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REVIEW

# Biosensors for the detection of waterborne pathogens

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**Abstract** Waterborne bacterial, viral and parasitic pathogens are a global health concern and their rapid and specific detection in contaminated potable water is of utmost importance. Biosensors using a variety of biorecognition molecules and transduction methodologies have been reported, and have the potential to enable highly sensitive detection of the analyte of interest in a short time with high specificity. However, there are several obstacles to the detection of waterborne pathogens—they tend to be present at very low concentrations in the environment and environmental samples contain numerous inhibitors of enzymatic reactions and interfering organisms and particulates. Here we present a review of the current state of biosensor technology with regard to the improvements needed over standard detection methods and the challenges presented by real environmental samples. Further, we identify future areas of focus necessary to realize novel detection devices capable of supplanting the gold standards of today.

**Keywords** Biosensor · Waterborne · Pathogen · Microfluidic

**Abbreviations**

| | |
|---|---|
| CFU | Colony-forming unit |
| CPE | Cytopathogenic effects |
| ELISA | Enzyme-linked immunosorbent assay |
| IMS | Immunomagnetic separation |
| NASBA | Nucleic acid sequence-based amplification |
| PCR | Polymerase chain reaction |
| PFU | Plaque-forming unit |
| PMT | Photomultiplier tube |
| RT-PCR | Reverse transcriptase PCR |
| SERS | Surface-enhanced Raman spectroscopy |
| SNT | Serum neutralization test |
| SPR | Surface plasmon resonance |
| UPT | Up-converting phosphor technology |
| US EPA | United States Environmental Protection Agency |
| WHO | World Health Organization |
| μTAS | Micro total analysis system |

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## Importance and challenge of waterborne pathogens

Lack of access to safe, potable water is a global health concern that, in combination with poor hygiene and sanitation facilities, affects more than half the population of the developing world. Almost 1 billion people rely on contaminated water sources, contributing to the 2.2 million annual deaths caused by diarrheal diseases [1], which the World Health Organization (WHO) estimates as at least 4% of the global disease burden [2]. This lack of access to safe water and proper sanitation facilities disproportionately effects children; those under five years old account for over 90% of the annual deaths from diarrheal diseases with about 5,000 children dying per day [3].

Waterborne pathogens are also a problem in developed nations. The annual number of cases of acute gastrointestinal illness in the United States caused by the consumption of contaminated water has been estimated to be from 4.26 million [4] to as high as 32.9 million [5]. Some of the largest outbreaks resulting from protozoan parasites, for

example *Cryptosporidium* and *Giardia*, have been in developed nations. The 1993 outbreak in Milwaukee, Wisconsin, is still the largest caused by *Cryptosporidium*; over 400,000 people were infected and 100 deaths were attributed to the contamination of the municipal water supply [6, 7]. A crisis hit Sydney, Australia, in 1998 when severe weather systems flooded the water treatment facilities with *Cryptosporidium* oocysts and *Giardia* cysts that were ultimately detected in filtered water triggering 70 days of boil water alerts [8]. In Western Europe, higher hygiene standards have led to reduced immunity to enteric viruses, for example hepatitis A, causing a spike in cases [9].

The causative agents of most waterborne diseases can be divided into three categories: bacteria, viruses, and parasites, the last consisting of protozoan and helminths. Those of greatest concern, shown in Table 1 adapted from the WHO [10], are those that are capable of causing severe

**Table 1** World Health Organization (WHO) List of Relevant Waterborne Pathogens[a]. List of bacterial, viral, protozoan, and helminth waterborne pathogens categorized by their relative infectivity, longevity under environmental conditions, and resistance to common disinfection procedures, namely chlorination

| Pathogen | Associated Disease | Relative Infectivity | Persistence in water | Resistance to Disinfection |
|---|---|---|---|---|
| Bacteria | | | | |
| *Burkholderia pseudomallei* | melioidosis | Low | May multiply | Low |
| *Campylobacter jejuni, C. coli* | gastroenteritis | Moderate | Moderate | Low |
| *Escherichia coli* - pathogenic | gastroenteritis | Low | Moderate | Low |
| *E. coli* O157:H7 (enterohaemorrhagic) | gastroenteritis, hemolytic-uremia | High | Moderate | Low |
| *Legionella* spp. | Legionnaires' disease | Moderate | May multiply | Low |
| Non-tuberculous mycobacteria | Pulmonary disease, skin infection | Low | May multiply | High |
| *Pseudomonas aeruginosa* | Pulmonary disease, skin infection[b] | Low | May multiply | Moderate |
| *Salmonella Typhi* | Typhoid Fever | Low | Moderate | Low |
| *Salmonella enterica* | Salmonellosis | Low | May multiply | Low |
| *Shigella* spp. | Shigellosis | High | Short | Low |
| *Vibrio cholerae* | Cholera | Low | Bioaccumulates | Low |
| *Yersinia enterocolitica* | gastroenteritis | Low | Long | Low |
| Viruses | | | | |
| Adenoviruses | gastroenteritis, respiratory infection | High | Long | Moderate |
| Enteroviruses | gastroenteritis | High | Long | Moderate |
| poliovirus | poliomyelitis | High | Long | Moderate |
| coxsackievirus | meningitis | High | Long | Moderate |
| Astroviruses | gastroenteritis | High | Long | Moderate |
| Hepatitis viruses A,E | Hepatitis | High | Long | Moderate |
| Noroviruses | gastroenteritis | High | Long | Moderate |
| Sapoviruses | gastroenteritis | High | Long | Moderate |
| Rotavirus | gastroenteritis | High | Long | Moderate |
| Protozoa | | | | |
| *Acanthamoeba* spp. | keratitis, encephalitis | High | May multiply | Low |
| *Cryptosporidium* spp. | Cryptosporidiosis | High | Long | High |
| *Cyclospora cayetanensis* | gastroenteritis | High | Long | High |
| *Entamoeba histolytica* | amoebic dysentery | High | Moderate | High |
| *Giardia lamblia* | Giardiasis (Beaver fever) | High | Moderate | High |
| *Naegleria fowleri* | Primary amoebic meningoencephalitis | Moderate | May multiply | Low |
| *Toxoplasma gondii* | Toxoplasmosis | High | Long | High |
| Helminths | | | | |
| *Dracunculus medinensis* | Dracunculiasis (Guinea worm disease) | High | Moderate | Moderate |
| *Schistosoma* spp. | Schistosomiasis | High | Short | Moderate |

[a] Adapted from WHO Guidelines for Drinking Water Quality [10]

[b] Immunocomprised individuals

disease, persist in the environment for a long time, and can withstand common disinfection methods, because of a resistant state such as a spore or vegetative phase for bacteria, the cysts, oocysts, and ova of parasites, and most viruses. In all cases, infected people and animals shed large numbers of microbes in feces, which can then directly contaminate water supplies through untreated or under-treated sewage or through accidental release. Additionally, the practice of using livestock waste for fertilization can result in contamination of reservoirs and wells via runoff or permeation into the groundwater [11]. As a result, some countries are considering restrictions on this practice based on pathogenic content modeled after current US EPA rules [12]. The use of untreated wastewater for irrigation of crops, particularly those consumed raw, can also result in exposure to pathogens and infection [13]. Contamination of marine environments can result in the bioaccumulation of bacteria, for example *Vibrio cholerae* [6], and viruses in shellfish [11, 14]. Many of these microbes remain infective for long periods in aqueous environments, enabling them to travel long distances from the initial source [15]. In this regard, viruses are of utmost concern. Their longevity enables them to contaminate deep aquifers—water sources long thought to be untouched by microbes and, thus, safe and pure [16]. As waterborne pathogens travel through the environment they are typically diluted to low, but clinically concerning, concentrations creating a detection challenge.

## Indicator organisms

In 1989, the United States Environmental Protection Agency (US EPA) established rules for water-quality monitoring, setting benchmarks, limiting the potential risk of infection to no more than 1 in 10,000 per year [17], that have served as a guideline for regulation in other countries [18]. The cornerstone of these regulations is the monitoring of total coliform bacteria in source and finished water to assess disinfection efficiency and trigger further testing for specific pathogens [19]. Coliform bacteria include bacteria that are typically found in feces, collectively called fecal coliforms, for example *Escherichia coli*, *Enterococcus* spp., and *Clostridium perfringens*, and are, therefore, indicators of fecal contamination, which in turn is an indicator of potential for pathogenic microbes [20]. This method is flawed, because significant pathogens persist in water far longer than coliform bacteria; examples include enteric viruses, which can be found in low concentrations in samples showing no other sign of fecal contamination. For this reason, some non-pathogenic viruses have been evaluated as indicators of contamination or to aid in the assessment of disinfection techniques [20, 21]. The use of these indicator organisms remains the gold-standard of monitoring and regulation, because of the prohibitive cost of testing for multiple, individual pathogens spanning a diverse array of microbe types.

## Standard techniques for detection of waterborne pathogens

Detection for bacteria, both fecal indicators and pathogens, and viruses are dominated by culture techniques and molecular biology methods. For bacterial detection, standard bacterial cell culture methods are used and the presence of bacterial growth can be measured via the development of colonies that may be counted, for plated cultures [22, 23]. Similarly, the presence of viral pathogens can often be determined via cell culture plaque assays. Here, a sample is added to a monolayer of mammalian cells that is then fixed in agar. The plates are then observed for plaques, regions of dead cells exhibiting cytopathogenic effects (CPE) [24]. It should be noted that 1 PFU does not equal 1 virion, rather it is a measure of the infectivity of the particular virus in the culture system used. Thus, 1 PFU can be thousands of virus particles. Culture methods are time-consuming, bacteria can take days to grow [25], and some viruses can take several weeks to develop plaques. Some viruses will never form plaques as they are very slow growing, do not produce CPE, or no cell lines have been determined on which they grow [26]. More rapid methods for coliform bacteria have been developed, marketed as Colilert and Colisure, that rely on chromogenic substrates added to culture media that yield a visible color change as it is cleaved by bacterial enzymes [27].

Immunological methods, for example serum neutralization tests (SNT), immunofluorescence, and enzyme-linked immunosorbent assays (ELISA) have been used for detection of waterborne pathogens. SNT is used for the serotyping of viruses and involves mixing a sample, extracted from a plaque assay, with antiserum and then assessing the decrease of infectivity by plaque assay [28]. These tests are extremely time-consuming as they require two rounds of cell culture. Immunofluorescence assays for viruses enable shortening of assay time compared with SNT because they eliminate the second round of cell culture and use fluorophore-labeled antibodies for detection via microscopy [29] or flow cytometry [30]. Assays based on immunofluorescence and immunomagnetic separation (IMS) are the gold standard for detection of protozoan parasites. The US EPA has established method 1623 for the combined detection of *Cryptosporidium* spp. and *Giardia* spp. [31] or method 1622 for *Cryptosporidium* spp. alone [32], both of which use IMS followed by immunostaining and fluorescent microscopy. ELISA has been used for the detection of waterborne pathogens [33, 34], but has not

been as popular for environmental samples as in clinical analysis [35]. Immunological methods suffer from the inability to determine infectivity, because living and dead organisms appear the same; in addition the methods are very time-consuming.

Since it was first published in 1988, the polymerase chain-reaction (PCR) [36], and modifications, for example reverse transcriptase PCR (RT-PCR), real-time, quantitative, nested, and multiplex PCR [37–42], have become indispensible tools for the detection and identification of pathogens. Other techniques, for example nucleic acid sequence-based amplification (NASBA), have also been used for detection of waterborne pathogens [43–46]. Compared with cell culture, molecular techniques are far more appealing because of the shorter time to result, typically no more than a few hours as opposed to days or weeks, and increased sensitivity [37]. Also, by enabling for the detection of sequences specific to only the pathogen of interest, molecular biological techniques enable discrimination unavailable in cell culture. However, particularly with regard to viruses, these methods do not provide information on the infectivity of the pathogen being detected. Further, the detection of a component of the pathogen rather than the intact organism or virus makes it difficult to retrospectively correlate to the number of cells, oocysts, cysts, or virions in the sample. In this review, such information is only presented when available in the original works discussed.

## Biosensors as novel detection methods

Biosensors are devices or assays that use a biorecognition element coupled to a signal transducer to detect an analyte of interest [47]. Common biorecognition elements include oligonucleotide probes, antibodies, enzymes, aptamers, cell-surface molecules [48], and phages [49]. Transduction methodology can be roughly divided into three primary types: optical, electrochemical, and mechanical. Biosensors have the potential for substantial improvements over standard methods and have been reported employing the full spectrum of biorecognition molecules and transduction methods for detection of waterborne pathogens, with oligonucleotide probes and antibodies being the most common.

## Criteria for biosensor development

New detection methods for waterborne pathogens must be superior to the current standards in one or more of three key characteristics: sensitivity, specificity, and speed. Also important, though secondary to these, are reductions in the susceptibility of the method to user error and the portability of the biosensor.

Sensitivity, in most cases, is defined as the ability to detect very few organisms in a sample (rather than the degree of discrimination between various concentration levels). How sensitive an assay must be depends on the specific pathogen's ability to cause disease and on governmental regulations and regulatory goals. For instance, as few as 10 viable *C. parvum* oocysts can cause infection in healthy adults [50] and the US EPA regulatory goal is the complete absence of these oocysts from finished drinking water [17]. Therefore, an effective new analytical method must meet this goal and be capable of detecting a single viable oocyst.

New methods can also seek to improve the specificity of detection with regard to discerning one closely related species from another or even viable organisms from dead. Some standard methods are very effective with regard to these two criteria. For example, cell culture methods can be used to detect a single CFU or PFU and inherently provide discrimination in respect of viability status. US EPA method 1622 can easily detect 10 *Cryptosporidium* spp. oocyst [51], but cannot discriminate between pathogenic and non-pathogenic species or viable and dead oocysts without additional analytical steps, for example staining. Further, all of these methods fail in respect of the third criterion, providing rapid results.

Novel methods can, do, and will make trade-offs with regard to these key areas, as do the standard methods. In developing a new assay these trade-offs are inherent in the selection of the detection methodology, especially between molecular biological and immunological. Molecular biological methods are often sensitive enough to detect a single organism but can sacrifice speed because they require time for the enzymatic amplification of the nucleic acid target and the processing of the sample in order to access the genetic material of the organism. For example, Taniuchi et al. reported a multiplex PCR reaction for protozoan parasites using Luminex beads with limits of detection of $10^2$ *Cryptosporidium* spp. oocysts, $10^3$ *Giardia lamblia* cysts, and $10^1$ *Entamoeba histolytica* trophozoites [52]. However, the assay includes an extensive DNA-purification procedure requiring well over an hour to complete. Immunological assays tend to be faster, because less sample pre-processing is required, but can sacrifice sensitivity. The lower limits of detection of immunoassays range from $10^3$ to $10^6$ cells, cysts, oocysts, or CFU per mL, but can yield results in as little as 10 min [53–59]. Both methods can be highly specific. However, the specificity of antibody-based detection is highly dependent on the availability of quality antibodies whereas nucleic acid amplification requires the ability to identify a unique sequence in the genome of the organism of interest and the design of suitable primers and probes.

## Obstacles to detection

Environmental samples pose a number of challenges to assay development because of to the numerous potential microbial, particulate, and other organic and inorganic contaminants. Also, as previously stated, waterborne pathogens, especially those that persist for long times, are typically present at low concentrations in environmental samples, itself an obstacle to detection. Taken together, these present a formidable hurdle to overcome; thus, sample pre-treatment that is compatible with the selected method of detection must be considered when comparing the relative merits of two assays and included in the development of a novel method.

The current standard for sample filtration and concentration is commercially available charged membrane filters through which large volumes of water can be processed, with various buffer changes required for the adsorption and elution of the analyte of interest [33, 42, 60]. Microfluidic devices for pre-processing have been developed exploiting similar principles. Balasubramanian et al. took advantage of the net surface charge of waterborne microbes in the development of a microfluidic electrostatic trapping device, consisting of a microchannel with electrodes as two of the walls, capable of concentrating *E. coli*, *Salmonella*, and *Pseudomonas* with over 99% capture efficiency [61].

Size-based microfluidic filtration techniques have also been implemented for pre-concentration. Taguchi et al. fabricated a stainless steel filter with conical pores integrated into a microfluidic device. Under negative pressure, a sample is pulled through the pores and larger particles are captured and the analyte of interest, here *C. parvum* oocysts, can be visualized and quantified by use of fluorophore-conjugated antibodies [62]. Microfluidic devices using electrokinesis and in-situ photopolymerized nanoporous membranes have been demonstrated for concentration of analytes [63] while enabling removal of smaller competing molecules [64]. Similar membranes have been combined into a single microfluidic device with downstream liposome-based detection in an immunoassay for a human norovirus surrogate, yielding an order of magnitude improvement in the analytical sensitivity [65]. Implementing a device replicating a macro-scale weir, Zhu et al. captured *C. parvum* oocysts or *G. lamblia* cysts where the channel depth dramatically reduces, again enabling detection via immunofluorescence [66].

Filtration methods are particularly useful when processing large sample volumes—on the scale of liters—and provide the much needed initial purification necessary when handling environmental samples. However, the miniaturization of filtration techniques into microfluidic devices will enhance the potential for fouling and clogging of the device and will limit their application to real samples. Further, filtration cannot provide the specificity of IMS, which has become an invaluable tool for both concentrating the analyte of interest and purifying the sample solution. The latter is of particular interest, because environmental samples often contain organic and inorganic compounds that can act as inhibitors of the enzymes required for molecular amplification techniques [67]. As magnetic particles are available in a range of sizes, IMS is also appealing for implementation in micro-total analysis systems. Microfluidic devices using IMS before PCR-based detection have been reported for *E. coli* O157:H7, with a detection limit of 0.2 CFU $\mu L^{-1}$ [68] and $10^2$ PFU $mL^{-1}$ enterovirus 71 [38, 39]. Many procedures can be used for washing magnetic particles in fluidic channels, most commonly capture by a stationary magnet in a continuous flow [69–73]. However, some novel methods have been reported. For example, Ramadan et al. developed a device with multiple magnets fixed to a single axel with the poles of alternate magnets arranged perpendicular to each other [74]. This axel is placed underneath a parallel microchannel containing magnetic particles in suspension under continuous flow; as the axel spins the particles are alternately concentrated and released, washing away contaminants and unbound analytes. Foregoing magnetic beads and directly immobilizing the capture antibodies inside a microchannel can minimize user error and reduce device complexity, Dharmasiri et al. reported a device capable of detecting only 6 CFU *E. coli* O157:H7 [75]. The use of IMS and other antibody-capture techniques as a pre-processing step in molecular biology-based assays very clearly indicates why immunoassays can be dramatically faster. Figure 1 compares the typical process flow for immunoassays and DNA biosensors using common signal-transduction techniques. Detection of nucleic acid sequences requires 2 to 3 additional pre-processing steps after immunoseparation, whereas the introduction of a second, labeled antibody creates the sandwich immunoassay commonly used with various detection platforms [59, 76–79]. (Detection complexes are depicted in Electronic Supplementary Material Fig. S1.)

## Future improvements for waterborne pathogen immunosensors

Key to the future potential of immunoassays for waterborne pathogens is an increase of the analytical sensitivity to levels more in line with the detection limits required to meet public health and water safety objectives. Lower limits combined with the already short time to results would make these biosensors indispensable. To achieve these increases in analytical sensitivity, methods for amplification of specific signals and reduction of the background noise must be investigated.

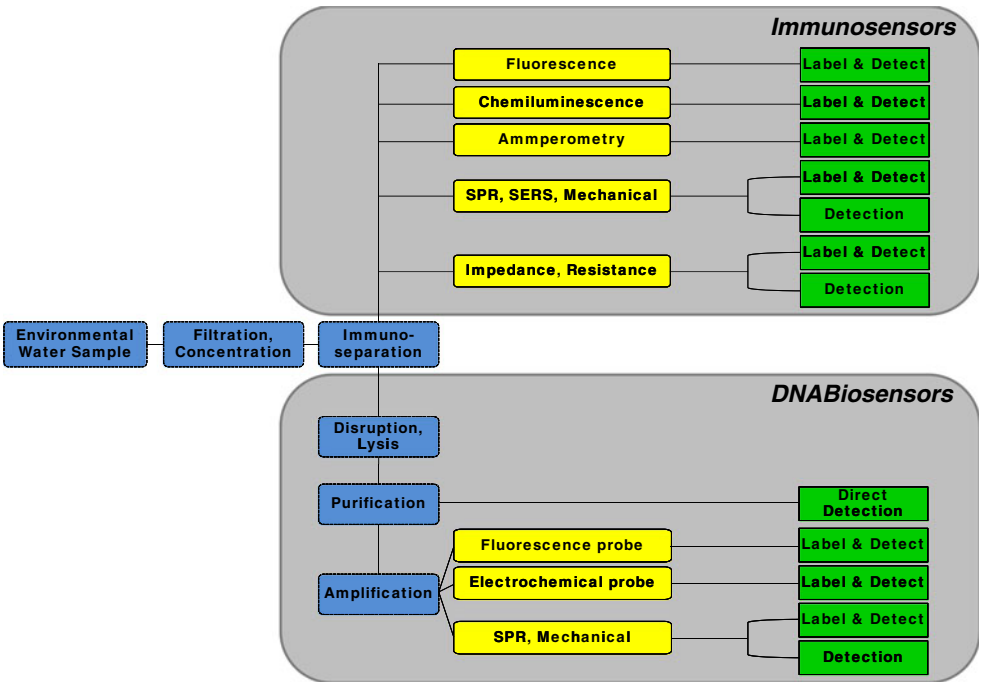


**Fig. 1** Typical process flow for detection of pathogens in environmental water samples. Irrespective of detection method, water samples must be passed through pre-processing steps (*blue boxes with dashed outlines*), including filtration and concentration, to retain all potential pathogens for analysis and reduce the volume of the sample proceeding through the process. Many assays will then use an immunoseparation step, for example IMS. Processes diverge at this point, with immunoassays requiring only labeling and detection (*green boxes*) and DNA biosensors calling for more pre-processing and, most often, enzymatic amplification. Common detection methods are shown in *yellow boxes*

Signal amplification using enzyme-labels, as in traditional ELISAs, is well established in biosensors [53]. However, as this amplification is reliant on the enzyme's action on a provided substrate over time, this method is less desirable. Liposome nanovesicles encapsulating a fluorescent dye, used as a marker, have been demonstrated to provide substantial, instantaneous signal amplification and high sensitivity compared with single fluorophore labels [80]. Biosensor immunoassays using liposomes for detection of whole *E. coli* O157:H7 have shown the benefit of liposome lysis for increasing analytical sensitivity; intact liposomes resulted in a limit of detection of $10^3$ CFU $mL^{-1}$ [81] whereas lysed liposomes produced readable signals for 1 CFU $mL^{-1}$ [82]. As liposomes can encapsulate a range of water-soluble molecules, they can be used for signal amplification with other transduction techniques, for example electrochemical detection [69, 70].

Exploring other transduction techniques, a wide range of signal amplification strategies can be chosen. For example, using a portable surface plasmon resonance (SPR) system, Soelberg et al. used antibody-linked magnetic beads to achieve 80-fold amplification of the signal in an assay for a microbial enterotoxin [76]. Here, because the change in the measured refractive index in SPR biosensors is relative to the size of particles binding to the surface, signal amplification can be produced simply with a large particle. Similarly, the increase in mass provided by gold nanoparticle labels has been used to produce signal amplification leading to four orders-of-magnitude improvement in sensitivity for surface acoustic wave biosensors [83, 84].

Interesting methodology for enhanced sensitivity is the integrating waveguide biosensor. Here, antibodies immobilized on the interior surface of a glass capillary tube and those conjugated to a fluorophore bind the pathogen of interest. Light is incident normal to the capillary for excitation whereas emitted light couples into the capillary waveguide and is collected by a photomultiplier tube (PMT). In this format, the sensitivity is enhanced as the specific signal increases with the length of the capillary while the background noise remains the same, yielding a two orders-of-magnitude decrease in detection limit compared with fiber optic biosensors [85]. Zhu et al. showed the integrating waveguide biosensor could detect as few as 10 cells of *E. coli* O157 per capillary [86], corresponding to a concentration of approximately $10^2$ CFU $mL^{-1}$.

The integrating waveguide biosensor indicates the importance not only of signal amplification but of reduction of background noise. Although in that system the noise simply does not increase, the development of systems that reduce the contribution of noise can reduce detection limits by increasing the signal-to-noise ratio. Biosensors that take advantage of the evanescent wave generated by total internal reflectance of light confined within an optical fiber or waveguide enable the specific excitation of fluorophores immobilized on the surface of the fiber and not those in the bulk sample, reducing potential noise. Extensive research on evanescent wave fluoroimmunoassays has been conducted at the US Naval Research Laboratory resulting in the commercialization of the portable RAPTOR system available from Research International. The RAPTOR allows four samples to be analyzed in parallel, with a

10-min assay time, and has been used to detect waterborne pathogens at concentrations such as $5\times10^4$ *G. lamblia* cysts $mL^{-1}$ [54], $10^5$ *C. parvum* oocyst $mL^{-1}$ [55], $10^4$ CFU $mL^{-1}$ *E. coli* O157:H7 [57], and $10^3$ CFU $mL^{-1}$ *S. enterica* Enteritidis [58], and fecal indicators, for example enterococci, in environmental samples at $10^5$ CFU $mL^{-1}$ [56].

Biosensors using the signal amplification and noise-reducing techniques described above can benefit from, or have benefited from, the increases in sensitivity commonly achieved with microfluidics or microfabricated sensor elements. As a result of working on a dimensional scale closer to the size of the microbial and viral pathogens found in water, limits of detection nearing the single cell may be possible. For example, with devices employing antibody-coated, 200-nm diameter polypyrrole nanowires individually suspended between two electrodes to create a field-effect transistor, Shirale et al. detected down to $10^{-3}$ PFU in 10 μL of an enterovirus surrogate in urban runoff water [87]. The binding of virions to the surface of the nanowire produces a measurable increase in resistance as the close proximity of negative moieties of the analyte depletes available charge carriers in the bulk of the wire. This effect is measurable for such a low virus concentration because of the nanoscale dimensions of the sensor [88].

## Improvements for DNA-based biosensors

Nucleic acid biosensors already provide the desired sensitivity for detecting waterborne pathogens present at low concentrations, with some reporting single-organism limits [52, 89]. The primary area to focus improvement efforts necessary for these biosensors is the overall assay time required to realize detection in real samples. Starting with environmental water samples requires, first, the purification and concentration of the pathogen of interest, followed by steps to disrupt the cell membrane, oocyst wall, spore coat, or viral capsid and purify the genetic material before amplification and detection. Clearly defined procedures and commercially available kits exist for these tasks, for example Dynal's IMS kits and beads and Qiagen's nucleic acid purification line of products. These pre-processing steps, while necessary, add time. Thus, investigations are necessary to shorten, eliminate, or provide alternatives to these methods.

Many pre-processing methods can benefit from miniaturization. Confining a reaction within a micro or nanoscale fluidic channel has great potential to reduce assay time, as the smaller volume reduces diffusion limitations [90], and to simplify assay procedures, by stringing multiple operations together into micro total analysis systems (μTAS) [91]. Microfluidic devices for some sample preparation steps have already been reported, including immunoseparation and pre-concentration as discussed previously, and for mRNA isolation [92], PCR [39, 93–98], and isothermal amplification reactions [99].

As is the case with immunosensors, methods that improve the analytical sensitivity are also of interest. However, as PCR-based methods already enable low limits of detection in respect of numbers of pathogenic organisms, methods that can provide similar limits without amplification should be the primary focus. This promises to reduce both assay time and complexity. Bio-barcode assays, for example, have been used to achieve signal amplification, as Zhang et al. demonstrated in an assay for *S. enterica* Enteritidis. Here, gold nanoparticles coated in a target-specific probe and fluorescein-labeled barcode DNA in a 1:100 ratio were used with magnetic beads in a sandwich hybridization assay capable of detecting 0.25 fmol target DNA [100]. This is similar to the limits achieved when using the aforementioned liposomal signal amplification in DNA-based sensors [70, 73]. However, this is not sensitive enough by itself for detection of unamplified target. Using multiple liposome-tagged probes in a lateral flow assay, Nugen et al. selected bacterial 16S rRNA as a potential target as 80% of the total RNA in a cell is rRNA [101]. This assay was capable of detecting 135 ng total bacterial RNA without enzymatic amplification in only 20 min [102], demonstrating the future potential of liposome-based signal amplification.

Another method that has proved capable of yielding specific signals in assays without enzymatic amplification is up-converting phosphor technology (UPT). Using inorganic microcrystals which emit visible light when excited by an infrared laser, UPT can produce specific signals with very low background because of a lack of autofluorescence [103]. With PCR amplification, assays using these reporter molecules were capable of detecting 3 amol of specific target DNA [104]. Zuiderwijk et al. used four probes, two labeled with biotin for capture of the desired target and two labeled with digoxigenin, and UPT-reporter molecules labeled with an anti-digoxigenin antibody [105]. With the assistance of the multiple probes, this assay was capable of detecting 1 ng genomic DNA, or approximately $10^6$ bacterial cells.

## Conclusion

Waterborne pathogens are a critical, global health problem and methods that can rapidly, sensitively, and specifically detect their presence in accordance with water safety regulations at clinically significant levels are imperative for the improvement of the health and quality of life for millions of people. Although DNA biosensors have proved

effective at detecting low concentrations, they typically require time-consuming purification processes upstream. Immunosensors require relatively fewer sample processing steps, thus less time. Through various signal amplification and background noise-reduction techniques, coupled with the improvements in sensitivity promised by miniaturization, antibody-based detection methods have great potential for rapid, sensitive analysis of all forms of waterborne pathogens.

Current and future research efforts must be focused on detection of pathogens in their actual environmental matrix and on the pre-processing steps needed. This includes miniaturization strategies, materials research, and emphasis on multiplexing so that ideally all relevant pathogens for a specific scenario can be detected at once. Taken together, this will produce novel methods capable of providing the necessary sensitivity, specificity, and speed to replace the current standards and, hopefully, improve access to safe drinking water and reduce the global health burden of waterborne disease.

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