VIRGINIA WATER RESOURCES RESEARCH CENTER

# Pathogen Research Symposium: Pathways and Monitoring in Natural and Engineered Systems


**SPECIAL REPORT**

Virginia Tech

**VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY**
**BLACKSBURG, VIRGINIA**
**2007**

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**Stephen Schoenholtz, Director**

# Pathogen Research Symposium:
# Pathways and Monitoring in Natural and Engineered Systems

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**January 2007**

**VWRRC Special Report SR32-2007**

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## 1. Pathogen Research Symposium – Objective and Format

The Pathogen Research Symposium Pathways and Monitoring in Natural and Engineered Systems was held on November 2, 2006 at Virginia Tech in conjunction with 2006 Virginia Water Science and Technology Symposium. The objective of the symposium was to facilitate a forum for discussing the state of research, research needs and regulatory issues related to pathogens in water. The pathogen symposium consisted of a keynote speaker and five invited panels. The symposium did not aim to cover all aspects of pathogens in water. Specific topics addressed by the panels were as follows:

Panel I. Methods of Detecting Cryptosporidium
Panel II. Pathogen Research at Virginia Tech
Panel III. Pathogen Research at Virginia's Universities/Colleges
Panel IV. Pathogens in Estuarine/Marine Environments
Panel V. State Agency Perspectives

Keynote Speaker
Dr. Valerie (Jody) Harwood, University of South Florida

Panel I
Discussion Leader: Dr. David Lindsay, Virginia Tech
Panelists: Dr. Ron Fayer, U.S. Department of Agriculture and Dr. Alan Lindquist, U.S. Environmental Protection Agency

Panel II
Discussion Leader: Dr. Nancy Love, Virginia Tech
Panelists: Dr. Joseph O. Falkenham III, Dr. Charles Hagedorn, and Dr. Ann Stevens, Virginia Tech

Panel III
Discussion Leader: Dr. Charles Hagedorn, Virginia Tech
Panelists: Dr. James Herrick, James Madison University; Dr. Hua Shen, Virginia State University; and Dr. Brooks Crozier, Roanoke College

Panel IV
Discussion Leader: Dr. Howard Kator, Virginia Institute of Marine Science (VIMS) – College of William and Mary
Panelists: Dr. Howard Kator, Dr. Martha Rhodes, and Dr. Corinne Audemard (VIMS)

Panel V
Discussion Leader: Dr. Kenneth E. Hyer, U.S. Geological Survey
Panelists: Mr. Alan Pollock, Virginia Department of Environmental Quality; Mr. Charles Lunsford, Virginia Department of Conservation and Recreation; and Dr. Wesley Kleene, Virginia Department of Health

This document is a summary of the presentations at the pathogen research symposium.

## 2. Summary of Presentations

# Overview of Pathogen Problems in Water

**Dr. Tamim Younos, Symposium Chair**
**Virginia Water Resources Research Center and Department of Geography, Virginia Tech**

Pathogens are disease causing agents that include various types of bacteria, viruses, protozoa, fungi, parasites and proteins. Pathogens enter water systems from various sources (Figure 1). They can be categorized as "known pathogens", when their effects on health are known, and "emerging pathogens" when they are newly discovered and adequate data for understanding their health effects are not documented.

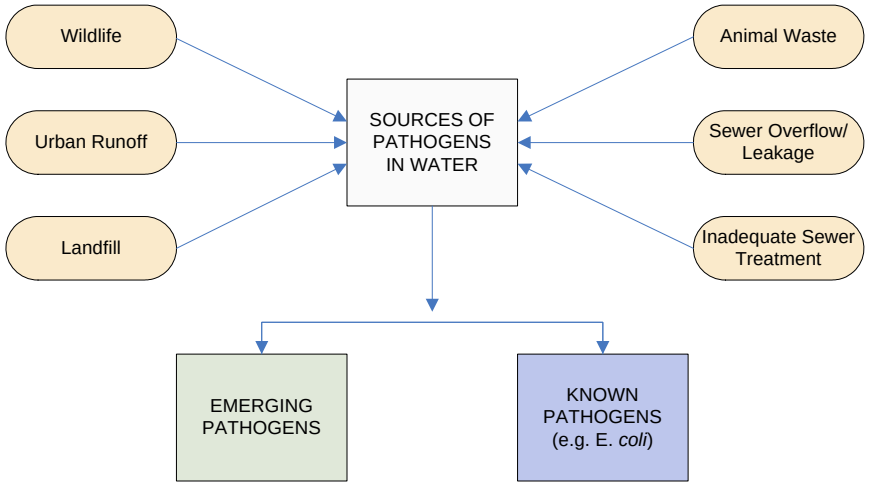


**Figure 1. Major Sources of Pathogens in Natural Waters**

Pathogens pose a risk to human health through the various uses of water (Figure 2). For example, contaminated irrigation water was speculated a possible pathway to recent outbreak of *E. coli* across the country. Bacterial pollution of beaches and swimming pools are well documented and pose a health risk through water contact and ingestion. Due to use of effective water treatment technologies, the occurrences of known pathogens are not normally expected in treated drinking water leaving water purification plants. However, recent research indicates problems associated with bacterial growth in water distribution systems and home plumbing. Aerosolization of some types of pathogenic bacteria in home-heated water system (bathing shower) are considered a health risk factor (pulmonary disease). Also, there are several documented cases of pathogenic effects on fish and shellfish that may impact human health through the food chain.

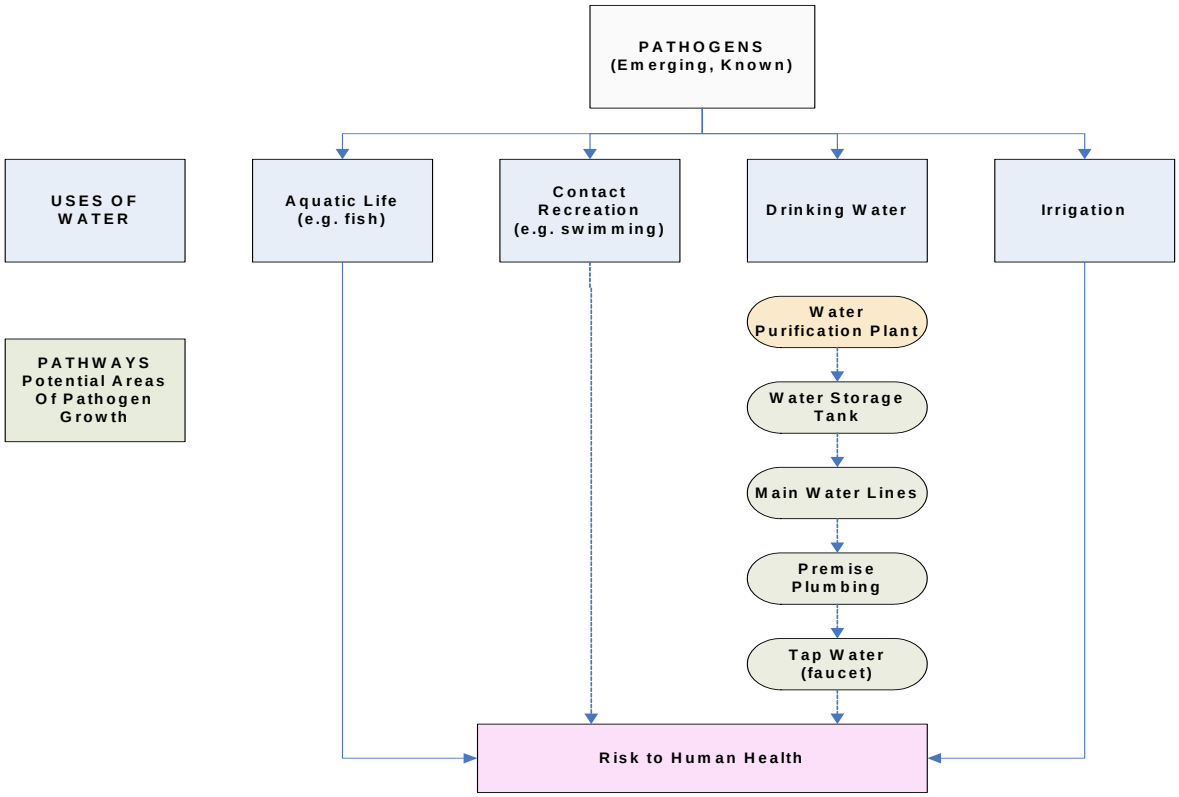


**Figure 2. Various Uses of Water and Pathogen Pathways in Natural and Engineered Systems**

The pathogen research symposium "Pathways and Monitoring in Natural and Engineered Systems" documented in this publication does not provide a comprehensive picture of pathogen issues is in water. The symposium was mostly focused on ongoing pathogen research in Virginia's colleges and universities and regulatory aspects. However, on specific issues discussed in this document, Virginia researchers are providing significant leadership in the national arena. Also, regulatory approaches in Virginia, i.e., issues related to the water quality standards and the total maximum daily load (TMDL) program are exemplary and can be used as a useful reference in other regions of the country.

## Keynote

### What's in your water? Pathogen Detection and Water Quality Monitoring for the 21st century

**Dr. Valerie J. Harwood, Department of Biology, University of South Florida**

As water usage is increasing, it is expected that 100% of available water will be necessary for human use by the year 2025. The challenges associated with an increasing need for water are to meet basic drinking water and hygiene needs, to protect ecosystems (especially in urban areas), to ensure an adequate food supply, to provide better treatment of waste, and to produce energy.

Assessment and improvement of drinking water quality is still a challenge, even in the U.S., because new pathogens and contaminants are being identified. Non-biological contaminants such as estrogens and pharmaceuticals have been identified with many questions still unanswered in terms of their impacts on ecosystem and human health in the long term. Biological emerging contaminants, such as viruses (over 50% of waterborne gastroenteritis is estimated to be caused by viruses!), bacteria such as *Helicobacter pylori*, and protozoa such as microsporidia may also have substantial impacts on human health. Recreational water quality issues are also increasing, with many of the etiological agents still unknown because of the difficulty of pathogen identification and source tracking.

Water quality monitoring and public health assurance is routinely performed by enumerating fecal indicator bacteria in both potable and recreational waters. Fecal coliforms are used as indicators based on the expectations that the only source of these organisms is directly from fecal contamination, and that the fate and transport of fecal coliforms reflects that of waterborne pathogens. Furthermore, environmental water standards are based on risk from human sewage contamination. However, conventional indicator organisms such as fecal coliforms and enterococci have many drawbacks in terms of recreational water quality assessment. Fecal indicator bacteria have many sources, including stormwater runoff and environmental reservoirs such as sediments. Many waterborne pathogens do not have the same fate and transport characteristics as fecal indicators, and these relationships are unknown for other pathogens. The ideal indicator organism may not be attainable, and we may have to rely upon several indicators, or some combination of indicator and pathogen testing in the future.

The rapid, accurate identification of these emerging contaminants and pathogens pose the following challenges to microbiologists and epidemiologists:

- Determine risks posed by various contamination sources
- Determine risks posed by various pathogens
- Explore the ecology of indicators and pathogens
- Develop better and faster detection technologies for indicators and pathogens

Some candidate “better mousetraps,” or detection and quantification methods, for these emerging pathogens include quantitative PCR (polymerase chain reaction), RT-PCR (reverse transcriptase-PCR) , multiplex PCR, NASBA (nucleic acid based sequence amplification) (isothermal method), transcription mediated amplification (isothermal and reverse transcriptase), and microarrays.

To help understand the sources, fate, and predictive relationships of pathogens, efforts are being made in mathematical modeling for prediction, microbial source tracking, and fecal source tracking (target chemicals instead of microbes). The ideal microbial/fecal source tracking target is: (1) unique to a particular host species or group; (2) all host individuals are carriers of the target; (3) it has wide geographic range and it is temporally stable; (4) the assay is inexpensive but reliable; (5) it is not just qualitative but also quantitative and levels should be correlated with some indicator organism. A new candidate for human fecal source detection is the human polyomavirus, which can be detected by PCR.

In summary, major research needs include:

- Quantitative risk assessment for a wide variety of waterborne pathogens
- Epidemiology studies of the relationship between indicator bacteria, pathogens, and human health outcomes in recreational waters that are not impacted by point sources
- Methods for rapid detection of pathogens and indicators
- Quantitative microbial source tracking methods for human and other sources of fecal contamination
- Improved modeling of microbial transport and fate in the environment

## Panel I: Methods of Detecting Cryptosporidium

**Dr. Ron Fayer (USDA)**

*Cryptosporidium* is a protozoan organism which causes the parasitic infection, cryptosporidiosis. *Cryptosporidium* goes through asexual and sexual phases during its cycle and eventually becomes oocysts (dormant form), the major mode of its transmission in drinking water. Whether or not humans develop severe symptoms of cryptosporidiosis when infected by oocysts depends on the physical and immunological state of individuals. Previously, there was thought to be only one species of *Cryptosporidium*, but currently, at least 16 species have been identified. Molecular techniques are necessary to distinguish between these species. About 40 genotypes of *Cryptosporidium* have been identified. Many of the genotypes have been found to infect humans. The major source of *Cryptosporidium* is fecal in origin. Identification of oocysts in samples is done by immunofluorescent techniques, but these techniques cannot tell if these oocysts are infectious or not or if they are of the human infective genotype.

*Cryptosporidium* is a known cause of many drinking water illnesses as well as possibly the cause of other smaller scale illnesses. The first detection of *Cryptosporidium* in recreational waters was in a bathing beach in Hawaii. Recreational water outbreaks are associated with public pools, lakes and rivers, and fountains. Many outbreaks are seen in pools that have been chlorinated. It has been found that *Cryptosporidium* oocysts are still infectious after 2 hours of exposure in 5.25% sodium hypochlorite. Oocysts have very high infectivity rates even in higher temperatures. Heat (heating to 74°C for 5 seconds kills oocysts) and some gases (most effective is ozone) are found to inactivate oocysts.

Other than human sources of infection, cattle/calves seem to be a significant source, especially in veterinary students and staff. Dairy cattle tend to have *Cryptosporidium* early on in age with the prevalence decreasing as the cattle age increases. Therefore, pre-wean cattle are the most infectious. Quite a few wildlife animals are also carriers of (or infected with) *Cryptosporidium* (ranging from 20-60% prevalence). Approximately 20% of shellfish are infected with oocysts indicating that coastal waters are contaminated by run-off containing oocysts.

Detection of oocysts is problematic. Microscopy is not very usable tool for oocysts detection. Molecular techniques such as IFA (immunofluorescence antibody) test and PCR (polymerase chain reaction) are more appropriate, with PCR being the better method (though not perfect and still need to optimize techniques).

Free-living predatory microorganisms such as some protozoa and biofilms (e.g., *P. aeruginosa*) engulf oocysts and may be used to remove oocysts from surface waters as a possible bio-control method.

**Dr. Alan Lindquist (USEPA)**

**Environmental Protection Agency Method 1623 for Detection of *Cryptosporidium* and *Giardia* in Water**

Method 1623 is a performance-based method for Detection of *Cryptosporidium* and *Giardia* in Water where deviation from the published manual may be made, but the deviation must be technically demonstrated to give results that are effectively equivalent to the published method.

Method 1623 involves filtering to remove organisms followed by elution with a surfactant based solution. Elute (pelleted) goes through immunomagnetic separation (IMS) where paramagnetic beads labeled with antibodies against cryptosporidium are used to separate *Cryptosporidium* spp. from environmental debris. After IMS, IFA (immunofluorescent antibody) staining is used to determine the presence of *Cryptosporidium* spp. oocyst. Use of immunofluotescent antibody staining microscopy alone results in non oocyst artifacts from environmental debris being misidentified as *Cryptosporidium* spp. oocysts. Differential interference contrast (DIC) microscopy, or fluorescent microscopy using staining with a dye that stains double stranded DNA (4'-6-diamidino-2-phenylindole, DAPI) are used to rule out artifacts that might be misidentified as *Cryptosporidium* spp. oocysts.

The performance basis changes in the method have two tiers of approval: tier 1 approval for the use in one laboratory, tier 2 for inclusion into the method so that the chance may be used in any laboratory. Tier 2 approved amendments to the method allow technological alternatives such as different filters, the use of continuous flow centrifugation for sample volume reduction and different immonofluorescent stains. More work in detection methods is necessary to reduce the costs associated and also to increase the precision and accuracy of detection.

Editor's Note
For details see the following USEPA publication:
U.S. Environmental Protection Agency. 2001. Method 1623: *Cryptosporidium* and *Giardia* in water filtration/IMS/FA. EPA 821-R-01-025. Office of Water, U.S. EPA, Washington, D.C.

## Panel II: Pathogen Research at Virginia Tech

### Dr. Joseph O. Falkenham III

### *Mycobacterium avium* Complex in Biofilms in Engineered Systems

*Mycobacterium avium* is an environmental opportunistic pathogen, a normal inhabitant of natural waters and drinking water distribution systems, and has been listed on the EPA's Candidate Contaminant List (CCL). They are opportunistic pathogens of humans, animals, and fish. They are very slow growing organisms with at the most, 1 generation/day. Slow growth makes them poor competitors but at the same time, they take a lot longer to die off and show evidence of adaptation to harsh conditions. The bacteria are very hydrophobic, which makes them impermeable to nutrients but makes them resistant to anti-microbial agents. Their hydrophobicity leads to their attachment to surfaces and proliferation (biofilm formation) because they can grow in waters with relatively low organic concentrations (> 50 µg AOC/L). Hydrophobicity also is a major contributor to *M. avium's* aerosolization and concentration in aerosols above waters. *M. avium* numbers increase in recirculating hot water distribution systems found in hospitals, office buildings and apartment houses. The disinfection phase of drinking water treatment actually selects for *M. avium* (because of resistance and slow death). Biofilm formation of *M. avium* increases their persistence in drinking water distribution systems and makes it harder for the elimination of the bacteria. Cells released from biofilms are more disinfectant- and antibiotic-resistant (for about a day).

With respect to the possible implementation of requirements for monitoring *M. avium* numbers there is a lack of familiarity with the microorganisms. Cultural isolation is not the best for *M. avium* because of slow growth rate, so molecular methods are necessary. They are disinfectant-resistant, but (fortunately!) are killed by UV at rates similar to those found for *Escherichia coli*. Showers are risk factors for *M. avium* pulmonary disease because of their aerosolization from water and the entrainment of *M. avium* rich biofilms into aerosols upon water flow.

Research needs relevant to *M. avium* include identification of pipe surface treatments to reduce *M. avium* biofilms, the need for cost-effective methods for reducing their numbers in waters, and the urgent need to develop effective antibiotic agents against this human and animal pathogen

### Dr. Charles Hagedorn

### Microbial Source Tracking

Microbial source tracking (MST) is being increasingly deployed in the United States. *E. coli* is used for MST in freshwater and Enterococci species used for other waters. *E. coli* and Enterococcus are both possible pathogens in the environment and in other communities such as hospitals.

MST is best done by combining methods, performing QA/QC on known source libraries periodically, and using alternative tracers.
Some research needs for MST: need to link different indicator bacteria and quantities of indicators with risks, need to link MST results to risks with the different indicators across the board, and need faster real-time methods for detection.

Editor's Note
For details about Dr. Hagedorn's research please see his Bacterial Source Tracking Website: http://filebox.vt.edu/users/chagedor/biol_4684/BST/BST.html

For details on MST applications see the USEPA publication:
U.S. Environmental Protection Agency. 2005. Microbial Source Tracking Guide Document. EPA/600/R-05/064. National Risk Management Research Laboratory. U.S. EPA Office of Research Development, Cincinnati, OH.

**Dr. Ann Stevens**

**Evaluating the Extent of Pollution-Induced Antibiotic Resistance in Environmental Bacterial Strains**

Chemical contaminations are known to change bacterial communities in the environment but what do they do to cellular physiology? Physiological changes in *P. aeruginosa* in response to PCP (pentachlorophenol) were measured through the use of a chemostat. Whole cell physiological measurements did not indicate stress in response to PCP, whereas sub-cellular (molecular) changes indicated multi-drug efflux transport genes being increasingly expressed in response to PCP. As many antibiotics are known to be effluxed out by these same transporters, further research showed that PCP exposure resulting in multi-drug efflux pump gene ultimately resulted in increased non-permanent antibiotic resistance. Other contaminants were shown to induce increased antibiotic resistance but it is not clear if it is solely due to the multi-drug efflux. More research is needed to determine the causes of increased antibiotic resistant due to chemical contamination in the environment.

## Panel III: Pathogen Research at Virginia's Universities/Colleges

**Dr. James Herrick, James Madison University**

**Environmental reservoirs of resistance and virulence: bacterial pathogens and antibiotic resistance gene transfer in sediments and sands.**

Research questions: How widespread are fecal pathogens (particularly *E. coli* and its relatives) and their genes in streams impacted by agricultural runoff? How persistent are fecal bacteria in stream sediments and how likely is it that these will harbor virulence and transmissible antibiotic resistance genes? What is the potential for the transfer of multiple antibiotic resistance and virulence genes to and from pathogens in streams?

In our laboratory at JMU, we are using genetic, molecular, and microbiological methods to study the occurrence, distribution, and antibiotic resistance of pathogenic bacteria in streams and beach sand. Our focus is currently on pathogenic *E. coli*, *Salmonella*, and related members of the Enterobacteriaceae. *E. coli* are not only important indicators of fecal contamination, but important pathogens, such as the enterohemorrhagic *E. coli* (EHEC), have apparently evolved from commensal populations and may be persistent and even grow in stream sediments and in beach sand. Persistence of fecal bacteria such as *E. coli* in waters is low but sediment persistence may be much higher. Using nested polymerase chain reaction (PCR), we have detected *stx-2*, the gene encoding a shiga-like toxin of EHEC, in DNA extracted from water in streams in the Harrisonburg area. We have developed a real-time polymerase chain reaction quantification system for *stx-1*, *stx-2*, *eae* (intimin) and other virulence factors of EHEC that is sensitive down to 18 ($\underline{+}$) 4 gene copies. While surveying for *Salmonella*, we have also recently cultured *Escherichia fergusonii*, the closest relative to *E. coli* and a new potential pathogen discovered in 1985, in an area on Smith Creek in Rockingham County, Virginia. An area of current focus is determining the potential for antibiotic resistance genes to be transferred to and from fecal pathogens introduced to streams. For example, we have used *E. coli* strains isolated from stream sediments and sand to capture, without previous cultivation, plasmids encoding resistance to multiple antibiotics, directly from bacteria native to stream sediments in the Shenandoah Valley. This suggests that there may be a substantial reservoir of resistance genes on transmissible plasmids that are capable of transferring to, and being expressed in *E. coli* that may be transiently passing through the stream ecosystem.

Persistent questions/Research needs:

- Are sediments and sands providing habitats for human pathogen survival and growth?
- Whether they survive or not, can pathogens exchange virulence and resistance genes with native bacteria?
- Could such exchange produce new combinations of genes of concern in potential pathogens?
- Are there previously unknown pathogens in our stream and recreational waters? How would we know?

**Dr. Hua Shen, Virginia State University**

## I. *Legionella pneumophila:* Detection & Biofilm

*L. pneumophila,* discovered during the 1976 American Legion outbreak, is a Gram negative bacterium, ubiquitous in the aquatic environments. It replicates in amoebae and other protozoa host, can be found in various man-made water systems and cause disease through aerosols generated from air conditioner, humidifier, decorative fountains, whirlpool spas or industrial cooling towers.

An opportunistic pathogen, the bacterium causes disease in individuals with weakened immunity, such as seniors, heavy smokers, individuals with chronicle lung disease, immune defect or immune suppressed patients. Large outbreaks get media attention, for example in Virginia, September 1996, a whirlpool spa display at a home improvement store caused 23 cases, and killed 2; 2001 in Spain, a hospital cooling tower caused 449 cases of the disease and 2 killed; September 2005, a Toronto senior house outbreak killed 17. The majority of diseases however, are sporadic community infections which is seriously under diagnosed and under estimated. In the United States up to 20,000 cases occur annually, mortality of disease is up to 25%. The bacterium thrives in warm & humid environments, thus changes in the ecological system and human population such as increased immune compromised population, may trigger the rise of infection. In 2003, CDC reported increased cases in Mid-Atlantic region, 178 cases versus 48 cases in 2002.

Disease control and prevention depends on rapid, sensitive, and quantitative detection method. The standard culture method depends on growth of the bacterium on a charcoal yeast extract medium which requires 5-10 days and misses the Viable But Non-Culturable (VBNC) bacterium that maintains the ability to cause the disease, thus sensitive and rapid alternative detection method is needed. One such a method is polymerase chain reaction (PCR) which detects marker DNA sequence specific to the organism, can detect VBNC and report result in 2 hour. Although detection speed and sensitivity is dramatically improved, PCR is a qualitative method. A newer version of PCR, Real-time PCR is rapid, sensitivity and also a quantitative detection method (Ballard *et al*. 2000). Using this method, we detected as few as 1 genome in a 50 μl of unconcentrated water. We plan to monitor *L. pneumophila* level using this method in the water of selected senior houses, and hospitals, or industrial cooling towers in Virginia for a two-year-period as these facilities host susceptible or large population. Such investigation will provide data to determine the acceptable level of the bacterium, and thus make guidelines for cost effective management and also protection from infection.

*L. pneumophila* is a fastidious organism as lab culture, but it's persistent in nature and man-made water systems. It is difficult to rid of the organism because of its ability to resist bacteriocidal, and grow in nutrient low clean water. Such property is likely due to its ability to form biofilm – a complex film forms at interfaces and consists of microbes and macromolecules released from these organisms. We wish to study the biofilm of *L. pneumophila*. Our questions are: i) what microorganisms exist in *L. pneumophila* biofilm?, ii) is there any host necessary in biofilm?, iii) what triggers biofilm formation?

iv) what signaling pathways lead to biofilm formation? Knowledge from this study can contribute to the development of new control strategy in man-made water systems, e.g. by coating the surface of water facilities with chemicals that blocks the signaling pathway.

## II. Drug-Resistant Bacteria and Bacteriophage Control

Antibiotics-resistant bacteria are serious problems in today's medicine because infection by drug resistant bacteria is difficult or have no treatment in multidrug resistant case. While most resistant bacteria arise from hospital, community resistant bacteria infections have been reported. However little is known about the community resistant bacteria. The widespread of resistant bacteria is due to selective pressure, use of antibiotic for treating human disease, live stock agriculture and antibiotics producing microbes in nature. Thus the community drug resistant bacteria can arise from hospital or environmental sources.

I am interested in understanding i) how wide spread is community resistant bacteria? - such data would be useful for monitoring the spread of resistant bacteria and evaluating control measure, ii) where do community resistant bacteria come from? hospital or environment source? We are currently performing a survey of resistant *Staphylococcus aureus* in healthy people by taking skin (fingers and nostril) samples from students and isolate resistant *S. aureus*. To understand the source of these resistant strains, we would like to compare our isolate with hospital and environmental isolates by DNA typing using pulse field gel electrophoresis.

Alternative treatment for drug resistant bacteria infection is needed. One of such treatment is using bacteriophage. Bacteriophage is viruses that infect, and multiply in the bacterial cell and destroy the bacteria in such process. Bacteriophage has been used for treating human disease in the former Soviet Union, Poland for decades, and proved to be safe. The advantage of bacteriophage over antibiotics are: i) highly specific - for example, a bacteriophage can kill *E.coli*O157H7 but not the general *E.coli* which is the normal bacterium in the intestine and does not cause disease, ii) no side effects -unlike antibiotics which may cause secondary infection due to disturbance of normal flora or liver damage due to long term use of antibiotic, iii) bacterial resistant to bacteriophage can be overcome because bacteriophage coevolves with its host. We have isolated two Salmonella bacteriophages from Petersburg Waste Water Treatment Plant and tested their effect on the control of Salmonella contaminated mustard and broccoli seeds, and consequently observed significant reduction of Salmonella counts. Recently, FDA approved the first bacteriophage to be used on meat to control bacterial (*Listeria*) contamination, thus this is a promising area of research. We plan to continue on bacetriophage isolation and testing on their control effect on food contamination and human disease.

**Dr. Brooks Crozier, Roanoke College**

**Microbial Source Tracking**

At Roanoke College, through our research and through collaboration with others, we have learned quite a bit about the assets and pitfalls of the most commonly used microbial source tracking (MST) methods. Some issues related to MST methods are: inconsistent banding patterns, statistics and controls, and difficult and expensive protocols. Our goal primarily is to develop and refine MST methods which will produce both better accuracy and precision, and are fast and inexpensive. We have employed both phenotypic and genotypic methods, with recent focus on DNA-sequence based source tracking in both *E. coli* and Enterococcus. We feel that strains of fecal indicator bacteria, at the level of specific DNA polymorphism, may reside in an animal host due to physiological differences among hosts and DNA sequence based techniques must use sequences that are selected for specific host animals. Finding genes of interest is the first step, followed by making PCR (polymerase chain reaction) primers for those genes, testing primers, and developing multiplex methods. We hope to increase knowledge in this area of research and simultaneously focus on the training of undergraduate students for graduate school, professional school or industry in microbiological and molecular biology techniques.

## Panel IV: Fecal Indicators and Pathogens in Estuarine/Marine Environments

**Dr. Howard Kator, Virginia Institute of Marine Science**

Research at VIMS focuses on a variety of problem areas that potentially affect human health. Because microbiology of marine waters has been and continues to be method-limited, evaluation of approved methods and development of alternative methods is a significant aspect of our research efforts. Fecal indicator bacteria have been used to regulate shellfish growing-waters since about 1925. An indicator is a surrogate for the potential presence of any or all of the pathogens found in a water sample. The indicator bacteria concept was initially derived and only applied to point sources of sewage contamination. It is problematic when applied to waters affected by nonpoint sources of fecal contamination. Approved marine/estuarine indicators are total coliforms (not much use) and fecal coliforms for shellfish growing waters, and *E. coli* (although EPA does not recommend its use for marine waters), and enterococcus for recreational waters. There are problems and questions with indicators applied to estuarine waters, such as "are indicators really predictive pathogen presence?" What is the fate and transport of these indicators? How is the predictive value of the indicator changed with contamination sources other than point sources? Do these (bacterial) indicators predict the presence of all types of pathogens, including viruses? How can indicators be validated in terms of human risk? Even with all these unanswered questions, we are still using fecal coliforms (which from a functional standpoint is *E. coli* for the most part) as the indicator for shellfish safety.

Consequently, research has focused on culturable candidate alternate indicators. Alternate fecal indicators studied have included *Streptococcus bovis*, *Rhodococcus* spp., *Bacteroides fragilis* and *B. fragilis* bacteriophage and FNRA male-specific coliphage. FRNA male-specific coliphage has been proposed as viral indicator and is a good candidate from various perspectives because it is similar in size and structure to norovirus, it is highly resistant to chlorination, is present in STP treated effluents and some animals, and occurs in genotypes that may be source-specific to some degree. Methods are needed for its concentration and rapid detection from marine waters.

A methodological shortcoming with all current indicators is the time required for a result. Recreational marine waters need rapid detection methods to be able to update swimming advisories accurately (quantitative PCR) and quickly. VIMS is now involved in development/validation of rapid methods for emerging shellfish-borne pathogens *Vibrio vulnificus* (opportunistic pathogen with 50% mortality rate), *V. parahaemolyticus*, and norovirus. *Mycobacterium* spp. are emerging pathogens in striped bass and may be a reservoir that can infect humans. We are now working on the detection of norovirus in sewage effluent and its persistence in estuarine water using real-time PCR.

**Dr. Martha Rhodes, Virginia Institute of Marine Science**

Mycobacterium infections in Chesapeake Bay striped bass were first observed in the late 1990's. Historically, mycobacterial infections in fish were termed fish "tuberculosis" because the symptoms were similar to those in human TB. However, the term has been discouraged and the term fish mycobacteriosis is preferred instead. Mycobacteriosis is not uncommon in captive, and aquacultured fishes but is observed less frequently in wild populations. External signs of infection may not be present. Mycobacteriosis is predominately a visceral disease, producing granulomatous lesions. Causative agents are frequently not isolated because of the slow growth rate of many mycobacteria. Previously, three *Mycobacterium* species, all human opportunistic pathogens, were recognized as the primary agents of fish mycobacteriosis.

The isolation of mycobacteria from fish viscera requires aseptic technique because background bacteria may overgrow the slowly growing mycobacteria. Tissue is homogenized, then spread plated and incubated for up to 3 months at room temperature. Spread plating is the preferred method of inoculation because it allows for detection of a mono- or poly-culture infection and permits quantification. Isolates are characterized using traditional biochemical methods. Speciation frequently requires the used of molecular techniques.

Mycobacterial infections in striped bass were prevalent (76%), especially in fish 3 years and older. *M. shottsii* a recently described species, was the most frequent mycobacterium recovered (76%). A second new species, *M. pseudoshottsii*, was also isolated and identified but was less prevalent (17%) than *M. shottsii*. *M. pseudoshottsii* isolates produced a lipid toxin similar to that produced by *M. ulcerans*, a mycobacterium causing "buruli ulcer" in humans. This newly described lipid toxin is called "mycolactone F"and produces cytotoxicity in mouse fibroblast cell culture. The extent to which these new mycobacteria found in striped bass can infect and cause disease in humans remains unknown.

**Dr. Corinne Audemard**

Quantitative PCR (qPCR) can allow a more rapid quantification of bacterial indicators and/or pathogens in environmental samples and organisms than cultural assays. The use of qPCR for environmental water samples however can be hindered due to the presence of nontarget organisms and compounds that can inhibit DNA extraction, DNA recovery and PCR. In this context, DNA extraction has to be optimized to minimize the presence of inhibitors to allow for more accurate and reliable quantification of the targeted organism. In our studies, different extraction kits (DNeasy tissue kit and Stool mini kit; both from Qiagen) were evaluated and showed different efficiencies of DNA recovery from environmental water samples. The Stool kit showed the best results and is currently used.

Our preliminary work with indicators for recreational marine waters showed that for both *Staphylococcus aureus* and *Escherichia coli*, new primers and probes will need to be

designed in order to increase the sensitivity of detection. For *Enterococcus faecalis*, genus specific probes will be tested in order to compare sensitivity of qPCR to standard assays.

Detection and quantification of norovirus in water required optimizing of concentration and RNA extraction techniques as well. Work will focus on the development of more sensitive qPCR methods and on reducing environmental samples processing time (including DNA extraction).

To detect and quantify *Vibrio* spp. in oyster tissues, we first chose to optimize and to apply quantitative PCR assays for *Vibrio* spp. in order to avoid an enrichment step that involved culturing and growing the microorganisms before quantification. The objective was to minimize confounding measurements of the relative quantities of the pathogens in the original sample due to unequal growth patterns. DNA was extracted, therefore, directly from oyster tissue homogenates and qPCR was used to quantify *Vibrio vulnificus* and *V. parahaemolyticus*. Although the assays chosen were designed to target all strains within each species, they were not sensitive enough to quantify *Vibrio* sp. in oyster tissues. A direct-colony lift plating method using various hybridization probes is now been tested.

## Panel V: State Agency Perspectives

### Mr. Allan Pollock, Virginia Department of Environmental Quality

Federal water use designations are stated in the Clean Water Act and the Federal Water Quality Standards Regulation. States must specify water uses, adopt criteria to protect uses, and state policies must be approved by EPA before implementation in the state. Safety criteria protect swimming but the most important concern is ingestion. The current approach for recreation bacteria criteria is *E. coli* and Enterococci instead of fecal coliforms. Shellfish waters use fecal coliform criteria. The 2006 assessment causes for rivers indicated that fecal coliforms and pathogen indicators are the most prevalent causes. In lakes and marine systems, dissolved oxygen and polychlorinated biphenyls (PCBs) are more prevalent and biological indicators were not as important.

Bacterial total maximum daily loads (TMDLs) studies are increasingly being conducted and completed. Significant management actions must be taken to follow the standards. Some issues are under consideration for triennial review, such as if both the geometric mean and single sample maximum must apply, if the violation rate is set to 10% or if it is flexible, assessing wet weather and combined stormwater overflow impacted waters, illness rates, and natural (wildlife) contribution. All Virginia waters are tested for primary contact. Non-attainment of bacteria criteria is the most common pollution impairment in Virginia's streams and rivers.

Editor's Note
For details about Virginia's water quality standards see the website:
http://www.deq.virginia.gov/wqs/

### Mr. Charles Lunsford, Virginia Department of Conservation and Recreation

As required by the state law, the second step in TMDL process in Virginia is to develop TMDL implementation plans once TMDL reports are completed. In terms of fecal coliform TMDLs, several agricultural best management practices (BMPs) such as livestock stream exclusion practices with riparian buffers and loafing lot management systems are practices that have been implemented in Virginia. In addition to the BMPs listed above, implementation of improved pasture management, vegetative buffers on cropland, manure and biosolids incorporation on cropland, and using retention ponds and bioretention filters are envisioned and needed. Overall, since the initial three implementation projects started in 2001, the percentages of the in-stream bacteria standard violations have decreased significantly in the impaired watersheds. However, as yet they have not decreased enough to be within a 10% or less of violation rate. Also, it is important to note that recently the bacterial TMDL standards changed from fecal coliforms to *E. coli*.

There are challenges to bacterial TMDLs implementation, such the amount of nonpoint source reduction needed to attain primary contact designation of all state waters and lack

of funding for on the ground BMP implementation. State is considering the next steps for three pilot implementation of projects such as: 1) down-size project implementation area by focusing on impaired streams where delisting the stream from the impaired waters list is within reach; 2) possible change of designated uses of water through a Use Attainability Analysis; 3) more intensive assessment of remaining pollution sources; and 4) increased regulation. Future research needs include: 1) assessing bacteria reduction efficiencies for several BMPs (*e.g.*, improved pasture management); 2) dynamics of in-stream bacteria growth and regrowth; 3) understanding the mechanism of sediment as a bacteria source; and 4) development of a tool box of bacterial (microbial) source tracking methods.

Editor's Note
For details related to bacterial TMDLs see the website:
http://www.deq.virginia.gov/tmdl/faq.html

**Dr. Wesley Kleene, Virginia Department of Health, Office of Drinking Water**

Disease-causing microbial contaminants tend to be the most concerning contamination in drinking water. The reason for this is that microbial contaminants create an acute response in populations that consume the contaminated water. Pathogens are bad!

The challenges with drinking water involve the limitations of the water supply system. The source water quality has a direct impact on the costs and treatment technologies that are available to the system. The maximum contaminant levels (MCLs) are set for the treated drinking water. The challenge of the drinking water systems is to provide pure drinking water that meets these MCL goals even though their source water may vary considerably. This is a significant challenge to small systems that account for over 90% of standards violations in the past 5 years.

When evaluating waterborne diseases; between 45-80% could be attributed to directly to pathogens as a source of the disease. Environmental Protection Agency (EPA) has recommended a multiple barrier approach of drinking water treatment to reduce the impact of pathogenic and chemical contaminants in the water supply. Subsequent to this approach, EPA has enacted a number of new rules that specifically address pathogens, source water quality, and other unique challenges in the drinking water industry. Current EPA regulations under the Safe Drinking Water Act require that drinking water utilities identify and quantify microbial contaminants in source waters. In addition, utilities are required to identify the best available technology for the treatment of contaminated source water.

During the evaluation and development of treatment technologies, the entire water quality must be considered. This is because the treatment effectiveness of certain techniques is impacted by other parameters of the water quality. For example, turbidity will have a significant impact on the deactivation of pathogens using ultraviolet (UV) technique. EPA rules that specifically address pathogens include total coliform rule, surface water

treatment rule, enhanced surface water treatment rules (source water monitoring requirement), LT1 (turbidity requirement), LT2 (focuses on Cryptosporidium and reduce disinfection byproducts in stage 2), groundwater rule (this is a new rule that recognizes distribution systems as an integral part of the waterworks).

Challenges ahead include aged infrastructure and regulatory alphabet soup, collaboration between the regulatory and research community, existence of very few approved and certified laboratories, and funding.

Editor's Note
For details about drinking water standards see the USEPA website:
http://ehso.com/ehshome/DrWater/drinkingwaterepastds.php