are extrapolated and used by EPA for decisionmaking. Dr. Stelma added that researchers attempted to remove *Aeromonas* from the CCL, but the mouse model is not accepted by risk assessors.

Dr. Chuanwu Xi commented that risk assessment also differs when dealing with a population, because there will be immunocompromised members of any population. Disparity among individuals must be considered in the development of models and thresholds.

Dr. Meschke stated that the real questions are: What do we want to answer? How much resolution is needed in the dose response data if the exposure data are better? Is it possible to develop a sensitive animal model that represents a sensitive case?

Dr. Parshionikar commented, in regards to the earlier discussion about chemical indicators, that under the Ground Water Rule coliphages and coliforms are being examined, as well as other indicators. Dr. Sobsey responded that he was pleased that coliphages had been added to the Ground Water Rule, but he would like to see more indicators. Researchers must look beyond traditional indicators and consider others that will improve analytical methods. There may be useful chemical indicators of fecal contamination that can be adapted to meet the needs of microorganism detection in water and incorporated into the toolbox of analytical tools.

Dr. Rublee commented that cheaper, easier, and faster methods of detection are good for researchers, but political considerations at the utility level also must be taken into account, including the burden being

placed on a water quality laboratory at the municipal level. It is not part of such a laboratory's mandate to find unique organisms in the water and identify them. The goal should be to place multiple diagnostics in a single test, creating a dual-use test that also tests for items that taxpayers consider important.

Dr. Sobsey stated that one goal should be to determine analytes associated with a pathogen's infectivity. It is possible that the wrong organisms have been investigated. *E. coli* may not be the best target because it is not a "superbug"; enterococci and *Clostridium* may be better targets. Have persistent microbial and chemical agents been considered enough in regards to human health? Value-added information for highly persistent organisms is possible and may inform decisions about what information is necessary. In terms of efficacy of treatment, *C. perfringens* is a better organism than *E. coli*. For example, many times following treatment, coliform bacteria are not present in sewage, but *C. perfringens* bacteria are. The rationales by which determinations are made to study organisms need to be continually reexamined. Dr. Stelma asked if adenovirus would be a good candidate organism because it is always present when human contamination has occurred and it is persistent in the environment. Dr. Sobsey agreed it would be and said that methods are available to detect adenovirus.

Dr. Meschke commented that there are two questions that frequently arise: What is the appropriate index versus an indicator? Why are researchers worried about the viability of indicator organisms if specific signatures are available?

A participant stated that large utilities have the abilities to respond to some things that small utilities do not. An important need is for inexpensive and easy methods that a small utility can use.

Ms. Srinivasan commented that the tools that have been talked about most have the lowest recovery rates and asked if this was an issue for risk assessments. Dr. Sobsey responded that it is and that no analytical method is perfect. The methods by which microorganisms are being measured are problematic. One example of this is the fact that different cell lines for enteric virus detection have different susceptibilities, and there is no standard cell line in use. The need for calibration is always present in analytical systems, including rate of recovery. One approach is to use more than one analytical method to better understand what the relationships are to determine details regarding health effects as well as exposures. The best decisionmaking will come from examining the range of analytical approaches available. Some utilities, however, do not want more sensitive detection methods because they will be out of compliance with drinking water standards.

Dr. Meschke stated that when recovery levels are reported, an overall percentage of recovery in all steps (e.g., detection, purification, concentration, etc.) is reported; this is inadequate. The efficiency and recovery of each step should be reported and examined. Dr. Fout commented that virus recovery levels usually are not reported for studies of virus levels in environmental waters, in spite of the fact that it is known that strong matrix effects occur for different types of water. Because recovery levels are not reported, it is difficult to know what a negative result means..

Dr. Rublee described standards in the toxic algae/harmful algal blooms community. In this community, one laboratory produced a standard toxin, and other laboratories obtained their supplies from this laboratory. Maintaining a stock of model organisms where the quality is constantly checked may be one method that the drinking water community could use to maintain a standard and make valid comparisons. This topic may be worth examination by a workshop or a work group.

Dr. Sobsey stated that there is one good precedent for spiking organisms in a real-world setting and using the results to validate water treatment methods. He cited the example of the Austrian rule that regulates UV disinfection technologies for which there is a standard protocol.

Dr. Meschke commented that standards and controls go beyond deciding which organisms to use, because some freshwater standards break down when applied to saltwater. One method of quality assurance would be to have periodic callbacks where laboratories submit their organisms for testing to ensure that the organisms they are using actually are the organisms they believe that they are using. At the recent American Society of Microbiology General Meeting, he learned about two standard bacteriophages that are supplied by the American Type Culture Collection (commonly known as ATCC) that are not what they are supposed to be. Dr. Sen added that the same problem was uncovered with a *Cryptosporidium* strain.

Ms. Page asked, from an exploratory and anticipatory standpoint, what were the top three areas of research on which scientists would like to focus. Dr. Sobsey replied that microbial ecology should be a focus. More information on the ecology of water-borne pathogens is needed. For example, the ecology of *E. coli* is not known. Dr. Stelma commented that the ecology of *E. coli* differs in tropical areas as compared to *E. coli* isolated from humans and cows.

Dr. Galambos asked if outbreaks that occur in drinking water are a breakthrough from treatment (e.g., an overwhelming load of organisms) or a function of the breakdown of the system. Dr. Sobsey responded that there were examples of both. Dr. Galambos suggested that one area of research be to determine where money should be spent so that these breakthroughs and breakdowns do not occur.

A participant commented that even in outbreak situations it is not possible to obtain samples from patients even after active attempts to do so; people with diarrhea generally do not go to the doctor. Therefore, starting at the source of the outbreak may be easier than using infected individuals to determine the agent. Dr. Sobsey described a biowipe that is being used in a study in South Africa that allows for the collection of enteric samples. The wipe is used and then mailed back to the researchers using special packaging included with the wipe. He will report the compliance rate in using these wipes when it is known. Collaborations with epidemiologists and public health nurses also can help manage confounders to studies.

Dr. Xi mentioned biofilms and explained that shear force causes microbes to be loosened from biofilms in pipes and enter the drinking water supply. Depending on the shear force, each household potentially has a different probability of releasing microbes into their own drinking water. This could be a household-specific problem. He added that it is easier to study microbial ecology than clinical microbiology because of human subject issues.

Dr. Fout stated that efficacy of treatment is an important issue for EPA, which is trying to address this dilemma. Although molecular methods are improving, they do not address viability and therefore do not address treatment efficacy. He asked if focus should be placed on a molecular test for viable organisms.

Dr. Sobsey commented that there are two important questions to consider: What kind of treatments need to be evaluated? How well can molecular methods for these treatments be designed? The real problems are with chemical (e.g., chlorine) and physical (e.g., UV) methods. How the sample is obtained and in what form are important factors in designing molecular methods. Not enough thought has been given to the collective steps of sample collection, processing, and final analysis to determine which target organisms are consistent with possible infectivity.

Dr. Stelma commented that the ratio of DNA to RNA before and after treatment could be examined. Another participant asked if RNA is an indicator of viability. Dr. Stelma responded that it demonstrates growth. Dr. Hashsham commented that knowing if an organism is able to grow is different from knowing if the organism is still infectious. Without tracking the growth, however, the difference between live and dead organisms cannot be determined.

A participant commented that one theory to explain viable but non-culturable (VBNC) organisms is that they are genetically programmed to remain dormant until conditions are right for growth. Another theory is

that these are organisms that are dying gradually, but if they are put in an animal, they can be resuscitated. Questions about these organisms include: At what point are they no longer able to be resuscitated? Is RNA the best method to determine if they are viable? Is the presence of ATP the best method by which to determine if they can be resuscitated or if they are dead? Dr. Hashsham responded that the definition regarding VBNCs was limited, but generally these organisms are not able to grow under conditions that are normal for normal organisms; some are able to be resuscitated following heat shock.

Dr. Sen asked if it was possible to perform an initial screen for DNA, and then if DNA is found, to heat shock the organisms to determine if they become viable. Dr. Hashsham replied that if one can continuously show that the organisms are all dead all the time, then it is easy to convince people that the water is safe to drink and that the treatment worked. But if the DNA is continuously seen, then the water source should be examined.

A participant asked if there were any good studies that have determined how long DNA survives after the organism dies. Dr. Hashsham responded that there were not, but he had plans to perform these types of experiments.

Dr. Oshida asked each panel member to provide at least two research areas that should be top priorities for the STAR Program. Dr. Hashsham replied that he would like to see projects relating to unknown etiology. Can a project focus on obtaining an outbreak metagenome to determine what is causing the outbreak? Why do so many outbreaks have an unknown etiology when sequences are known? Determining risks from biofilms and examining alternative indicators also should be priority.

Dr. Rublee described his ideas for priority projects: (1) Microbial ecology covers a large spectrum, but it is a good research area. (2) If given a charge to find alternate bioindicators, the community will be innovative in its response. (3) If a good question can be asked, survey-based work should be encouraged. (4) A multiple-diagnostic, "yes-or-no" test should be developed for the water quality end user to use to determine if more definitive tests should be ordered. (5) Leveraging of resources by working with other programs and agencies is important. (6) The program should encourage enhanced communication by developing listservs and workshops.

A participant cited the example of a recent National Science Foundation (NSF) initiative regarding active nanoparticles, nanomaterials, and nanosystems and asked if a meaningful and helpful approach might be to release a specific Request for Proposals (RFP) that requires collaboration between biologists and engineers. Dr. Meschke responded that it is already difficult to obtain funding without an interdisciplinary approach and then described his priority research areas. He would like to see mathematical and statistical approaches incorporated into research projects and all data to be transparent, with more just one data point presented. He also finds the development of an appropriate suite of indicators to be a priority research area.

Ms. Page asked how the panel viewed creating STAR Centers to focus on these research areas. Dr. Meschke commented that Centers have certain strengths but are administratively heavy. One alternative approach is to allow the submission of four or five supporting proposals, each with a different PI but with linked projects that will share reagents, data, and so forth.

Dr. Sobsey added that a shorter term wish would be improved water-borne disease surveillance that is leveraged to better understand exposures. Public health nurses could be recruited to contact researchers when there is a suspected outbreak, and the microbiology/epidemiology equivalent of a "SWAT" team could respond to investigate the outbreak, acquire immediate samples at the source, and so forth. Additionally, surveillance systems should be designed to capture information that is otherwise hard to obtain. Providing resources at the state level to develop a richer system of disease surveillance may lead to better surveillance of endemic water pathogens.

Dr. Parshionikar asked if it would be of value to have an overview document detailing past outbreaks, including causes (e.g., breaches, regulatory problems, agents, etc.). Dr. Meschke responded that this is being compiled within EPA and includes size of the utility involved in the outbreak, cause, and so forth. Dr. Sobsey stated that this type of information would be beneficial to retrospectively mine additional data that could inform the whole process of designing an active surveillance system. A participant added that there was a recent workshop in Nashville at which there was much discussion about current efforts to compile such information.

Dr. Meschke stated that there was a medical surveillance database called BioWatch/Bioshield that was enacted as a result of homeland security and asked if it would be possible to access data from this program. Dr. Sobsey responded that there are obstacles to tapping into such databases, as the data are proprietary for the Department of Defense and the Department of Homeland Security. It may be necessary to justify attempts to determine water-borne disease levels.

Dr. Sobsey commented that the wrong water source may be being investigated. Contamination may come from irrigation system failures (e.g., the recent outbreak in spinach) and not from drinking water. A participant added that changes in rainfall also affect these systems. Dr. Sobsey replied that this issue goes back to the issue of microbial ecology and seasonal water quality changes as a result of rainfall.

Dr. Rublee stated, in regards to the center or correlated grants topic mentioned earlier, that this type of initiative could be good if it was kept nimble, but he would not recommend placing all research dollars into program or center grants. Archiving of samples is another potential topic of exploration.

A participant asked how long-term storage affects the target organism. Information regarding viability is needed, as well as experiments that determine the levels of degradation over time. These type of experiments will take a long time, so starting sooner rather than later will be beneficial. Dr. Hashsham confirmed that the samples do degrade to some extent. A participant added that if there is not good quality control when the samples are archived, then the results obtained from even the most robust method are meaningless. She stated that she was not able to compare the results of any of the research presentations at the workshop because there were no common target organisms, reagents, methods, primers, probes, and so forth. Dr. Sen responded that a quality control document was implemented to ensure some standards are met regarding sequencing, probe hybridization, and so forth. It is unrealistic to expect that identical primers and probes will be utilized across laboratories. Dr. Rublee added that this is an interagency issue and all agencies should be involved in the discussion.

A participant asked about the archival cards that Dr. Meschke presented in his talk the previous day. He asked if these cards are stable enough to quantify RNA in addition to detection. Dr. Meschke responded that it is possible to recover enough RNA from the cards for amplification, but he is unsure if it can be quantitated.

Dr. Sobsey suggested that the National Health and Nutrition Examination Survey (NHANES) archiving methods be investigated. EPA was involved with NHANES, so there must be some expertise in archiving samples within the Agency. Models of successful archiving may be available and should be explored. Dr. Rublee recommended that the culture collection experts be added to this discussion.

Ms. Page asked panel members to identify three areas for which there is not current information that may become a problem in the future. Dr. Sobsey replied that it may be helpful to perform a gap analysis to determine which areas have the least helpful information and then design STAR RFPs in response to these gaps.

Dr. Hayes asked if NCER is able to reserve funds to subsidize unsolicited research proposals. Ms. Page responded that she did not know if those type of funds would be available in the future, but if they do become available, she would recommend placing the funds aside for this purpose.

A participant stated that the NSF has a method by which it funds pilot projects that may uncover new information at a level of 20 percent. If the project is considered successful, it will then receive full funding.

Dr. Rublee stated that programs that fund time-sensitive projects (e.g., those following Hurricane Katrina) should have minimal review so that rapid response is possible.

A participant commented that EPA used to be able to fund unsolicited proposals up to $500,000, but that is no longer possible.

Dr. Sobsey suggested building a team to obtain a range of information that is needed, because these opportunities do not exist currently. If centers are established, they should be given finite funding and a specified amount of time to perform the mandated research. At the end of this time, the projects should be completed, and a review will be held to determine if the center should be funded for a new set of projects. Ms. Page said that this type of arrangement was what she had described earlier, but she has received mixed opinions about whether this is a good idea. Dr. Meschke added that his version of this idea was that a series of PIs interact with each other to accomplish a set of common goals while maintaining their own projects. Centers can become unwieldy and dysfunctional with the focus shifting toward administration and the review process; once this occurs, they are no longer research-driven. Dr. Sobsey agreed and commented that the program project model was better because it decreases the amount of administration and bureaucracy associated with a center. Although it increases the burden on the PIs, it is less expensive and more efficient, and the program projects have a fixed beginning and end. A participant also agreed that he was not a proponent of centers because they steal research time for administrative activities.

Dr. Xi commented that some projects cannot clearly define which agency is appropriate for funding, so collaborations between agencies are very helpful in these situations. Dr. Sobsey added that there had been a good combined NSF/EPA grant program, and he was sorry when it was canceled. Dr. Stelma explained that the program was designed to be short-lived while the NSF instructed EPA on how to manage grants. This was the forerunner to the STAR Program. Dr. Rublee added that the ECOHAB (Ecology and Oceanography of Harmful Algal Blooms) Program is a joint effort between EPA, NSF, the National Oceanic and Atmospheric Administration, and state agencies.

Dr. Hashsham asked Dr. Parshionikar whether EPA has reasons behind having particular organisms on the CCL. He stated that the past CCL had organisms listed without giving any reasons for having them on the CCL. Dr. Parshionikar responded that EPA was trying to make the CCL3 more transparent and that there are specific reasons why the organisms were on the list. Dr. Mark Rodgers commented that many outside the EPA may not like the reasons but there are reasons. Dr. Sobsey commented that the CCL3 does not reflect what researchers want. Dr. Shay Fout commented that ORD had given several comments to OW regarding CCL3.

Dr. Sobsey suggested that the communications between EPA headquarters and the laboratories and centers be improved. Dr. Ernst responded that the trend within the Agency is to encourage the regulatory personnel and the research personnel to communicate; there have been strong efforts to increase communication. Sometimes disagreements occur, but an attempt at progress is being made, including at the upper levels of the administration.

Dr. Sen described the EPA ETC Action Teams that have been formed as a result of a congressional mandate; she leads the Rapid Detection of Microbial Contamination of Water Action Team. Although most of the teams concentrate on solving immediate problems with available technology, her team moved away from the technology-driven focus and concentrates instead on a problem-driven focus. She asked for ideas

for pilot projects that could demonstrate rapid detection in water and encouraged any interested participants to join the group, which meets once a month. Dr. Sobsey suggested a pilot project that demonstrated that rapid response for surveillance is possible in a situation in which a quantitative measure of pathogens in the source water is desirable. Currently, rapid mobilization and the immediate acquisition of water samples to identify the pathogen and the exposure concentration are problems. States and other venues need to allow samples to be taken immediately and then collaborate with and provide supportive information to researchers trying to determine the source of the outbreak. The Centers for Disease Control and Prevention (CDC) cannot do this because the states must invite their participation, and states often are hesitant to do so because of the ramifications of calling the CDC.

Dr. Sobsey stated that various analytical options should be kept on the table, especially options that allow collaborations. Analytical approaches should be compared to determine which are worth pursuing. Dr. Rublee stated that there is already a model for this from the ECOHAB program. CDC funded various state programs, including the North Carolina Department of Environment and Natural Resources, which in turn established a Rapid Response Team that collects samples at the source of an outbreak and sends them to between two and eight different laboratories with different methods to compare the methods and determine the source species of the algal outbreak. Dr. Sobsey commented that there was an epidemiological component to this project. Dr. Rublee added that states with such programs include Maryland, Virginia, North and South Carolina, Florida, New York, and New Jersey.

Dr. Meschke stated that initial pilot projects should be left up to the PI to propose because there will be many novel ideas. Many PIs have the ideas for projects that can be completed in a 6- to 12-month timeframe, but there is never any funding for that type of project.

A participant commented that technology cannot be utilized properly until the underlying problems are known and understood. Another participant commented that no company will commercialize any products because there is no mandate to do so. A participant stated that only microbiology is being taken into account, but chemistry also must be. Chemistry affects the ability of the pathogens to grow in the environment; geographic chemistry and water parameters greatly affect the recovery of microbes by various methods (e.g., one method generally yields about a 60 percent recovery rate in most waters but only a 10 percent recovery rate in a few others). A multidisciplinary approach that includes microbiologists, chemists, ecologists, and mathematical modelers will eventually need to be pursued to better understand how all of these parameters affect public health.

Dr. Sen thanked the participants for attending the workshop and adjourned the meeting at 11:40 a.m.