RECLAMATION

Managing Water in the West

eDNA testing for Pathogens in Reused Water

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Mission Statements

The U.S. Department of the Interior protects America's natural resources and heritage, honors our cultures and tribal communities, and supplies the energy to power our future.

The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

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14. ABSTRACT *(Maximum 200 words)* Testing for the presence of pathogens in reused water is of critical important to maintaining human health. This scoping project had two goals. First, to create a list of pathogens of concern and look at how indicator organisms are used to test for the pathogens. Second, to perform a literature search for methods used to detect pathogens, in particular the DNA methods that are available. Future research will hopefully apply the methods that were found during the course of this scoping project to the analysis of pathogens in reused water.		
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Acronyms and Abbreviations

DNA-Deoxyribonucleic acid
eDNA- environmental DNA
NGS- next generation sequencing
PCR- polymerase chain reaction
RDLES- Reclamation Detection Laboratory for Exotic Species

Executive Summary

The detection of pathogens in water is of vital importance for human health. Outbreaks of waterborne illness continue to occur both in the United States and worldwide, and detecting pathogens is an ongoing challenge. There are several issues associated with pathogen detection in water: low pathogen densities, large volumes of water, detection of multiple organisms at the same time, and the detection of live organisms [1]. Molecular methods, such as testing for the presence of the DNA or environmental DNA (eDNA) in the water, offer a way to overcome some of these issues.

The purpose of this scoping project was to determine the most current molecular methods for the detection of pathogens in water. Additional methods of interest were found during the course of this literature search, including next generation sequencing (NGS) and microarray. The first goal of this project was to produce a list of the pathogens of concern and a brief discussion of the indicator organisms that are used as proxies in their detection. Second, a brief summary of the various methods that are used for the detection of pathogens.

Assays such as the coliform test, have been developed as indicator tests of bacterial contamination in water. The downside of the coliform assay is that it takes a long time to complete and it does not indicate presence of viral or protozoan pathogens. Thus multiple indicator assays are needed.

In addition to human pathogens it is also important to work towards the detection of animal and plant pathogens. The presence of a plant pathogen in irrigation water is of major concern to farmers [1]. The development of assays that can detect multiple organisms simultaneously is an area of ongoing research. It is possible that molecular methods can be utilized to analyze a water sample for all pathogens of interest.

When this scoping project was started it was assumed that the detection of eDNA by polymerase chain reaction would be the most direct method to detect waterborne pathogens. For the purposes of this report, eDNA is defined as both the intracellular and extracellular DNA that an organism leaves behind in the environment. Over the course of this literature review it was found that instead of using just basic PCR, other methods, such as next generation DNA sequencing and microarrays would be more encompassing as methods for the detection eDNA from a wide range of organisms simultaneously. Even with the advantages of molecular assays, there are issues that will have to be overcome with these methods. For example, there are several factors that can influence the detection of

eDNA- temperature, sunlight (UV), salinity, and also biotic factors such chemicals in the sample that can inhibit detection.

Creating a single over encompassing method that allows for the collection, processing, and analysis of all the pathogens in a water sample is a major undertaking. Some of the technologies that are currently in use and being developed are just starting to grasp that goal. Determining if a pathogenic organism is viable is a key issue for all of the monitoring methods. In addition, having the technology in place at the facility for real time monitoring is another goal that is being worked towards.

Based on the literature searches for this project several technologies, such as microarray and next generation DNA sequencing were identified as potential methods for future projects. Further research into biosensors could also be performed to assess how this technology could be applied to future projects. In addition, a research project was proposed for 2017 that will look at antibiotic resistance and bacteria in water. The technologies identified in this scoping project, in particular next generation sequencing, could be applied to this project.

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Main Report

Introduction

Testing and analyzing reused water for the presence of pathogens is important to maintaining human, animal, and plant health. Outbreaks of illness due to waterborne bacteria, viruses, and protozoa occur worldwide. This scoping project had two parts. First, to create a list of pathogens of concern and a brief discussion of the indicator organisms that are used as proxies for their detection. Second, to briefly summarize the various methods that can be used for the detection of pathogens. Over the course of this project several lessons were learned that will be applied to future research. The importance of testing water for biological contaminates is of great importance.

Pathogens of Concern

There are many waterborne bacteria, protozoa, helminthes, and viruses that can cause human health issues (Table 1). Monitoring and testing for all of the organisms is currently not practical or feasible with current detection methods. Coliforms, *Escherichia coli*, and enterococci are all used as indicators of contamination.

The EPA has five criteria that make an organism an ideal indicator of fecal contamination (www.epa.gov). 1) Whenever there are enteric pathogens present, the indicator organism should also be present. 2) The indicator organism has to be useful for all types of water. 3) The indicator organism must survive longer than the enteric pathogens. 4) It should not be able to grow in water. It should grow only within a warm blooded organism. 5) And finally, it should be found in the intestines of warm-blooded animals. Finding organisms that satisfy all five of these criteria is difficult.

Also, an indicator organism for bacteria contamination does not mean that a protozoan outbreak will be detected. For example, in 1993 over 1.61 million people became ill in the Milwaukee cryptosporidium outbreak[2]. The coliform tests that were performed would not have shown any indication that there was an issue because this outbreak was caused by a protozoa. Having molecular assays that can detect a broad range of organisms will help overcome the issues of using indicator organisms.

Table 1**:** List of bacteria, protozoa, helminthes, and viruses that can be present in water and cause human health concerns (From Keele and Pucherelli 2016).

Bacteria			
Acinetobacter spp.	*Aeromonas spp. (aeromonads)*	*Atypical mycobacteria*	*Atypical mycobacteria*
Burkholderia pseudomallei	*Campylobacter jejuni*	*Clostridium perfringens*	*Enteropathogenic Escherichia coli (not all strains of E. coli are harmful)*
Helicobacter pylori	*Legionella (L. pneumophila)*	*Leptospires (Leptospira and Leptonoma)*	*Mycobacterium avium intracellular (MAC)*
Pseudomonas aeruginosa	*Salmonella typhii*	*Shigella (4 spp.) (S. dysenteriae, S. flexneri, S.boydii, S. sonnei)*	*Staphylococcus aureus*
Vibrio cholera	*Yersinia enterocolitica*		

Protozoa			
Balantidium coli	*Cryptosporidium*	*Cyclospora*	*Entamoeba histolytica* (most prevalent worldwide)
Giardia lamblia	Microsporidia	*Naegleria fowleri*	
Cyclospora			

Helminthes			
Ancylostoma duodenale	*Ancylostoma*	*Ascaris lumbricoides*	*Dracunculus medinensis*
Echinococcus granulosis	*Enterobius vermicularis*	*Fasciola*	*Necator americanus*
Strongyloides stercoralis	*Taenia (spp.)*	*Trichuris trichiura*	

Viruses			
Adenovirus	Astrovirus	Caliciviruses (including Norovirus and Sapovirus)	Coronavirus
Coxsackieviruses	Echoviruses	Enteroviruses	Hepatitis A and E
Norwalk agent	Parvovirus	Reovirus	Rotavirus

Methods of Detection

Aw and Rose (2012) state that high-density microarrays, quantitative real-time PCR (qPCR) and pyrosequencing which are considered to be breakthrough technologies borne out of the 'molecular revolution' are at present emerging rapidly as tools of pathogen detection and discovery[3]. To assess the types of detection methods that are available a literature search was performed using the Mendeley reference program. These references are compiled in Appendix 1 at the end of this report.

There is a range of detection methods that include traditional methods such as coliform measurements to more advanced molecular methods such as microarray and next generation DNA sequencing. This literature search showed that eDNA could be employed using both polymerase chain reaction, microarray, and next generation DNA sequencing. The DNA present in the environment, or eDNA can be both intracellular or extracellular. It also does not indicate if the organism was alive or dead when the sample is collected. The sensitivity and time it takes to perform each of these assays varies and ranges from a few hours to several days.

For eDNA analysis a water sample is taken, DNA extracted, and the sample is analyzed with PCR primers to detect the organism(s) of interest. eDNA can be used to detect pathogens that affect both humans and other organisms. For example, Huver et al (2015), designed an assay to test for a pathogenic parasitic nematode that affects amphibians [4]. This assay can be useful to monitor the spread of pathogens that can infect endangered amphibians.

There are PCR, qPCR, isothermal amplification, next generation DNA sequencing, and microarray that can be used that all can be applied to the analysis of eDNA. Each method can designed to meet a specific need. For example, PCR and qPCR assays can be multiplexed to detect more than one pathogen at a time [5]. The next step of using next generation DNA sequencing allows for the analysis of all the organisms of interest at one time. There are now commercial companies that are offering next generation sequencing services and analysis to determine the pathogens that are present in water samples.

Another available technology is isothermal amplification assays [6], where DNA is amplified at a constant temperature. This technology can be used easily in the field and is an ideal technology for water treatment facilities because it does not require any specialized equipment. In addition, once the assay is developed and validated, training others on how to perform the assay is straightforward.

The microarray assay uses a chip to capture the DNA from the organism of interest. The creation of microbial diagnostic microarrays (MDM) is a promising technology because each chip can be used to detect thousands of organisms at the same time [1]. These are all DNA based technologies that can be applied to the detection of the organisms that are present in the water. The issue with all of these methods is that they do not determine if the pathogen is viable.

Other methods, such a biosensors [7],[8] and enzymatic assays [9] can be used to detect whole organisms. With these methods viability of the pathogenic organism could be assessed. One of the downsides is that these assays will be for a specific set of organisms. For all of these technologies, the time that it takes to analyze the sample is critical to obtaining results that can be helpful, especially if there is an issue detected.

Lessons Learned

Several lessons were learned during this scoping project. First, keeping up to date with the literature is key in this field because there are many different researchers and methods being developed to detect pathogens in water. This is also a very wide field because of the diversity of organisms involved. The detection methods have to encompass bacteria, protozoa, and viruses.

Also, there are currently many different methods already available for the detection of pathogens in water. These methods all have differences in their sensitivity, time for analysis, and cost. Also, the equipment and training needed to perform these assays varies. Deciding which method best serves a particular site or situation best is an important decision. The research into of having detection methods that are present within a facility that can detect a wide range of pathogens is of great interest.

Future Directions

During this literature search several technologies were identified that could be useful in future projects. For example, instead of performing a PCR to detect the eDNA from a single pathogen, the use of next generation DNA sequencing to detect all of the pathogens should be explored. This technology could be very useful in assessing whole microbial communities that are present at a water reuse facility. Biosensor technology also shows promise and should be considered for future research.

The Reclamation Detection Laboratory for Exotic Species (RDLES) conducts eDNA assays for invasive and endangered species and the lessons learned from performing these assays can be translated to the detection of pathogens. The

collection methods might differ, but the analysis methods are very similar. There is a clear parallel between the two areas of research. PCR can be used to detect specific pathogens, but there are other methods, such as microarray and next generation sequencing that can be used to detect a whole range of organisms at once

The research is trending towards developing technology that allows technicians at water treatment facilities to monitor in real time for pathogens that are in the water. This will help to ensure that if there is a pathogen present, measures can be taken to control the contamination.

Conclusions

The detection of pathogens in water is an ongoing concern for both human, animal and plant health. One of the goals of this project was to learn about pathogen detection methods that could potentially be performed at RDLES. The PCR and next generation sequencing methods are the ones that will most likely be explored to be added to the capabilities of RDLES. As detection methods continue to improve it will be possible to detect smaller numbers of pathogens in water.

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