Analytical Innovations for the Detection of Biological Microconstituents

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In order to ensure public safety, regulatory agencies must face the challenges posed by emerging pathogens and constituents in drinking-water sources and finished water. Among these constituents are man-made products like pharmaceutical compounds, hormones, antibiotics, and endocrine disruptors.

Agents like plant hormones and other naturally occurring compounds may be a concern but can not be appropriately termed "pollutants," so the term "microconstituents" has been used to describe all the agents, since it describes man-made and naturally occurring agents that are a concern to the water industry (Table 1).

The Environmental Protection Agency (EPA) has the task of identifying, evaluating, and prioritizing microconstituents for potential regulation. Some of these microconstituents are already included in the EPA's Contaminant Candidate List (CCL), the primary source of contaminants for the agency's drinking water program, which includes research, monitoring, and future regulatory actions (EPA 2004a). Current knowledge of these microconstituents (i.e., occurrence, viability, environmental transport, dose responses, disinfection and removal) is limited, however, and in many cases there are no available methods that would help in gathering information.

These issues have propelled the water research community to develop new tools to help in gathering data and analyzing microconstituents. This article offers an overview of the current and new approaches being developed to detect biological microconstituents. It will discuss the efforts undertaken by Orange County Utilities to develop standardized operating procedures (SOP) using new molecular methods for the detection of *Bifidobacterium*, enteric viruses, and viable *Giardia* in water matrices.

Do We Have the Means to Detect Biological Microconstituents?

While monitoring programs have been largely successful for reducing the number of waterborne disease outbreaks, three major weak areas remain for detecting pathogens and indicators. Culture-based methods for detecting bacteria have been developed only for about 2 to 5 percent of the total number of bacteria in the world (Maier 2000). Culture-based methods are available for some viruses, but they are expensive and time-consuming and they require extensive training and expertise.

Current protozoan pathogen detection methods can only detect presence or absence in water samples. For these reasons, it is important to continue efforts to develop and improve detection methods and monitoring programs to protect public health.

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It is important for the water industry to have the means to detect biological microconstituents, including non-cultivable pathogens, pathogen indicators, toxins, and mutagens; therefore, significant advances have been made in the development of techniques and analytical innovations.

The new trend in method development has been geared toward the field of molecular biology (EPA 2004b). These techniques identify microorganisms at the deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) level by using polymerase chain reaction (PCR)-based procedures.

Molecular techniques have the capability to discriminate and identify specific genes from a wide variety of organisms, and have the potential to detect a single nucleic acid molecule. By targeting a gene of a specific organism, these methods have been able to detect microorganisms in water samples with a high degree of specificity and sensitivity. The organism's target of interest can be DNA, RNA or even proteins molecules.

Gene probes are used to help capture and identify nucleic acids specific to an organism, while highly specific antibodies are used to detect the organism's proteins. Gene probes and antibodies are commonly labeled with fluorescent or chromogenic molecules that aid in the detection and visualization of results (Embrey 2002, Konrad 2003).

While some culture methods rely on colorimetric reactions to provide qualitative results in the form of presence/absence tests, molecular methods rely on capturing and amplifying genes (Konrad 2003). Variations of culture and molecular methods can be used to obtained quantitative results.

The widely used most probable number (MPN) approach can be used for both culture and molecular methods (Clesceri 1998, EPA 2004b). In addition, the quantification

Chemical	Biological		
	Bacteria	Protozoa	Viruses
Endocrine Disruptors	*Aeromonas*	*Cryptosporidium*	Echovirus
Estrogenic Compounds	*Helicobacter*	*Giardia*	Adenovirus
Polybrominated Diphenyl Ethers	Pathogenic *E. coli*	*Toxoplasma*	Calicivirus
NDMA	*Mycobacterium avium* complex (MAC)	Microsporidia spp.	Coxsackievirus
Personal Care Products	Cyanobacteria (and their toxins)		
Pharmaceuticals	*Legionella*		
Antibiotics			
Alkylphenols			
Disinfection By-Products			
Heavy Metals			

TABLE 1: Listing of Chemical and Biological Microconstituents

Feature	Culture	Molecular
Specificity	Very	Very
Sensitivity	Not very	Very
Qualitative	Yes	Yes
Quantitative	Yes	No (semi-quantitative)
Viability	Yes	No (varies)
Interference	No	Yes
Cost	Less costly (varies)	More costly
Skills	Routine procedures (varies)	Highly skillful procedures

TABLE 2: Comparison of culture and molecular-based methods for detecting biological microconstituents in water

of bacteria can be achieved with filtration procedures and selective chromogenic media formulations. On the other hand, the quantification of microorganisms by molecular methods requires the use of specialized equipment and more skillful procedures involving the use of fluorescently labeled gene probes in a real-time PCR format (EPA 2004b).

One of the drawbacks of DNA and some RNA-based methods is that they can detect the nucleic acid of organisms even if they are dead and/or if their free-floating stable nucleic acid is present in the water sample (Venter 2000, Pedley 2003). To help answer the question of occurrence vs. viability, new strategies have been developed that rely on the detection of the microorganism's messenger ribonucleic acid (mRNA).

Only viable organisms, when challenged by stressing conditions (e.g., heat and oxidants), are capable of activating genes to help them survive the stress. Gene activation results in the production of gene-specific mRNA molecules that can be detected utilizing common molecular biology procedures (Embrey 2002, Konrad 2003). Table 2 compares some features of culture and molecular-based methods.

There is a great variety of molecular method techniques, including DNA sequencing, polymerase chain reaction (PCR), reverse transcription–PCR (RT-PCR), real-time PCR/RT-PCR (Embrey 2002, Konrad 2003), microarrays (gene chip) (NRC 2001, Straub 2003), fluorescence in situ hybridization (FISH) (Wilkinson, 1999), enzyme-linked immunosorbent assay (ELISA) (Crowther 2001), immunomagnetic separation (IMS), and immunofluorescence assay (IFA) (EPA 2001). Regardless of the molecular technique format, they are all based in the specific identification of nucleic acids and/or proteins.

Molecular techniques allow for more sensitive ways to detect and speciate microorganisms. Data obtained using these techniques would provide valuable information needed to develop better water quality management programs.

Table 3 describes current and new approaches that researchers are using for the detection of biological microconstituents. Even though many of the molecular approaches are yet to be standardized and approved, the EPA and other organizations are currently funding projects that may result in the development of analytical innovations for the detection of microconstituents.

Examples of Molecular-Based Method Development for Detection of Microconstituents

Molecular biology shows promise for improving microconstituent detection because the laboratory formats and techniques are flexible and numerous. Common cultures techniques can be combined with

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TABLE 3: Current and New Detection Approaches for Biological Microconstituents

Microconstituent	Human Health Effects	Detection Method	Method Development Status
Aeromonas	Gastroenteritis, septicemia	USEPA Method 1605 (culture-based)	Current UCMR program for occurrence underway
Helicobacter	Peptic ulcers and gastritis	**NM**	Can be cultured, but it is slow growing. Only detected in water by PCR approach
Mycobacterium avium complex (MAC)	Pulmonary infection, cervical lymphadenitis (in children), Chrohn's Disease	**NM** Culture and molecular approaches available	AWWARF Project #3016 for improving MAC detection method
Cyanobacteria (blue green algae), other freshwater algae, and their toxins	Gastroenteritis, respiratory effects, skin irritations, allergic responses, and liver damage	**NM** Immunogenic (ELISA) and molecular approaches	Toxins can be detected using GC-MS, LC-MS & HPLC UV.
Legionella	Severe pneumonia, gastrointestinal symptoms and in some cases death	**NM** Immunogenic (IFA) and molecular approaches	AWWARF Project #2657, detection by cultivation, PCR and flow cytometry
Cryptosporidium	Chronic diarrhea and in some cases death in susceptible hosts	USEPA Method 1622 (with immunomagnetic separation)	Tissue culture and PCR
Giardia	Chronic diarrhea	USEPA Method 1623 (with immunomagnetic separation)	Occurrence only by PCR and real time PCR. No molecular method to detect viability
Microsporidia	Gastroenteritis, eye infections, genitourinary track infection, respiratory infection	**NM**	Tissue culture and PCR
Adenoviruses	Gastroenteritis	**NM** ICC-PCR approaches	AWWARF Project #3029 & #2591, detection by real-time RT-PCR and PCR
Caliciviruses	Gastroenteritis	**NM** RT-PCR and sequencing	AWWARF Project #345, detection by immuno-capture RT-PCR
Coxsackieviruses	Meningitis, myocarditis, gastroenteritis & hepatitis	EPA/600/4-84/013 for detecting Enteroviruses	Immunofluorescence or ICC-PCR required for genus differentiation. AWWARF Project #2591, detection by immuno-capture RT-PCR
Echoviruses	Diarrhea, common cold, otitis	EPA/600/4-84/013 for detecting Enteroviruses	Immunofluorescence or ICC-PCR required for genus differentiation

*Sources: EPA 1998; EPA 2001a, EPA 2001b, EPA 2002; FDA 2003; Franzen 2001; Hardin 2002; Maier 2000; NERL 2002; Fields 2002, Oehrle 2003.

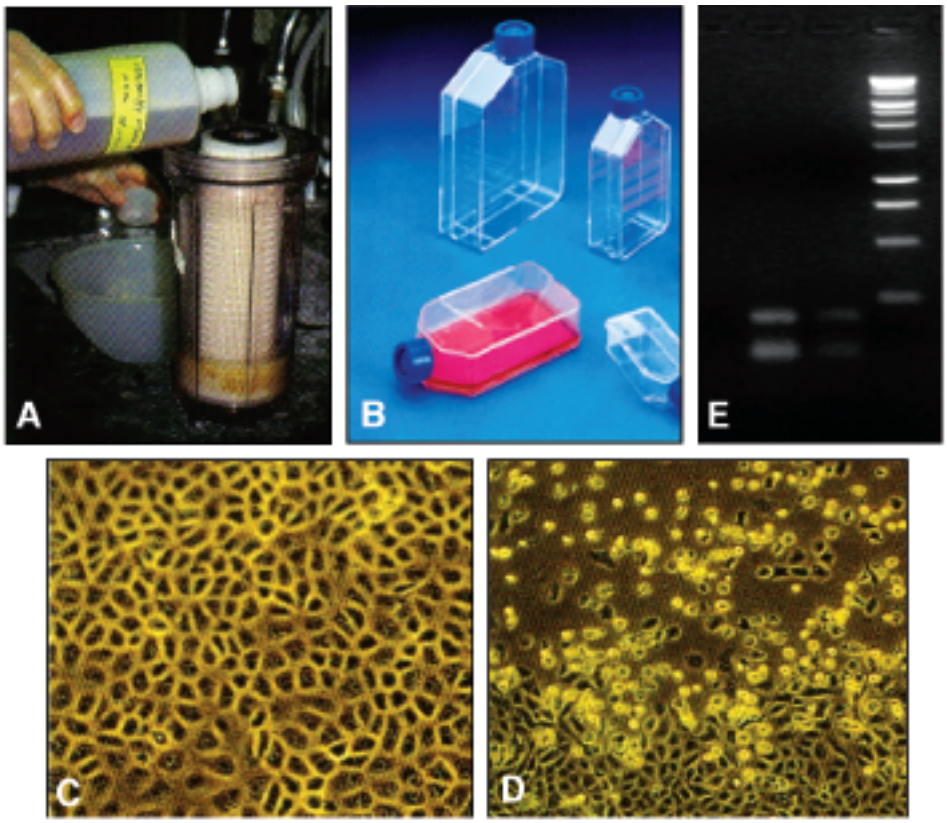

Figure 1

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DNA, RNA, and immunogenic methods to help gather more information; therefore, the research community is constantly exploiting this flexibility in order to find the most reliable way of detecting microconstituents.

Once a given set of techniques has proven to be reliable, then a standard operating procedure (SOP) can be written to specify the steps needed for the new detection method. Following are examples of how molecular biology approaches are being used to detect enteric viruses, *bifidobacteria*, viable *Giardia*, and environmental estrogens.

Enteric Viruses

The Integrated Cell Culture-PCR (ICC-PCR) method to detect enteric viruses was developed for research purposes in 1996 (Reynolds 1996). In this method, specialized 1-MDS Virosorb filter cartridges are used to collect viruses from water samples. Viruses are later eluted and concentrated using beef extract and centrifugation (Figure 1).

The premise behind the ICC-PCR method is the combination of cultural and molecular techniques for increased sensitivity and specific detection of enteric viruses. The method uses Buffalo Green Monkey kidney (BGM) host cells, followed by the specific amplification of the 16S rRNA gene from potential entero viruses.

As a result of virus-host interactions, viral populations are enhanced while cytopathic effects on the cell monolayer (cell death) are visually detected using an inverted light microscope (Figure 1); however, there have been reports of enteric virus that do not cause cytopathic effects and were only detected when their DNA was amplified by PCR (Gilliam 2001 & 2004). It is evident that the combination of cell culture with PCR techniques provides a clearer picture of the presence of enteric viruses in water samples.

The Orange County Utilities (OCU) Laboratory is currently developing an SOP to standardize the ICC-PCR procedure and intends to submit it for NELAC approval. This method is currently under evaluation for enteric viruses in environmental samples.

Bifidobacterium

Human feces are generally perceived as constituting a greater human health risk than animal feces; however, no reliable epidemiological evidence supports this view (Sinton 1997). Despite relative risks, the ability to identify sources would be beneficial in the overall management of water quality. Unfortunately, the most commonly used fecal indicator microorganisms are found in both humans and animals and don't allow sources to be differentiated (Sinton 1997).

Recently there has been interest in the genus *bifidobacterium* as a potential new fecal contamination indicator, since they are the most prevalent bacteria in the human intestinal tract and because their biochemistry is different than other waterborne bacteria. Special culture media formulations and specific PCR protocols targeting the 16S rRNA gene are able to differentiate between human and animal *bifidobacterium* species (Matsuki 1998 & 2004, Sinton 1997).

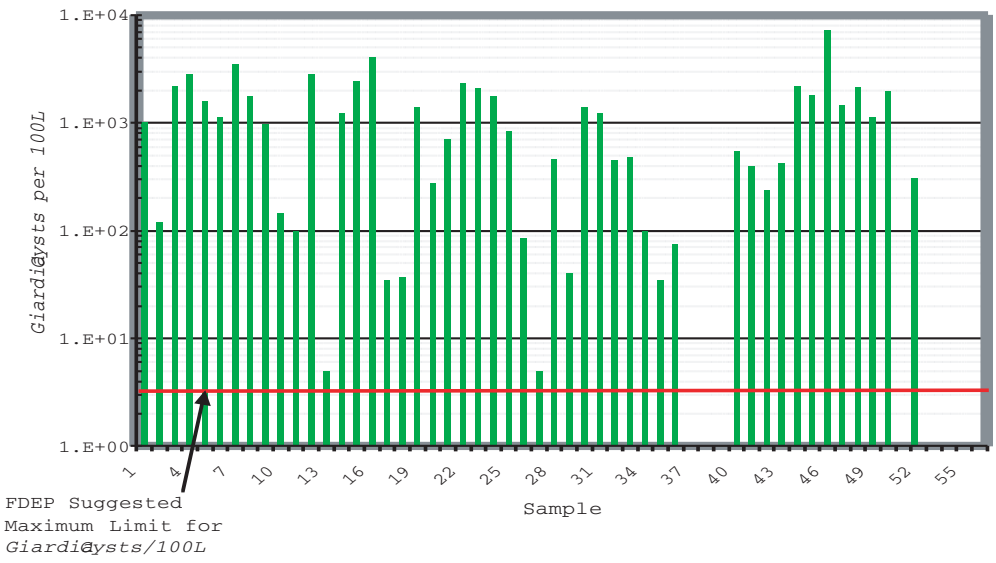

FIGURE 2: Giardia levels in finished reclaimed water in Central Florida

Giardia

The Florida Department of Environmental Protection (FDEP) requires monitoring for *Giardia* cysts and *Cryptosporidium* oocysts in reclaimed water (Florida Administrative Code 62-610) and recommends using USEPA Method 1623 (EPA 2001b). Guidelines have been developed to establish maximum allowable cyst and oocysts levels in reclaimed water, but they are based on the results of occurrence data, and not viability or infectivity.

Slifko et al. (2004) showed *Giardia* cyst and *Cryptosporidium* oocyst levels in reclaimed water that were higher than previously reported, with an average of 1090 cysts/100L (n=56) in 13 reclamation plants in Central Florida (Figure 2). More than 85 percent of the samples analyzed contained more than 50 *Giardia* cysts/100L after advanced secondary or tertiary treatment followed by high-level disinfection using chlorination.

Unfortunately, without viability or infectivity assessment, it is not possible to know if the detected oocysts and cysts may be inactivated by wastewater and reclamation treatment processes and may not be a public health risk. Animal infectivity methods can be used to assess the viability of both *Cryptosporidium* and *Giardia*, but the techniques are impractical and too expensive for routine analyses. While tissue culture can be used to determine *Cryptosporidium* oocyst viability (Rochelle 2002, Slifko 2002), there is no current method for evaluating if cysts detected in water samples are alive or dead.

Based on the premise that only viable *Giardia* cysts are capable of genetically and metabolically reacting to stressing conditions, a viability method can be based on the detection of mRNA molecules (by RT-PCR) synthesized by the thophozoites inside the cysts. Preliminary research using published DNA primers targeted the *Giardia's* heat shock protein 70 gene (Abbaszadegan 1997); however, results from several laboratories were inconsistent (Kaucner 1998, Robles 2004). False-positive blank samples and detection specificity for *Giardia* cysts were questionable (Robles 2004).

Currently, efforts involve pursuing different genetic targets. In order to identify candidate genes, they must meet two criteria: 1) genes demonstrated to be inducible by stressing conditions, and 2) genes with sufficient genetic variability that would allow for *Giardia* cysts speciation (genotyping).

This is very important since there have been reports demonstrating the preference of certain *Giardia* genotypes (e.g., assemblages A and B) for human hosts (Monis 2003, Thompson 2004). A molecular-based viability method that can distinguish living from dead *Giardia* cysts as well as differentiating genotypes that infect humans would be useful for evaluating if environmentally detected cysts are alive and potentially infectious. The method also has potential for survival and disinfection studies.

Environmental Estrogens

Endocrine disruptors are exogenous chemicals or mixtures that alter the function(s) of the endocrine system and thereby cause adverse effects to an organism, its progeny or (sub)populations (Symons 2000). Both natural estrogen and synthetic compounds that mimic estrogen are considered endocrine disruptors of concern, since it is unclear whether their concentrations in the environment and finished reclaimed water are sufficient to cause adverse physiological effects (Snyder 1999). Even though chemical analyses such as conventional high-pressure liquid chromatography (HPLC) have been used to detect estrogenic compounds in aqueous matrices (Snyder 1999), they do not provide any information regarding biological uptake, bioconcentration and biotoxicity.

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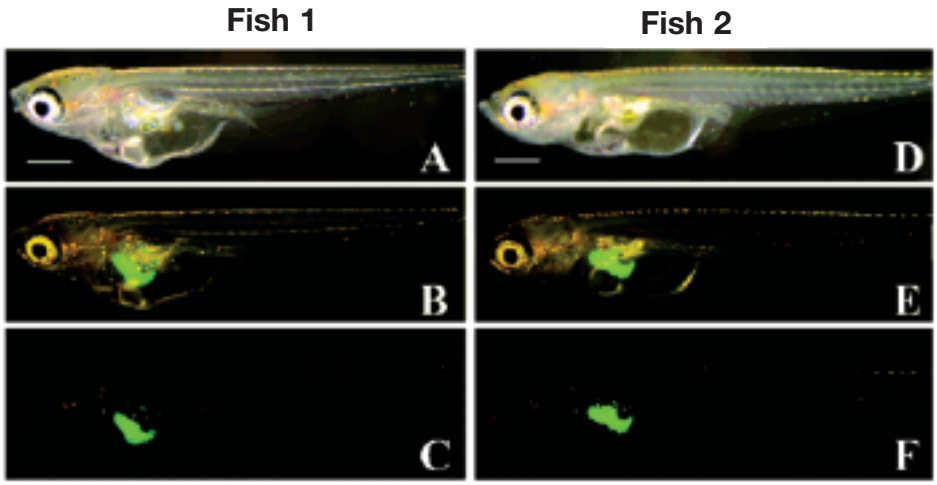

Figure 3

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Recent developments of in-vivo biotests have the potential to offer meaningful data regarding the bioconcentrations and biological effects of estrogenic compounds. Kurauchi et al. (2005) described the use of genetically modified (transgenic) medaka fish larvae to detect estrogen-like substances (ELSs). The researchers introduced a version of the green fluorescent protein (GFP) gene that was activated by ELSs. As a result, the liver of ELS-exposed larvae fluoresced green and could be seen easily through the semi-transparent body of the fish larvae using a fluorescence stereoscopic microscope (Figure 3).

This molecular and in-vivo approach has been used in dose response experiments and has the potential to offer biological data regarding the effects of estrogenic compounds.

Final Thoughts

Before implementing new water-monitoring regulations and water-quality management programs, it is important to gather the necessary knowledge regarding current biological microconstituents. Because of the limitations of current detection methods, new laboratory methods and vanguard strategies are being developed. Researchers have taken advantage of common culture procedures and coupled them with innovative molecular approaches in order to generate comprehensive detection strategies.

Even though this technology has not yet been standardized for water industry applications, it shows great promise and is the new direction for water-quality monitoring. As more data is collected and interpreted, we will better understand how microconstituents could potentially affect our environment and the general public health.

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