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Review article

Towards a unified system for detecting waterborne pathogens

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Abstract

Currently, there is no single method to collect, process, and analyze a water sample for all pathogenic microorganisms of interest. Some of the difficulties in developing a universal method include the physical differences between the major pathogen groups (viruses, bacteria, protozoa), efficiently concentrating large volume water samples to detect low target concentrations of certain pathogen groups, removing co-concentrated inhibitors from the sample, and standardizing a culture-independent endpoint detection method. Integrating the disparate technologies into a single, universal, simple method and detection system would represent a significant advance in public health and microbiological water quality analysis. Recent advances in sample collection, on-line sample processing and purification, and DNA microarray technologies may form the basis of a universal method to detect known and emerging waterborne pathogens. This review discusses some of the challenges in developing a universal pathogen detection method, current technology that may be employed to overcome these challenges, and the remaining needs for developing an integrated pathogen detection and monitoring system for source or finished water.
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Keywords: Unified system; Waterborne pathogen; Current technology

1. Introduction

1.1. Global impact of waterborne disease

Throughout the world, many people do not have access to safe drinking water. As a consequence, there is significant morbidity and mortality due to disease-causing organisms in water. It is estimated that nearly one-fourth of all hospital beds in the world are occupied by patients with complications arising from infection by waterborne organisms (Gerba, 1996). Citing the *WHO/UNICEF Global Water Supply and Sanitation Assessment 2000 Report*, Water For People estimates that nearly 6000 people, mostly children, die every *day* because of water related diseases (http://www.water4people.org/default.htm). Even in the United States, an estimated US$20 billion per year in lost productivity has been attributed to diseases caused by waterborne pathogens (Gerba, 1996).

1.2. Known and newly recognized waterborne pathogens

Known agents of waterborne disease include viruses, bacteria, protozoa, and helminthes. Representative examples from each of these groups are listed in

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Table 1
Examples of major groups and genera of waterborne and water-based pathogens (modified from Gerba, 1996)

Group	Pathogen	Diseases caused
Viruses	Enteroviruses (polio, echo, coxsackie)	meningitis, paralysis, rash, fever, myocarditis, respiratory disease, and diarrhea
	Hepatitis A and E	infectious hepatitis
	Human Caliciviruses	
	Norwalk viruses	diarrhea/gastroenteritis
	Sapporo	diarrhea/gastroenteritis
	Rotavirus	diarrhea/gastroenteritis
	Astroviruses	diarrhea
	Adenovirus	diarrhea (types 40 and 41), eye infections, and respiratory disease
	Reovirus	respiratory and enteric
Bacteria	*Salmonella*	typhoid and diarrhea
	Shigella	diarrhea
	Campylobacter	diarrhea-leading cause in foodborne outbreaks
	Yersinia enterocolitica	diarrhea
	Escherichia coli O157:H7 and other certain strains	diarrhea, can lead to hemolytic uremia syndrome as a complication in small children.
	Legionella pneumophila	pneumonia and other respiratory infections
Protozoa	*Naegleria*	meningoencephalitis
	Entamoeba histolytica	amoebic dysentery
	Giardia lamblia	chronic diarrhea
	Cryptosporidium parvum	acute diarrhea, fatal for immunocompromised individuals
	Cyclospora	diarrhea
	Microsporidia includes	chronic diarrhea and wasting, pulmonary, ocular, muscular and renal disease
	Enterocytozoon spp.	
	Encephalitozoon spp.	
	Septata spp.	
	Pleistophora spp.	
	Nosema spp.	
Cyanobacteria	*Microcystis*	diarrhea from ingestion of the toxins these organisms produce microcystin toxin is implicated in liver damage
	Anabaena	
	Aphantiomenon	
Helminths	*Ascaris lumbricoides*	ascariasis
	Trichuris trichiora	trichuriasis-whipworm
	Taenia saginata	beef tapeworm
	Schistosoma mansoni	schistosomiasis (affecting the liver, bladder, and large intestine)

Table 1 (Gerba, 1996). While some agents are well recognized, others have only recently been identified from the broad category of cases classified as "waterborne disease outbreaks of unknown etiology" (AGI, Fig. 1). For example, considerable attention is now focused on cyanobacterial toxins and their removal by

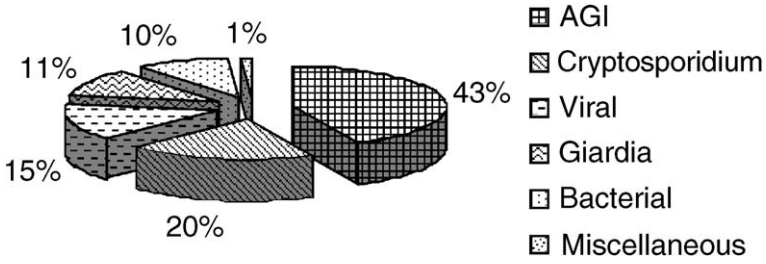

Fig. 1. Etiological agents associated with cases of waterborne diseases (AGI = acute gastroenteritis of unknown etiology). Source: Gerba, 1996.

conventional drinking water treatment (Karner et al., 2001). A number of world organizations are considering guidelines for the maximum allowable concentration of cyanobacterial microcystins in drinking water (WHO, 1999; NHMRC, 2000), and the United States Environmental Protection Agency (USEPA) has placed algal toxins on the Drinking Water Candidate Contaminant List (USEPA, 1998). The protozoan *Cyclospora cayetanenis* is another recently described human pathogen (Ortega et al., 1993) that has been isolated from food (Herwaldt et al., 1997) and wastewater (Sturbaum et al., 1998). The Microsporidia includes at least five genera that were originally recognized as pathogens in immuno-compromised (usually HIV infected) patients (Weber et al., 1994). Their presence in water and wastewater has been implied (Dowd et al., 1998), implicating water as a potential reservoir or transmission vehicle for these organisms.

1.3. Prevention of waterborne disease

Several nations, such as those countries in the European Union and the United States, have the financial resources to prevent waterborne disease outbreaks. Source water protection, advances in water treatment, and real time monitoring of water quality parameters are some of the preventative measures that may one day relegate specific pathogen monitoring to those situations where a waterborne disease outbreak is likely (Allen et al., 2000). Cooperation between the water industry and government is cited as one of the reasons for declining waterborne diseases attributed to treated surface water in the United States (Barwick et al., 2000). Global organizations are also assisting developing nations to provide safe drinking water to all people.

Despite advances in preventing waterborne disease, severe outbreaks still occur, even in developed nations like the United States (*Cryptosporidium*, Milwaukee, 1993), Canada (*Escherichia coli* O157:H7, Walkerton, Ontario, 2000), the United Kingdom and Europe (several outbreaks of *Cryptosporidium*). Thus, specific detection methods are still required in order to trace the origin of etiological agents, identify lapses in water treatment, and identify new quality control processes and procedures.

1.4. USEPA and standard methods (American Public Health Association) for pathogen detection in water

1.4.1. Viruses

Viruses are often the most dilute pathogens in water. Volumes in excess of 100 l for surface water sources and 1000 l for drinking water are frequently required in order to be reasonably confident in an assay. The currently accepted sample collection method is filtering source or finished water through positively charged 1-MDS filters (USEPA, 1993). Viruses are trapped on the filter by electrostatic charge and are released by elution with beef extract or an amino acid solution. Viruses eluted into the solution are precipitated by acid flocculation (USEPA, 1993) or polyethylene glycol (PEG, Schwab et al., 1996). The viruses are then resuspended into sodium phosphate buffer, filter sterilized to remove bacteria and other debris, and detected on monolayers of mammalian cells (usually simian origin). Variability in the efficiency of the method across different water matrices, co-concentration of substances toxic to cell monolayers, and the inability of some viruses (notably the human caliciviruses) to infect known cell lines are some of the known limitations of these methods for virus recovery and detection.

1.4.2. Protozoa

The methods for the recovery and detection of protozoa also require large sample volumes. In USEPA Method 1623 (USEPA, 2001) for the detection and enumeration of *Giardia* and *Cryptosporidium*, 10 l of surface water is passed through a depth filter (cartridge style Hach, or foam pad) to capture the parasites (nominally 3–18 μM in diameter). The filters are eluted with a detergent solution, concentrated by centrifugation, and parasites separated from the matrix using immunomagnetic techniques. The purified oocysts are then immobilized on glass slides and stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies and counterstained with DAPI. Detection and enumeration is accomplished by manually scanning the entire surface of the slide for objects that have the size, shape, and fluorescence typical of these organisms. For each suspected object, the operator then confirms their presence using Differential Interference Contrast (DIC) microscopy to scan for internal structures

typical of these organisms. Finally, the object of interest is examined using DAPI fluorescence microscopy to determine the number of nuclei that are stained (*Cryptosporidium* oocysts may have four sporozoite nuclei per oocyst and *Giardia* may have two trophozoite nuclei per cyst). Despite better reproducibility of this method compared to the method used for the USEPA's Information Collection Rule, there is still a high degree of skill and operator involvement needed to analyze multiple samples (Allen et al., 2000).

1.4.3. Bacteria

In the United States, members of the family *Enterobacteriaceae* have served as indicators of microbial contamination of drinking water. Defined substrate media formulations have made it possible to differentiate between total coliforms and *E. coli*, and to quantify their concentrations in a source or treated water (APHA, 1998). In comparison to the viral and protozoan techniques, the methods for detecting indicator bacteria are relatively simple. If the monitoring requirement is simple presence/absence, an operator adds a 100-ml water sample to a bottle containing defined substrate (Colilert and Colisure are two common brand names). If total coliforms are present, the organisms cleave a choromgenic analog of lactose that turns the media from clear to yellow (Colilert test; indicative of beta galactocidase activity). Methylumbelliferryl galactocide (MUG) is also present in this media, and is specifically cleaved by *E. coli*. If MUG is cleaved, the media appears fluorescent blue under long-wave UV light. Monitoring for indicator organisms with these simple techniques has been the traditional test for microbiological quality of drinking water, but a number of known deficiencies exist. For example, protozoan and viral pathogens have been found in water when indicator organisms were absent (Gerba, 1996). Even *E. coli* O157:H7 escapes detection with the indicator methods, because approximately 50% of the O157:H7 strains do not cleave MUG (FDA, 1995).

1.4.4. Alternative detection methods

Because of known deficiencies in the current methods, numerous culture-independent, molecular assays are under continuous development for individual pathogens in each of the major pathogenic groups (viruses, bacteria, protozoa). These include immunofluorescent antibody techniques (Yu, 1998; Dowd et al., 1999), fluorescent in situ hybridization (Buswell et al., 1998; Franks et al., 1998), magnetic bead cell sorting (Porter and Pickup, 1998; Pyle et al., 1999), electrochemiluminescence (Yu and Bruno, 1996), amperometric sensors (Perez et al., 2001), gene probes (Bej et al., 1990; Kreader, 1995) and innumerable polymerase chain reaction (PCR), RT-PCR and real-time PCR methods (Toranzos and Alvarez, 1992; Puig et al., 1994; Tsai et al., 1994; Bassler et al., 1995; Mayer and Palmer, 1996; Stinear et al., 1996; Deng et al., 1997; Rochelle et al., 1997a,b; Sluter et al., 1997; Kaucner and Stinear, 1998; Sheridan et al., 1998; Di Giovanni et al., 1999; Dowd et al., 1999). Obviously, neither the EPA nor Standard Methods (APHA, 1998) for pathogen detection in water nor the references cited here encompass the full range of possible pathogen detection techniques. What is evident from even a cursory review of the literature, however, is that detecting etiological agents in source or finished water is frequently problematic because (1) pathogens are very dilute, (2) established protocols for sample collection, concentration, and identification do not have the requisite method-level sensitivity to detect dilute agents; and most importantly, (3) the plethora of competing methods are non-comparable to each other, to approved regulatory methods, or to comparative analysis across the different groups of pathogens (i.e. viruses, bacteria, protozoa). The resulting confusion and technical schizophrenia surrounding pathogen detection in water therefore highlight the need for a unified, standardized, integrated biodetection method and system.

1.5. Integrated biodetection systems

Integrated water quality biodeteciton systems and/or unified methods for pathogen detection are only now receiving attention at scientific meetings and conferences. Inherent in the discussions (and debates) are questions such as:

- What is the detection objective? Is it enumeration? Presence/absence? Viability and infectivity? All of the above?
- What is an appropriate level of risk? Is it one virion in 1000 l of water?

- What are the performance specifications (e.g. false positive/false negatives) for the method or system?
- What volume of water needs to be collected, processed, *and examined* to be confident in the result and declare a source to be safe for public consumption?
- Is it necessary to specifically detect the full range of pathogens that may be present in a water supply?
- Can the proposed method be automated, or operated and interpreted by a non-specialist?

These questions (and others) are easily translated into specific technical challenges associated with water sampling, pathogen quantitation, and multiplexed detection, all of which are interlinked in the analytical method. Converting the disparate techniques and detection methods described above into an integrated biodetection system, however, also implies the seamless integration of biochemistry and instrumentation that span sample collection, sample preparation, detection and data synthesis/output functions of direct relevance to end user (Fig. 2). The detection objective is required in order to define and guide the appropriate selection of techniques that allow one to quantify microbiological properties of the original environmental sample, but the detection objective clearly constitutes more than a "detector." Thus, we illustrate some of the specific technical challenges facing unified pathogen "detection" and the development of a fully integrated water quality monitor.

2. Technical challenges

2.1. Physical parameters that impede universal collection

Pathogens of interest in drinking water range in size from 0.01 to 100 μm in diameter (Gerba, 1996 and Table 2). This size difference alone presents unique challenges for universal sample collection. For viruses, the current method uses positively charged filters (1-MDS) with a nominal pore size of 0.2 μm (200 nm). However, it is the charge of the filter that is the governing factor in trapping viruses, not the pore size. This filter works reasonably well for viruses, but may not be the best filter for recovery of bacteria or parasites due to the physical structure and tortuous flow path of the device that may concentrate and compact sediment with bacteria and parasites (Juliano and Sobsey, 1997). Subsequent to filtration and elution, low-speed centrifugation of the eluant will concentrate bacteria and protozoans, but ultracentrifugation is required in order to concentrate

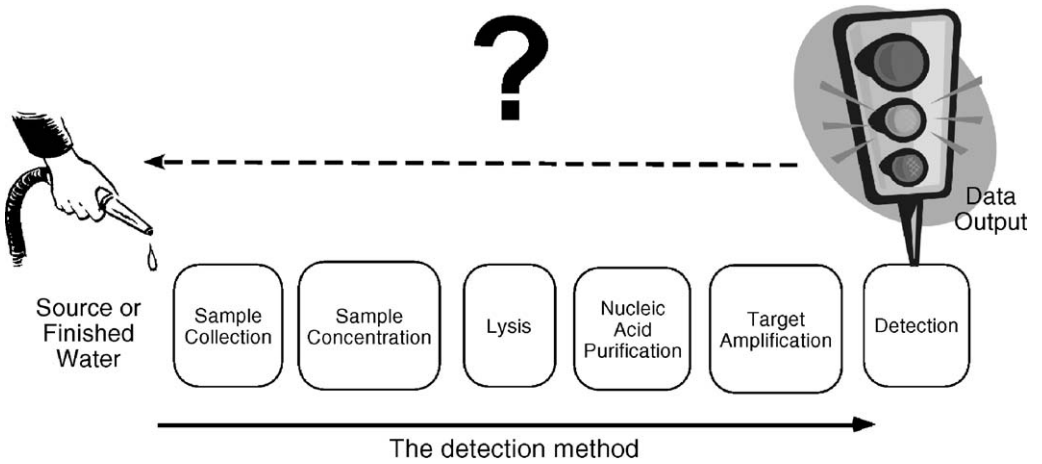

Fig. 2. The biodetection process. Most nucleic acid detection methods require some or all of the manipulations illustrated here. An integrated system requires the same operations, with the added challenge of automating the biochemistry that seamlessly integrates the different steps of the detection method. Even with the plethora of available detection methods (see text), the uncertainty (and lack of quantitation) associated with each of the operations makes it difficult to relate the detector output (red light/green light) to the starting concentration of microbial pathogen in the original sample.

Table 2
Characteristics of waterborne and water-based pathogens (source: Gerba, 1996)

Organism	Size (μm)	Shape	Environmentally resistant stage
Viruses	0.01–0.1	variable	virion
Bacteria	0.1–10	rod, spherical, spiral, comma	spores or dormant cells
Protozoans	1–100	variable	cysts and oocysts
Helminths	1–10^9	variable	eggs
Cyanobacteria	1–100	coccoid and filamentous	cysts

viruses. Thus, acid flocculation of the proteins or precipitation using polyethylene glycol (PEG) is typically performed in order to concentrate viruses from filter eluants. Unfortunately, centrifugation and precipitation are not conducive to automation within an integrated detection system. Thus, what analytical principle and/or method can simultaneously collect and concentrate viruses, bacteria and protozoa?

2.2. Expected pathogen concentrations in the environment

The absolute and relative concentration of pathogens in water varies significantly in both time and space, as illustrated in Table 3 (USEPA, 1988), an issue that becomes important when the etiological agent has an infectious dose of one particle (e.g. enteroviruses). The difference between total coliform counts and specific parasites, for example, can vary by a factor of 10^6–10^9, a scaling and sampling problem that is exacerbated as the pollution source becomes more dilute. That is, water quality detection objectives coupled with the disparate concentrations of etiological agents in the environment imply a concomitant requirement to sample and process large volume water samples (in excess of 100 l) to detect low copy numbers of certain target pathogens. What analytical principles and/or methods can be (semi-) automated in such a manner that very dilute and/or concentrated organisms are captured and detected from very large volume water samples?

2.3. Chemical and matrix effects

Concentrating or recovering organisms from large volume water samples has been shown to co-concentrate inhibitors that interfere with downstream molecular and/or fluorescent detection methods (Schwab et al., 1996; Tebbe and Vahjen, 1993). In addition to interferents at the point of detection, the physical quantity of sediment recovered during filter elution often makes it impossible to examine all of the concentrated material. Due to physical loading from suspended sediments, as little as 100 ml equivalent of water may be examined from a 100-l water sample, a problematic situation discovered for protozoan monitoring and subsequent data collection during the USEPA's Information Collection Rule (ICR; Allen et al., 2000). That is, a report of 1 *Cryptosporidium* oocyst per 100 ml is equivalent to 1000 oocysts per 100 l, which would require a water utility to continuously monitor drinking (finished) water. However, a single 100-ml sample is not statistically representative of the ICR-required 100-l sampling volume, such that the inferred pathogen load is wholly inaccurate relative to the environmental sample (Fig. 2, question mark). Thus, regulatory compliance becomes impossible based on a 100-ml equivalent volume. What methods or technologies are available that can separate particles of interest (viruses, bacteria, protozoa) from suspended sediments within the context of an integrated system while providing statistically relevant sample volumes?

2.4. Detection objectives

Water quality detection objectives generally fall into three general categories: (1) presence/absence methods to alert officials that a problem may exist, (2) enumeration of the target pathogen or indicator organism(s) to meet regulatory requirements or provide data for microbial risk assessments, and (3) methods to determine if the target pathogen is viable

Table 3
Estimated levels of enteric organisms in sewage and polluted surface water in the United States (source: USEPA, 1988; Gerba, 1996)

Organism	Concentration per 100 ml	
	Raw sewage	Polluted stream water
Coliforms	10^9	10^5
Enteric viruses	10^2	1–10
Giardia	10	0.1–1
Cryptosporidium	10–10^3	0.1–10^2

and/or infectious. These monitoring or detection objectives are not necessarily congruent. For instance, EPA Method 1623 for *Giardia* and *Cryptosporidium* is principally an enumeration method. The fluorescent antibodies used to label the cysts and oocysts are genera-specific, and the DAPI stain for nucleic acid material was found to significantly overestimate viability and infectivity compared to "gold standard" neonatal mouse infectivity assays (Bukhari et al., 2000). Thus, the principal utility of Method 1623 is microscopic enumeration and confirmation for the presence of *Giardia* spp. and *Cryptosporidium* spp. in a water sample, but it cannot be used to make conclusions about viability or infectivity.

Differing detection objectives have a corollary challenge that faces the biologist or biochemist. That is, cell cultures, DNA assays, antibody-based assays, vital dye stains, and the molecular techniques described above are also non-congruent. Microorganisms exist in a viable but non-cultural state, and many cannot be detected (or accurately quantified) by standard culture techniques (Baudart et al., 2001). DNA testing alone does not provide evidence for viability or infectivity. And it is very difficult to develop specific antibodies that do not cross-react with non-target species or strains. Thus, what biological assay can simultaneously detect (presence/absence), quantify and assess the viability of waterborne organisms? Can a unified biochemical approach be developed within the context of the integrated system in Fig. 2? Is there a common platform (biochemistry and devices) that can simultaneously address the non-congruent detection objectives of varied users?

3. Towards an integrated water quality monitoring system

From the foregoing discussion, we assert that an idealized water quality biodetection system should be capable of addressing the multitude of detection objectives described above. The system should also simultaneously detect viruses, bacteria and protozoa. While additional operational requirements could be added to the list of desired system features, designing such a system will require specific resolution of the aforementioned technical challenges. Further, concentrated pathogens may require additional processing steps before a specific detection technique can be successfully employed (e.g. Fig. 2). Given the different detection objectives described in Section 2.4, we believe that nucleic acid analysis (as opposed to other molecular techniques) holds the most promise for simultaneously satisfying the disparate endpoints. In particular, nucleic acid signatures can be absolutely specific for the target organism; nucleic acids can be quantified even in the absence of culture-based techniques; and RNA may provide evidence for viability. Additionally, nucleic acid methods may allow the detection of injured or damaged cells that can not be resuscitated using culture-based methods. We acknowledge that the analysis of viral nucleic acids does not necessarily provide evidence for infectivity. On the other hand, the mere presence of a (viral) contaminant provides evidence that an infectious particle may be present. Thus, we highlight some of the leading, candidate technologies that, in our view, satisfy most of the criteria for a universal biodetection system or method as set forth above, emphasizing a nucleic acid-based integrated system.

3.1. Large volume sampling and filtration

Hollow fiber filters are commonly used to separate colloidal particles from a fluid stream. The smallest colloid that can be separated from a fluid is dependent on the molecular weight cutoff (MWCO) rating for the filter, with typical cutoffs from several thousand (6000 MWCO; Oshima et al., 1995) to 100,000 daltons (Berman et al., 1980). The filters work primarily by a cross flow circulation pattern (as opposed to tortuous path filtration). Fluid that passes through the filter (the permeate) is discarded while particles larger than the MWCO are retained (the retentate). Using a two-step hollow fiber ultrafiltration method, for example, Olszewski et al. (2001) was able to concentrate a 100-l sample of Rio Grande river water (turbidities up to 159 NTU) to 250 ml; using 10 ml of concentrated water, the authors actually assayed 4 l equivalent of Rio Grande water with a cumulative recovery efficiency of 56%. There are also reports where ultrafiltration is used to remove toxins (Evans-Strickfaden et al., 1996) and concentrate viruses from source or finished water (Berman et al., 1980; Divizia et al., 1989; Klein et al., 1971; Oshima et al., 1995), illustrating how a hollow-fiber filter can be used as a

front end for many different water quality monitoring applications.

With increasing pressure to monitor for all types of pathogens that may be present in a water sample, the additive costs of performing a separate analysis for each organism of interest, and with recent improvements in the design of hollow-fiber filters, there is renewed interest in using hollow-fiber filters to simultaneously collect and concentrate all pathogens in a water sample with high (40–80%) efficiency (Juliano and Sobsey, 1997; Kuhn and Oshima, 2001; Olszewski et al., 2001; Simmons et al., 2001). The hollow fiber filter maintains the physiological integrity of a target organism by keeping particles suspended in the retentate, unlike a conventional depth filter that results in sample compaction and dessication. For traditional cell or animal culture infectivity studies, then, a hollow-fiber filter may increase overall method sensitivity and reproducibility. From the integrated system perspective, however, hollow fiber filters also offer several important advantages over other concentration techniques. For example, the MWCO of a hollow-fiber filter allows soluble or smaller inhibitors to pass into the permeate which is ultimately discarded (Wilson, 1997). This is a practical feature of tremendous value for subsequent, nucleic acid-based detection methods. Keeping particles in suspensions eliminates the need to elute particles (viruses, bacteria, protozoa) from a membrane. Hollow-fiber filters can also be backflushed and re-used (Kuhn and Oshima, 2001; Olszewski et al., 2001), which would allow the sample concentration step to be economically and efficiently (semi-) automated.

3.2. Automated sample purification and processing

Many advanced, integrated nucleic acid diagnostic devices are under development in academic, government, and commercial laboratories, driven primarily by clinical diagnostics and drug discovery applications (Schena et al., 1996; Belgrader et al., 1998; Burns et al., 1998; Cheng et al., 1998; Drmanac et al., 1998; Waters et al., 1998; Woolley et al., 1998; Schmalzing et al., 2000; Schneider et al., 2000). While these devices may be appropriate as detection elements (in Fig. 2), water quality systems must be capable of processing very large sample volumes (Section 2). Thus, most advanced "detection" devices lack the physical dimensions and sample throughput required for environmental sample processing.

One interesting aspect of the sample preparation challenge is that many of the biological separations required for the detection and characterization of waterborne pathogens are based upon interactions of the analyte (whole cells, oocysts, viruses, nucleic acids, proteins) with a surface. Microparticles, in particular, are increasingly applied to biological separations and detection, including whole cell concentration (Berry and Siragusa, 1997; Di Giovanni et al., 1999), cell lysis (More´ et al., 1994), nucleic acid purification (Chandler et al., 1999), solid-phase PCR amplification (Toranzos and Alvarez, 1992), and detection (DiCesare et al., 1993; Yu and Bruno, 1996; Seo et al., 1998). This is not to say that a particle (or more generally, a reactive surface) is required to perform each of the biochemical steps in the integrated method or process, only that the operations and chemistries required to satisfy many water quality detection objectives are compatible with microparticle-based systems. Thus, microparticles can form the basis of a unified analytical principle and method.

The convergence of microparticle chemistry, microfluidic systems, molecular biology, and environmental microbiology has recently resulted in an interesting set of renewable surface techniques for automated sample processing (Fig. 3; (Chandler et al., 2000a,b)), concepts that we have exploited specifically for nucleic acid analysis and detection in environmental samples. Renewable surface systems take advantage of scaleable fluidics and custom-designed flow cells to automatically pack, perfuse, observe, and discard microparticles, providing the necessary fluidics platform for handling microliter to liter sample volumes typically required for the isolation, separation and detection of pathogens in source or finished water. A key feature of direct relevance to a water monitoring system is that a renewable column flow cell *itself* can be used to separate residual, suspended sediments co-concentrated with pathogenic agents prior to microbial and/or nucleic acid purification techniques. Renewable column designs are further able to manipulate most (commercial) microparticle matrices typically utilized in bench-top procedures, including polymer, silica, hydrogel, and magnetic particles ranging in size from 50 nm to 300

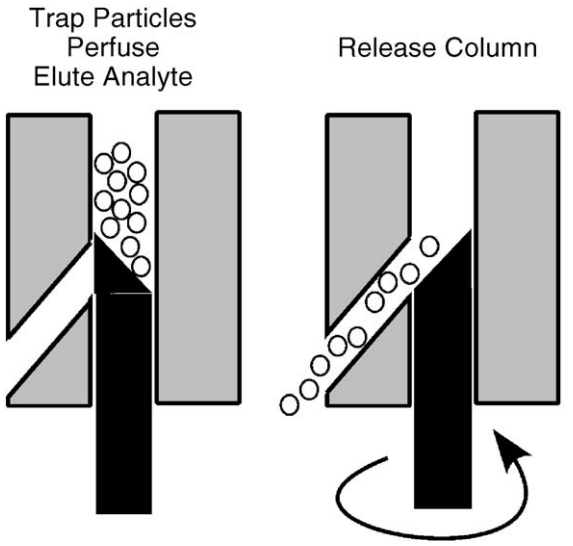

Fig. 3. The renewable microcolumn concept relies upon the manipulation of microparticle suspensions in a fluidic system, and retaining those particles in a suitable flow cell for sample preparation and/or detection (inject, perfuse). After analysis, the microparticle matrix is removed from the system and replaced for the next sample (release). Renewable surfaces prevent degradation, contamination, or fouling of the affinity matrix, and are therefore compatible with highly specific affinity binding reagents. Direct observation of nucleic acid binding events is possible on-column, or with an appropriate downstream detector.

μm in diameter. Thus, many bench-top assays can be easily re-formatted and automated within the fluidics system. For example, nucleic acids have been purified directly from soil and sediment extracts on either affinity (Chandler et al., 1999) or total (Bruckner-Lea et al., 2002) nucleic acid binding resins. DNA purification and PCR amplification from soil extracts has been physically and biochemically coupled within a renewable surface system (Bruckner-Lea et al., 2002). Whole cell immunocapture has been automated and biochemically coupled to multiplexed PCR and microarray detection (Chandler et al., 2000a,b). These examples are only illustrative of how the biochemical processes required for an integrated detection system (Fig. 2) can be configured to connect environmental samples to advanced nucleic acid detectors in a fully automated (and potentially autonomous) system. Other techniques amenable to automation in the renewable surface fluidics platform include total nucleic acid purification and labeling methods of the type required for nucleic acid analysis on microarrays (Bavykin et al., 2001).

3.3. Multiplexed detection with microarrays

Increasing public visibility, concern over emerging pathogens, bioterrorist activity, and a congressionally mandated microbial Contaminant Candidate List (CCL), have resulted in new regulatory and monitoring requirements for specific microorganisms and a concomitant need for new, near real-time, quantitative and on-line analytical techniques. Importantly, every published method or alternative detection technique developed thus far is specific for an *individual* microorganism, such that 10 (or more) independent methods/protocols are required simply to survey water supplies for organisms on the CCL, irrespective of bioterrorist threats or non-pathogenic microorganisms that also affect water quality.

The solution to detecting multiple pathogens in a sample *with a single, not split sample, assay* is a technical challenge regardless of the chosen platform or molecular recognition element (nucleic acids, proteins). However, microarrays represent an important advance and potential solution to the challenge of specific, multiplexed detection. Simply stated, microarrays are high-density dot blots. Probes can be arrayed on glass microscope slides (Guo et al., 1994), gel element pads (Bavykin et al., 2001), and nylon membranes using a robotic device, or synthesized on-chip through photolithographic techniques (McGall et al., 1996). Thousands to millions of gene sequences may be arrayed in an area as small as 1×1 cm. A number of biotechnology companies now sell whole genome arrays for *E. coli*, *Pseudomonas aeruginosa*, yeast, *Drosophila*, rat, and human, with forthcoming microarray products for other model systems.

Unfortunately, a "water quality" microarray has not yet been developed that satisfies the multiple detection objectives stated above. Nonetheless, there are a growing number of reports illustrating how microarrays can be used for environmental biodetection purposes. Ye et al. (2001) discuss the applications of DNA arrays for microbial systems (in general), and cite several studies involving detection of clinically important pathogens. Guschin et al. (1997) describe a gel-pad array technology for the detection of 16S

rRNA from pure cultures of environmentally relevant bacteria. This work was continued by Bavykin et al. (2001) who developed a portable microarray reader (and attendant sample preparation biochemistry) for field-deployment of the gel-pad technology. Small et al. (2001) described the direct detection of intact rRNA on oligonucleotide microarrays, including the hybridization and detection of target organisms directly from an unpurified soil extract (i.e. limited sample preparation). The significance of these studies lies in the direct detection of RNA without invoking a PCR amplification step, a technological advance that circumvents inhibitors (Tebbe and Vahjen, 1993) and common biases (Polz and Cavanaugh, 1998) of the PCR for multi-species (or community profiling) detection.

Other environmentally oriented applications of microarray technology include studies by Call et al. (2001) who optimized multiplex PCR for 4 *E. coli* virulence factor genes and used an oligonucleotide microarray to differentiate between O157:H7 genotypes, other pathogenic *E. coli* and non-pathogenic strains. The prototype *E. coli* chip was then coupled to the automated, immunomagnetic separation of O157:H7 directly from poultry carcass rinse (Chandler et al., 2000a,b). Chizhikov et al. (2001) performed multiplex PCR targeting six virulence factor genes present in *E. coli* O157:H7, *Shigella*, and *Salmonella*, and resolved these products on an array. Specificity of the microarray assay was high compared to the analysis of PCR products on agarose gels. Finally, Straub et al. (2002) used an *hsp70*-targeted oligonucleotide microarray to simultaneously genotype *Cryptosporidium* species and strains with single-nucleotide mismatch discrimination. While these studies show how microarrays can be applied to detect and genotype relevant water-quality pathogens, the PCR represents a potential integrated systems bottleneck to higher-order multiplexing and simultaneously meeting the disparate water quality detection objectives.

4. Making a better system

We have made the argument that an integrated system for detecting multiple pathogens in source and drinking water is much more than just the endpoint "detector," and we have illustrated several technologies and methods that could be integrated into a prototype, universal, multiplexed detection system that satisfies multiple user requirements. And while significant advances have been made to detect and identify new pathogens that are the causative agents of the "AGI" (Fig. 1), relatively little attention has been paid to the system-level perspective and attendant technology that would allow for the specific detection of all possible pathogens that may be present in a water supply at a given time. As more is learned about newly discovered pathogen threats in water, and new methods to determine source attribution for existing pathogens are developed, it becomes difficult to justify ascribing a waterborne disease outbreak to one etiologic agent as is currently done. This is especially true if the reason for an outbreak was a failure of the water treatment train. It begs the question: if the agent that has been implicated made it through the system, what else made it through the system? Configuring the same method within an integrated system would provide a monitoring tool to warn public utilities before individuals become symptomatic, and eliminate the need for retrospective epidemiological investigations.

Clearly, the regulatory requirements for allowable maximum contaminant levels (MCLs) are quite demanding, and represent a significant challenge to any method or system. This paper describes several technologies that can be integrated into a unified method and system, but it is unlikely that the first iteration of the method (or system) will meet MCL requirements. To achieve the requisite method-level sensitivities will likely require additional, fundamental progress in several areas, including improved sample extraction and purification efficiencies, eliminating PCR from the analytical process, optimizing the sensitivity and specificity direct hybridization and detection (to compete with PCR detection limits for low-abundance targets), and quantifying the efficiency of each analytical step in real time. Nevertheless, a unified method and system for microbial monitoring in water is a realistic possibility with important regulatory and public health implications.

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