WASTEWATER SECURITY: CONTAMINANT PRIORITIZATION AND RAPID PCR METHODS FOR PATHOGEN DETECTION

by

Catherine Adria Hardison

Bachelor of Science, Rensselaer Polytechnic Institute, 2002

Submitted to the Graduate Faculty of

School of Engineering in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2007

UNIVERSITY OF PITTSBURGH

SCHOOL OF ENGINEERING

This thesis was presented

by

Catherine Adria Hardison

It was defended on

March 28, 2007

and approved by

Dr. Ronald Neufeld, Professor, Civil and Environmental Engineering Department

Dr. Stanley States, Water Quality Manager, Pittsburgh Water and Sewer Authority

Thesis Advisor: Dr. Leonard Casson, Associate Professor, Civil and Environmental Engineering Department

WASTEWATER SECURITY: CONTAMINANT PRIORITIZATION AND RAPID PCR METHODS FOR PATHOGEN DETECTION

Catherine Adria Hardison, M.S.

University of Pittsburgh, 2007

Since September 11, 2001, there has been a renewed focus on the security of vital infrastructure, such as wastewater treatment and collection systems. Terrorism, combined with the potential destruction in the wake of natural disasters, has led to an all hazards approach when evaluating contaminants that could be introduced into a wastewater treatment and collection system either accidentally or intentionally.

Prioritization is an evaluation technique that can prove useful to wastewater treatment plants (WWTPs) in providing a list of contaminants that would cause the most deleterious effects to the plant and its surrounding area. A framework was developed to focus on four endpoints that would be of the greatest concern to WWTPs. It was applied to 78 contaminants, including biological agents, flammable chemicals, radioactive elements and decontamination agents. Those contaminants having the highest weighted score for each endpoint were considered the greatest threat to the physical treatment plant and its unit processes, the plant's workers, and human and animal populations in contact with its receiving waters.

The prioritization process can provide utilities with information on which contaminants should be screened for throughout the plant and collection system. One such method for biological agents, such as *Bacillus anthracis*, is real-time, rapid cycle polymerase chain reaction (PCR). This rapid analytical technique allows for preliminary detection of pathogen presence in a much shorter time than is required for bacteria culturing. PCR used jointly with a field

concentration method greatly improved the sensitivity of the screening process, with a detection limit of 20 cfu/mL in concentrated secondary effluent, as compared to 3.50×10^3 cfu/mL in the unconcentrated sample. The detection limits of *Salmonella typhimurium* exceeded that expected from volume reduction with log recoveries of 3.53 and 2.24 compared to theoretical log recoveries of 2.40 and 2.10 for river water and secondary effluent, respectively. PCR cannot be applied for *Brucella* spp. screening due to cross-reactions with other bacteria present within the wastewater system, but for other pathogens, PCR may be a viable option for screening in treated and untreated wastewater.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to thank my committee members, Drs. Ronald Neufeld and Stanley States, for taking the time to read and comment on this thesis. I want to acknowledge especially the tireless effort of Dr. Leonard Casson, my thesis advisor. His input, both editorial and academic, was greatly appreciated. I would also like to extend my thanks to the staff of the Pittsburgh Water and Sewer Authority's Water Quality Laboratory, especially to Georgina Cyprych and Shawnelle Jackson, for their help in the preparation and execution of the experiments performed for this thesis.

1.0 INTRODUCTION

The events of September 11, 2001 have led to a heightened sense of need for security within the United States. The protection of buildings and infrastructure elements, in addition to human life, are of the utmost importance, the most critical of which are potable water treatment, storage, and distribution systems, and wastewater collection and treatment systems. To this end, a drinking water component was incorporated into PL 107-188, the "Public Health and Security and Bioterrorism Preparedness and Response Act," passed by Congress in June 2002. In addition, the Homeland Security Presidential Directive-9 (HSPD-9) was issued in January 2004. It established a national policy for the defense of agricultural, food, and water systems (both drinking water and wastewater) in the United States against terrorist attacks and other emergencies. These proclamations provided the environmental science and engineering community with a public and Federal mandate to develop and design analytical techniques and integrated physical protection systems to protect water and wastewater infrastructure systems against intentional contamination, damage, and destruction.

Terrorism is not the only concern for defense against contamination events. An all hazards approach has been developed to take into account natural disasters, whose aftermath can pose a serious risk to the infrastructure of a community, as seen with Hurricane Katrina. Wastewater treatment plants can be put out of commission by these events, and once brought online again, these facilities may have to deal with large amounts of contamination due to runoff

and flooding. These incidents may require an immediate emergency response to rapidly analyze for possible contaminants within the system.

Accordingly, a research project was developed to provide high quality information for municipal wastewater collection and treatment facilities and to help them better prepare to safely respond to, remediate, and recover from intentional and accidental contamination events. This analysis also extends to secondary contamination that may result from other acts of intentional contamination not focused on wastewater systems. An example would be decontamination water generated by cleanup efforts at intentionally contaminated buildings. This overall goal was divided into the following specific objectives:

- Identify contaminants that would be of concern for public and worker health, process upset, physical damage or destruction of wastewater infrastructure, and pass through to the environment;
- Develop methods that address the fate, transport, and removal of these contaminants in typical municipal wastewater treatment plants;
- Assess practical and currently available technologies that could be used to detect, remove, degrade, or inactivate contaminants;
- Evaluate and compile available data to determine and prioritize critical areas of uncertainty; and
- Address these areas of uncertainty by developing guidance for emergency operating procedures, treatment process modifications, and potentially new treatment technologies.

The research described herein is based on the objectives discussed above and will specifically address:

- The application of a prioritization framework to contaminants to determine those that would be of the greatest interest to municipal wastewater treatment plants;
- An assessment (Phase I) of raw water, finished drinking water, and following various stages of treatment, wastewater to screen for viruses, neurotoxins, and pathogenic bacteria and protozoa using real-time, rapid cycle PCR. These tests would be used to determine any background interferences in the water samples of interest;
- A determination of the detection limits (Phase II) of the R.A.P.I.D.® PCR using samples spiked with *Salmonella typhimurium* by comparing PCR results with traditional plate culturing techniques;
- The use of a concentration method (Phase III) to evaluate the R.A.P.I.D.® PCR with respect to *Salmonella* recovery and improved detection limits.

Although there have been no documented incidents of intentional contamination of wastewater treatment plants and collection systems in connection with terrorism, the possibility of a terrorist attack still exists. There have been numerous accidental and intentional discharges that have caused significant damage to municipal wastewater treatment plants and their collection systems. These events can be used as a basis for what could potentially happen if a contamination incident, whether direct or secondary, were to occur. The threat of terrorism, combined with the potential destruction caused by natural disasters, has made it necessary to provide utilities with a prioritization protocol to determine which contaminants would be of the greatest threat to wastewater collection and treatment systems. This assessment will aid in the development of reliable rapid screening procedures for those contaminants of interest. The screening will then provide wastewater facilities with preliminary data to take the appropriate measures for risk assessment and operational responses.

2.0 BACKGROUND

To assess the viability of rapid analytical methods for wastewater, an evaluation of potential security issues regarding wastewater was performed. A number of rapid analytical techniques exist for possible use in contaminant detection: rapid immunoassays, rapid enzyme tests, and field real-time, rapid cycle PCR for pathogen detection, and field gas chromatography-mass spectrometry for the detection of volatile organic chemicals. The research presented herein will focus on rapid polymerase chain reaction and its feasibility in detecting pathogenic contaminants in wastewater. *Salmonella typhimurium* is used as a model bacterium for assessing detection ability and limits in these experiments.

2.1 WASTEWATER SECURITY ISSUES

The likelihood of an attack on a wastewater collection system and treatment plant is minimal. However, the USA PATRIOT Act has defined critical infrastructure as "systems and assets, whether physical or virtual, so vital to the United States that the incapacity or destruction of such systems and assets would have a debilitating impact on security, national economic security, national public health or safety, or any combination of those matters" (Office of Homeland Security). Since public utilities are a vital component of a community's infrastructure, the result of such an attack would have a severe impact on public perception and could be detrimental to

the surrounding community. The destruction of other infrastructure components, such as roadways and drinking water treatment and distribution systems, may cause more fear within the public, making them more desirable targets. However, if a wastewater collection and treatment system were destroyed or needed to be taken offline due to accidental or intentional contamination, daily activities, such as taking showers and using the bathroom, would be disrupted, and basic household chores like washing clothes and cleaning dishes could not be performed.

Contamination of wastewater collection and treatment facilities and the potential disruption of treatment processes may not be the direct public health threat that drinking water treatment contamination would be, but the consequences could be just as dire. Instead of a single outcome that far outweighs other possible outcomes, as with drinking water and public health, there are four endpoints that would have equal weighting with respect to a wastewater contamination event. For example, if the contaminated effluent were released into receiving waters, downstream drinking water treatment facilities may be affected. The recreational use of the receiving waters may be seriously compromised, and there could be potential negative effects on the flora and fauna of the receiving waters and surrounding riparian areas. The health of workers at the treatment plant can be compromised by the release of volatile chemicals into the air. Unit processes can be disrupted, causing sewage to remain untreated and released into receiving waters, causing further environmental damage. Physical damage and destruction of the treatment plant and collection system would also be of concern due to flammable chemicals causing explosions. Other contaminants can also remain within the infrastructure, requiring significant remediation efforts.

Contamination can result from a direct attack on the wastewater collection and treatment system. An attack of this nature would be the most damaging and destructive attack on a wastewater treatment system, but also the least plausible. A far more probable scenario is the contamination of wastewater treatment plants due to the flushing of contaminated drinking water systems or the decontamination of buildings, as with the anthrax attacks on the Senate buildings in 2001. Biological agents may be assimilated into activated sludge flocs; radioactive elements may remain in the sludge of primary and secondary treatment; and flammable chemicals that can be metabolized by bacteria during secondary treatment may pose as a fire and explosion hazard or employee health risk by being volatilized during aeration. Other nonflammable chemical agents may adhere to the infrastructure of the wastewater treatment plant components. Another possibility is the use of decontamination chemicals to treat other chemical and biological agent attacks that find their way to the wastewater treatment plant via drainage systems. Little is known of the fate and transport of these chemicals in wastewater processes, but due to the disinfecting nature of these substances, the effects on biological treatment processes may cause the treatment plant to become nonfunctional. They may also be highly corrosive, causing damage to infrastructure components.

Several incidents where harmful chemicals have entered wastewater treatment plants and their collection systems have been documented in the last 30 years. While they are not classified as terrorist attacks, they provide an example of what may happen when a contaminant is released into a wastewater treatment system.

The Hexa-Octa Incident in 1977 began with the intentional dumping of toxic chemicals in a manhole and would end up setting a legal precedent in the United States (Louisville/Jefferson County Metropolitan Sewer District). Employees at the Morris Forman

treatment plant in Louisville, Kentucky, noticed a noxious chemical odor that was coming from the plant. After an investigation of over a week, it was determined that the offending chemicals were hexachloropentadiene (Hexa) and octachlorocyclopentene (Octa), two highly toxic substances used in the manufacturing of pesticides. This discovery resulted in the shutting down of the treatment plant for three months, and over 100 million gallons of raw sewage were released directly into the river each day. The sewer lines responsible for carrying the waste to the treatment plant took another two years to remediate, requiring the raw sewage carried by them to be diverted away from the treatment plant and discharged directly in the river.

The source of the contamination was traced to a local chemical disposal company, whose employees dumped the waste down a manhole. Subsequently, the president of the company and two of its employees were charged and eventually found guilty of polluting a waterway. The originators of the waste later agreed to compensate the local sewer district for costs associated with sewer and treatment plant cleanup and employee medical care.

A 1981 incident in Louisville, Kentucky led to extensive damage to the city's wastewater collection system, resulting in millions of dollars of repairs to the sewer lines and subsequent damage of streets and buildings. A spark from a car caused an explosion that rippled through two miles of road, damaging the surface and leaving large craters in place of manholes. The source of the explosion was traced to a soybean processing plant that spilled thousands of gallons of hexane, a highly flammable solvent, into the sewer lines. The resulting vapor mixture was within the limits to be explosive, and the spark from the car was just the ignition source needed to cause the explosion. Repairs to the sewer lines took about 20 months to complete, while work on the damage to surrounding streets and buildings took several additional months. While the chemical release was accidental, the company responsible for the spill pled guilty to four counts

of violating environmental laws, paid its fine, and later, agreed to pay damages to the sewer district.

In April 1992, another sewer explosion, in Guadalajara, Mexico, was the result of pipe corrosion (Suburban Emergency Management Project). A previously installed water pipeline leaked onto a gasoline line that lay underneath. The corrosion of the gasoline line allowed vapors to accumulate in the sewers. Nine separate explosions occurred throughout the city, killing 206 and injuring 1,460 others. The blasts also damaged 1,148 buildings.

More recently, in June 2006, a release of 25 gallons of potassium thiocyanate into a Philadelphia sewer system led to fish kills downstream of the discharge of the wastewater treatment plant and the closure of the city's drinking water intake valves (Bauers 2006). The chemical, commonly used in the development of vaccines and antibiotics, was traced to Merck & Co., Inc. However, due to its hazardous nature, it should not have been released into the sewers. A day after the release, the receiving treatment plant, Upper Gwynedd Township Wastewater Treatment Plant, noticed fluctuations in the chlorine levels in the discharge. Numbers of dead fish were later noticed downstream of the effluent outlet. Due to these events, testing was performed and showed that a cyanide compound was present in the waters. It was thought that the potassium thiocyanate reacted with the chlorine used during disinfection to create cyanogen chloride, a chemical that is highly toxic to fish.

These incidents are examples of what may happen if an accidental or intentional contamination event occurred. The health of the treatment plant's workers may be in jeopardy; components of the treatment plant and collection system may be destroyed; treatment processes may deviate from typical conditions; and flora and fauna may suffer in the event of contaminant pass through. The research presented herein focuses on developing a prioritization score for

potential wastewater contaminants evaluated at each of these endpoints. The results of the prioritization can then help municipal wastewater authorities determine which contaminants would be the most dangerous if introduced into a wastewater treatment facility or collection system. To this end, effective and rapid screening procedures are needed in the event of accidental, intentional or secondary contamination. Polymerase chain reaction (PCR) is one method that can characterize the presence of target pathogenic material in a sample and can be used in the event of suspected biocontamination.

2.2 POLYMERASE CHAIN REACTION

Polymerase chain reaction is a molecular biology technique that allows for the replication and amplification of a specific DNA sequence. Developed in 1983 by Kary Mullis, this method is now commonly used in cloning, DNA sequencing, the tracking of genetic disorders, and forensic analysis (Nelson and Cox 2005). The technology of PCR is based on the premise that every living organism has its own unique genomic sequence made up of nucleotides. These nucleotide sequences may have many similarities, as is the case with *Salmonella typhimurium* and *Escherichia coli*, which are 60-70% identical on the DNA level (Bej et al. 1994; Maloy and Edwards). To use PCR to detect a specific DNA sequence, shorter sequences known as primers need to be designed so that there are enough nucleotide mismatches with other DNA sequences that may be present within a sample. Primers consist of 18 to 28 nucleotides that are complementary to sequences on either side of the target DNA (Bitton 2005). Their proper design can ensure the selectivity of the PCR process. The nucleotide mismatches will prevent DNA polymerase from extending the whole DNA sequence even if there is a single base pair

mismatch. Environmental analysis applications include the detection and identification of specific bacteria in wastewater and sludges and the tracking of genetically engineered microorganisms used in bioremediation processes. It can also detect foodborne and waterborne pathogens and viruses in many environments.

There are three steps which form a single cycle of PCR. DNA denaturation occurs as the temperature is increased and allows the DNA to uncoil and separate into two strands by breaking the hydrogen bonds between base pairs. The temperature is then decreased, which causes the synthetic primers to anneal to either side of the target DNA sequence. The primers serve as a starting point for the DNA polymerase, resulting in the replication, or extension, of the desired DNA sequence. Extension is performed by *Taq* polymerase, a thermostable enzyme that is extracted from bacteria that live in hot springs (Bitton 2005). It is able to withstand the high temperatures required for DNA denaturation without denaturing itself. The result is the amplification of the target DNA, and the cycle is repeated.

The cycling of the denaturing, annealing, and extending steps is automated by using a thermocycler, which controls the temperature required for each of these steps. Typically, the PCR process is run for 30 cycles lasting about 3 hours. An entire PCR run results in the exponential accumulation of the target DNA. The PCR products can then be analyzed using gel electrophoresis, which can determine the size and purity of the amplicon.

The PCR method is highly sensitive; only one piece of the target DNA is necessary for proper amplification resulting in billions of copies at the end of the process. While it can detect the presence of an organism within a sample, it cannot determine the viability of an organism as DNA does not readily degrade after death. This fact is important to note, because the confirmation of a pathogen's presence may be the result of residual DNA from non-viable

organisms. Contamination is also a possibility with PCR, because it is not a closed system. The reaction products must be directly applied to the gel electrophoresis to be analyzed.

In the event of wastewater system contamination, time is of the essence. Traditional PCR methods may not be suitable for field applications, and the time required to perform conventional PCR may not be quick enough when trying to mitigate the effects of contamination. Advances have been made in PCR technology, which allow for shorter cycles and contained product analysis. Thermocyclers using this real-time, rapid cycle PCR technology have also been developed for use in the field.

2.3 REAL-TIME RAPID CYCLE PCR

Real-time rapid cycle PCR is based on the technology of traditional PCR, but as the name suggests, the cycles are shorter, and PCR product analysis occurs during the reaction. Reactions are performed in glass capillary tubes that have a high surface area-to-volume ratio, so that quick temperature changes are possible. A typical cycle time for rapid cycle PCR ranges from 20 to 60 seconds (Wittwer 2001). The temperature is quickly ramped up and down, and then held briefly, allowing all three steps to occur simultaneously. This temperature profile is in contrast to conventional PCR methods where the temperature is held for each step, so that thermal equilibrium is reached.

During sample preparation, fluorescent dyes are added that may be attached to probes or primers. Some dyes may also attach to the PCR product itself (Saunders 2004). The fluorescent dyes are specific to the target DNA sequence, and the strength of the fluorescent signal is dependent on the amount of amplicon within the capillary tube. The amplicons within the

capillary tube are constantly monitored during cycling by measuring the product's fluorescence after extension. The strength of the fluorescent signal can aid in quantifying the amount of template DNA in the sample.

The set-up for real-time, rapid cycle PCR is similar to conventional PCR systems in that a thermocycler is used to control the temperature profiles required for cycling. The optics integrated into the thermocycler for real-time, rapid cycle PCR emit a light source for dye excitation and have the ability to collect the resulting fluorescent emissions (Logan and Edwards 2004). Data acquisition and analysis is provided by a computer connected to the thermocycler. The capillaries in which the PCR process takes place are a closed system. Amplification and fluorescent detection take place within the reaction vessel, requiring no post-PCR manipulations (e.g., gel electrophoresis) and thereby prevents the many possible sources of contamination.

The R.A.P.I.D.® (Ruggedized Advanced Pathogen Identification Device) Cycler is a militarized version of the LightCycler, one of the first real-time PCR platforms made for commercial use. Air is used as the heating and cooling medium. The samples are more uniformly heated and cooled, speeding up the PCR process; a run of 40 cycles takes about 30 minutes to complete (Saunders 2004). The light source for the system is a single blue light emitting diode (LED). The light emitted from the excited fluorophores is collected in three discrete channels. These channels are able to detect emitted light at varying wavelengths.

The R.A.P.I.D.® system makes use of hybridization probes for detection of target DNA sequences. Two probes are designed to recognize and attach to internal sequences within amplified DNA. One probe is labeled at the 3'-end with fluorescein, a fluorescence resonance energy transfer (FRET) donor. The other probe has LC-Red 640, a FRET acceptor, attached at the 5'-end. The number in the dye name (e.g. LC-Red 640 and LC-Red 705) corresponds to the

wavelength emitted by the dye when excited. The 3'-end is modified by phosphorylation to prevent extension. During hybridization, the probes come into close proximity of one other. When fluorescent energy is present, the fluorescein transfers energy to the LC-Red 640, resulting in a signal that can be detected by the optics of the R.A.P.I.D.® Cycler. This is then compared to signals from positive and negative control samples to determine the presence of the desired DNA sequence in unknown samples.

To ensure the PCR process runs smoothly, additional reagents must be added during sample preparation. Bovine serum albumin, or BSA, is used to ensure that the DNA does not adhere to the sides of the glass capillaries. It also aids in stabilizing the enzyme (Innis and Gelfand 1990). To guarantee adequate extension by the *Taq* polymerase, deoxyribonucleotide triphosphates (dNTPs) are included in the solution. Magnesium chloride is another necessary reagent for PCR, as the magnesium ion maximizes the activity of the *Taq* polymerase (Gelfand and White 1990). Many manufacturers of real-time PCR devices produce commercial pre-made master mixes that contain all the necessary primers, probes, enzymes and buffers at their optimal concentrations. This speeds up sample preparation time and allows assay uniformity.

To be a viable method for wastewater screening, the PCR process must be able to proceed without hindrance from the wastewater matrix. This quality is important in determining PCR's ability to screen for a wide variety of pathogens. The sensitivity of PCR product detection must also be within levels that are of public health significance. *Salmonella typhimurium* can be used as a bacterial model to determine the detection limits due to its ease of culturing and lower virulence compared to other pathogens of interest. Its use also does not require special laboratory safety equipment or additional laboratory licensing.

2.4 *SALMONELLA* SPP.

Salmonella spp. are a genus of bacteria in the family Enterobacteriaceae. They are classified as facultatively anaerobic, non-spore-forming, gram-negative bacilli and are usually motile (Covert 1999). There are over 2,000 *Salmonella* serotypes, or strains, some of which are pathogenic, causing salmonellosis. In humans, the infection may cause gastroenteritis, enteric fever, and septicemia. A severe type of enteric fever, typhoid fever, is caused by *S. typhi*, a strain that is only pathogenic to humans. Other serotypes of interest, *S. enteritidis* and *S. typhimurium*, are both common causes of food poisoning in the United States. *S. enteritidis* is most commonly associated with chickens because of the ease in which they are infected. The infection is asymptomatic for chickens, resulting in its ability to travel up the food chain and infect humans. *Salmonella* has also been used as a biological warfare agent, when during World War II, the Japanese used the pathogen to contaminate the food and water of Chinese cities (Burrows and Renner 1999).

Salmonella infection in humans typically results from the ingestion of contaminated water or food. Once consumed, the bacterium passes through the stomach to the intestine where it can bind to the intestinal walls. With the use of special proteins, it is able to pass through the intestinal wall and enter the blood stream, eventually traveling to the liver, spleen and gall bladder. Infection of these organs may lead to focal lesions resulting in meningitis, endocarditis, pneumonia, or osteomyelitis (Covert 1999). The bacterium can also continue to multiply in these organs and travel back in the intestine to perpetuate the illness within its host.

Typical carriers of *Salmonella* include wild and domestic animals, especially poultry and swine. Pets, including cats, may be asymptomatic carriers. Humans can also serve as reservoirs

for infection without showing any symptoms. Some birds and animals may even be chronic carriers of the bacteria.

Salmonella infection can occur through the ingestion of food or water that has been contaminated with feces of infected hosts or by eating infected meat. The median infective dose (ID_{50}) for humans via ingestion ranges from 10^4 to 10^5 organisms, corresponding to a drinking water concentration of about 100 cfu/L for consumption of 15 L/day (Burrows and Renner 1999). Infection may occur with as little as 15-20 organisms depending on the age and health of the host and the virulence of the infecting strain (United Stated Food and Drug Administration).

The *Salmonella* bacterium is able to survive in many environments. It can remain stable in seawater for nine days, up to five months in ice, and in fresh water for about eight days (United States Environmental Protection Agency, Burrows and Renner 1999). When in fresh water, the organism can enter a viable, yet unculturable state. This quality may contribute to its long-term survival. It can live for weeks in sewage; 80% of activated sludge effluents contain *Salmonella*. *Salmonella* may also pass through to the non-chlorinated wastewater effluents of wastewater plants with concentrations typically ranging from 1 to 1,100 colony-forming units (cfu)/100 mL (Covert 1999). The bacteria can be rapidly inactivated by chlorine, as is the case with many other waterborne pathogens and indicator organisms. Sodium hypochlorite solutions of 1% or lower concentrations are suggested to inactivate this organism (United States Environmental Protection Agency). A chlorine residual of at least 0.2 mg/L is recommended to provide adequate protection in drinking water treatment systems. High concentrations of salt, over 30% NaCl, have also been able to inactivate *Salmonella typhi* within one day (New Zealand Food Safety Authority).

The traditional method for *Salmonella* detection is to use selective enrichment and plating media to isolate the target organism from other bacterial cells. This technique is long and labor-intensive and may take several days to complete. Other assays for the detection of *Salmonella* include fluorescent antibodies, latex agglutination, enzyme-linked immunosorbent assay (ELISA), motility enrichment, and DNA hybridization (Rahn et al. 1992; Nguyen et al. 1993). These techniques are much quicker in their ability to detect the target organism, but lack specificity due to cross-reactions with bacteria that carry the same antigenic determinants. To that end, PCR techniques have been developed to target specific and unique DNA sequences within the *Salmonella* genome. Genes, such as *invA*, *B*, *C*, and *D*, allow *Salmonella* to enter epithelial cells in the body and lend to its pathogenesis (Rahn et al. 1992). Given the specificity of PCR, targeting nucleotide sequences within these genes allow for the accurate detection of the pathogen.

3.0 PRIORITIZATION

Prioritization is a method to analyze contaminants that may be introduced into wastewater treatment and collection systems. Four endpoints were determined to be most critical in wastewater applications. Each endpoint was evaluated using the same eight criteria. A weighted score was calculated for each endpoint. These scores were then compiled to determine which contaminants would be of the highest priority if found in wastewater treatment and collection systems.

3.1 MATERIALS AND METHODS

The contaminant universe chosen for evaluation using the prioritization framework developed in this research were those that would be of concern to wastewater treatment and its infrastructure and collection systems. The list of contaminants evaluated is shown in Appendix A. Contaminants that would be a priority for drinking water systems was the starting point in developing this list. These contaminants were drawn from the Select Agent List developed by the Centers for Disease Control and Prevention (CDC), and lists provided by the Water Information Sharing and Analysis Center (Water ISAC) and the U.S. Army. Other contaminants considered for the wastewater prioritization were flammable chemicals as classified by the

Occupational Health and Safety Administration, radioactive elements, and decontamination agents (Perry 2003).

The primary concern for drinking water contaminants is their effect on public health through exposure by ingestion. For wastewater, the concerns are much broader in nature, being broken down into four endpoints:

- Worker and Public Health
- Process Upset
- Physical Damage and Destruction
- Pass Through

Worker and public health took into account the effects of inhalation exposure of a given contaminant on the individual treatment plant worker and the surrounding community. Process upset considered the effects of the contaminant on primary, secondary, and tertiary treatment and disinfection. Physical damage and destruction dealt with the effects of fire or explosion on a wastewater treatment plant or collection system. The consequences of infrastructure contamination by agents that may persist and adhere to infrastructure components were also considered. Pass through was evaluated by assessing the deleterious effects of a contaminant on the flora and fauna of receiving waters and potential contamination of downstream drinking water facilities.

These four endpoints were evaluated based on criteria that the Environmental Protection Agency (USEPA) developed for the drinking water prioritization (Allgeier 2005). Eight criteria were chosen to be pertinent to wastewater treatment (Table 3.1). An appropriate description was assigned to each criterion with respect to wastewater treatment and collection systems. Each criterion was assessed with respect to the each of the four endpoints developed for wastewater

(Figure 3.1). A literature review was performed for each contaminant to gather information pertaining to each of the criteria. Emphasis was placed on open, reputable sources, such as government websites and reference handbooks on industrial chemicals.

Business rules of scoring, as defined by the USEPA, were applied to each criterion ranging from 1 to 5, from least significant to most (Table 3.2). These rules were developed to provide a quantitative approach to contaminant analysis with respect to the prioritization criteria. They were also designed to encompass a wide range of possibilities. Scoring was based on information found for each contaminant regarding the criterion in question. For example, the scoring basis for the availability criterion ranged from contaminants that could be easily obtained (e.g., gasoline, common household products) to contaminants that can only be obtained from secure facilities, such as the majority of radioactive elements. These criteria were given a score of 5 and 1, respectively. The other scores were determined by varying the degree of availability of the contaminant. This same logic was applied to the other criteria being evaluated. Contaminants for which no or ambiguous data were found with respect to the criterion in question, a score of 3 was given.

Table 3.1. Criteria descriptions as they apply to wastewater contamination

Criterion	Description
Availability	The ease with which the contaminant can be obtained, synthesized or harvested from natural, industrial or commercial sources.
Potency	The amount of a contaminant that would be required to contaminate a reference volume of water at a flammable/explosive level or a toxic/infectious dose. This criterion considers the flammable nature, toxicity, infectivity and purity of the contaminant. This criterion may be defined differently for different endpoints.
Persistence	The time that a contaminant remains toxic, infectious or flammable after introduction into a wastewater collection and treatment system.
Introduction/ Dispersion	The ease with which a contaminant can be handled and effectively introduced and dispersed into a wastewater collection and treatment system.
Process Removal (Inactivation/ Treatability)	The removal or inactivation of a contaminant by typical wastewater treatment unit processes (primary treatment, secondary treatment, disinfection and sludge/biosolids treatment and disposal).
Storability	The time that a contaminant remains toxic, flammable or infectious while in storage (prior to intentional contamination of a wastewater collection and treatment system).
Outcomes (Human Illness)	The severity of human health effects for wastewater treatment plant workers exposed to the contaminant or the extent of the physical damage/destruction or process disruption associated with the presence of the contaminant in the wastewater collection and treatment system. Additional Outcomes have been developed to describe the extent of process upset and contaminant pass through.
Public Perception	Public perception of the risks associated with the contaminant (e.g., the fear that the public has developed toward anthrax following the US Mail attacks of 2001), the inconvenience associated with the disruption or destruction of the wastewater collection and treatment system, or the presence of wastewater (i.e., using sewage as a weapon).

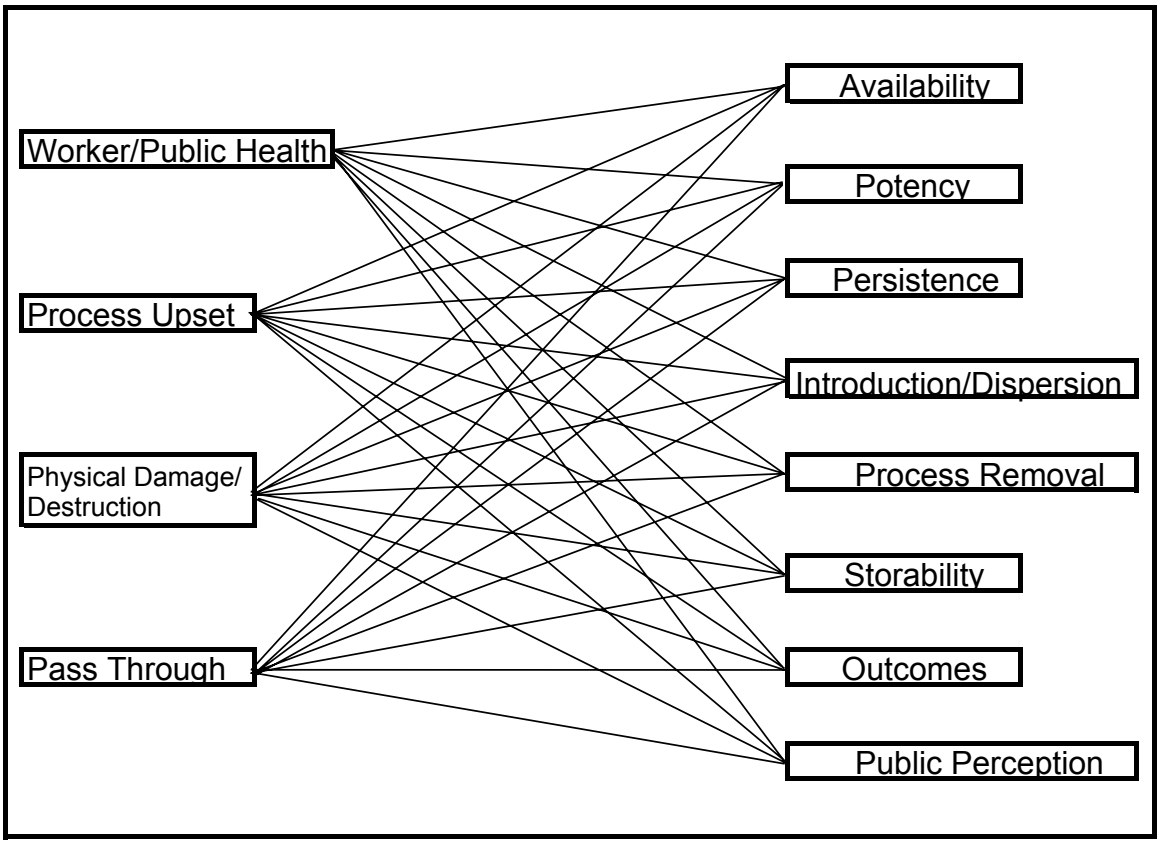

Figure 3.1 Framework Flow Chart

Table 3.2. Business Rules for Scoring of Each Criterion

Criterion	Score	Basis for Score
Availability	1	Contaminant only exists in secure facilities and could not be generated without access to specialized information/equipment.
	2	Contaminant is secured, but precursors may be available and synthesis is possible but difficult.
	3	Controlled material with limited production.
	4	Controlled material with widespread application.
	5	Readily available from commercial, industrial, or natural sources (or easily synthesized).
Potency *(Note, potency tables have been developed for LD_{50}, Flashpoint, etc. similar to the ID_{50} information shown here and the flammable potency chart on Page 6)*	1	High ID_{50}, High Flashpoint, High Lower Explosive Limit, High LD_{50}, High LC_{50} (e.g., ID_{50} > 100,000 microbes per ml)
	2	ID_{50} between 10,000 and 100,000 microbes per ml
	3	ID_{50} between 1,000 and 10,000 microbes per ml
	4	ID_{50} between 100 and 1,000 microbes per ml
	5	Low ID_{50}, Low Flashpoint, Low Lower Explosive Limit, Low LD_{50}, Low LC_{50} (e.g., ID_{50} < 100 microbes per ml)
Persistence	1	Contaminant is known to degrade to harmless end products immediately upon contact with water or wastewater.
	2	Contaminant remains viable in water or wastewater for up to two hours.
	3	Contaminant remains viable in water or wastewater for between two hours and one day.
	4	Contaminant remains viable in water or wastewater for between one day and one week.
	5	Contaminant remains viable in water or wastewater for more than one week, or produces more hazardous end products in water and wastewater than the initial contaminant.

Table 3.2. Business Rules for Scoring (continued)

Criterion	Score	Basis for Score
Introduction/Dispersion	1	Difficult to introduce and specialized equipment is required to introduce or disperse the contaminant.
	2	Industrial equipment is required to introduce or disperse the contaminant.
	3	Commercial equipment is required to introduce or disperse the contaminant.
	4	Household equipment is required to introduce or disperse the contaminant.
	5	Easily introduced and no equipment is required to introduce or disperse the contaminant. The contaminant is easily introduced and dispersed.
Process Removal *(Inactivation/Treatability)*	1	Contaminant is removed from the wastewater in the pretreatment or primary treatment processes.
	2	Contaminant is removed from the wastewater in the biological treatment process/secondary sedimentation
	3	Contaminant is removed or inactivated by conventional wastewater treatment disinfection processes. *Unknown Process Removal = 3*
	4	Contaminant is only removed or inactivated by tertiary treatment or specialized unit processes.
	5	Contaminant is not removed during wastewater treatment unit processes.
Storability	1	Not capable of being stored
	2	Half-life/infectivity of the contaminant in storage is less than 1 day.
	3	Half-life/infectivity of the contaminant in storage is between 1 day and 2 weeks.
	4	Half-life/infectivity of the contaminant in storage is between 2 weeks and 6 months.
	5	Half-life/infectivity of the contaminant in storage is greater than 6 months.
Outcomes (Result of Contamination, additional outcomes have been developed to describe the extent of human illness and the possible outcomes of pass through)	1	No adverse effect expected.
	2	Minor effects expected. Simple or normal operational adjustments are required to deal with the contaminant.
	3	Manageable effects expected. Treatment processes must be altered or changed. No remediation is required.
	4	Serious effects expected. Public notification is required. Treatment plant processes are upset and remediation is required.
	5	Catastrophic effects expected. Treatment
Public Perception	1	The public is unaware of the substance or problems with the substance.
	2	Common substance not linked to terrorist activities
	3	Exotic substance not linked to terrorist activities
	4	Common substance linked to terrorist activities
	5	Exotic substance linked to terrorist activities

A method of scoring for potency and outcome was established for each of the endpoints because of the different factors in evaluating these criteria. Worker impact potency was scored by evaluating the occupational exposure levels as required by OSHA and the National Institute for Occupational Safety and Health (NIOSH). This included Permissible Exposure Limit (PEL) as required by OSHA, and the Recommended Exposure Limit (REL) and Immediately Dangerous to Life or Health (IDLH) concentration as suggested by NIOSH. These limits were put in place to establish allowable airborne concentrations of chemicals to ensure the health and safety of workers. Both the long-term (PEL and REL) and short-term (IDLH) exposure concentrations were scored for the prioritization as these numbers put the potential inhalational toxicity of a substance into quantifiable terms (Tables 3.3 and 3.4). In both instances, the scoring for the PELs, RELs, and IDLHs increased as the concentration of the exposure limit decreased. The long-term exposure limits were increased by an order of magnitude to determine the scoring for varying concentrations. The procedure for the IDLH was slightly different in that the ranges for individual scores were larger. This difference took into account the fact that larger concentrations were documented for short-term exposure limits. The IDLH takes into account the acute toxicity of a substance, and in the event of volatile chemical contamination of a wastewater treatment system, the IDLH would be of greater importance as the attack would be more likely be a one-time instance rather than be sustained over a long period of time.

Table 3.3. Business Rules for Potency Scoring for PEL and REL

Score	ppm
1	> 1,000
2	100 to 1,000
3	10 to 100
4	1 to 10
5	< 1

Table 3.4. Business Rules for Potency Scoring for IDLH

Score	ppm
1	> 5,000
2	1,000 to 5,000
3	100 to 1,000
4	1 to 100
5	< 1

A different parameter was needed to account for the potency of pathogenic agents with respect to the worker and public health endpoint. The documented ID_{50} (the infective dose needed to cause illness in 50% of the exposed population) was used to score these biological agents. Typically, these numbers are based on exposure by ingestion. This route of exposure would not be likely with wastewater, but many of the pathogens in question can be aerosolized, leading to their ability to be inhaled, thus causing infection. In this instance, ID_{50}'s based on inhalation were evaluated and scored (Table 3.5). Again, the highest score was given to the lowest concentration of microbes, and scoring increased as the concentration increased by an order of magnitude.

Table 3.5. Business Rules for Potency Scoring for ID_{50}

Score	ID_{50} Value (microbes per 100 mL)
1	> 100,000
2	10,000 to 100,000
3	1,000 to 10,000
4	100 to 1,000
5	<100

Flammable agents that may be intentionally introduced into a wastewater treatment system could lead to the physical damage and destruction of the collection system and unit operations within the plant. Two properties of volatile chemicals, the flash point and lower explosive limit (LEL), were used to develop a scoring method for their potency. Each property

was placed on a separate axis of a two-dimensional table, which would be used to calculate a composite potency score. The flash point, the lowest temperature at which a liquid's vapor can form an ignitable mixture with air, was scored from 1 to 5 based on the ambient temperatures found in wastewater treatment facilities and distribution systems. Flash points below 10°C were scored a 5, as this was lowest temperature documented in many cold weather wastewater treatment facilities (U.S. Army and Air Force 1987). A score of 4 was assigned to flash points between 10°C and 30°C, representative of the higher end of temperatures present in wastewater treatment facilities (Tchobanoglous et al. 2002). For flash points above 30°C, scores of 3 to 1 were arbitrarily assigned with lower scores given to higher temperatures.

Scoring for the LEL, defined as the percentage of vapor of a volatile needed to create an explosive mixture with air, was determined for percentages ranging from below 1% to those above 12%. Those LEL's 2% and below were scored a LEL factor of 5 corresponding to the increased likelihood of explosion when lower concentrations of the vapor of a volatile substance are necessary. To score the other LEL's, these factors were decreasingly assigned in 3% increments as the LEL increased. This resulted in a LEL factor of 4 for 3% and an LEL factor of 1 for a lower explosive limit above 12%.

A composite potency score was calculated by averaging the factors for the lower explosive limit and flash point and rounding up to the next integer (Table 3.6). For example, a substance with a flash point of 40°C, corresponding to a factor of 3, and a LEL of 2% (a factor of 5) would result in an overall score of 4 (Equation 3.1).

$$\text{Flammable Potency} = (\text{Flash Point Factor} + \text{LEL Factor}) / 2 \quad (3.1)$$

Table 3.6. Flammable Contaminant Potency Scoring Chart

	Flash Point Factor														
	1	> 180	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	1	170	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	1	160	3	3	3	3	3	3	2	2	2	2	2	2	1	1
Flash Point	1	150	3	3	3	3	3	3	2	2	2	2	2	2	1	1
°C	1	140	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	1	130	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	1	120	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	1	110	3	3	3	3	3	3	2	2	2	2	2	2	1	1
	2	100	4	4	4	3	3	3	3	3	3	2	2	2	2	2
	2	90	4	4	4	3	3	3	3	3	3	2	2	2	2	2
	2	80	4	4	4	3	3	3	3	3	3	2	2	2	2	2
	3	70	4	4	4	4	4	4	3	3	3	3	3	3	2	2
	3	60	4	4	4	4	4	4	3	3	3	3	3	3	2	2
	3	50	4	4	4	4	4	4	3	3	3	3	3	3	2	2
	3	40	4	4	4	4	4	4	3	3	3	3	3	3	2	2
	4	30	5	5	5	4	4	4	4	4	4	3	3	3	3	3
	4	20	5	5	5	4	4	4	4	4	4	3	3	3	3	3
	5	10	5	5	5	5	5	5	4	4	4	4	4	4	3	3
	5	0	5	5	5	5	5	5	4	4	4	4	4	4	3	3
	5	-10	5	5	5	5	5	5	4	4	4	4	4	4	3	3
	5	< -20	5	5	5	5	5	5	4	4	4	4	4	4	3	3
			<1	1	2	3	4	5	6	7	8	9	10	11	12	>12
		LEL Factor	5	5	5	4	4	4	3	3	3	2	2	2	1	1
			Lower Explosive Limit (Volume % in air)													

The potency of contaminants for the endpoints of process upset and pass through was determined by their concentration in water, measured in parts per million (ppm). Scoring for these criteria increased as concentration decreased, and the scoring for this data is identical to that of the ID_{50} (Table 3.7). The potency for process upset was determined by the documented toxicity of *Pseudomonas spp.*, a major component of activated sludge flocs (Bitton 2005). The documented ecotoxicity of freshwater fish was used to score the potency of contaminants, with the lower concentrations assigned a higher score.

Table 3.7. Business Rules for Potency Scoring for LC_{50}, Lethal Concentration for 50%

Score	ppm
1	> 100,000
2	10,000 to 100,000
3	1,000 to 10,000
4	100 to 1,000
5	< 100

Weighting factors were developed for each of the eight criteria to determine overall relative importance of each criterion with respect to the endpoint in question as compared to the other seven criteria. The analysis was based on the analytic hierarchy and network process in which each criterion was compared to the others and was assigned a numerical score based on importance (Saaty 2005). The scores for each of the criteria were summed, and then normalized. The weighting factors developed for each endpoint are presented in Table 3.8. The criteria considered being of the utmost importance for worker and public health, process upset, and physical damage and destruction is the overall outcome of the contamination. A contamination event may result in worker illness and death with possible effects on the surrounding community. Process upset may cause untreated sewage to be released into receiving waters, requiring extensive remediation efforts within the treatment plant and areas downstream. The effects of contaminants that may be responsible for physical damage and destruction may be the complete destruction of the treatment plant or its collection system, requiring extensive remediation and rebuilding. Individual components of the treatment and collection system can also be damaged in which remediation and clean-up would be necessary to bring them back online.

Public perception was considered to be the most important criterion in the event of contaminant pass through into receiving waters. Contaminants may result in fish kills and the harming of flora and fauna in the surrounding areas of the receiving waters. Downstream water

treatment plant intakes may also have to be closed due to the contamination. While these scenarios are possible outcomes of the pass through of a contaminant, the public perception of such events would be considered more important. Each of these outcomes may result in considerable public outrage and media coverage and a general mistrust of the utility. If the contamination was due to an intentional attack, these outcomes may also result in public fear as well.

Table 3.8. Weighting Factors for Prioritization Framework

Criteria	Worker/Public Health	Process Upset	Physical Damage/Destruction	Pass Through
Availability	**0.18**	**0.24**	**0.16**	**0.03**
Potency	**0.16**	**0.11**	**0.06**	**0.06**
Persistence	**0.04**	**0.10**	**0.05**	**0.16**
Introduction/Dispersion	**0.04**	**0.11**	**0.06**	**0.03**
Process Removal	**0.02**	**0.09**	**0.04**	**0.16**
Storability	**0.04**	**0.07**	**0.02**	**0.03**
Outcomes	**0.28**	**0.26**	**0.32**	**0.20**
Public Perception	**0.24**	**0.02**	**0.29**	**0.33**

The composite prioritization score for each endpoint was determined by multiplying the weighting factor for each criterion by the numerical score assigned to each factor. These numbers were then summed (Equation 3.2).

$$Score = Av*W1 + Po*W2 + Pe*W3 + ID*W4 + Prem*W5 + St*W6 + Out*W7 + Pp*W8 \quad (3.2)$$

Where:

Av - numerical value assigned to the criteria of Availability

Po - numerical value assigned to the criteria of Potency

Pe - numerical value assigned to the criteria of Persistence

ID - numerical value assigned to the criteria of Introduction/Dispersion

Prem - numerical value assigned to the criteria of Process Removal

St - numerical value assigned to the criteria of Storability

Out - numerical value assigned to the criteria of Outcomes

Pp - numerical value assigned to the criteria of Public Perception

W1 - Weighting Factor for Availability

W2 - Weighting Factor for Potency

W3 - Weighting Factor for Persistence

W4 - Weighting Factor for Introduction/Dispersion

W5 - Weighting Factor for Process Removal

W6 - Weighting Factor for Storability

W7 - Weighting Factor for Outcomes

W8 - Weighting Factor for Public Perception

The composite score developed for each endpoint for a single contaminant could then be compared to those scores of other contaminants to determine those that would be the highest priority for screening in a wastewater treatment plant and collection system.

3.2 RESULTS AND DISCUSSION

A total of 78 contaminants were analyzed within the prioritization framework. Weighting factors were applied to the scores to come up with a composite score for each of the four endpoints analyzed in this scheme. The composite score for each endpoint ranges from 1 to 5, with 5 indicating a highly hazardous contaminant with respect to the endpoint in question. The prioritization of contaminants is to show that the higher the overall score for each endpoint,

the higher the priority of screening for that contaminant in a wastewater treatment system. Data sheets for the most dangerous contaminants with respect to specific endpoints are presented in Appendix B.

The first endpoint to be analyzed was worker and public health. The primary factors in scoring this endpoint is the potency of the contaminant as determined by the OSHA and NIOSH guidelines for inhalation exposure and the effects of acute inhalation exposure to the contaminant. The ten most important contaminants with their composite scores are listed as follows (Table 3.9):

Table 3.9. Top ten contaminants of concern for worker and public health

CONTAMINANT	Worker/Public Health Score
Botulinum Toxin	4.39
VX	4.33
Cyanide Salts	4.00
Arsenic	3.91
Mercury	3.91
Bacillus anthracis	3.83
Cobalt - 60	3.80
Malathion	3.75
Cesium - 137	3.65
Strontium - 90	3.64

Wastewater contaminants that would be most harmful to worker and public health cannot be classified into a single category. Radioactive isotopes, heavy metals, biological and chemical weapons, and highly toxic chemical compounds are all represented on this list. Those that would be most important to screen for would be those that are easily aerosolized. This would be primary route of exposure for treatment plant workers and members of the surrounding community. Spore-forming bacteria, like *Bacillus anthracis* and *Clostridium botulinum* (the bacteria responsible for producing Botulinum toxin), can be easily aerosolized and have been weaponized for wartime use in the past.

The radioactive isotopes such as Cobalt-60, Cesium-137, and Strontium-90, do not pose much of an inhalation threat. They are not easily aerosolized and pose more of a chronic radiation exposure threat. However, if large quantities are present, radiation sickness may occur in exposed workers.

Possibly the most deadly of chemical agents, VX and cyanide salts, pose the greatest human health risk if they were to be volatilized into the air by a treatment process. Very small quantities of these chemicals, if inhaled, can cause death.

Process upset is characterized by the ability of a contaminant to disrupt or upset the various treatment processes within a wastewater treatment plant. This will most likely occur in biological processes, such as secondary treatment and nitrification and denitrification processes, in which microorganisms are used. These contaminants may retard the respiration and growth of microbes, thus rendering them unable to remove the organic and inorganic materials in wastewater. Other contaminants may cause deflocculation, causing problems in settling tanks and the pass through of untreated sewage throughout the plant. Upset can also occur during disinfection, where contaminants may react with the chlorine, increasing the chlorine demand of the process and possibly creating more dangerous chemicals in the process. Those contaminants of interest are listed in Table 3.10.

Table 3.10. The top eleven contaminants with respect to Process Upset

CONTAMINANT	Process Upset Score
Malathion	4.31
Mercury	4.22
Calcium Hypochlorite	4.21
Pentane	4.21
1-Chloro-2,4-Dinitrobenzene	4.20
Cadmium	4.19
Lead	4.11
Carbon Disulfide	4.08
Phenol	4.03
Fuel Oils	4.01
2,4-Dinitrophenol	4.01

Heavy metals, such as mercury, cadmium, and lead, can be easily integrated into the flocs during secondary treatment, causing deflocculation and decreases in COD removal. Because they are not metabolized by microorganisms, remediation efforts would be necessary to remove the metals from the plant.

2,4-Dinitrophenol (DNP) and 1-chloro-2,4-dinitrobenzene (CNDB) are industrial chemicals that can severely inhibit the nitrification process. Both also inhibit the respiration rates of mixed liquor, leading the reduction in COD removal. 2,4-DNP is especially toxic to anaerobic systems.

Malathion and carbon disulfide, a pesticide and an intermediate in pesticide production respectively, have been shown to inhibit the nitrification process. Both may also increase the chlorine demand of wastewater disinfection. Malathion can be neutralized by chlorination, and this is a common tertiary process for its removal from wastewater streams. However, carbon disulfide also reacts readily with chlorine to form carbon tetrachloride, a documented carcinogen.

Calcium hypochlorite is a disinfectant commonly used in swimming pools and drinking water applications. Because of its use as a disinfectant, its introduction into wastewater

treatment systems may severely hinder secondary treatment by killing the microorganisms necessary for the process. Remediation would be necessary after its introduction.

The third endpoint analyzed was the physical damage and destruction of the wastewater treatment plant and collection system. This may result from the buildup of vapors above the lower explosive limit within sewers requiring only a small spark to ignite, as previously documented. Some contaminants, due to their chemical properties, may adhere to infrastructure components of the treatment plant. They may also persist throughout the wastewater treatment system without being degraded, and plant shutdown and remediation efforts would be necessary. The contaminants of most concern according to their composite score are (Table 3.11):

Table 3.11. Top ten contaminants with respect to treatment plant physical damage and destruction

CONTAMINANT	Physical Damage / Destruction Score
VX	4.23
Bacillus anthracis	4.22
Kerosene	4.03
Gasoline	4.03
Octane	3.97
Isopropyl Alcohol	3.94
Hexane	3.91
Styrene	3.90
Acetone	3.90
Arsenic	3.83

Most prominent on this list are flammable organics that can easily be introduced into collection systems through open sewers. Once they enter the treatment plant, they may be degraded during secondary treatment or stripped from the wastewater during aeration due to their volatility. This phenomenon can raise the concentrations of these chemicals to explosive and/or flammable levels and also may pose as a further threat to worker and public health. The introduction of styrene may be particularly dangerous, because it polymerizes easily upon contamination. This could result in the clogging of pipelines. The monomer has also been responsible for several

industrial explosions. It also forms peroxides quite easily upon exposure to oxygen, which are also quite explosive.

Pathogens are common constituents of flocs in wastewater, but due to grazing by protozoa and disinfection, generally do not survive long enough to reach the effluent. Results of testing indicate that *Bacillus anthracis* spores may be able to withstand the rigors of wastewater treatment. Spores may become integrated within the floc structures of secondary treatment, and they are highly resistant to chlorine disinfection. Given that the spores can survive in aqueous environments for decades, extensive remediation would be necessary for their removal.

VX, due to its chemical structure, can adsorb to organic carbon, prevalent within wastewater, and adhere to infrastructure components of the treatment plant and collection system. Its high toxicity makes remediation of the plant a necessity in order to prevent VX from passing through into receiving waters and possibly entering drinking water installations downstream.

The final endpoint to be analyzed was the pass through of the contaminant into the receiving waters of the wastewater treatment plant. Of greatest concern are the effects of the contaminants on wildlife, especially fish, and plant life. If the contaminant is not removed by the receiving waters, there is also the potential that it may enter the raw water intakes of the drinking water facilities, becoming a greater threat to the human population. Contaminants that would pose the greatest risk to the surrounding environment according to its composite score are (Table 3.12):

Table 3.12. Top eleven contaminants with respect to treatment plant pass through

CONTAMINANT	Pass Through Score
VX	4.83
Cyanide Salts	4.38
Bacillus anthracis	4.10
Aldicarb	4.03
DDT	4.02
Plutonium-238	3.86
Pentane	3.80
Lead	3.80
Arsenic	3.76
Cyclohexanol	3.75
1-Chloro-2,4-Dinitrobenzene	3.75

This list is comprised of pathogens, pesticides, radioactive elements, chemical warfare agents, organic chemicals, and other highly poisonous chemicals. VX is by far the most toxic compound according to its composite score of 4.83. It is especially harmful to fish, birds, and wildlife, with only very minute quantities needed to cause death.

VX chemistry is very similar to that of organophosphates, compounds commonly used for the basis of pesticides. These are typically cholinesterase inhibitors, severely affecting the nervous system at very small doses. Aldicarb, in the carbamate family, is a pesticide that acts in the very same way, having the same effects on wildlife as that of VX. DDT is another pesticide that can have dire consequences for the fauna living in receiving waters and other animals that use its waters. DDT is also able to bioaccumulate in the tissues of animals, making its effects persistent and long lasting.

Lead is another contaminant with the ability to bioaccumulate in wildlife. It has no metabolic purpose and acts a toxicant in fish and animals. Other contaminants, such as cyanide

salts and arsenic, are not likely to bioaccumulate, but will be likely to responsible for fish kills if released into receiving waters.

Organic chemicals like pentane and 1-chloro-2,4-dinitrobenzene can also be toxic to aquatic organisms. Due to pentane's hydrophobic structure, it will form a slick on the surface of receiving waters, preventing oxygen from dissolving into the water, potentially smothering aquatic life.

Plutonium-238 is considered one of the most poisonous radioactive substances known to man. Its introduction into receiving waters is expected to cause great ecological stress on aquatic flora and fauna and those living in areas bordering the waterways. Land that has been contaminated by this isotope of plutonium is generally deemed unsuitable for public use.

As previously stated, *Bacillus anthracis* can cause disease in animals. Its spores would be able to persist in the receiving waters without being inactivated. Fish and vegetation are not expected to be affected by its pass through.

There is no one specific type of contaminant that can be considered harmful with respect to the four endpoints. Biological and chemical agents, flammable organics, heavy metals, and radionuclides exhibit properties that would be detrimental if introduced into a wastewater treatment and collection system. A means to quickly identify these contaminants needs to be developed for use by emergency response teams. One such technique is real-time, rapid cycle PCR for use in the detection of biological agents.

4.0 PATHOGEN SCREENING AND DETERMINATION OF DETECTION LIMITS OF REAL-TIME RAPID CYCLE PCR USING *SALMONELLA TYPHIMURIUM*

A series of three evaluations were performed to determine the efficacy of using real-time rapid cycle PCR as a screening tool for wastewater. Screening tests were performed to determine the presence of interferences within the wastewater matrix. Secondly, experiments using *Salmonella typhimurium* were designed to determine the detection limits of the R.A.P.I.D.® PCR System by comparing the PCR results with traditional plate culturing techniques. Lastly, a field concentration method was applied to river water and secondary effluent to determine any improvements in bacteria recovery by culture technique and PCR detection.

4.1 MATERIALS AND METHODS

4.1.1 Phase I – Screening for pathogens

Three steps were necessary to perform real-time PCR using the R.A.P.I.D.® System: DNA extraction and purification, PCR reaction set-up, and R.A.P.I.D.® PCR set-up. Each step was performed in three separate areas to prevent the possibility of cross-contamination, as this could lead to erroneous results (i.e., false positives).

DNA extraction was performed using the 1-2-3 DNA™ Purification Kit (IdahoTech). Extractions were prepared from six water samples:

- Distilled and Deionized Water (Millique)
- Allegheny River Water
- Finished Chlorinated Drinking Water (Pittsburgh Water and Sewer Authority)
- Chlorinated Secondary Effluent (Allegheny Valley Joint Sewage Authority)
- Mixed Liquor (AVJSA)
- Primary Effluent (AVJSA)

Three samples, filtered drinking water, river water, and secondary effluent, were also concentrated using a field portable unit involving a hand pump and a disposable filter. The filtered drinking water was concentrated from a volume of 2 L down to 2 mL for a 1000-fold decrease in volume. An initial volume of 500 mL was chosen for concentrating the river water and secondary effluent due to the high turbidity of both samples. The filter unit consisted of a 300-mL polypropylene funnel and a 47-mm mixed cellulose-ester membrane filter with a pore size of 0.45 microns. The sample to be concentrated was filtered through the membrane using a hand pump that used suction to draw the water through. Once the entire sample was filtered, the membrane was removed using forceps and sterile technique and placed into a sterile 15-mL polystyrene centrifuge tube. Two (2) milliliters of a surfactant containing 0.05% turgitol was added to the centrifuge tube, and the tube was vortexed at maximum speed for about a minute using a field portable vortex. This allowed for the removal of all particles and possible microorganisms trapped on the filter. The filter was then removed using sterile forceps and squeezed dry. The resulting eluate was then used to prepare DNA extractions for the screening tests.

The bead tubes provided in the 1-2-3 Kit contained glass microbeads along with lysis buffer and molecular grade water. Prior to extraction, the bead tubes were centrifuged for 30

seconds to ensure its contents were at the bottom of the tube. A volume of 100 µL of sample was used for each DNA extraction, regardless of water type, and was added to each bead tube. The samples were beat for five minutes using a Vortex Genie 2 with the Turbomix 2.0 mL adapter (Scientific Industries). This aided in breaking the cells possibly present in the sample, releasing the DNA. Using a P-1000 pipette (Gilson), 350 µL of Buffer 1 was added to the bead tube. Buffer 1 is a high salt buffer containing guanidine HCl, Triton X-100 (a nonionic surfactant used to solubilize cell membrane proteins [Roche Applied Science]), and isopropanol. The bead tube was centrifuged again for 30 seconds. The liquid was extracted using the P-1000 pipette, being careful not to draw up the glass beads, and transferred to a spin filter placed in a receiving tube. This assembly was then centrifuged for 2 minutes to allow all the liquid to pass through to the receiving tube, thus binding the DNA to the spin filter.

The spin filter was placed in a new receiving tube, and the second washing step was performed. A volume of 550 µL of Buffer 2, denatured ethanol, was pipetted into the new spin filter-receiver assembly. The assembly was then centrifuged for 2 minutes to allow the second buffer to pass through the spin filter. This step removed humic and fulvic acids that could be present in the sample, and subsequently could interfere with the PCR reaction and analysis. After centrifugation, the spin filter was placed in a new receiver tube and centrifuged again for 3 minutes. This removed any residual ethanol that could hinder the PCR reaction.

The third step involved eluting the bound DNA from the spin filter into solution. The spin filter was placed into a new receiver tube, and 400 µL of Buffer 3, a solution of Trizma HCl and EDTA Disodium, was pipetted into the assembly. This was allowed to sit for 2 minutes and then was centrifuged for an additional two minutes. The spin filter was thrown away, and the remaining solution containing the DNA was stored at 2 - 4°C.

After the DNA extractions, each water sample was analyzed for 11 pathogens to determine their background presence in water and wastewater. The assays performed are as follows:

- *Bacillus anthracis* (Anthrax),
- *Clostridium botulinum* Type A,
- *Brucella* spp.,
- *Yersinia pestis* (Plague),
- *Francisella tularensis* (Tularemia),
- *Salmonella* spp.,
- *Escherichia coli* O157,
- *Listeria monocytogenes*,
- *Campylobacter* spp.,
- *Cryptosporidium* spp.
- Variola (Small Pox)

Freeze-dried reagents from Idaho Technology, Inc. were used for the PCR reaction setup. For each pathogen, there were positive and negative control vials and unknown reagent vials spiked with the appropriate DNA primers for the target DNA sequence. The positive and negative controls were reconstituted with 40 μL of sterile PCR grade water, while the unknowns were prepared using 40 μL of the previously extracted DNA. The reconstituted vial contents were transferred into two capillary tubes and briefly centrifuged to ensure that the contents were in the bottom of the tube.

The prepared capillary tubes were placed into the carousel of the thermocycler according the Load Window of the R.A.P.I.D.® program. Each run was preprogrammed into the computer with the appropriate numbers of cycles and required temperature profile for DNA denaturation and amplification. The fluorescence of each sample was measured by the instrument and recorded by the computer. At the end of the PCR run, the results were analyzed by the manufacturer-provided software, making a qualitative "Present/Not Detected" determination of the target pathogen's presence.

4.1.2 Phase II – Detection Limits of *Salmonella* in River Water and Wastewater Matrices

The detection limits for the rapid cycle real-time PCR assay for *Salmonella* spp. were determined using four water samples:

- Allegheny River Water
- Chlorinated Secondary Effluent (Allegheny Valley Joint Sewage Authority)
- Mixed Liquor (AVJSA)
- Primary Effluent (AVJSA)

Each water sample was spiked with *Salmonella typhimurium* and then analyzed using PCR and traditional culturing methods. The results of these techniques were then compared to determine the detection limits of the R.A.P.I.D.® system.

The bacteria culture was derived from a commercial strain (ATTC14028) and was cultured overnight at 37°C in 150 mL of nutrient broth. A quarter of this stock solution, about 37.5 mL, was pipetted into 500 mL of autoclaved water sample to be tested. Seventeen additional flasks containing 500 mL of water sample were autoclaved for five minutes to remove background bacteria that may skew the experiment results. Serial dilutions were prepared in these flasks in 0.5-log increments from 10^{-1} to 10^{-9} using the initial *Salmonella*-spiked solution as a basis. From each dilution, DNA was extracted and purified for PCR analysis. The dilutions were also cultured for *Salmonella* by spreadplating 0.1 mL onto nutrient media. Aliquots of 0.1 mL and 0.2 mL were plated for dilutions 10^{-6} and higher. The plates were incubated for 24 hours at 37°C, and the colonies that grew were counted the next day. The extracted DNA was analyzed in duplicate using *Salmonella* assay kits, where a present/not detected result could be

determined. These results were compared to the qualitative results of the plate counts, and a detection limit for the PCR method could be determined.

4.1.3 Phase III – Detection Limits of *Salmonella* in River Water and Secondary Effluent using a Concentration Method

The concentration method previously described in Phase I was applied to determine any improvement in the detection limits of the PCR method. This technique was previously applied to finished, but not yet chlorinated, drinking water by concentration from 2 L to 2 mL (States et al. 2006). In this study, the river water sample was concentrated from 500 mL to 2 mL. Due to its high turbidity and visible suspended solids, only 250 mL of the secondary effluent was able to be concentrated for this experiment. All water samples were autoclaved for 5 minutes prior to being spiked with the *Salmonella* culture. Dilutions were plated for both the unconcentrated and concentrated samples as described in the Phase II Materials and Methods section. DNA was extracted for each of the dilutions as previously described in the Materials and Methods section for Phase I. These extractions were then analyzed for *Salmonella* spp. using the R.A.P.I.D.® PCR. The presence of Salmonellae was confirmed by one or both capillary tubes testing positive. The lowest dilution at which there was a Salmonellae presence in the concentrated sample was compared to the bacterial count at the same dilution in the unconcentrated sample. This comparison determined how much bacteria in the original sample could be detected by the PCR when concentrated.

4.2 RESULTS AND DISCUSSION

4.2.1 Phase I – Screening for pathogens

As discussed in the Materials and Methods section, screening tests for various pathogenic organisms were performed on nine drinking water and wastewater samples using the R.A.P.I.D.® PCR. Two unique PCR assay kits contained tests for eight pathogens. The BioThreat Screening Kit (IdahoTech) tested for *F. tularensis*, *B. anthracis*, *Y. pestis*, and *Brucella* spp., while the Pathogen Test Kit (IdahoTech) screened for *E. coli* O157, *Listeria monocytogenes*, *Salmonella* spp., and *Campylobacter* spp. These assays were performed on each individual water sample. Individual assays were also performed for *Cryptosporidium* spp., *Clostridium botulinum* Type A, and Variola. Each screening test was performed in quadruplicate with the contents of two vials being divided into four capillary tubes total. All PCR results were evaluated using the software supplied by IdahoTech and reported in a qualitative Present/Not Detected format. Samples were considered positive when at least one capillary tube returned a confirmation of pathogen presence. As an example, the results of the *Salmonella* screening tests are provided in Table 4.1. Each water matrix was analyzed for *Salmonella* spp. in quadruplicate, and the results were reported as the number of hits per number of capillary tubes analyzed. The same format was used in analyzing the other pathogens being screened, and all screening tests results are shown in Appendix C.

Table 4.1. PCR Results of the Screening Tests for *Salmonella* spp.

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/21/2006	0/4
River Water	6/23/2006	0/4
Primary Effluent	8/4/2006	0/4
Mixed Liquor	8/25/2006	0/4
Secondary Effluent	8/4/2006	0/4
Concentrated Filtered Water (2000 mL)	9/26/2006	0/4
Concentrated River Water (500 mL)	8/25/2006	0/4
Concentrated Secondary Effluent (500 mL)	10/12/2006	0/4

The screening tests were designed to determine if any background interference from pathogenic material existed. No interferences were found in the drinking water samples. Assays on Primary Effluent, Mixed Liquor and Concentrated Secondary Effluent yielded a confirmation of the presence of *Brucella* spp. (Table 4.2). All four capillary tubes yielded a positive result for the Primary Effluent sample. The Mixed Liquor sample gave a confirmation of two hits. Tests of Concentrated Secondary Effluent also yielded a positive result, with three out of four capillary tubes having the target DNA present. To confirm that the positive results reported were not the result of the DNA contamination, DNA extractions of Millique water and Primary Effluent were compared using the BioThreat Screening Kit, which contains the *Brucella* spp. screening test. The results confirmed that there was no contaminant present in the deionized water sample (no hits out of two samples), but present in the primary effluent (two hits out of two samples).

Table 4.2. PCR Results of Screening Tests for *Brucella* spp.

Water Matrix	Date Tested	PCR Results
Distilled Water	6/27/2006	0/4
Finished Water	6/29/2006	0/4
River Water	8/10/2006	0/4
Primary Effluent	8/14/2006	**4/4**
Mixed Liquor	8/14/2006	**2/4**
Secondary Effluent	8/10/2006	0/4
Concentrated Filtered Water (2000 mL)	9/29/2006	0/4
Concentrated River Water (500 mL)	9/29/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	**3/4**
Comparison of Distilled Water and Primary Effluent	8/15/2006	DI: 0/2 PE: **2/2**

Brucella spp. are Gram-negative bacteria typically found in livestock and responsible for causing undulant fever in humans. The bacterium was weaponized by the United States in the 1940s, but these stockpiles have since been destroyed. It has also been classified as a Select Agent by the Centers of Disease Control and Prevention due to its potential to pose a severe threat to public health and safety. Because of this classification, the likelihood of this bacterium being present in wastewater systems without prior warning or knowledge is highly improbable. There were no incidents of *Brucella* contamination reported to the local health department. An explanation for this supposed presence is that the PCR assay may be cross-reacting with other bacteria present in the three wastewater matrices. The manufacturer of the assay kit makes note that the *Brucella* assay may also detect *Ochrobactrum anthropi*. It has been documented that this pathogenic

bacterium has been isolated from activated sewage sludges (Leal-Klevezas et al. 2005). *Ochrobactrum anthropi* is also phylogenetically related to bacteria in the *Brucella* genus.

The concentration method was shown to improve the detection of bacteria. The screening results for *Brucella* were negative for the unconcentrated Secondary Effluent sample. By concentrating the sample 250-fold, the bacterium that cross-reacts with the *Brucella* primers was able to be detected by the PCR. There was no confirmation of *Brucella* spp. and/or *Ochrobactrum anthropi* in the unconcentrated sample. In comparing these two instances, the concentration of the secondary effluent allowed for the improved detection of this pathogenic bacteria.

It was observed that the number of positive results decreased for the wastewater samples. The primary effluent yielded four positive results, while the mixed liquor had two confirmations, and none was present in the secondary effluent. An explanation for this phenomenon is that concentration of *Ochrobactrum anthropi* decreases through each stage of wastewater treatment based on the PCR results of the screening tests. While the screening assays were not used to quantitatively analyze the samples, the sensitivity of the PCR is dependent of the concentration of target DNA, and therefore the bacterial concentration of the sample. This reduction in bacterial concentration could be due to protozoa grazing and the target bacteria settling in the sludge of each stage, both resulting in the removal of bacteria from the sample streams. The three positive results of the concentrated secondary effluent screening assays indicated that the bacteria was not completely removed during treatment but were reduced to levels that could not be detected by the PCR in the initial screening tests.

During the initial screening of pathogens, many samples had positive results for pathogens that should not be present in the sample, and a number of negative controls returned a

positive result. This trend was most apparent for the Millique water samples, where the water is passed through a number of filters to ensure that it is distilled and deionized. Data logs were sent to IdahoTech to be analyzed to determine the problem; and it was found that there was a large amount of contaminant DNA present within the system. According to IdahoTech, this contamination led to the false positives during data analysis. Contamination could have been due to improper disposal procedures for the DNA samples once amplified and the thermocycler carousal being exposed to amplicon due to broken capillaries during placement and removal. Because DNA can persist for years without degrading, decontamination steps were taken per IdahoTech's instructions using DNAZap (Ambion, Inc.), two acidic solutions that are able to degrade nucleic acids. This decontamination process, along with changing the location of the three stations for the DNA extraction, sample preparation, and sample amplification, helped to ensure that further samples would not become contaminated. The hood specifically designed for PCR sample preparation was also purchased to ensure a clean workspace to reconstitute the reagents. The PCR hood is also equipped with UV light, which is able to dimerize nucleotide bases, thus making the DNA unable to replicate during the PCR process.

4.2.2 Phase II – Detection Limits of *Salmonella* in River Water and Wastewater Matrices

Four samples, river water and three wastewater components, were spiked with *Salmonella typhimurium*, and analyzed using the culture and PCR technique. The results, presented in Table 4.3, compared the detection limits of each procedure. The detection limits of the culture technique for each water sample were determined by plate counts done for each of the dilutions. The PCR detection limits were determined by analyzing the DNA of each of the dilutions with the *Salmonella* spp. assay kit. The lowest dilution at which there was a positive result was

compared to the same dilution in the plate counts. From the results of the plating, the bacterial concentration of the dilution could be calculated.

Table 4.3. *Salmonella typhimurium* detection limits comparison of using culturing and PCR

		Salmonella Detection Limits (cfu/mL)	
Water Sample	**Date**	**Culture**	**PCR**
River Water	8/24/2006	100	1.42×10^5
River Water	9/13/2006	45	5.5×10^5
Primary Effluent	10/6/2006	45	2.10×10^4
Mixed Liquor	10/20/2006	1200*	4.00×10^3
Secondary Effluent	9/22/2006	40	2.20×10^5

* Plating of the mixed liquor samples led to severe overgrowth. This was smallest number of colonies counted where counts were possible.

By comparing the results of culturing and PCR, this table demonstrates that the traditional method of culturing *Salmonella* is far more sensitive than the PCR technique. Typical detection limits ranged from 10^3 to 10^5 CFU/mL for the PCR, while plating allowed bacteria to be detected at much lower levels, ranging from 40 to 1200 cfu/mL. Both methods allow for detection of *Salmonella* at levels lower than its median infective dose of 10^4 to 10^5 organisms, with the culture method being much more sensitive with respect to screening pathogens for public health purposes. In the event of intentional contamination and threats of such contamination, a 24-hour incubation time may be too long to determine a pathogen's presence when time is of the essence. Less than two hours are required to complete the DNA extraction and analysis for the R.A.P.I.D.® PCR. This timeframe will be much more imperative if more virulent species are under consideration.

One potential drawback with using PCR to detect virulent species is that the detection limits of the device may be well above the median infection dose. Some pathogens, such as anthrax spores, have very low infectious doses, and their detection by PCR may not be suitable

for public health needs. In this case, the culture technique may be more desirable in the detection of these species.

Typical culturing of *Salmonella* requires the use of selective enrichment or plating media that allows for the isolation of colonies in samples of mixed microbial flora. To use nutrient broth agar plating media, the wastewater samples were autoclaved to kill off bacteria present, so that the presence of their colonies would not skew the results of the plate counts. The autoclave time was reduced to 5 minutes, from a standard runtime of 20 minutes, to prevent scaling on the glassware. There were no problems with the plate counts for the majority of the samples, but problems did arise with the plating of the straight samples from the serial dilutions of the mixed liquor sample. There was significant growth on these plates due to the direct plating of the flocs present in mixed liquor. Because of the large quantities of bacteria present in mixed liquor, especially in the flocs, the autoclave time was not long enough to inactivate the bacteria. The colonies on these plates also had a different appearance than those of *Salmonella typhimurium*. The higher dilution plates did not have this issue, possibly because the mixed liquor bacteria remained in the flocs while the *Salmonella* remained in the free liquid.

The detection limits of *Salmonella* in river water and the secondary effluent were similar in order of magnitude, with an average detection limit of 1.40×10^5 cfu/mL in river water and 2.20×10^5 cfu/mL in chlorinated secondary effluent. There was a decline in the detection limits of the primary effluent and mixed liquor samples, by one order and two orders of magnitude, respectively. This may be due to interferences in these two samples that may not be as prevalent in the other two samples. Humic and fulvic acids can have a two-prong effect on the effectiveness of the PCR. These substances have been shown to inhibit polymerase activity at high concentrations (Tsai and Olson 1992). Humic acids can also interfere with the fluorescent

signals of the thermocycler as these compounds are spectrally active and will become excited at the wavelengths used for detection (Zipper et al. 2003).

Humic and fulvic acids result from the decomposition of organic matter. They occur in natural waters and, due to the nature of wastewater treatment, in the effluents and mixed liquor. The secondary treatment process is where the majority of the decomposition of organic matter occurs. Because of this phenomenon, humic and fulvic acids would be a significant component of the slurry. The detection limit of *Salmonella* in this matrix was 4.00×10^3 CFU/mL, about a 1000-fold difference from the concentrations from river water and secondary effluent assays (Table 4.4). The results of primary effluent tests had an order of magnitude difference, 2.10×10^4 CFU/mL. Both samples show that there is a substantial amount of humic and fulvic acids in each; enough to affect the readings of the R.A.P.I.D.® PCR. The results show that the mixed liquor sample would have the highest concentration of these substances, followed by the primary effluent. While humic and fulvic acids would be present in both streams, these decomposition products would be higher in the mixed liquor, where biological processes are the primary means of waste removal, as opposed to primary treatment, which depends on physical phenomena.

Table 4.4. Detection limits of PCR for unconcentrated samples

	Salmonella Detection Limits (cfu/mL)
Water Sample	**PCR**
River Water	1.42×10^5
River Water	5.5×10^5
Primary Effluent	2.10×10^4
Mixed Liquor	4.00×10^3
Secondary Effluent	2.20×10^5

The second step of the DNA extraction process, the ethanol wash step, is used to remove the humic and fulvic acid interferences. A volume of 550 µL is added to the spin filter and

passed through via centrifugation. This quantity may be suitable to remove small quantities of humic and fulvic acids from an assay, but because of the nature of many environmental samples, a larger volume may be needed to get more accurate results via PCR.

4.2.3 Phase III – Detection Limits of *Salmonella* in River Water and Secondary Effluent using a Concentration Method

Two samples, river water and secondary effluent, were concentrated through a 0.45-μm filter to determine improvements in *Salmonella* recovery and detection in both culture and PCR analyses. Due to the high turbidities of the samples, only 500 mL of river water and 250 mL of secondary effluent were able to be filtered. Filtered products for both samples were reconstituted to 2 mL. This resulted in a 250- and 125-fold volume reduction and 2.40 and 2.10 theoretical log reduction, respectively.

To determine the effectiveness of the concentration method, the log difference was calculated for each dilution by comparing the unconcentrated and concentrated plate counts. The average log recovery for the river water sample was 2.11 log units, while the *Salmonella* recovery in the secondary effluent was 1.41 log units. Both recoveries were below the theoretical recovery of the concentration method. This phenomenon may result from bacteria adhering to glassware and the filtration device. Also, all the cells trapped on the membrane filter may not have been removed during the elution process.

Table 4.5. Evaluation of the Concentration Method Using PCR and Culture

		Unconcentrated Sample *Salmonella* Detection Limits (cfu/mL)		Concentrated Sample *Salmonella* Detection Limits (cfu/mL)		Improvement in detection following concentration (log units)	
Water Sample	**Date**	**Culture**	**PCR**	**Culture**	**PCR**	**Culture**	**PCR**
River Water (250x)	12/9/2006	60	2.70×10^5	0.60	80	2.00	3.53
Secondary Effluent (125x)	12/20/2006	20	3.50×10^3	0.63	20	1.50	2.24

The detection limits of the concentration process were determined by finding the concentrated sample dilution that was detectable by both culturing and PCR. These results were then compared to the plate count results of the unconcentrated sample to determine at what *Salmonella* concentration in the original sample could be detected by concentration (Table 4.5). After concentration, the PCR detection limits of the river water and secondary effluent were within the same order of magnitude, 80 and 20 cfu/mL, respectively. These findings were compared to detection limits of the unconcentrated samples, which were found to be 2.70×10^5 cfu/mL for river water and 3.50×10^3 cfu/mL for the wastewater matrix. The disparity in the detection limits in the two unconcentrated samples can possibly be attributed to interferences (primarily humic and fulvic acids) that may be in higher concentrations in the secondary effluent. These would lead to positive PCR readings at lower dilutions, thus allowing for a smaller number of organisms to be detected by the PCR fluorescence.

The improvement in *Salmonella* detection was calculated by determining how many log units recovery occured with the concentration method. In comparing the results of culturing the concentrate to the average log recovery observed from plating, the recovery of cells was not aided or abetted significantly. There was very little difference between the log units of the

average cell recovery and those numbers calculated for the concentration method, and both were well below the theoretical cell recovery in log units.

The concentration method displayed a marked improvement in *Salmonella* detection by PCR. The log improvement for both samples was greater than the theoretical log recovery via concentration. The improvement in detection for river water following concentration was 3.53 log units, over a 1-log improvement than the theoretical. The improvement in recovery for the secondary effluent was only 0.14 log units greater than the theoretical, but the difference between the two samples can be attributed to the lower unconcentrated PCR detection limit of the secondary effluent. One possible explanation for greater log-unit recovery may be that the membrane filter collected both live and dead cells. This phenomenon would not affect the cultured concentrates, because this technique would only take into account viable cells. Because DNA does not degrade readily in the environment, the dead cells concentrated in the sample would also contribute to the PCR results. This would account for a portion of the improvement of recovery in the concentrated sample.

The concentration method for both samples has the ability to detect *Salmonella* at levels of public health significance. The literature suggests that the median infective dose can be a little 15-20 cells and as many as 10^5 organisms (Burrows and Renner 1999). These numbers apply to the ingestion of the microorganisms, but pathogens may be more immediately incapacitating when inhaled. This property would be more pertinent in a wastewater treatment plant, where pathogens can be easily aerosolized, but the likelihood of ingestion is highly improbable. Select agents, such as *B. anthracis* and *Y. pestis*, can be and have been aerosolized for weapons use in the past. The detection limits of these bacteria could not be researched due to safety concerns and legal restrictions. The size of the *Salmonella typhimurium* bacterium (0.5 x

2.5 μm) is comparable to that of *B. anthracis* spores (0.8 x 1.5-1.8 μm) and *Y. pestis* organisms (0.5-1.0 x 1-2 μm). The membrane filter used for concentration has 0.45 μm pores, making it able to trap these pathogens as well. Due to the virulence of these species, concentration at the volumes tested may not be sufficient enough to bring detection to within public health standards. With increased volumes though, the concentration method in tandem with PCR should improve detection in the event of intentional or accidental contamination.

5.0 SUMMARY AND CONCLUSIONS

Prioritization of contaminants can prove to be a useful tool for wastewater treatment authorities. It will provide these utilities with a basis of which contaminants would pose the greatest threat to treatment plants and their collection systems. Screening methods can be then developed and implemented to rapidly detect these threats to plant security and safety in the event of a contamination incident. The prioritization can also aid in the development of contingency plans for treatment and remediation, if necessary. The information in data sheets may also provide guidance in ways to decontaminate affected systems.

Real-time rapid cycle PCR is one such method that can be used by wastewater utilities to effectively and efficiently screen for pathogens that may be introduced into a treatment system. While selective culturing may be the most accurate in determining the presence of pathogens, PCR analysis in tandem with the concentration method may aid in screening for bacteria at levels of public health significance. This technique may be particularly useful for the detection of Select Agents, such as *Bacillus anthracis* (one of the highest-ranking contaminants according to the prioritization), which require special laboratory biosafety requirements for culturing. Screening for *Brucella* spp. in wastewater may still require culturing for confirmation due to bacteria ubiquitous in treatment processes, whose DNA cross-reacts with primers of *Brucella* spp. during the PCR process. The further development of primers that prevent this cross-reaction will be necessary for PCR's use in the of *Brucella* spp. in the future, but is outside the scope of

this paper. The concentration method was applied to *Salmonella typhimurium*, but it is anticipated to be applicable for other types of bacteria and protozoa, given that the cells are larger than the filter pore size used. The PCR detection limits of the concentrated sample exceeded that expected due to the reduction in volume. This method can also be easily used onsite due to the apparatus's light weight and portability, making it a viable option in field operations. If necessary, larger volumes could be concentrated to improve pathogen detection, but may require more time for filtration. In situations where intentional contamination may be suspected, the concentration method along with real-time rapid cycle PCR will provide essential preliminary screening that will aid in protecting public health, wastewater treatment plant processes and infrastructure, and wildlife that may inhabit the surrounding area and live in the receiving waters.

Acts of terrorism and natural disasters can be equally destructive to critical infrastructure (e.g., water treatment and distribution systems and wastewater collection and treatment systems). The prioritization of contaminants will provide emergency response teams with the tools necessary to determine which contaminants would be a priority for screening purposes. The framework may also provide emergency responders with data to mitigate the effects of contaminants on wastewater treatment and collection systems. The use of real-time rapid cycle PCR will allow emergency responders to quickly identify if and what type of biological agents are present, so that the proper remediation efforts are enacted. These efforts will bolster the protection of vital infrastructure and help prevent irreversible damage to wastewater treatment processes and structures and ensure the health of the surrounding community.

APPENDIX A

CONTAMINANTS EVALUATED USING THE PRIORITIZATION FRAMEWORK

The following list of contaminants was evaluated using the prioritization framework developed for wastewater. The contaminants were divided into rounds for ease of evaluation.

Round 1
- Anthrax (*Bacillus anthracis*)
- Cesium-137
- Cyanide Salts
- Ethylene Glycol
- Gasoline
- Malathion
- Paraquat

Round 2
- Ammonia
- Arsenic
- Botulinum Toxin
- Hexane
- Kerosene
- Phenol
- Strontium-90

Round 3
- Acetone
- Aldicarb
- *Brucella suis*
- Chlorine Dioxide
- Cobalt-60

Round 3 (continued)
- Mercury
- Sodium Hypochlorite
- Staphylococcal Enterotoxin B

Round 4
- Benzene
- Ethylbenzene
- Fuel Oils
- m-Xylene
- o-Xylene
- p-Xylene
- Pentane
- Toluene

Round 5
- Americium-241
- Calcium Hypochlorite
- Cyclohexanol
- DDT
- Glycerine
- Lead
- Nitrobenzene
- Paraformaldehyde

Round 6 (Class 1A Flammables)

- 1,1-Dichloroethylene
- Ethylamine
- Ethyl Chloride
- Ethyl Ether
- Isopentane
- Isopropyl Chloride
- Methyl Formate
- Propylene Oxide

Round 7 (Class 1B Flammables)

- 1,2-Dichloroethylene
- Carbon Disulfide
- Ethyl Acetate
- Ethyl Alcohol
- Isoamyl Acetate
- Isoamyl Alcohol
- Isobutyl Acetate
- Isopropyl Acetate
- Isopropyl Alcohol
- Methyl Acetate
- Methyl Alcohol
- Methyl Ethyl Ketone
- Methyl Propyl Ketone

Round 7 (continued)

- n-Butyl Acetate
- Octane
- Propyl Acetate
- sec-Butyl Acetate
- tert-Amyl Alcohol
- tert-Butyl Acetate
- VM&P Naphtha

Round 8 (VA Tech Process Upset)

- 1-Chloro-2,4-Dinitrobenzene
- 1-Octanol
- 2,4-Dinitrophenol
- Cadmium
- Cyclohexanone
- Dichloromethane

Round 9

- Isophorone
- Plutonium-238
- Styrene
- Sulfuric Acid
- Turpentine
- VX

APPENDIX B

PRIORITIZATION ANALYSIS OF SELECTED CONTAMINANTS

The following tables provide an example of the application of the prioritization framework. The contaminants chosen are the top two most hazardous for each endpoint according to the prioritization. Each table has comments specific to the contaminant for each criterion with respect to the endpoint in question. The criterion score is based on these comments in cojunction with the prioritization framework.

Table B.1. Application of the Prioritization Framework to *C. botulinum* with respect to the Worker/Public Health Endpoint

Criterion	Worker/Public Health		
	Weighting Factor	Score	Comments
Availability	0.18	2	[BT01] *Clostridium Botulinum* is a spore forming obligat whose natural habitat is the soil and can be easily isolated. Iraq has admitted to producing 19,000 L of botulinum toxin. This amount constitutes more than three times the amount needed to kill the current human population.
Potency	0.16	5	[BT02] Lethal dose for a 70 kg person is estimated to be between 0.7 and 0.9 µg/kg for inhalation.
Persistence	0.04	4	[BT02] Spores may survive boiling for up to 4 hours. Botulinum toxin is inactivated in fresh water within 3 to 6 days.
Introduction/Dispersion	0.04	5	Easily introduced and dispersed into the wastewater collection and treatment system. No special equipment is required to introduce or disperse the contaminant.
Process Removal	0.02	3	[BT02] Spores are readily killed by chlorine (either as chlorinated water or as hypochlorite). Spores are resistant to ultraviolet light. [BT03] A reverse osmosis field study reported >99.988% removal of botulinum toxin from raw water spiked with the toxin. Treatment systems using charcoal should effectively remove the toxin. Botulinum toxins are inactivated by 0.5% hypochlorite for 10-15 minutes. Botulinum toxins are > 99.7% inactivated by 3 mg/L free available chlorine in 20 minutes, and are 84% inactivated by 0.4 mg/L free available chlorine in 20 minutes.
Storability	0.04	5	[BT02] Spores are resistant to dessication and can survive for years in a dry state.
Outcomes	0.28	5	[BT02] The fatality rate for food borne botulism is 5-10% and 15-44% for wound botulism. It is assumed that similar rates would exist for inhalation of botulinum toxin.
Public Perception	0.24	5	Exotic substance linked to terrorist activities.
Composite Score		**4.39**	

Comments:

[BT01] http://jama.ama-assn.org/cgi/content/full/285/8/1059

[BT02] http://www.idsociety.org/Template.cfm?Section=Botulism1&CONTENTID=11058&TEMPLATE=/ContentManagement/ContentDisplay.cfm

[BT03] WCIT Profile for Botulinum toxin

Table B.2. Application of the Prioritization Framework to VX with respect to the Worker/Public Health Endpoint

Criterion	Worker/Public Health		
	Weighting Factor	Score	Comments
Availability	0.18	2	VX01VX is a man-made chemical warfare agent classified as a nerve agent. It is an odorless, tasteless, boily liquid that is amber in color. Nerve agents are the most toxic and rapidly acting of known chemical warefare agents. They are similar to pesticides called organophosphates in how they work and what kinds of harmful effects are caused. It was originally developed in the United Kingdom in the early 1950s and is not found naturally in the environment. VX02It is stated that VX is produced in a manner very similar to that of modern phosphorous-based pesticides today, and therefore is extremely easy to manufacture. Anyone creating modern pesticides could be secretly producing VX liquid. VX03They are known to be stored by several nations, including the U.S. VX09The U.S. stopped production and shipment of the chemical agent
Potency	0.16	5	VX03The airborne exposure limit (as recommended by the Surgeon General's Working Group, U.S. Department of Health and Human Services) is 0.003 µg/m^3 TWA for the workplace. VX04The TLV (U.S. military) and VX05airborne exposure limit TWA ("DA Pam 40-8, Occupational Health Guidelines for the Evaluation and Control of Occupational Exposure to Nerve Agents GA, GB, GD, and VX") are both 0.00001 mg/m^3. VX06The estimated inhalational LCt_{50} for humans has been stated as 10 and VX0730-50 mg min/m^3. VX06As little as one drop of VX on the skin can be fatal.
Persistence	0.04	5	VX08If released into water, VX is expected to adsorb to suspended solids and sediment. Based on its pKa, VX will exist in the protonated state in the environment and cations generally adsorb to organic carbon more readily. Volatilization from water surfaces is not expected to be an important fate process. It will decompose slowly by hydrolysis, particularly at low temperatures. Recent experiments at 21°C, gave a pH independent hydrolytic half-life of 57 days. Earlier results had indicated that the hydrolysis was base-catalyzed. VX04The hydrolysis products of VX include EA2192, which is nearly as toxic as VX and is hydrolysed over 1,000 times more slowly. Its solubility in water is 30 g/L at 25°C.
Introduction/Dispersion	0.04	2	VX12For emergency situations, wear a "moon suit" consisting of a positive pressure, pressure-demand, full-facepiece self-contained breathing apparatus (SCBA) or a pressure-demand supplied air respirator SCBA with escape cylinder in combination with a fully-encapuslating, chemical resistant suit capable of maintaining a positive air pressure within the suit. VX07Masks, including self-contained breathing apparatus masks, alone do not previde adequate protection against VX.
Process Removal	0.02	5	VX09The Chemical Weapons Convention requires destruction of chemical weapons by 2007. The Army is proposing to pretreat stockpiles of VX nerve agent at its Newport Chemical Agent Disposal Facility in Newport, Indiana, and then transport the resulting hydrolysate across the country to the DuPont Chambers Works facility at Carney's Point, Salem County, NJ for final treatment and disposal into the Delaware River. VX10This DuPont facility is the largest commercial wastewater treatment facility in North America. There, DuPont proposes to treat the wastewater from the neutralization of the VX nerve agent with a new patented wastewater treatment technology using powdered activated carbon. VX11Limited pilot-scale testing has demonstrated the ability of supercritical water oxidation to achieve high destruction efficiencies for the organic constituents of VX hydrolysate. VX04Oxidation using common bleach and superchlorinated bleach is also said to decontaminate. VX04The estimated log Kow is 2.06.
Storability	0.04	5	VX12VX should be stored in tightly closed containers in a cool, well-ventilated area away from heat. VX04It is relatively stable at room temperature. Unstabilized VX of 95% purity decomposes at a rate of 5% per month at 71°C. At pH 12, the toxic by-product has a half-life of about 14 days; in 90 days there is about a 64-fold reduction.
Outcomes	0.28	5	VX04VX is on the Superfund Extremely Dangerous Substances List. It is a lethal cholinesterase inhibitor in liquid or vapor form. There is only a slight difference between a mild and fatal doses. Symptoms from inhalation may occur within minutes or hours. Skin and eye exposure cause a very rapid onset of symptoms. Death usually occurs within 15 minutes after absorption of a fatal dose. VX03In general, the manifestation of toxic effects is fastest via inhalation or ingestion. Manifestation of nerve agent exposure includes runny nose, chest tightness, pinpoint pupils, shortness of breath, excessive salivation and sweating, nausea, vomiting, abdominal cramps, involuntary defecation and urination, muscle twitching, confusion, seizures, paralysis, coma, respiratory paralysis, and death. Incapacitating effects occur within 1 to 10 minutes and fatal effects within 4 to 18 hours. Fatigue, irritability, nervousness, and memory defects may persist for as long as 6 weeks. VX07Immediate treatment includes intravenous or intramuscular injection of 2 mg atropine sulfate. This should be followed by additional injections of atropine
Public Perception	0.24	5	VX07VX was synthesized and used by the Aum Shinrikyo organization in various attacks throughout Japan in the Nineties. It is an exotic substance linked to terrorist activities.
Composite Score		**4.33**	

Comments:

VX01 http://www.bt.cdc.gov/agent/vx/basics/facts.asp
VX02 http://www.milnet.com/chembio.htm
VX03 http://www.atsdr.cdc.gov/tfactsd4.html
VX04 http://www.bt.cdc.gov/agent/vx/erc50782-69-9.asp
VX05 http://www.ilpi.com/msds/vx.html
VX06 http://www.atsdr.cdc.gov/MHMI/mmg166.html
VX07 http://www.cbwinfo.com/Chemical/Nerve/VX.shtml
VX08 http://toxnet.nlm.nih.gov/
VX09 http://www.delawareriverkeeper.org/factsheets/vx_nerve_agent.htm
VX10 http://www.keepmedia.com/pubs/EnvironmentNewsService/2005/05/23/868464?extID=10026
VX11 http://www.nap.edu/books/0309060435/html/
VX12 Ed. Pohanish, Richard P. Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens, Fourth Edition. 2002.
VX13 http://www.state.nj.us/health/eoh/rtkweb/2673.pdf
VX14 WCIT Profile for VX

Table B.3. Application of the Prioritization Framework to Malathion with respect to the Process Upset Endpoint

Criterion	Process Upset		
	Weighting Factor	Score	Comments
Availability	0.24	5	[MA01]Malathion is a widely used organophosphate insecticide.
Potency	0.11	5	[MA05]An IC_0 of 10 mg/L can inhibit nitrification and denitrifcation (rotating disc and activated sludge).
Persistence	0.10	4	[MA03]Malathion's rapid degradation in soils with high microbial activity and many water bodies results in reduced environmental exposures relative to other organophosphate insecticides. [MA04]Hydrolysis half-life ranges from ~1.5 days at pH 8 and up to 21 weeks at pH 6. In raw river water, 90% of malathion was biologically degraded within two weeks. [MA02]No solubility in water.
Introduction/Dispersion	0.11	5	Easily introduced and dispersed into the wastewater collection and treatment system. No special equipment is required to introduce or disperse the contaminant.
Process Removal	0.09	2	[MA05]The aerobic biodegradation half-time was 0.05 days with an initial concentration of 2 mg/L using a mixture of nonacclimated sludge, field soil, and river sediment as inoculum. Activated carbon and calcium hypochlorite are also effective for removal with 90% and 100% removal, respectively.
Storability	0.07	5	[MA02]Keep in a well-ventilated room. Malathion reacts violently with strong oxidants and can attack iron, some plastics and rubber.
Outcomes	0.26	4	Malathion may disrupt some tertiary treatment processes. Remediation may be necessary.
Public Perception	0.02	2	Malathion is a common substance not linked to terrorist activities.
Composite Score		**4.31**	

Comments:

[MA01] http://www.cdc.gov/niosh/pel88/121-75.html
[MA02] http://www.hazard.com/msds/mf/cards/file/0172.html
[MA03] www.epa.gov/pesticides/op/malathion/efedresp.pdf
[MA04] http://www.speclab.com/compound/c121755.htm
[MA05] http://www.knovel.com

Table B.4. Application of the Prioritization Framework to Mercury with respect to the Process Upset Endpoint

Criterion	Process Upset		
	Weighting Factor	Score	Comments
Availability	0.24	5	[HG01]Mercury is a naturally occuring metal found in various forms. [HG05]It is a silvery, mobile, odorless liquid, that is [HG06]the byproduct of cinnabar ore mining and gold extraction projects. Elemental metallic mercury is used to produce chlorine gas and caustic soda, and is also used in thermometers, dental fillings, and batteries. Methyl mercury is mainly produced by bacteria in soil and water and [HG08]has no practical use. [HG08]Most products containing inorganic mercury compounds have been banned.
Potency	0.11	3	Unknown.
Persistence	0.10	5	[HG01]Methyl mercury may be formed in water and soil by bacteria. Increased amounts of elemental mercury in the environment will increase the amount of methyl mercury produced. Methyl mercury is considered much more toxic than inorganic forms of mercury. [ME02]Mercury is not soluble in water. Its vapor pressure is 0.26 Pa at 20°C.
Introduction/Dispersion	0.11	5	Easily introduced and dispersed into the wastewater collection and treatment system. No special equipment is required to introduce or disperse the contaminant. [HG07]The EPA requires the reporting of mercury spills or releases of 1 pound or more.
Process Removal	0.09	2	[HG03]Mercury can be precipitated to low levels using carbonate, phosphate, or sulfide. Metallic mercury is soluble in water at 25 μg/L which is above the regulatory limit. Mercury can be removed with ion exchange. Granulated carbon is often used to polish treated solutions with varying success. [HG04]Average % removal by: Primary Treatment - 10%, Activated sludge - 60%, Trickling filter - 50%, and Tertiary Treatment - 67%.
Storability	0.07	5	[HG05]Mercury must be stored to avoid contact with chloride dioxide, nitric acid, nitrates, ethylene oxide, chlorine and methylazide to prevent violent reactions. It should be stored in a cool, well-ventilated area.
Outcomes	0.26	4	There has been no documented incidents of process upset, but mercury does react violently chlorine and chlorine dioxide, two common disinfectants. The effects are expected to be manageable. There could be possible alteration with the treatment process.
Public Perception	0.02	3	Mercury is a common substance not linked to terrorist activities, however there is public awareness regarding its presence in drinking waters and bioaccumulation in fish.
Composite Score		**4.22**	

Comments:

[ME01] http://www.atsdr.cdc.gov/tfacts46.html
[ME02] http://www.cdc.gov/niosh/ipcsneng/neng0056.html
[ME03] http://www.rwaterguy.com/removal_of_mercury_from_wastewat.htm
[ME04] U.S. EPA's *Guidance Manual on the Development and Implementation of Local Discharger Limitations Under the Pretreatment Program* , December1987, 3-58

Table B.5. Application of the Prioritization Framework to VX with respect to the Physical Damage and Destruction Endpoint

Criterion	Physical Damage/Destruction		
	Weighting Factor	Score	Comments
Availability	0.16	2	[VX01]VX is a man-made chemical warfare agent classified as a nerve agent. It is an odorless, tasteless, boily liquid that is amber in color. Nerve agents are the most toxic and rapidly acting of known chemical warefare agents. They are similar to pesticides called organophosphates in how they work and what kinds of harmful effects are caused. It was originally developed in the United Kingdom in the early 1950s and is not found naturally in the environment. [VX02]It is stated that VX is produced in a manner very similar to that of modern phosphorous-based pesticides today, and therefore is extremely easy to manufacture. Anyone creating modern pesticides could be secretly producing VX liquid. [VX03]They are known to be stored by several nations, including the U.S. [VX09]The U.S. stopped production and shipment of the chemical agent in the late 1960s.
Potency	0.06	3	[VX05]VX has a flash point of 159°C. [VX12]The boiling point is 298°C (decomposition). Explosive limits have not been documented.
Persistence	0.05	5	[VX08]If released into water, VX is expected to adsorb to suspended solids and sediment. Based on its pKa, VX will exist in the protonated state in the environment and cations generally adsorb to organic carbon more readily. Volatilization from water surfaces is not expected to be an important fate process. It will decompose slowly by hydrolysis, particularly at low temperatures. Recent experiments at 21°C, gave a pH independent hydrolytic half-life of 57 days. Earlier results had indicated that the hydrolysis was base-catalyzed. [VX04]The hydrolysis products of VX include EA2192, which is nearly as toxic as VX and is hydrolysed over 1,000 times more slowly. Its solubility in water
Introduction/Dispersion	0.06	2	[VX12]For emergency situations, wear a "moon suit" consisting of a positive pressure, pressure-demand, full-facepiece self-contained breathing apparatus (SCBA) or a pressure-demand supplied air respirator SCBA with escape cylinder in combination with a fully-encapuslating, chemical resistant suit capable of maintaining a positive air pressure within the suit. [VX07]Masks, including self-contained breathing apparatus masks, alone do not previde adequate protection against VX.
Process Removal	0.04	5	[VX09]The Chemical Weapons Convention requires destruction of chemical weapons by 2007. The Army is proposing to pretreat stockpiles of VX nerve agent at its Newport Chemical Agent Disposal Facility in Newport, Indiana, and then transport the resulting hydrolysate across the country to the DuPont Chambers Works facility at Carney's Point, Salem County, NJ for final treatment and disposal into the Delaware River. [VX10]This DuPont facility is the largest commercial wastewater treatment facility in North America. There, DuPont proposes to treat the wastewater from the neutralization of the VX nerve agent with a new patented wastewater treatment technology using powdered activated carbon. [VX11]Limited pilot-scale testing has demonstrated the ability of supercritical water oxidation to achieve high destruction efficiencies for the organic constituents of VX hydrolysate. [VX04]Oxidation using common bleach and superchlorinated bleach is also said to decontaminate. [VX04]The estimated
Storability	0.02	5	[VX12]VX should be stored in tightly closed containers in a cool, well-ventilated area away from heat. [VX04]It is relatively stable at room temperature. Unstabilized VX of 95% purity decomposes at a rate of 5% per month at 71°C. At pH 12, the toxic by-product has a half-life of about 14 days; in 90 days there is about a 64-fold reduction.
Outcomes	0.32	5	[VX12]VX may burn, but it does not readily ignite. [VX12]Poisonous gases including oxides of nitrogen and sulfur are produced in fire. Vapors are heavier than air and will collect in low areas. This chemical should be kept out of a confined space, such as a sewer, because of the possibility of explosion. All ignition sources should be removed. [VX14]VX is considered an infrastructure threat because of its high toxicity combined with its ability to adhere to infrastructure surfaces and its ability to corrode metals.
Public Perception	0.29	5	[VX07]VX was synthesized and used by the Aum Shinrikyo organization in various attacks throughout Japan in the Nineties. It is an exotic substance linked to terrorist activities.
Composite Score		**4.23**	

Comments:

[VX01] http://www.bt.cdc.gov/agent/vx/basics/facts.asp
[VX02] http://www.milnet.com/chembio.htm
[VX03] http://www.atsdr.cdc.gov/tfactsd4.html
[VX04] http://www.bt.cdc.gov/agent/vx/erc50782-69-9.asp
[VX05] http://www.ilpi.com/msds/vx.html
[VX06] http://www.atsdr.cdc.gov/MHMI/mmg166.html
[VX07] http://www.cbwinfo.com/Chemical/Nerve/VX.shtml
[VX08] http://toxnet.nlm.nih.gov/
[VX09] http://www.delawareriverkeeper.org/factsheets/vx_nerve_agent.htm
[VX10] http://www.keepmedia.com/pubs/EnvironmentNewsService/2005/05/23/868464?extID=10026
[VX11] http://www.nap.edu/books/0309060435/html/
[VX12] Ed. Pohanish, Richard P. Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens, Fourth Edition. 2002.
[VX13] http://www.state.nj.us/health/eoh/rtkweb/2673.pdf
[VX14] WCIT Profile for VX

Table B.6. Application of the Prioritization Framework to *Bacillus anthracis* with respect to the Physical Damage and Destruction Endpoint

Criterion	Physical Damage/Destruction		
	Weighting Factor	Score	Comments
Availability	0.16	3	[AX01]Since Anthrax is an animal disease that may occur in many parts of the world, soil samples may make anthrax readily available at numerous locations worldwide. Additionally, approximately 1,500 international microbiological repositories sell anthrax cultures. *Bacillus anthracis* can be produced with common biological production equipment.
Potency	0.06	4	Specific remediation procedures for a wastewater collection and treatment system are not in the open literature. Over 80 million dollars was spent on the remediation of one post office to remediate the anthrax mail attacks in 2001.
Persistence	0.05	5	[AX01]Anthrax spores can remain viable for two years. The spores have been known to remain viable for decades in aqueous environments.
Introduction/Dispersion	0.06	5	Easily introduced and dispersed into the wastewater collection and treatment system. No equipment is required to introduce or disperse the contaminant.
Process Removal	0.04	3	Spores are resistant to chlorine. Sodium hypochlorite as a sporicide is applicable under an emergency exemption: The sporicidal effectiveness of hypochlorite solutions depends on the concentration of free available chlorine and pH. (1) Chemical Sterilization: Method: Free available chlorine. Concentration: 2.4 -2.3 mg/L available Cl_2, pH 7.2, 22°C. Inoculum size: 1.1 x 10^5 spore suspension of B. anthracis. Time: 1 hour. Efficiency: >99.99 % killed (1 spore/mL survived). (2) Chemical Sterilization: Method: Sodium hypochlorite (NaOCl). Concentration: 0.05 %, pH 7.0, 20°C. Inoculum Size: Spore suspension of B. subtilius globigii, representing 1.6 - 2.2 x 10^9 CFU/mL. Time: 30 minutes. Efficiency: 99.99 % killed. (3) Chemical Sterilization: Chlorine dioxide (ClO_2). Concentration: 6-7 mg/L, 20% -40% RH, 23°C. Inoculum sixe: 10^6 spores/biologic indicator.
Storability	0.02	5	[AX01]Anthrax spores can remain viable for years. The spores have been known to remain viable for decades in aqueous environments.
Outcomes	0.32	4	Serious effects expected. Treatment plant/ collection system is destroyed or inactivated. Remediation would be required to remove or inactivate the spores. Specific remediation procedures for a wastewater collection and treatment system are not in the open literature. Over 80 million dollars was spent on the remediation of one post office to remediate the anthrax mail attacks in 2001.
Public Perception	0.29	5	Exotic substance linked to terrorist activities. Much public fear exists regarding anthrax following the anthrax attacks on the USMail in 2001.
Composite Score		**4.22**	

Comments:

[AX01] Davis, J., Johnson-Wingar, A. *The Anthrax Terror DOD's Number-One Biological Threat* Aerospace Power Journal Winter 2000

[AX02] WaterISAC

[AX03] www.cdc.gov/ncidod/EID/vol5no4/cieslak.htm

[AX04] WCIT Profile for Anthrax

Table B.7. Application of the Prioritization Framework to VX with respect to the Pass Through Endpoint

Criterion	Pass Through		
	Weighting Factor	Score	Comments
Availability	0.03	2	[VX01]VX is a man-made chemical warfare agent classified as a nerve agent. It is an odorless, tasteless, boily liquid that is amber in color. Nerve agents are the most toxic and rapidly acting of known chemical warefare agents. They are similar to pesticides called organophosphates in how they work and what kinds of harmful effects are caused. It was originally developed in the United Kingdom in the early 1950s and is not found naturally in the environment. [VX02]It is stated that VX is produced in a manner very similar to that of modern phosphorous-based pesticides today, and therefore is extremely easy to manufacture. Anyone creating modern pesticides could be secretly producing VX liquid. [VX03]They are known to be stored by several nations, including the U.S. [VX09]The U.S. stopped production and shipment of the chemical agent in the late 1960s.
Potency	0.06	5	[VX09]According to an Ohio EPA study, VX at a level of 20 $\mu_{g/L}$ after 17.4 hours killed half of the striped bass exposed. In light of this study and numerous other concerns, an agency toxicologist 'strongly recommended' against discharge of treated VX hydrolysate into the local POTW and waterbody until there was 'more information about the possible toxic effects of the treated hydrolysate discharge on aquatic life'. [VX08]The estimated BCF is 8.
Persistence	0.16	5	[VX08]If released into water, VX is expected to adsorb to suspended solids and sediment. Based on its pKa, VX will exist in the protonated state in the environment and cations generally adsorb to organic carbon more readily. Volatilization from water surfaces is not expected to be an important fate process. It will decompose slowly by hydrolysis, particularly at low temperatures. Recent experiments at 21°C, gave a pH independent hydrolytic half-life of 57 days. Earlier results had indicated that the hydrolysis was base-catalyzed. [VX04]The hydrolysis products of VX include EA2192, which is nearly as toxic as VX and is hydrolysed over 1,000 times more slowly. Its solubility in water is 30 g/L at 25°C.
Introduction/Dispersion	0.03	2	[VX12]For emergency situations, wear a "moon suit" consisting of a positive pressure, pressure-demand, full-facepiece self-contained breathing apparatus (SCBA) or a pressure-demand supplied air respirator SCBA with escape cylinder in combination with a fully-encapuslating, chemical resistant suit capable of maintaining a positive air pressure within the suit. [VX07]Masks, including self-contained breathing apparatus masks, alone do not previde adequate protection against VX.
Process Removal	0.16	5	[VX09]The Chemical Weapons Convention requires destruction of chemical weapons by 2007. The Army is proposing to pretreat stockpiles of VX nerve agent at its Newport Chemical Agent Disposal Facility in Newport, Indiana, and then transport the resulting hydrolysate across the country to the DuPont Chambers Works facility at Carney's Point, Salem County, NJ for final treatment and disposal into the Delaware River. [VX10]This DuPont facility is the largest commercial wastewater treatment facility in North America. There, DuPont proposes to treat the wastewater from the neutralization of the VX nerve agent with a new patented wastewater treatment technology using powdered activated carbon. [VX11]Limited pilot-scale testing has demonstrated the ability of supercritical water oxidation to achieve high destruction efficiencies for the organic constituents of VX hydrolysate. [VX04]Oxidation using common bleach and superchlorinated bleach is also said to decontaminate. [VX04]The estimated log Kow is 2.06.
Storability	0.03	5	[VX12]VX should be stored in tightly closed containers in a cool, well-ventilated area away from heat. [VX04]It is relatively stable at room temperature. Unstabilized VX of 95% purity decomposes at a rate of 5% per month at 71°C. At pH 12, the toxic by-product has a half-life of about 14 days; in 90 days there is about a 64-fold reduction.
Outcomes	0.20	5	[VX08]It is suggested that the potential for bioconcentration in aquatic organisms is low. [VX12]If material or contaminated runoff enters waterways, notify downstream users of potentially contaminated waters. [VX14]VX is not expected to have a negative impact on vegetation. It is toxic to fish, birds, and other wildlife.
Public Perception	0.33	5	[VX07]VX was synthesized and used by the Aum Shinrikyo organization in various attacks throughout Japan in the Nineties. It is an exotic substance linked to terrorist activities.
Composite Score		**4.83**	

Comments:

[VX01] http://www.bt.cdc.gov/agent/vx/basics/facts.asp
[VX02] http://www.milnet.com/chembio.htm
[VX03] http://www.atsdr.cdc.gov/tfactsd4.html
[VX04] http://www.bt.cdc.gov/agent/vx/erc50782-69-9.asp
[VX05] http://www.ilpi.com/msds/vx.html
[VX06] http://www.atsdr.cdc.gov/MHMI/mmg166.html
[VX07] http://www.cbwinfo.com/Chemical/Nerve/VX.shtml
[VX08] http://toxnet.nlm.nih.gov/
[VX09] http://www.delawareriverkeeper.org/factsheets/vx_nerve_agent.htm
[VX10] http://www.keepmedia.com/pubs/EnvironmentNewsService/2005/05/23/868464?extID=10026
[VX11] http://www.nap.edu/books/0309060435/html/
[VX12] Ed. Pohanish, Richard P. Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens, Fourth Editi
[VX13] http://www.state.nj.us/health/eoh/rtkweb/2673.pdf
[VX14] WCIT Profile for VX

Table B.8. Application of the Prioritization Framework to Cyanide Salts with respect to the Pass Through Endpoint

Criterion	Pass Through		
	Weighting Factor	Score	Comments
Availability	0.03	3	[CY07]Not publicly available, [CY10]but quantities are readily available to contaminate wastewater systems. [CY08]Facilities in the U.S. producing sodium cyanide in 2005 include: Cyano Co. in Nevada and Du Pont Chemical Company in Tennessee. Cyanide substances also occur naturally in the fruits, seeds, roots and leaves of numerous plants, and cyanide is released to the environment from natural biogenic processes from higher plants, bacteria and fungi. Used as a pesticide and in mining, manufacturing and non-manufacturing industry.
Potency	0.06	5	[CY09]The adjusted oral LD_{50}'s for various mammals range from 14 mg/m^3 (13.2 mg/L) for rabbits to 56 mg/m^3 (52.8 mg/L) for mammals.
Persistence	0.16	4	[CY03]Cyanide salts in surface water will form hydrogen cyanide. The half life of hydrogen cyanide in the atmosphere is 1 to 3 years. Most cyanides in water will form hydrogen cyanide and evaporate. Some cyanide in the water will be transformed into less harmful chemicals by microorganisms or will form a complex with metals such as iron. [CY05]At pH values less than 9.2 most of the cyanide in water is present as hydrogen cyanide.
Introduction/Dispersion	0.03	5	Easily introduced and dispersed into the wastewater collection and treatment system. No special equipment is required to introduce or disperse the contaminant.
Process Removal	0.16	5	[CY03]Some of the cyanide salts will form hydrogen cyanide which will evaporate. [CY04]One of the major sources of cyanides in natural waters is from the discharges of industrial wastewater treatment facilities. [CY05]Generally cyanic compounds do not readily dissolve in water and can be removed by sedimentation or filtration. Free chlorination using hypochlorite will remove cyanide in water, if the pH is equal to or greater than 10; however if the pH is less than 10 the reaction may produce cyanogen chloride, which is sometimes considered even more toxic than molecular hydrogen cyanide. Aeration conducted at room temperature on water with a pH level above 9.2 may be effective for removing hydrogen cyanide and cyanogen chloride. If the pH level is below 9.2, the cyanide removal efficiency will increase compared to higher pH values. [CY10]Water can be treated to remove cyanide with chlorine combined with caustic, hypochlorite (for small quantities), or ozone.
Storability	0.03	5	Can be stored in a dry place for extended periods.
Outcomes	0.20	3	[CY04]Cyanide salts are toxic to fish, but will not build up in their bodies. Cyanide releases into surface waters will form hydrogen cyanide and evaporate.
Public Perception	0.33	5	Exotic substance linked to terrorist activities.
Composite Score		**4.38**	

Comments:

[CY01] http://www.cdc.gov/niosh/npg/npgd0562.html
[CY02] http://www.atsdr.cdc.gov/MHMI/mmg8.html
[CY03] http://www.atsdr.cdc.gov/tfacts8.html
[CY04] http://wwwatsdr.cdc.gov/toxprofiles/tp8-c1-b.pdf
[CY05] Whelton, A., Jensen, J., Richards, T., Val via, R., *The Cyanic Threat,* Civil Engineering pgs 50-54, and 84.
[CY06] Handbook of Toxic and Hazardous Chemicals and Carcinogens. Second Edition.
[CY07] http://www.epa.gov/opprd001/factsheets
[CY08] http://www.atsdr.cdc.gov/toxprofiles
[CY09] http://www.cdc.gov/niosh/idlh/cyanides.html
[CY10] WCIT Profile for Sodium Cyanide

APPENDIX C

SCREENING TEST (PHASE I) RESULTS

The following tables are the results of the screening tests for all the pathogens tested. The results are reported by the number of positive results, or hits, per number of capillaries screened.

Table C.1. *Bacillus anthracis* screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/29/2006	0/4
River Water	8/10/2006	0/4
Primary Effluent	8/14/2006	0/4
Mixed Liquor	8/14/2006	0/4
Secondary Effluent	8/10/2006	0/4
Concentrated Filtered Water (2000 mL)	9/29/2006	0/4
Concentrated River Water (500 mL)	9/29/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	0/4

Table C.2. *Brucella* spp. screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/29/2006	0/4
River Water	8/10/2006	0/4
Primary Effluent	8/14/2006	**4/4**
Mixed Liquor	8/14/2006	**2/4**
Secondary Effluent	8/10/2006	0/4
Concentrated Filtered Water (2000 mL)	9/29/2006	0/4
Concentrated River Water (500 mL)	9/29/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	**3/4**

Table C.3. *Yersinia pestis* screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/29/2006	0/4
River Water	8/10/2006	0/4
Primary Effluent	8/14/2006	0/4
Mixed Liquor	8/14/2006	0/4
Secondary Effluent	8/10/2006	0/4
Concentrated Filtered Water (2000 mL)	9/29/2006	0/4
Concentrated River Water (500 mL)	9/29/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	0/4

Table C.4. *F. tularensis* screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/29/2006	0/4
River Water	8/10/2006	0/4
Primary Effluent	8/14/2006	0/4
Mixed Liquor	8/14/2006	0/4
Secondary Effluent	8/10/2006	0/4
Concentrated Filtered Water (2000 mL)	9/29/2006	0/4
Concentrated River Water (500 mL)	9/29/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	0/4

Table C.5. *Salmonella* spp. screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/21/2006	0/4
River Water	6/23/2006	0/4
Primary Effluent	8/4/2006	0/4
Mixed Liquor	8/25/2006	0/4
Secondary Effluent	8/4/2006	0/4
Concentrated Filtered Water (2000 mL)	9/26/2006	0/4
Concentrated River Water (500 mL)	8/25/2006	0/4
Concentrated Secondary Effluent (500 mL)	10/12/2006	0/4

Table C.6. *E. coli* O157 screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/21/2006	0/4
River Water	6/23/2006	0/4
Primary Effluent	8/4/2006	0/4
Mixed Liquor	8/25/2006	0/4
Secondary Effluent	8/4/2006	0/4
Concentrated Filtered Water (2000 mL)	9/26/2006	0/4
Concentrated River Water (500 mL)	8/25/2006	0/4
Concentrated Secondary Effluent (500 mL)	10/12/2006	0/4

Table C.7. *L. monocytogenes* screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/21/2006	0/4
River Water	6/23/2006	0/4
Primary Effluent	8/4/2006	0/4
Mixed Liquor	8/25/2006	0/4
Secondary Effluent	8/4/2006	0/4
Concentrated Filtered Water (2000 mL)	9/26/2006	0/4
Concentrated River Water (500 mL)	8/25/2006	0/4
Concentrated Secondary Effluent (500 mL)	10/12/2006	0/4

Table C.8. *Campylobacter spp.* screening results

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/27/2006	0/4
Finished Water	6/21/2006	0/4
River Water	6/23/2006	0/4
Primary Effluent	8/4/2006	0/4
Mixed Liquor	8/25/2006	0/4
Secondary Effluent	8/4/2006	0/4
Concentrated Filtered Water (2000 mL)	9/26/2006	0/4
Concentrated River Water (500 mL)	8/25/2006	0/4
Concentrated Secondary Effluent (500 mL)	10/12/2006	0/4

Table C.9. *C. botulinum* Type A screening tests

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/30/2006	0/4
Finished Water	9/14/2006	0/4
River Water	9/14/2006	0/4
Primary Effluent	9/14/2006	0/4
Mixed Liquor	9/14/2006	0/4
Secondary Effluent	9/14/2006	0/4
Concentrated Filtered Water (2000 mL)	11/9/2006	0/4
Concentrated River Water (500 mL)	9/14/2006	0/4
Concentrated Secondary Effluent (500 mL)	11/9/2006	0/4

Table C.10. *Cryptosporidium* spp. screening tests

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/30/2006	0/4
Finished Water	6/30/2006	0/4
River Water	6/30/2006	0/4
Primary Effluent	9/14/2006	0/4
Mixed Liquor	9/14/2006	0/4
Secondary Effluent	9/14/2006	0/4
Concentrated Filtered Water (2000 mL)	12/12/2006	0/4
Concentrated River Water (500 mL)	9/14/2006	0/4
Concentrated Secondary Effluent (500 mL)	12/12/2006	0/4

Table C.11. *Variola* screening tests

Water Matrix	Date Tested	**PCR Results**
Distilled Water	6/30/2006	0/4
Finished Water	6/30/2006	0/4
River Water	6/30/2006	0/4
Primary Effluent	12/4/2006	0/4
Mixed Liquor	12/4/2006	0/4
Secondary Effluent	12/4/2006	0/4
Concentrated Filtered Water (2000 mL)	12/4/2006	0/4
Concentrated River Water (500 mL)	12/4/2006	0/4
Concentrated Secondary Effluent (500 mL)	12/4/2006	0/4

BIBLIOGRAPHY

Allgeier, Steve. Personal communication. 2005.

Bauers, Sandy. "Merck Faces Fish-Kill Probe." *Philadelphia Inquirer*, June 23, 2006. http://www.philly.com/mld/inquirer/14881232.htm.

Bej, Asim K., Meena H. Mahbubani, Martin J. Boyce, and Ronald M. Atlas. "Detection of *Salmonella* spp. in Oysters by PCR." *Applied and Environmental Microbiology* 60, no. 1 (January 1994): 368-373.

Bitton, Gabriel. *Wastewater Microbiology*. 3rd ed. Hoboken, NJ: John Wiley & Sons, Inc., 2005.

Burrows, W. Dickinson, and Sara E. Renner. "Biological Warfare Agents as Threats to Potable Water." *Environmental Health Perspectives* 107, no. 12 (December 1999): 975-984.

Covert, Terry C. "*Salmonella*." In *Waterborne Pathogens: Manual of Water Supply Practices – M48*, edited by David Talley, 107-110. Denver, CO: American Water Works Assocation, 1999.

Edwards, Kirstin, Julie Logan, and Nick Saunders, eds. *Real-Time PCR: An Essential Guide*. Norfolk, UK: Horizon Bioscience, 2004.

Gelfand, David H. and Thomas J. White. "Thermostable DNA Polymerases." In Innis, Gelfand, Sninsky, and White, *PCR Protocols*, 129-141.

Innis, Michael A., David H. Gelfand, John J. Sninsky, and Thomas J. White, eds. *PCR Protocols*. San Diego, CA: Academic Press, Inc., 1990.

Innis, Michael A. and David H. Gelfand. "Optimization of PCRs." In Innis, Gelfand, Sninsky, and White, *PCR Protocols*, 3-12.

Leal-Klevezas, Diana Sara, Octavio Martínez-de-la-Vega, Ector Jaime Ramírez-Barba, Björn Osterman, Juan Pablo Martínez-Soriano, and June Simpson. "Genotyping of *Ochrobactrum* spp. by AFLP Analysis." *Journal of Bacteriology* 187, no. 7 (April 2005): 2537-2539.

Logan, J. M. J. and K. J. Edwards. "An Overview of Real-Time PCR Platforms." In Edwards, Logan, and Saunders, *Real-Time PCR: An Essential Guide*, 13-29.

Louisville/Jefferson County Metropolitan Sewer District. "A Time of Crises." *MSD*. http://www.msdlouky.org/aboutmsd/history20.htm.

Maloy, Stanley and Rob Edwards. "*Salmonella* Information." Salmonella.org. http://www.salmonella.org/info.html.

Meuer, Stefan, Carl Wittwer, and Kan-ichi Nakagawara, eds. *Rapid Cycle Real-Time PCR: Methods and Applications*. Berlin: Springer Verlag, 2001.

Nelson, David L. and Michael M. Cox. *Lehninger Priniciples of Biochemistry*. 4th ed. New York: W. H. Freeman and Company, 2005.

New Zealand Food Safety Authority. "*Salmonella Typhi*." *Microbial Pathogen Data Sheets*. http://www.nzfsa.govt.nz/science/data-sheets/salmonella-typhi.pdf.

Nguyen, Anh V., Mazhar I. Khan, and Zhiqiang Lu. "Amplification of *Salmonella* Chromosomal DNA Using the Polymerase Chain Reaction." *Avian Diseases* 38 (1994): 119-126.

Office of Homeland Security. "National Strategy for Homeland Security." http://www.dhs.gov/xlibrary/assets/nat_strat_hls.pdf (published July 2002).

Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galán, C. Ginocchio, R. Curtiss III, and C. L. Gyles. "Amplification of an *invA* Gene Sequence of *Salmonella typhimurium* by Polymerase Chain Reaction as a Specific Method of Detection of *Salmonella*." *Molecular and Cellular Probes* 6 (1992): 271-279.

Roche Applied Science. "Triton X-100." http://www.roche-applied-science.com/pack-insert/1332481a.pdf.

Saaty, Thomas L. *Theory and Applications of the Analytic Network Process: Decision Making with Benefits, Opportunities, Costs, and Risks*. Pittsburgh: RWS Publications, 2005.

Saunders, N.A. "An Introduction to Real-Time PCR." In Edwards, Logan, and Saunders, *Real-Time PCR: An Essential Guide*, 1-11.

States, Stanley, Jennifer Wichterman, Georgina Cyprych, John Kuchta, and Leonard Casson. "A Field Sample Concentration Method for Rapid Response to Security Incidents." *Journal AWWA* 98, no. 4 (April 2006): 115-121.

Suburban Emergency Management Project. "SEMP - #356: The Guadalajara 1992 Sewer Gas Explosion Disaster." http://www.semp.us/biots/biot_356.html.

Tchobanoglous, George, Franklin L. Burton, and H. David Stensel. *Wastewater Engineering: Treatment and Reuse*. 4th ed. New York: McGraw-Hill, 2002.

Tsai, Yu-Li and Betty H. Olson. "Detection of Low Numbers of Bacterial Cells in Soils and Sediments by Polymerase Chain Reaction." *Applied and Environmental Microbiology* 58. no. 2 (February 1992): 754-757.

United States Environmental Protection Agency. "*Salmonella typhi*." Water Contaminant Index Tool. http://www.epa.gov/.

United States Food and Drug Administration. "*Salmonella* spp." *Bad Bug Book*. http://www .cfsan.fda.gov/~mow/chap1.html.

United States Joint Departments of the Army and Air Force. *Arctic and Subarctic Construction: Utilities*. Technical Manual TM 5-852-5/AFR 88-19, Vol. 5, August 1987.

Wittwer, Carl. "Rapid Cycle Real-Time PCR: Methods and Applications." In Meuer, Wittwer, and Nakagawara, *Rapid Cycle Real-Time PCR*, 1-8.

Zipper, Hubert, Christiane Buta, Katrin Lämmle, Herwig Brunner, Jürgen Bernhagen and Frank Vitzthum. "Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments." *Nucleic Acid Research* 31, no. 7 (2003): e39.