A Comparative Study of Three Bacterial Source Tracking Methods and the Fate of Fecal Indicator Bacteria in Marine Waters and Sediments

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ABSTRACT

E. coli and *Enterococcus* were used to determine the fate and survival of fecal indicator bacteria (FIB) in sand and sediments. The microbial source tracking (MST) methods antibiotic resistance analysis (ARA), *Bacteroides* human-specific primer test, and fluorometry were compared against the FIBs to determine how reliable each method was in detecting the presence of human fecal contamination. Two phases (Summer 2009 and 2010) were evaluated based on the type of contamination event. A combined sewage overflow (CSO) event was simulated in Phase I, where large amounts of influent were added to sand and bay water columns over 1 to 4 days. In 2010, a low volume sewage leak was simulated in which smaller doses of influent were added to sand and bay water columns over a period of 5 to 15 days. Within each of the phases, both non- and re-circulated columns were also evaluated.

Evaluation of FIB survival indicated that *Enterococcus* was able to stabilize and re-grow in the water and at the sediment/water interface within the Phase I non-circulated columns. *E. coli* was unable to re-grow and/or stabilize within any environment. Comparisons between the ARA and the FIBs revealed a large majority of isolates identified as coming from either bird or wildlife sources. Human sources were identified but at much lower concentrations than expected. *Bacteroides* results indicated strong relationships between the increase of FIB

concentrations and the presence of the human-specific *Bacteroides*. Fluorometry results did not indicate any relationship with the FIBs. Unexpectedly, fluorometry readings increased as time progressed indicating that another compound was present that fluoresced at the same wavelength as optical brighteners (OBs).

This project was one of the first to study the differences related to two different pollution events (CSO vs. sewage leak) while also evaluating what happens to pollution as it settles into the sediment. It was also unique because it compared bacterial (ARA), molecular (*Bacteroides*), and chemical (fluorometry) MST methods.

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Chapter 1. Introduction

The availability of clean water, for both recreation as well as drinking, is of great importance in the United States and worldwide. In 2004, there were an estimated 21,604 publicly owned treatment works, or wastewater treatment plants operating in the U.S. (USEPA, 2008). This is in addition to the millions of privately owned septic systems throughout the country. Both are to ensure that the wastewater we discharge from our homes and businesses is clean enough to ensure the safety of all that use it. In addition to wastewater, there are 3,740 beaches currently being monitored throughout the United States. These beaches are being monitored for all types of pollution, including fecal pollution to ensure the safety of beach-goers (USEPA, 2009). This need for monitoring was established by The Clean Water Act.

The founding document for the Clean Water Act (CWA) was first implemented in 1948 as The Federal Water Pollution Control Act. The CWA was then amended in 1972 and 1977 where the latter changed the name to what is now known as the Clean Water Act (USEPA, 2010a). The CWA regulates many facets including the discharge of pollutants, implementing pollution control programs, establishing water quality standards, and addressing the problems associated with nonpoint source pollution (USEPA, 2010b). In doing so, the CWA allows the Environmental Protection Agency (EPA) to be the regulating force behind these issues.

The CWA allows the EPA to regulate the quality of recreational waters. The EPA is responsible for establishing water quality standards for all surface waters. Water quality standards are a set of criteria that a body of water must meet in order to maintain a clean, unpolluted status. If a body of water does not meet the water quality standard, other measures must be taken in order to establish a remediation protocol. Total Maximum Daily Loads (TMDLs) are established for any body of water that is deemed polluted or impaired. These

TMDLs are a set of criteria that constitute the maximum amount of pollution (whether it be bacterial or chemical in nature) allowed in a body of water on any given day (Garcia, 1999). TMDLs are established to not only evaluate the impaired water on a regular basis but also in hopes of reducing the pollution (USEPA, 2008).

One of the major issues with current water quality assessments is that large numbers of fecal indicator bacteria (FIB) are often found by disturbing the sediments where samples are taken, especially at recreational beaches (Haller, 2009). Such FIB populations are typically much higher in sediments than in the water column, but state regulatory agencies focus on sampling the water column, as specified by USEPA monitoring requirements, and often do not sample and test the sediments. Sediment FIB populations have become a priority issue for the USEPA because anyone using such waters for recreational purposes is exposed to the sediment populations, and higher numbers of FIBs are well correlated with greater risk from water-borne pathogens (USEPA, 2007). These sediment populations are usually of unknown origin and the conditions under which these FIBs accumulate (and possible re-growth), and how long they might persist if the sources could be eliminated, are not well understood.

The goals of this study are to: (1) determine under what concentration of raw sewage amendments FIB populations stabilize; (2) determine how long stabilization will last after the last sewage amendment; (3) determine if re-growth can be detected in the sediment populations; and (4) compare the three microbial source tracking (MST) methods; fluorometry, *Bacteroides* human primer test, and antibiotic resistance analysis (ARA); amongst one another and with both FIBs (*E. coli* and *Enterococcus)* to examine possible correlations between the methods themselves and between the methods and the FIB populations.

Two phases of the experiment were set up over two consecutive summers. Phase I simulated a combined sewage overflow (CSO) scenario; whereas in Phase II simulated a low volume sewage-main leak. In the underground column facility, samples were taken at three locations within the columns. Bacterial counts of *E. coli* and *Enterococcus* species were assessed, ARA was performed on *Enterococcus* species, flourometry readings were taken on all water samples and assessment of *Bacteroides* human specific gel-electrophoresis were performed. In addition, electrical conductivity (EC), pH, and dissolved oxygen (DO) were monitored.

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Chapter 2. Literature Review

I. Current Recreational Water Issues

The Clean Water Act was first developed to monitor chemical pollution in recreational and drinking waters in the United States (USEPA, 1972). Later amendments were added to extend the coverage to include fecal pollution on the same waters. These additions were made because it was discovered that high pollution rates in water were positively associated with illness in humans who have had contact with that water (Cheung, 1990; Corso, 2003). As of 2008, the Environmental Protection Agency (EPA) had received a notification from all 30 coastal states. A notification action is simply a posted announcement of a hazardous environment. This particularly refers to bacterial levels being above recognized standards. Of those 30 coastal states, 32% had at least one advisory or closure (USEPA, 2009). These high bacteria levels are associated with fecal contamination and can come from a variety of sources. Since the microorganisms (bacteria, viruses, and protozoa) found in human fecal waste can include a wide variety of pathogens, human waste has the most potential to cause illness. Because of these pathogens, the source of pollution is typically differentiated between human and non-human waste.

Non-human waste contamination can come from a number of sources depending on the environment surrounding the pollution. In areas populated by agriculture, a major source of non-human pollution is the cattle industry--both free-ranging cattle with open access to water such as streams and rivers and also poorly run feed lots where large populations of cattle are confined in a small area. Within these poorly run cattle operations most of the waste can enter bodies of water through runoff. Large-scale swine and chicken farms are also a source of non-human contamination. Many swine and poultry farms utilize large manure pits to store their waste

materials. These pits are potential sources of groundwater pollution (Krapac, 2002; Mallin, 1997). In areas where there is a large migratory bird population, such as Canadian geese and shore birds, fecal contamination can come primarily from these animals (Levesque, 1993).

When considering human fecal contamination, it primarily comes from the inadequacies of the wastewater treatment infrastructure. Two major areas of concern are aging sewer systems and combined sewage overflows (CSO) associated with wastewater treatment facilities. Aging sewer infrastructure is a major contributor of human fecal contamination in both surface and underground waters. Many sewer systems are over 50 years old and have had only limited upgrades since then. The American Society of Civil Engineers gave the United States a "D-" for wastewater infrastructure (ASCE, 2010). In 2002, the Congressional Budget Office (CBO) estimated that the cost to improve the nation's wastewater infrastructure would be between $13.0 and $20.9 billion (CBO, 2002). Combined Sewage Overflows (CSOs) are another major issue in cities around the United States. CSOs are systems in which sewers and runoff are combined before heading towards a wastewater treatment facility. The issue is that when major rain events occur, the wastewater treatment facilities are unable to handle the volume of water coming into the facility so they dump the untreated water directly into streams, rivers or other bodies of water. There are approximately 772 communities or 40 million people in the U.S. that are serviced with CSOs (USEPA, 2008). Every year there are around 75,000 CSO events that discharge between 3 and 10 billion gallons of untreated water that lead to 5,500 illnesses through recreational waters (USEPA, 2010a).

On-site systems, such as septic systems, also pose an issue concerning proper disposal of wastewater. In 2009, the U.S. Census Bureau estimated that 20% of households use an on-site system (USCB, 2009). Unfortunately, poor regulation and inadequate maintenance of these

systems inadvertently leads to failed on-site systems that pose a potential health problem by leaking into ground and surface waters (USEPA, 2010b).

A. Fecal Indicator Bacteria

Fecal indicator bacteria (FIB) are a grouping of microorganisms that are thought to be directly associated with pathogenic fecal microorganisms. These FIBs were chosen as indicators of fecal pollution in water because of their small probability of causing disease and inability of reproducing outside of the host (Field, 2007), but recent results have called these characteristics into question (Anderson, 2005; Filip, 2009). FIBs are used to determine if a water sample has been contaminated with fecal pollution, although it is not an indication of the source of the pollution. But because these organisms have been reliable in determining if pollution is present, the FIBs have been used as the basis for many different microbial source tracking (MST) methods.

B. The Fecal Indicator Dilemma

The two major FIBs, *E. coli* and *Enterococcus*, are now coming into question as to whether or not they are the most reliable organisms to determine fecal contamination (Leclerc, 2001; Tallon, 2005). There have been several reports of persistence in the environment, and some studies indicate that sand and sediment could be a possible reservoir for FIBs.

In a report by Davies et. al. in 1995, they compared survival rates of *C. perfringens*, fecal streptococci and *E. coli* in the presence of bacterial predators in marine and freshwater sediment. *C. perfringens* is a popular FIB that is used mainly in Europe. In this study, it was found that *C. perfringens* persisted in the sediment and the presence of predators had no effect on its survival. They showed that both fecal streptococci and *E. coli* were capable of growing without the presence of predators in the environment. But when predators were present, natural die-off

occurred. Davies et. al. also discovered a high proportion of viable but non-culturable (VBNC) bacteria in the sediment. VBNC bacteria can be capable of causing disease, but are not detectable by the popular culture-dependent methods. Davies et. al. study was the first to look at VBNC bacteria persistence in sediment as a possible source of disease. Further reports have validated that persistence and re-growth are possible in sand and sediment environments(Hartz, 2008; Yamahara, 2007).

One of the major issues with current water quality assessments is that large numbers of fecal indicator bacteria (FIB) are often found by disturbing the sediments where samples are taken, especially at recreational beaches (Alm, 2006; Anderson, 2005; Haller, 2009). Such FIB populations are typically much higher in sediments than in the water column, but state regulatory agencies focus on sampling the water column and ignoring the sediments, as specified by EPA monitoring requirements. Sediment FIB populations have recently become a priority issue for the EPA because anyone using such waters for recreational purposes is exposed to the sediment populations, and higher numbers of FIB are well correlated with greater risk from water-borne pathogens (USEPA, 2008). These sediment populations are usually of unknown origin and the conditions under which these FIBs accumulate (and possible re-growth), and how long they might persist if the sources could be eliminated, are not well understood.

II. Current Microbial Source Tracking Methods

Currently, there is no single MST method that stands out as the best one for all circumstances. There are several methods that are currently being utilized and many more that are being validated and tested for reliability (Fig. 1). Though, in reality, there will probably never be just one method that is reliable enough to use alone. This is because there is no single indicator or method that does not have a drawback. For instance, *E. coli* and enterococci have

been detected living outside the host organism for extended periods of time. Concerning host-specific molecular methods, there is still much to be understood regarding the specificity of the markers, the behavior of individual DNA-based markers under environmental conditions including their rates of decay relative to pathogens (Field, 2007), and the potential for the markers to become indigenous to the environment, similarly to FIB. So for these reasons, MST proves most accurate when using a variety of several methods to determine the sources of fecal contamination.

When MST was first being developed in the mid-1990s, molecular methods were far from being useful at that time. Therefore, the most logical route utilized the same methods of cultivation used for the detection of fecal pollution with FIBs. These culture-dependent methods were developed using the unique characteristics of these fecal organisms—such as antibiotic resistance, metabolism or concentrations/ratios (Fig. 1). In order to catalog some of the characteristics, libraries had to be developed to compare to unknown organisms. These libraries constantly have to be updated and reviewed to introduce new bacterial characteristics. Because libraries are labor and time intensive, it didn't take long for researchers to begin testing methods that were library independent. During the same time, one molecular culture-dependent method, pulsed field gel electrophoresis (PFGE) was developed that proved to be equally reliable (Schwartz, 1983). Figure 1 illustrates the major types of library-dependent and independent methods.

A. Library-dependent methods

As mentioned before, the most logical step for determining the source of fecal pollution would involve using some of the FIBs that have, thus far, proven reliable by utilizing their unique properties. Therefore several culture-dependent methods were developed--although, these methods were not the only ones being explored. As technology has advanced, culture-independent and culture-dependent methods were also developed (Fig. 1).

A.1. Antibiotic Resistance Analysis (ARA)

ARA is a popular culture-dependent MST method that has been useful for many years (Hagedorn, 1999; Sayah, 2005; Wiggins, 1999). ARA was developed as a relatively inexpensive method that could be readily learned to assess the sources of fecal pollution. Antibiotic resistance in bacteria was first explored in a clinical setting but soon was realized as an important feature in fecal bacteria (Gilardi, 1971; Washington, 1969). Since antibiotics are readily given to humans in a clinical setting and animals such as cattle, chicken, and horses in the form of feed supplements to increase growth and productivity, it seemed rather obvious that the fecal bacteria from these different sources would have different sets of antibiotic resistance patterns.

ARA involves isolation of a fecal indicator, such as enterococci species. The bacteria are exposed to a variety of antibiotics at varying concentrations. There is no set group of antibiotics that are used with every isolate. Depending on the type of known fecal isolates collected in the area, determines the type of antibiotics used for ARA against unknown sources. Table 1 shows some of the antibiotics used in ARA analysis. The ability to grow (resistance vs. susceptibility) in the presence of these antibiotics is observed. Based on this growth vs. non-growth, ARA creates a unique pattern that can be compared to a library of known patterns for the organism to identify the source of contamination.

Because of its ease of replication and inexpensive materials, ARA has been widely accepted as valid method of MST. But because of the nature of bacteria, antibiotic resistance can change relatively quickly. Mutations can take place in many stages of the MST methods (Samadpour, 2005). During the exposure of new antibiotics within the source of the fecal pollution—such as new antibiotics introduced in animal feed or in humans as more antibiotics are introduced to patients with infections (Simpson, 2002). Mutations can also occur during cultivation or storage of the bacterial isolates. What is not known is how antibiotic resistance for the fecal indicators that are typically used for ARA, can change over time in the environment. Such mutations can totally invalidate the library used to find the source and could require constant reevaluation of each organism used. Along those same lines, organisms can have different patterns of antibiotic resistance based on geographical location. This requires individualized patterns, based on location, in a library. This amount of time required to update a library is a definite shortcoming of ARA.

A study by Graves et. al. revealed that ARA could successfully be used in a field setting in northern Virginia. Water samples were collected from a stream nearby a small community of houses which were serviced by individual septic systems. The stream was listed as impaired and their work found fecal contamination that exceeded the recreational water standard. They built a library of sources from the most common animal sources as well as human sources from that area. Once the library was developed, they began testing the water samples against the library and found 94.6% correct classification rate for human isolates. They also found 93.7% correct classification rate for livestock and 87.8% correct classification rate for wildlife species. This study clearly showed that ARA could be applied to a field setting and be used to find the correct source of fecal contamination as long as the library shortcomings are addressed.

A.2. Carbon Utilization Profiles

Carbon utilization profiles (CUPs) have also been used to determine the source of fecal pollution. CUPs were initially used in determining the identification of an unknown organism. One such commercial CUP system is Biolog. The Biolog system consists of a 96 well microtiter plate. Each well contains a different source of metabolite. The wells also contain an indicator that changes color (clear to purple) if the bacteria utilize the carbon source in that well. After incubation, the bacteria create a unique pattern of growth vs. non-growth. This pattern can then be compared to a library of known organisms and can be identified.

Biolog has been used for the identification of organisms in a clinical setting (Holmes, 1994). But in 2003, Hagedorn et. al. used Biolog to distinguish between human and non-human sources of fecal pollution. They collected 365 *Enterococcus* isolates from known sources. These isolates were identified using Biolog, They then used discriminate analysis (DA) to determine distinct patterns for human, livestock, wildlife, and domestic pet sources. Once a library of known patterns was created, they then collected stream samples where specific sources of contamination were suspected. The results concluded that Biolog could successfully distinguish between sources and be utilized as a method of MST (Hagedorn, 2003). Unfortunately, Biolog requires a computer system to identify and search the library. This system can be extremely expensive and is an upfront cost that many are unable to afford.

A.3. Pulsed Field Gel Electrophoresis (PFGE)

Around the same time that ARA was first being utilized as a MST method, PFGE was also being studied as a possible MST method (Schwartz, 1983). PFGE involves using agarose gel electrophoresis with alternating pulsed electric currents. PFGE requires the use of extensive libraries in which organisms have very unique genetic fingerprints (Farber, 1996). PFGE was

used in the first published account isolating an outbreak of the O157:H7 *E. coli* (entero-hemorrhagic *E. coli*, EHEC) strain associated with swimming (Samadpour, 2002). An advantage of PFGE is that it is extremely sensitive to even the smallest genetic differences (Scott, 2002). But this can also be a disadvantage in that it is actually too sensitive and requires an extensive library if used for MST in large or complex watersheds (Lu, 2004).

A.4. Ribotyping

Ribotyping is another genotypic method utilizing the differences in DNA using restriction enzyme digestion, electrophoresis, and Southern blotting (Farber, 1996). Ribotyping was first used as an MST method in 1999, where they used the *Hind*III restriction enzyme protocol to distinguish between human and non-human *E. coli* (Parveen, 1999). Ribotyping has proven to be a successful MST method because of its continued success in reproducibility (van Belkum, 2001). Unfortunately, ribotyping can be expensive and labor intensive (Scott, 2002). It also requires some specialized skills that would require training (Carson, 2003).

A.5. Polymerase Chain Reaction (PCR)

PCR has also been utilized as a fingerprinting library-dependent method. Repetitive element PCR (Rep-PCR) uses a primer that targets repetitive, palindromic DNA sequences that are unique to specific species (Versalovic, 1991). Unknown sequences are compared to the library of individual sequences in order to find the source. There have been several studies that have found varying results in reliability with Rep-PCR (Baldy-Chudzik, 2003; Dombek, 2000; Lipman, 1995). But Rep-PCR has proven to be an advantage for MST because is it not as labor intensive as PFGE and ribotyping, is less expensive than ribotyping, and does not require DNA purification (Carson, 2003; Dombek, 2000; USEPA, 2005). However, a few studies have

reported issues with reproducibility (Scott, 2002; Seurinck, 2005b). With any molecular method, equipment expenses can be great and spending such upfront costs can be hard to justify.

A.6. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a molecular method that can separate PCR products of the same length using their DNA sequences by manipulating their melting points using a polyacrylammide gel (Muyzer, 1993). Unlike other molecular methods, the fingerprints produced using DGGE are of whole populations of organisms instead of individual species (Wintzingerode, 1997). Since DGGE is relatively new to MST; most of the studies to date involve trying to locate a target area in genomes that produce the best distinction between sources (Buchan, 2001; Farnleitner, 2000). Four genes (*uidA, mdh, phoE,* and *uidA*-4) have proven successful in linking environmental pollution in environmental samples to the source of pollution (Esseili, 2008; Sigler, 2006). Because DGGE is a new area of research for MST, there is still much that is unknown about its efficiency. Another issue with DGGE surrounds the technical difficulty of the procedure and the time-consuming efforts involved (Meays, 2004).

B. Library-independent methods

Library-independent methods were first developed to eliminate the dependence and time-consuming process of maintaining a library. Libraries require the constant updating because of several elements. Since bacteria have a relatively high rate of mutation, libraries can become obsolete over time and geographic distance. Also, with time more bacteria are being discovered and more thoroughly investigated, increasing the need for updating libraries.

Within the classification of library-independent, there are methods that require cultivation of the organism being used and those that don't (culture-dependent vs. culture-independent, Fig. 1) (Byamukama, 2005; Shanks, 2007). Most library-independent methods are also culture-

independent for the mere fact that they are also molecular methods that don't require cultivation. There are a few culture-dependent methods that are currently being studied.

B.1. Fecal coliform/fecal streptococci ratio

One of the first library-independent MST methods investigated looked at the ratio of fecal coliforms to fecal streptococci (Geldreich, 1976; Geldreich, 1969). They determined that a ratio larger than 4 (FC/FS) was an indication of human pollution. This ratio was later debunked and is no longer considered to be differential between human and non-human pollution. It was later found that the different ratios were due to differential die-off (Feachem, 1974). Even though this is not a suitable MST method, it can provide supplementary information about the characteristics of fecal pollution (Farnleitner, 2010).

B.2. *Enterococcus* species profiles

There have also been studies conducted using *Enterococcus* species as reliable organisms to determine the source of fecal pollution. Most studies involved using the biochemical or molecular typing characteristics and were therefore library-dependent based (Kuntz, 2004; Wheeler, 2002). There have been a few studies that investigated *Enterococcus* through molecular PCR-based methods (Harwood, 2004; Layton, 2010). It was the latest study by Layton et. al. that found that there is no one *Enterococcus* species that is source specific.

As technology has greatly improved, so have the methods for MST. Specific genetic markers have been utilized in a variety of ways in the realm of MST. Khatib et. al. (2002) used the heat labile toxin IIA gene in enterotoxigenic *E. coli* (ETEC). They developed primers and methods specific to that gene. Both human and animal fecal samples were collected and tested using PCR methods. Once minimum detection levels were established and it was clear that no cross-reactivity was present, they tested waste samples in order to validate the method. They

found that one marker (cow) in particular was highly specific for cattle pollution. When testing for this toxin gene in wastewater, they found that this toxin was specific to cattle. Using this information, it was suggested that this toxin marker could be used for the detection of cattle fecal pollution (Khatib, 2002). Because the toxin is only shed in infected animal, it is not known the percentage of cattle that possess this marker so therefore it may not be as source specific as it needs to be.

Other species of bacteria and even viruses have been suggested and used for molecular-based MST methods. Human polyomoviruses and adenoviruses have been successfully demonstrated as potential MST methods (McQuaig, 2009; Pina, 1998). In addition to viruses, bacteria that were once not suitable for MST, due to difficulty in cultivating, were able to be considered for MST using these molecular methods. Such is the case for *Bacteroides* species. Also, bacteriophages, or viruses specific to infecting bacteria, could also be considered as suitable MST methods. Figure 1 lists the library-independent methods that have been most thoroughly studied to date.

B.3. *Bacteroides*

Bacteroides species were first thought to be good indicator organisms in the mid 1980s. *Bacteroides* species are anaerobic organisms that are normally found in fecal pollution. These organisms are normally found in much higher concentrations than other fecal indicators such as *E. coli* and *Enterococcus* species. But because of their anaerobic nature, they are harder to cultivate and grow using traditional methods.

One of the first studies examining *Bacteroides* species as a potential general indicator organism was by Fiksdal et. al. (1985). In this study, they examined *Bacteroides fragilis* in particular. They found that *B. fragilis* declined in the water samples much sooner than *E. coli* and

Streptococcus faecalis. But using a method involving fluorescent antiserum found that *B. fragilis* was detected after 8 days in an aerobic environment (Fiksdal, 1985). But because of the issues surrounding cultivation, including the anaerobic nature and VBNC species, it was soon realized that *Bacteroides* was not an ideal fecal indicator organism. Although *Bacteroides* is not a suitable organism for indicating fecal pollution, with the advances in molecular technology, it was realized that *Bacteroides* had potential in the realm of MST.

Bacteroides species were first examined and tested for specific DNA sequences that could be associated with specific host species. Bernhard and Field (2000a) looked at several sequences from both human and cattle samples. Within these sequences, they found several DNA subunits that were specific to both humans and cattle (Bernhard, 2000b). This was a starting point for looking at these specific genetic sequences as an indicator of a source of fecal pollution. In a separate study by Bernhard and Field (2000b), they performed a more in-depth study on the PCR products that would be suitable for an inexpensive MST method. Here they found that the HF8 primer clusters, found in the previous study, were indeed specific to human sources (Bernhard, 2000a). The HF8 cluster includes the forward primer (HF183) that was used in this current study.

Within the realm of using PCR methods with human-specific *Bacteroides*, endpoint PCR, quantitative PCR (qPCR) and real-time PCR (rtPCR) have been developed. Endpoint PCR is the traditional PCR/gel electrophoresis method in which the end result is a presence or absence of the sequence in question. This method is reliable as a MST method in that it detects whether the source of the pollution is human or not. And because it has proven reliable, both qPCR and rtPCR are being explored as a possible methods to detect a certain level of pollution--in other words, using *Bacteroides*, in a sense, as a FIB (Seurinck, 2005a; Shanks, 2009). Quantitative

and real-time PCR's end results can detect or quantify the amount of *Bacteroides* in the sample. With some guidelines illustrating the maximum amount of *Bacteroides* that can present within a sample, both qPCR and rtPCR could be successful methods to determine the level of fecal pollution within a body of water.

Although qPCR and rtPCR can be used for MST purposes, it is not necessary to determine the source of pollution. Therefore, endpoint PCR is the more feasible method. Since the discovery of the human-specific *Bacteroides* sequence, endpoint PCR studies have been developed to validate the method as a reliable tool to incorporate into the MST methodology (Bernhard, 2000a; Kreader, 1998).

A study in 1998 used endpoint PCR to determine the fate and survival of *Bacteroides distasonis* both in the Ohio River and in laboratory experiments (Kreader, 1998). Kreader found that based on whether or not predation existed (Ohio River vs. laboratory) and temperature differences, *B. distansonis* survived for about 2 weeks at 4°C and persisted in the laboratory experiments for about 1 week without the presence of predators. These eukaryotic predators (such as flagellates, ciliates, and metazoans) are a part of the natural ecosystem and can consume bacteria as a primary food source (Beers, 1971; Heinbokel, 1979; Takahashi, 1978). This study investigated the changes in temperature playing an important role in detecting *Bacteroides* PCR products over time.

Although using *Bacteroides* as a MST method has proven reliable, more studies need to investigate the sensitivity and survival of *Bacteroides* both in the laboratory and environment. Survival of *Bacteroides* DNA in the environment is also unknown. Also, other unique primers could prove to be more reliable than those already discovered. Further studies could provide evidence for using *Bacteroides* as a more reliable FIB.

B.4. Bacteriophages

Bacteriophages are specific viruses that can only infect bacteria. These organisms can be extremely specific, only infecting one specific species of bacterium. Coliphages are one such example. Coliphages only infect the species *Escherichia coli*. Coliphages were suggested for use as a possible MST method because they are associated with fecal pollution and can persist in the environment (Borrego, 1990; Friedman, 2009; Skraber, 2004). Because bacteriophages don't require the cultivation of the organism, rapid methods have been developed that can save countless hours (Love, 2007; Luna, 2009). Reports have shown that the popular F-RNA coliphages are isolated in less than 10% to 35% of human fecal samples, however. (Long, 2005; Schaper, 2002).

C. Chemicals

Chemicals have recently been suggested and tested as possible MST methods (Hagedorn, 2009). Caffeine has been suggested as a possible MST method (Peeler, 2006). Caffeine is metabolized in the human system, but a small amount is excreted. This small amount can be detected in waters and can be used in addition to other MST methods. Other chemicals that are often associated with human waste have been considered as potential MST methods. Pharmaceutical products, such as ibuprofen and carbamazepine, have been determined as possible methods for MST (Buser, 1999; Clara, 2004). Chemicals used in perfumes, soaps, detergents, air fresheners, and even cosmetics have been suggested as markers of fecal pollution (Buerge, 2003). Fecal sterols/stanols, such as cholesterol and its metabolites, can be found in the human pollution and used in MST (Macdonald, 1983). Fluorometry or the detection of optical brighteners has also become a possible method for detection of fecal pollution.

C.1. Fluorometry/Optical Brighteners

Optical brighteners (OBs) or fluorescent whitening agents (FWAs) are compounds present in many everyday household products. Toilet paper and laundry detergents are prime examples. The purpose of OBs in these products is to whiten items such as clothes and toilet paper. Sewer or onsite systems such as septic systems combine all waste water coming from homes and businesses. So therefore if leaks occur, these OBs are released along with the fecal contamination. Therefore, searching for OBs in or around leaking systems is a good determinant for the presence of human fecal pollution.

Because OBs fluoresce at a certain wavelength (436 nm), fluorometry was developed to detect these compounds in water samples. Fluorometry is most commonly used on site, detecting plumes of OBs in a body of water. Hagedorn et. al. (2005) used fluormetry to determine if the dominant sources of pollution in condemned Virginia shellfish beds were human in nature. The investigators traveled along the coastline of the shellfish beds continuously taking water samples by pumping the water through the fluorometer. Throughout their testing, they discovered that some locations where other conventional MST methods showed human pollution were negative for fluorometry. But in every instance where fluorometry tested positive, the other MST methods tested positive for human pollution. In other words, they determined that fluorometry could be a great addition to MST (especially in rapid initial screenings) but should be used in combination with microbial-based MST methods (Hagedorn, 2005).

III. Current issues involving sand/sediment pollution

One of the most current revelations about water quality and MST is that the FIBs have been found to colonize beach sands and sediments (Alm, 2006; Anderson, 2005; Haller, 2009). Because this is a relatively new discovery, we know little about the FIBs that inhabit such sands

and sediments. This raises huge concerns for public health safety. It is not known if these bacteria can cause disease in human populations or not. If so, we do not know at what concentrations these bacteria are considered harmful. There are currently no regulations or testing criteria for sand and sediment FIB populations.

Within the species *E. coli*, there are several strains that can be extremely dangerous to humans. O157:H7 *E. coli* has been in recent news causing severe outbreaks and hospitalizations in relation to spinach and hamburger products (CDC, 2006; CDC, 2009). This *E. coli* strain is also called Entero-hemorrhagic *E. coli* (EHEC) because it causes bloody diarrhea in infected individuals. Other strains include Entero-invasive- (EIEC), Entero-toxigenic- (ETEC), and Entero-pathogenic- (EPEC) *E. coli*. All of these strains are pathogenic and can cause considerable disease in humans. The survival of these strains in sediment is unknown and therefore of importance in preventing disease.

A. Possible explanations for survival

It has been widely known that bacteria can colonize many areas due to adaptations such as the formation of biofilms. These biofilms create an environment that is conducive to bacterial growth providing protection from desiccation and harsh chemicals, such as antibiotics and antiseptics, while providing nutrients necessary to sustain survival and even growth. This formation of biofilms on surfaces such as the inside of sewer lines and associated with groundwater could play an important part of FIBs survival in sand and sediment (Banning, 2003; Daims, 2000). Although it is not clear why FIBs might adapt to this form of survival, it is possible for biofilms to develop in sand and sediment and allow bacteria to survive and perhaps re-grow.

Again, the other large issue with fecal bacteria is the viable but non-culturable (VBNC) state. During periods of stress, either brought on by starvation or other environmental factors, bacteria can enter this VBNC state where they focus only on survival and not necessarily on reproduction. These bacteria are still capable of causing disease and returning to a culturable state if the conditions are right (Colwell, 1996; Lleo, 2001; Signoretto, 2008). The implications surrounding this are substantial. Without being able to cultivate these organisms, other methods such as molecular markers must be employed to determine if these organisms are present. This applies to both pathogenic organisms as well and the fecal indicators. Without the knowledge of all organisms present in the environment, it is impossible to determine the potential for causing disease.

B. Evidence for sand/sediment persistence

In recent years there have been several examples of bacteria, such as FIBs, surviving in sand and sediment throughout the United States and world-wide. One of the first studies was performed by Alm et. al. (2003). They observed the abundance of both *Enterococcus* species and *E. coli* in wet sand. This was the first indication that sand could be a possible reservoir for FIBs and possibly pathogenic fecal organisms (Alm, 2003). Around the same time, *E. coli* and total coliforms were found in sediments at lake marinas in Oklahoma and Texas (An, 2002).

In Florida, fecal coliforms and *Enterococcus* species were found to persist in microcosms filled with the sub-tropical waters found in that area. Anderson et. al. (2005) established 6 microcosms that all contained natural water and sediments collected from the area (either freshwater or Gulf of Mexico). These microcosms were aerated to simulate more natural conditions. They were then inoculated with dog feces, untreated wastewater, or soil that was near contaminated water. Although it was not evident that growth occurred, there was evidence

of varied persistence based on the type of environment the organism was in. Fecal coliforms persisted longer in freshwater than *Enteroccocus* species and they persisted longer in the sediment than in the water column (Anderson, 2005).

In other countries outside the United States there has also been evidence of FIB survival. Pote et. al. (2008) conducted a study using the water and sediments from Vidy Bay, Lake Geneva in Switzerland. Like the previous study microcosms were used to simulate natural conditions. Either lake water or effluent from a sewage treatment facility was added to the microcosms along with sediments. Membrane filtration was performed to quantify the bacterial concentrations at different temperatures (10, 20, and 25°C) over time (days). It was found that the sediments of Vidy Bay were indeed reservoirs for FIBs and disturbing the sediments would allow the bacteria to resuspend in the water column, increasing the potential for causing disease among recreational water users. The important factors surrounding the FIBs survival were high amounts of organic matter and median temperatures. The bacterial counts were highest in the microcosms containing the sewage treatment effluent kept at 20°C (Pote, 2009).

IV. Summary

Currently there are many issues surrounding recreational water quality. Introduction of fecal waste from failing wastewater infrastructure and non-human sources into streams, rivers, lakes, and oceans has created the need for water quality monitoring. While monitoring for pollution is important and the first step in protecting against disease, MST is also important in finding the source of the pollution in order to remedy the situation. Since the development of the first MST methods, many technological advances have allowed improved MST methodologies. Along the way, many MST methods have been questioned as to whether or not they are reliable. Many of those methods include utilizing the popular FIBs (such as *E. coli* and *Enterococcus*

species) that are used in monitoring water quality. Evidence is building showing the possible persistence and/or re-growth of these organisms in sand and sediments. And because there are no current standards concerning the limit of these organisms in sand and sediments, the implications concerning health safety are unknown. Therefore, it is important to understand the complex environment in sand and sediments and the roles that these organisms play in the environment. Once researchers begin to understand, they can begin to determine what role they play in public health safety.

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Table 1. Popular antibiotics and concentrations used in ARA (USEPA, 2005).

Type of Antibiotic	Concentrations (µg/ml)
Amoxicillin	4-128
Ampicillin	10
Bacitracin	10-100
Cephalothin	10-50
Chloramphenicol hydrochloride	4
Chlortetracycline hydrochloride	20-80
Chlortetracycline	20-80
Doxycycline hydrochloride	4
Erythromycin	5-50
Gentamicin	1-20
Kanamycin monosulfate	3-50
Monensin	5-250
Moxalactam	0.2-1
Nalidixic acid	3-25
Neomycin sulfate	3-50
Norfloxacin	0.1
Oxytetracycline hydrochloride	20-100
Penicillin G	20-200
Polymixin B	1-10
Rifampicin	2-16
Sstreptomycin sulfate	0.2-5
Sulfathiazole	500
Tetracycline hydrochloride	4-64
Trimethoprim:sulfamethoxazole (1:19 ratio)	0.2-5
Vancomycin	2.5-30

Figure 1. Illustration of Library- dependent and independent methods.

Chapter 3. Materials and Methods

I. Material collection

Both sand and water were collected at Festival Beach (Havens Beach) located in Matthews County, Virginia (37° 26'08.92" N 76° 15'08.52")in 2009 and 2010 (Fig. 2). All materials were transported back to the Virginia Tech campus. The water was stored at 4°C until it was needed to set up the columns. In 2009, the sand was dried in a greenhouse facility for at least 48 hrs. For the 2010 experiment, the same sand used in 2009 was re-used after sterilizing for 30 min. at 121°C and drying in a 80° C oven overnight. In both cases, the drying was to insure that any resident bacteria would be killed before inoculation of the columns.

Figure 2. Festival Beach located in Matthews County, VA. This is the site of water and sand collection (beach in upper left of picture).

II. Column setup

The column setup was the same for both Summer 2009 (Phase I) and 2010 (Phase II) experiments. The columns were constructed of PVC (Sch 40 PVC) pipe that contained a rubber covering at the bottom (Fig. 3). This was sealed to prevent any leakage. The PVC pipe's dimensions were 20.3 cm in diameter and 61.0 cm in length. The upper end of the PVC pipe was left open to attach the sampling apparatus and to collect samples. A plywood covering was custom made and fitted over the open end of the columns (Fig. 4), to keep rain and debris out of the columns. In each column, 15 cm of sand was added to the bottom.

Figure 3. Close-up of PVC pipe columns. Notice the black rubber covering on the bottom of the pipes

Figure 4. Column facility with custom-made plywood coverings.

Once the columns were filled with sand, each was fitted with a sampling apparatus (Fig. 5). This apparatus was constructed out of a bar of Plexiglas that ran the diameter of the pipe and attached at either end with duct tape. In the center of the bar, three tubes were attached with zip ties. These tubes were used as the housing for a more flexible and smaller tube that was inserted into the larger tubing. At the bottom of the larger tubing, small screens were attached to keep

them free of sand so samples could be collected with ease. This smaller tubing was the actual tubing used to draw up the sample. Each tube setup was labeled with A, B, and C. These labels corresponded to the three different sampling depths inside the column. Sampling point A corresponded to the middle of the water column. Sampling point B was located at the water/sediment interface and sampling point C was located in the middle of the sediment.

In both Phase I and II, a total of 24 columns were used. In these 24 columns, half (12) were fitted with a re-circulating apparatus. This consisted of a water pump located below each of the columns. The pump was immersed in bay water inside a reservoir bucket and fitted with two tubes. The first tube was attached to the pump that fed water into the column at approximately 13 cm above the sand. This tubing was fed through the roof in the bunker and attached to a clear pipe that fed down into the column. The second was attached to an outlet that was drilled at 26 cm above the sand as an overflow line. This line led back into the reservoir bucket. The other half (12) of the columns were not fitted with any re-circulation device (stagnant water conditions).

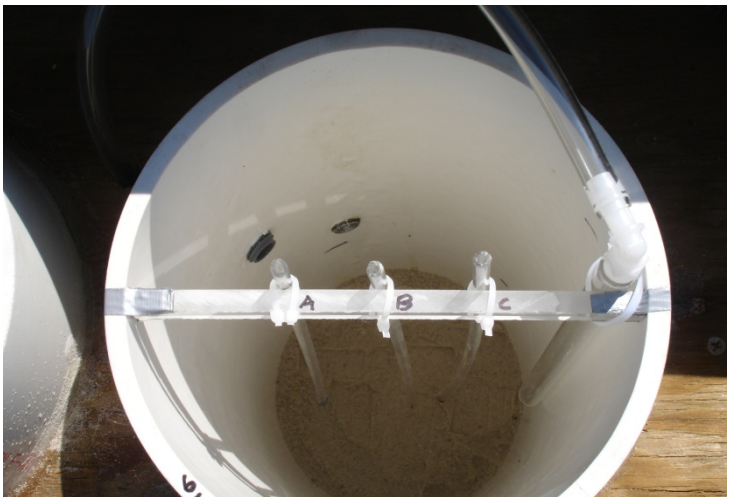

Figure 5. Sampling apparatus. A, B, and C refer to the location of the sampling depth within the column.

A. Phase I: Summer 2009

For Phase I of the project, in the summer of 2009, 12 re-circulated columns were set up first. Each column had its own water reservoir with a water re-circulation pump (Fig. 6). Ten liters of water was added into each column. This was accomplished by adding the water to the top of the column, gently, to avoid disrupting the sand at the bottom of the column. A total of 12 L of water was added to the reservoir buckets. Re-circulation rates were set up to achieve a dissolved oxygen content of 90 % saturation in the columns. This reading is consistent with readings taken at Festival Beach. To adjust the rate to achieve the desired dissolved oxygen (DO), a clamp was attached to each tube that fed water from the reservoir to the column. This clamp was adjusted until the DO meter read 90 % saturation.

Figure 6. Picture of reservoir bucket. The large tubing is the overflow and the small clear tubing in the back is pumping the water back into the column.

In the second part of Phase I, again 12 columns were set up. Instead of re-circulating the water, the water was allowed to stand and become stagnant. As before, 15 cm of sand was added to each of the columns. Water was then added to the columns to achieve an initial height of 43 cm above the sand. Once the water was allowed to infiltrate the sand the actual measurement was 36 cm above the sand. This was determined as an acceptable height for the water based on the amount of influent that would need to be added to the columns. Also, the total amount of sample was determined to ensure that there would be enough water for all samples needed. The total amount of bay water per column was 10 L.

B. Phase II: Summer 2010

In Phase II, the column setup was relatively the same as in Phase I. The non-circulated columns were run first, while in Phase I the re-circulated columns were run first. The same protocol was used for the amount of sand and water placed in the columns and reservoir buckets. The sand used in Phase I was autoclaved, dried and used in the columns in the Phase II experiments. Fresh water was collected from the same location at Festival Beach (Fig. 1). For the re-circulated columns, the same DO rate (90%) was used.

III. Treatments

Once the columns were filled with water, they were allowed to settle for several days. This allowed for all of the sand/sediment that was disturbed during the filling process to settle down out of the water column. Once the appropriate amount of time had been established, raw influent was collected at a pumping station for Pepper's Ferry Regional Wastewater Treatment Plant located in Fairlawn, VA. The influent was immediately transported to the column facility where amendments could be added to the columns beginning on Day 0.

In Phase I, the treatments were established as addition of influent spread out over a relatively short number of days. The amendment schedule was created to imitate the conditions surrounding a Combined Sewage Overflow (CSO) where large amounts of raw influent are dumped into a body of water, such as after a large rain event in many cities in the eastern and midwest U.S. that are served by CSO systems. Each column received the same total of influent (2.2 L) over a 4 day period. Treatments were done in triplicate and three control columns (no influent) were also included. The first treatment was an initial large dose of 2.2 L of influent on Day 0. Treatment 2 involved an addition of 1.1 L on Day 0 and 1.1 L on Day 1. Treatment 3 involved inoculating three columns with 550 mL of influent on Day 0, Day 1, Day 2 and Day 3. All control columns also received a 2.2 L addition of bay water. In Phase I, the influent was added to the reservoir bucket to simulate a more realistic CSO event (Fig. 7). This could not be accomplished with the non-circulated columns, so the additions were slowly added to the actual columns themselves.

Figure 7. Adding wastewater influent to the reservoir buckets.

In Phase II, the treatments were added to the various columns to simulate a low volume sewage-main leak. This involved adding treatments at smaller amounts over a longer period of time in comparison to the Phase I treatments. Because the *Bacteroides* human-specific test is sensitive to concentrations, a sample of influent was taken and processed before the start of Phase II. DNA extractions, PCR amplification, and gel electrophoresis were performed on the influent using different volumes to determine the minimum dilution necessary to produce a positive result. The minimum concentration was determined to be 22%. So for all treatments, a total of 2.2 L was added to the columns. For Treatment 1, 440 mL of influent per day was added over a total of 5 days. Treatment 2 consisted of 220 mL per day introduced over 10 days. For Treatment 3, 146.67 mL per day was added for a total of 15 days. Again, all control columns were given an additional 2.2 L of bay water.

For the re-circulated columns in Phase II, 22% of influent (4.84 L) was also added to the treatment columns. For Treatment 1, 968 mL per day of influent was added over a 5 day period. In Treatment 2 columns, 484 mL per day of influent was added over 10 days. For the Treatment 3 columns, 322.67 mL per day of influent was added over 15 days. All control columns received an additional 4.84 L of bay water.

IV. Sampling

Samples were taken at all three sampling locations (A, B, and C) over a 21 to 30 day period. Intense sampling was accomplished during the first few days because it was thought that the majority of change would occur in the first week. Sampling then gradually spread out over the next few weeks. Sampling was performed on Day 0, 1, 2, 4, 7, 10, 16, 21 and on Phase II columns on Day 30. Initial sampling size was 180 mL from each sampling location in the column, but was later reduced to 120 mL. Samples were collected using a 60 mL plastic

syringe. The syringe was attached to the smaller tubing on sampling apparatus and the liquid was drawn up into the syringe (Fig. 8). The sample was then emptied into a 250 mL clean sampling bottle. This procedure was done three times to achieve a total of 180 mL sample. The samples were then immediately taken back to the lab for filtration and stored at 4°C.

In addition to taking samples, DO (% saturation), electrical conductivity (EC) (mS/cm), temperature (°C), and pH were measured in each of the columns. DO, EC, and temperature were measured each time a sampling occurred. pH was measured at the beginning (Day 0) and on the last day of sampling.

Figure 8. Taking a sample from the columns with a syringe.

A. Processing Samples

A.1. Membrane-filtration and FIB counts

All samples were filtered through a membrane-filtration device. The device consisted of a membrane filter assembly attached to a 1.0 L flask. The flask was attached to a pump that suctioned the liquid through the filter (cellulose nitrate, 0.45µm pores, 47 mm diameter) and into

the flask below catching all potential bacteria on the filter. Filters were then placed on two types of plate media. Membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI) was used to differentiate between *Enterococcus* and non-*Enterococcus* genera. The mEI plates were incubated at 41°C for 24 hrs. *Enterococcus* colonies exhibited a blue halo on the surrounding filter. Modified membrane-thermotolerant *Escherichia coli* Agar (Modified mTEC) was used to select for and differentiate between *E. coli* and other species. The mTEC plates were incubated at 44.5°C for 24 hrs. *E. coli* colonies were either red or magenta in color. These two methods are in accordance with the EPA's guidelines for membrane filtration (USEPA, 2002a; USEPA, 2002b).

After incubation, all plates were inspected for growth. All suspected colonies were counted and recorded. To determine the colony forming units (CFU) per 100 mL of water the following calculation was used:

$$\text{CFU/100 mL} = \frac{\text{Number of colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

The numbers were recorded and used to monitor the microbial populations in each of the columns.

A.2. Fluorometry

Fluorometry was used on all samples collected to detect for the presence of optical brighteners (OBs). OBs are present in all laundry detergents and are a great indicator of human pollution. Fluorometry is a quick and easy determination of human waste and is a presence/absence test (Hagedorn, 2005).

For each of the samples 5 – 7 mL of sample was placed into a clean glass sampling tube. Each sample was placed into the fluorometer and allowed to stabilize for a few seconds before taking the reading. Once the initial readings were taken, all samples were placed under UV light for 4 hrs. After the 4 hr UV incubation, all samples were placed in the fluorometer once more and readings were taken. In order to establish the amount of OBs in the sample the final reading was subtracted from the initial reading:

$$OBs\ (FSU) = FSU_{initial} - FSU_{final}$$

Where FSU stands for Fluorometric Sampling Unit.

A.3. *Bacteroides* DNA extraction, PCR, and gel-electrophoresis

Assessment of the presence of human-specific *Bacteroides* occurred periodically throughout the sampling period. The human-specific *Baceroides* test utilizes two primer pairs (HF183/Bac708 and Bac32/Bac708) which distinguish between human and non-human respectively (Bernhard, 2000a). DNA extraction, PCR amplification, and gel-electrophoresis were performed on all samples at Day 0 and 21 (or 30 for non-circulated samples). If the human-specific *Bacteroides* was present at Day 0 then the analysis was done to see if it was also present on the last day of sampling. For any sample where that was not the case (not present on Day 21 or 30), then the sample from the previous sampling period was evaluated. For example, if the human-specific *Bacteroides* was found in the middle of the water in Column 1 (1A) on Day 0 but not on Day 21, then the sample from 1A on Day 16 would be tested for human-specific *Bacteroides*. If the sample from Day 16 was negative, then Day 7 would be tested. If Day 16 was positive, then no further testing would be necessary. This process would continue until all human-specific *Bacteroides* that were found on Day 0 were accounted for.

All samples were filtered using the sample filtering device as mentioned above. The filters were then placed into 15 mL sterile plastic conical tubes. DNA extraction was performed using an amended procedure from a Qiagen QIAmp Fecal DNA Extraction Kit (Crozier, 2009). ASL buffer (1.4 mL) was added to the tube containing the filter. The tube was vortexed for 1 min. After vortexing, samples were then placed in a 70°C water bath for 5 or 6 min. The later time was used if the samples were refrigerated beforehand. One and two-tenths mL of the sample was then placed in a microcentrifuge tube and an InhibitEX Tablet was added to the tube. The tube was vortexed until the tablet had absorbed most of the liquid. The tube was then centrifuged for 3 min. to create supernatant at the top of the tube. This supernantant was pipetted off the top of the pellet and this liquid was added to a new sterile microcentrifuge tube containing 15 µL of Proteinase K. Two-hundred µL of AL buffer was added and contents were vortexed for 15 sec., then the tube was incubated at 70°C for 10 min. Once incubation time passed, 200 µL of 96 - 100% ethanol was added to the tube and vortexed. The content of the tube was pipetted into a QIAmp Spin Column. The spin column was centrifuged for 1 min. It was then placed into a new collection tube. Five-hundred µL of AW1 buffer was added to the top of the column. The column was centrifuged again for 1 min. Again, the spin column was placed into a new collection tube and 500 µL of AW2 buffer was added to the top of the column. The column was centrifuged for 3 min. After centrifugation, the spin column was placed into a sterile 1.5 mL microcentrifuge tube. Two-hundred mL of AE buffer was added to the top of the column. The column was allowed to incubate at room temperature for 1 min. The column was then centrifuged for 1 min. The liquid that filtered into the 1.5 mL microcentrifuge tube now contained the DNA needed for PCR. The top of the spin column and all previous collection tubes were discarded.

PCR was then performed on the same samples in the same manner as those in the DNA extraction. In other words, PCR was performed on Day 0 samples and Day 21 (or 30 for non-circulated columns). If the human-specific *Bacteroides* was found on Day 0 but not the last day of sampling, PCR was performed on the previous sampling day until all *Bacteroides* found on Day 0 were accounted for. A PCR master mix was prepared by using the formula in Table 2. The forward primer, Bac32, was used as control that contained a DNA sequence that is present in all *Bacteroides* species. HF183 forward primer was used as the human-specific primer for *Bacteroides.* The reverse primer in both instances was Bac708. Table 3 illustrates the sequences of each of these primers.

A total of 2 PCR products were obtained from each sample. One contained the *Bacteroides* DNA specific to humans (HF183) and the other contained the *Bacteroides* DNA sequence found in all species (Bac32). Two sterile PCR tubes were obtained. One was labeled HF183 and the other Bac32. To each of the tubes, 22.5 mL of the corresponding PCR master mix was added. Obtaining the sample DNA extraction, 2.5 mL of the liquid was added to each of the two tubes. Each was placed into their corresponding gradient thermal cycler (Eppendorf, Mastercycler). The protocol for each thermal cycler is located in Table 4. Once the cycles were completed, the PCR product was either frozen for future use or immediately assayed with gel-electrophoresis.

Gel-electrophoresis was performed using the PCR products obtained earlier using Biorad Gel Electrophoresis components. A 1 % agarose gel was prepared using 0.5 g of agarose and enough cold 1X TAE to bring the total mass to 50 g. The mixture was boiled and allowed to cool until the flask was cool enough to handle. The contents were then poured into a gel tray

with plastic comb added to allow for the wells necessary to add the PCR products. This gel was allowed to solidify in the refrigerator for at least 10 min. if not overnight.

Once the gel had solidified, manipulation of the PCR products began. On a piece of Parafilm, a 2 mL dot of 6 X blue running dye was placed. To each dye dot, 5 mL of PCR product was added. The entire 7 mL mixture was then pipetted into one of the wells in the solidified agarose gel. The process continued until all wells were filled, leaving room for two controls and a 100 base pair (bp) ladder for reference. The two controls included PCR product that was created using DNA extracted from the raw influent. The gel was then placed into the electrophoresis apparatus. This apparatus was a box that contained electrodes at either end of the gel. Once the gel is loaded, enough cold 1 X TAE was added to the apparatus to just cover the top of the gel. The lid was then placed on top of the gel and the machine was allowed to run at 120 V (volts) for 45 minutes. During this process the DNA segments traveled through the agarose gel by the aid of electricity. The DNA separates throughout the gel based on its molecular size and therefore leaves a band at a specific point on the gel. The bp ladder was used to determine the size of the bands present.

After the electrophoresis was finished, the gel was then stained with 2 mL of 10,000 X SYBR green dye in 20 mL of 1 X TAE for 20 min. Once the gel was stained, the bands were observed using UV transilluminator light in a dark room. The human-specific bands occur at 520 bp on the gel whereas the general *Bacteroides* (BAC32) band could occur anywhere between 119 and 222 bp (Ahmed, 2009; Bernhard, 2000b). A photograph was taken of each gel.

A.4. Carbon Utilization Profiles

Using the mEI membrane-filtration plates, a total of 90 colonies were selected from Phase I and 75 selected from Phase II. The colonies selected were spread out throughout the

entire sampling period to get a view of the population changes from start to finish. All of these colonies were selected from the mEI plates and inoculated onto a nutrient-rich Tryptic Soy (T-soy) Agar (TSA) plate and incubated at 35°C for at least 24 hrs. A freezer stock solution was made using 70% nutrient broth to 30% glycerol. One and one-half (1 ½) mL of the solution was added to a 2 mL sterile plastic freezer vial. This was repeated for all colonies. The TSA plates containing the *Enterococcus* colonies were swabbed and the swab was placed into each 1.5 mL solution, inoculating it heavily. The freezer vial stock cultures were frozen to use for BIOLOG identification and ARA.

Identification of 90 *Enterococcus* colonies was performed by using BIOLOG GP2 MicroPlate system (Hayward, CA). This system utilizes a carbon source profile and has proven successful in identifying *Enterococcus* species (Graves, 2009). Beyond identifying species of bacteria, BIOLOG has been shown a successful MST method (Hagedorn, 2003). An *Enterococcus* colony was inoculated onto a Biolog Universal Growth (BUG) plus blood agar plate at 35°C for 24 hours. From the BUG plate, a BIOLOG Inoculating Fluid tube was heavily inoculated to match a turbidity standard that is provided with the BIOLOG materials. Three or four drops of thioglycolate solution were then added to the fluid. This ensures the breakup of bacterial clumps. All the fluid was then poured into a plastic tray provided. Using a multi-channel pipettor, 150 mL of the fluid was added to each of the 96 wells in the BIOLOG plate. The plate was then incubated at 35°C for 24 hrs. Once the incubation period was over, the BIOLOG plate was then read by the BIOLOG plate reader. Genus and species identification was acquired within 1-2 min., and a printout of each identification was obtained. Of the 90 colonies (cultures) from each Phase of the experiment, only 72 colonies (from each Phase) were identified as an *Enterococcus* species.

A.5. Antibiotic Resistance Analysis (ARA)

Although only 72 colonies from Phase I and II samples gave a positive result using BIOLOG, all original colonies were included in ARA. When utilizing ARA, BIOLOG is usually not employed before-hand as an identification method (Graves, 2002). So this was the reasoning behind using all colonies.

In order to perform ARA, cultures must be grown up in a 96 well plate. Each well contains a different culture in Enterococcosel broth (BD, Sparks, MD). The plates are incubated at 35°C for 24 hours. Once incubated, a 48 prong replica plater is used to aseptically inoculate 31 T-soy agar plates (including a positive control) containing different concentrations of various antibiotics. The plates are incubated at 35°C for 48 hours. Table 5 shows the concentrations and antibiotics used.

Once the antibiotic plates have incubated, each culture is inspected for growth at every concentration of every antibiotic. The growth of each culture on the plates containing antibiotics was compared to the control plates. If the growth was equal, the culture was considered to be resistant to that antibiotic and concentration. Cultures that were positive for growth were recorded as a "1" and those that did not were recorded as a "0".

V. Statistical Analysis

Statistical analysis was performed on all MST methods using SAS-JMP statistical software (version 8.0, SAS Institute Inc.). Discriminant analysis was utilized to determine the rate of correct classification for ARA (Harwood, 2000). Isolates used in ARA were compared to a library of known organisms collected mainly in northern Virginia. Logistic regression was used to compare the *Bacteroides* human-specific primer test to FIB concentrations (Ahmed, 2008). Fluorometry results were compared with FIB concentrations to determine if fecal

pollution existed. Fluorometry results that were above 100 FSU were considered positive for fecal pollution (Dickerson Jr., 2007).

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Table 2. Components of PCR Master Mix in µL.

Promega PCR Mix	BSA	Forward Primer	Reverse Primer	Nuclease Free Water
125	10	10	10	70

Table 3. DNA primer sequence for *Bacteroides.* Bac32 is the sequence for all *Bacteroides* and HF183 is for the human-specific *Bacteroides*. Bac708 in both cases is the reverse primer.

Primers	Oligonucleotide Sequence (5' – 3')	bp
Bac32F	AAC GCT AGC TAC AGC CTT	700
Bac708	CAA TCG GAG TTC TTC GTC	
HR183	ATC ATG AGT TCA CAT GTC CCG	520
Bac708	CAA TCG GAG TTC TTC GTC	

Table 4. PCR Protocol for each of the two primer sets.

Cycles	Bac32/Bac708		HF183/Bac708	
	Temp °C	**Time**	**Temp °C**	**Time**
1	95	5 min	95	5 min
35	94	1 min	94	30 sec
	53	1 min	59	1 min
	72	1.5 min	72	2 min
1	72	5 min	72	10 min

Table 5. Antibiotics and concentrations used in ARA.

Antibiotic Used:	Code	Concentration (µg/mL)
Chlortetracycline	CTC	60
		80
		100
Oxytetracycline	OTC	20
		40
		60
		80
		100
Streptomycin	STR	40
		60
		80
		100
Cephalothin	CEP	10
		15
		30
		50
Erythromycin	ERY	10
		15
		30
		50
Tetracycline	TET	10
		15
		30
		50
		100
Neomycin	NEO	40
		60
		80
Vancomycin	VAN	2.5
Amoxicillin	AMX	2.5

Chapter 4. Results and Discussion

I. Water monitoring

A. Dissolved oxygen (DO)

DO measurements were taken throughout the sampling period to monitor changes due to re-circulation vs. non-circulation and the different treatments administered. Table 6 shows the average DO measurements for Phase I, non-circulated columns. At the beginning of sampling (Day 0 and 1), all treatment columns behaved similar to the controls. As the bacteria became established, the DO in the treatment columns began to decline and differed from the control columns from Day 2 until Day 10. After Day 10, DO measurements in the treatment columns began to rise and were similar to the control columns. Bacterial metabolism could account for the decline in DO readings during the middle of the sampling period (Amon, 1996). Figure 9 (A) illustrates the decline in DO readings as the bacteria reduced the available O_2 in Treatments 1, 2, and 3.

The re-circulated columns in Phase I performed similarly to the non-circulated column (Table 7). On Days 0 and 1, there was no difference between the control columns and the different treatments. From Day 2 to Day 10, the treatment columns were different from the controls. What is interesting is that the control columns had DO readings lower than the treatment columns for Days 2, 4, and 7 whereas the opposite trend was true in the non-circulated columns. This difference between the treatments and controls may not be due to the introduction of influent into the treatment columns but merely due to experimental design. After Day 16, all columns (controls and treatments) were not significantly different from one another. DO readings stayed relatively stable throughout the sampling period because re-circulation added

oxygen to the water at a constant rate. Figure 9 shows a graphic representation of the DO measurements for both non-circulated and re-circulated columns in Phase I.

DO measurements in Phase II, non-circulated columns behaved somewhat differently from those in Phase I, but the same trends were observed. There was a little more variation between treatments and the controls (Table 8). As with the Phase I columns, no difference was detected between the controls and treatments until Day 4. From Day 4 to Day 16, the treatment columns were different from the controls. On Day 4, Treatment 1 and 3 columns were different from one another but this difference did not occur again until Day 16. This fluctuation that did not occur in Phase I columns could be due to the different dosage regiment (CSO event vs. low-volume sewage leak). By Day 22, all columns showed DO readings that were not different from one another. On Day 36, the last day of sampling, Treatment 3 columns showed DO readings that were different from the control columns. This reading could correlate with re-growth of bacteria, but not from either of the FIBs (*E. coli* or *Enterococcus*) that were tested. The differences found during the middle of sampling are not large and are mostly likely due to experimental design and not differences between the three treatments.

Like the comparison between Phase I and II non-circulated columns, DO readings in Phase II re-circulated columns showed more variation between treatments than those in Phase I Table 9). On Day 2, Treatment 1 and 3 were different from one another. Treatment 3 columns had higher DO readings than Treatment 1 columns. This difference did not continue through Day 4 and 7, but by Day 10 resurfaced. By Day 16, all columns had returned to similar DO readings until the end of sampling on Day 22. As with the non-circulated columns, this variation between treatments corresponds to the difference in pollution events (Phase I and II). Figure 10 shows the DO readings for Phase II non- and re-circulated columns.

The same trends in DO readings were seen throughout both Phases. In the case of the non-circulated columns, bacterial metabolism decreased the DO as influent was being introduced and as bacteria died off the DO increased. In both Phase I and II re-circulated columns the re-circulating water caused the DO readings to stay constant throughout the sampling periods.

B. Temperature

Like DO, changes in temperature can be directly related to bacterial metabolism. As the bacteria metabolize nutrients, they give off energy that could be measured in the form of an increase in temperature in the bacteria's surroundings (Madigan, 2000). Differences in temperature were not seen in any of the re-circulated columns (Tables 11and 13), but slight differences were seen in the non-circulated columns. Comparisons between temperature to DO readings in the Phase I re-circulated columns revealed no correlation between the two ($r = -0.181$).

In the Phase I non-circulated columns, differences occurred on Days 1 and 7 (Table 10). On Day 1, both Treatment 2 and 3 temperatures were different than the control and Treatment 1 columns. Also, on Day 7, Treatment 2 columns were different from the control columns. In both of these cases, the difference in temperature does not seem to be related with bacterial metabolism. If that was the case, Treatment 1 had the highest introduction of influent and therefore the highest introduction of bacteria on Day 0 and would have the most potential for an increase in temperature due to bacterial metabolism. While the temperature in Treatment 2 and 3 columns were lower than Treatment 1, Treatment 1 columns were not different than the control columns. On Day 7, Treatments 1 and 2 had ceased for about one week whereas Treatment 3 had just ended on Day 3 (4 days prior). If bacterial metabolism was responsible for the difference in temperature, Treatment 1 and 3 columns would have been different from the control columns.

Because the non-circulated and re-circulated columns in Phase I were setup back to back and not run simultaneously, temperatures in the non-circulated columns were lower than those in the re-circulated columns (Fig. 11).When comparing the change in DO readings with temperature changes in the non-circulated columns a strong correlation between the two was not evident (r = 0.172).

In Phase II non-circulated columns, differences in temperature occurred at Day 2 and 10 (Table 12). On Day 2, only Treatment 2 columns were different from the control columns. In this case, the temperature in the Treatment 2 columns was slightly higher than the control columns. This could be an indication of bacterial metabolism causing the rise in temperature but one would expect the other Treatments (1 and 3) to also have different temperatures at Day 2. Comparing means on Day 10 temperatures indicates that the only difference is between Treatment 2 and 3 columns. This does not really correspond to metabolic activity because there is no difference between these treatments and the control columns. Phase II, non-circulated columns were set up first in the month of May and therefore had lower average temperatures than the re-circulated columns that were set up after sampling had ended on the non-circulated columns (Fig. 12). Comparing temperature in the non-circulated columns with DO readings does not show a strong correlation (r = -0.268). No strong correlation existed between the change in temperature and the change in DO in the re-circulated columns either (r = 0.0279).

C. Electrical Conductivity (EC)

EC was measured on all columns throughout the sampling periods to monitor the salinity and dissolved materials in the water. As the salinity and/or dissolved materials increase, the EC also increases (Bruckner, 2010). In both Phases, EC readings exhibited the same trends over

time. As influent was added, the salinity of the bay water was diluted and therefore EC readings for the treatment columns began lower than the control columns.

On Day 0, in Phase I non-circulated columns, the EC readings in each treatment were different from each other and the control columns (Table 14). As time progressed, the differences between the treatments and controls became less and less apparent. By the end of sampling there were no differences between the treatments, only between the treatments and control columns.

In Phase I re-circulated columns, EC measurements began with differences between Treatment 1 and Treatments 2 and 3 and between all treatments and the control columns (Table 15). Again, Treatment 1 was one large introduction (2.2 L) of influent on Day 0, Treatment 2 consisted of 1.1 L of influent introduced on Days 0 and 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3. As time progressed, variation between treatments and controls lessened until Day 16 when there was no difference between any treatment and the control columns.

In both the re-circulated and non-circulated columns, evaporation was evident by the increase in EC readings over time. When comparing Phase I re-circulated and non-circulated columns, the re-circulated columns showed more of an increase in EC over time than the non-circulated columns (Fig. 13). The increased evaporation in the re-circulated columns corresponds well with the increased temperature of those same columns.

Phase II non-circulated columns also showed the same trend in EC readings over time as Phase I columns (Table 16). As time progressed, all treatments were not significantly different from one another but all were significantly different from the control columns. What is interesting with the Phase II non-circulated columns is that the EC readings decreased up until

Day 16 where they made a slight increase until the last day of sampling (Fig. 14). Looking back at the weather during this sampling period (May 2010), there were several days of rain that could have added to the dilution of the water and caused lower EC readings.

The average EC readings for Phase II re-circulated columns behaved similarly to the Phase I re-circulated columns (Table 17). No variation was present at the beginning of sampling. But as influent was added to the columns, differences began to occur within the treatments and between the treatments and control columns. By the end of sampling, there were no differences between any of the treatments or control columns. As with the re-circulated columns from Phase I, Phase II re-circulated EC measurements increased over time due to evaporation.

D. pH

pH was measured at the beginning and end of each sampling cycle. The pH for Phase I non-circulated columns were not different between treatments or controls (Table 18). The same was true for the Phase I re-circulated and Phase II non-circulated columns (Tables 19 and 20). Figure 15 illustrates the average pH for Phase I non- and re-circulated columns. The average pH measurements for Phase II re-circulated columns did show a difference between the control columns and Treatment 1 columns on Day 0 (Table 21). But on the last day of sampling, there was no difference between treatments or control columns. Figure 16 illustrates the average pH for the non- and re-circulated columns for Phase II on the first and last day of sampling.

II. Fecal Indicator Bacteria (FIB) counts

FIB counts were measured throughout the sampling period in both Phase I and II. Phase I columns simulated a CSO event in which large volumes of influent were added to each of the three treatments. Phase II columns simulated a low-volume sewage leak where small volumes of influent were added to the treatment columns for extended periods of time.

Within both Phases, *Enterococcus* and *E. coli* counts were looked at closely to determine if stabilization and/or re-growth occurred. Stabilization would occur after the last introduction of influent and would be characterized by prolonged CFU/100 mL numbers for more than one sampling day. Re-growth would be characterized by a spike in CFU/100 mL after the last introduction of influent. It would only be considered re-growth if it occurred throughout the three duplicate treatment columns and not dependent on just one alone.

A. Phase I

A.1. Non-circulated

Enterococcus counts in Phase I non-circulated columns closely followed the introduction of influent by the three treatments (Fig. 17). *Enterococcus* counts spiked either on Day 0 or Day 1 for all three treatments in the water column (A). Counts then very quickly declined by either Day 2 or 4. Treatment 1 (1L on Day 0) counts then increased at Day 7 and stabilized until the end of sampling. The same can be said for the other 2 treatments. An increase in *Enterococcus* counts for Treatments 2 (500 mL over 2 days) and 3(250 mL over 4 days) occurred on Day 16 and stabilized until the end of the sampling time. There was a spike in *Enterococcus* counts for the control columns on Day 21, which indicated contamination in one of the columns.

E. coli counts also closely followed the introduction of influent (Fig. 19). Unlike the *Enterococcus* counts, *E. coli* quickly declined in the water column (A) and did not experience stabilization or re-growth. *E. coli* in all treatments had declined by Day 4.

Enterococcus counts in the sediment/water interface (B) behaved similarly to those in the water column (A) (Fig. 17). Counts spiked on Day 1 for all three treatments and declined sharply thereafter. All three treatments experienced a second *Enterococcus* spike and gradual

stabilization until the end of sampling. The spikes indicate re-growth of *Enterococcus* because they occurred after the last introduction of influent.

E. coli counts in the sediment/water interface (B) again closely followed the introduction of influent and the delay associated with settling out of the water column (Fig. 19). Counts spiked at both Day 2 for Treatment 3 or Day 4 for Treatments 1 and 2. With all three treatments, *E. coli* was evident until Day 21. From the time that the counts spiked until *E. coli* was undetectable, a steady decline was evident indicating that no stabilization or re-growth occurred.

As *Enterococcus* settled out of the water column and began to collect in the sediment (C), counts spiked on Day 1 for Treatment 1 and Day 2 for Treatments 2 and 3 (Fig. 17). *Enterococcus* then declined and stabilized until Day 30 for Treatments 2 and 3. Treatment 1 experienced a large spike in *Enterococcus* starting at Day 4 and peaking at Day 21. The spike was due to one column and therefore was most likely due to a contamination issue (wildlife or human error) and not re-growth.

E. coli in the sediment (C) spiked between Day 1 and 2 (Fig. 19). The decline in counts was much more dramatic than those in the sediment/water interface. *E. coli* counts were evident until Day 16 with all three treatments, then undetectable after that.

A.2. Re-circulated

Enterococcus counts in Phase I re-circulated columns followed closely with the introduction of influent (Fig. 18). Because the counts in the re-circulated columns were lower than those in the non-circulated columns, a close-up was needed (Fig. 18). Counts in the water column (A) began high as influent was being introduced. Treatment 1 (2.2 L on Day 0) counts started high on Day 0, but declined rapidly by Day 2. Treatment 2 (1.1 L over 2 days) experienced a spike in counts on Day 1 and decline steadily by Day 4. Treatment 3 (550 mL

over 4 days) experienced a spike in counts on Day 2 and declined rapidly by the next sampling (Day 4). All treatments experienced a steady decline in numbers and remained low without any indication of re-growth or stabilization.

E. coli counts in the re-circulated columns were also much lower than those in the non-circulated columns and required a closer view in order to see the trends (Fig. 20). No evidence in re-growth or stabilization of *E. coli* was present in the water column (A). Counts closely followed the introduction of influent. Counts for Treatment 1 had declined by Day 2 and for Treatments 2 and 3 had declined by Day 4.

Enterococcus was present in the sediment/water interface (B) on Day 0 for all three treatments (Fig. 18). Treatment 1 counts did not spike, but experienced a steady decline by Day 4. Treatment 2 experienced a large spike in *Enterococcus* counts on Day 2 which was also present on Day 4. Since this spike was due to only one of the three duplicate columns, this was most likely due to contamination of the column and not due to re-growth. Treatment 3 counts spiked on Day 1 and steadily declined until Day 10. After all three treatments declined, they remained low until the end of sampling. Therefore, there was no indication of re-growth or stabilization.

No growth or stabilization was evident for *E. coli* in the water/sediment interface (B) (Fig. 20). There was a delay in the count spike for Treatment 3 (Day 2). But all *E coli* counts declined sharply and were not evident by Day 7.

Enterococcus counts in the sediment (C) shows the delayed spikes indicating the settlement of the influent out of the water and into the sand (Fig. 18). Counts in all three treatments spiked between Days 1 (Treatment 3) or 2 (Treatments 1 and 2). After *Enterococcus* counts spiked for Treatment 1 on Day 2, they steadily declined until Day 16. Treatment 2 counts

declined rapidly by the next sampling (Day 4), but experienced another spike on Day 7. Because the spike was due to only one of the triplicate columns, contamination was likely the cause. Also the counts had declined rapidly by Day 10 and did not show any evidence of stabilization. After Treatment 3 experienced a spike in counts on Day 1, they declined rapidly by Day 2 and did not indicate re-growth or stabilization.

E. coli counts in the sediment (C) also followed closely the delay of the introduction of influent (Fig. 20). All three treatments experienced a spike on Day 2 and immediate decline by Day 4. Again, re-growth or stabilization of *E. coli* was not evident.

B. Phase II

B.1. Non-circulated

Enterococcus counts for the non-circulated columns did not survive long in the water column (A) (Fig. 21). The concentration of *Enterococcus* was directly proportional to the amount of influent added to each of the treatments. By Day 2 counts had dropped off dramatically but maintained their populations until Day 7 for Treatment 1 (440 mL over 5 days), Day 10 for Treatment 2 (220 mL over 10 days), and Day 16 for Treatment 3 (146.67 mL over 15 days). All but one treatment stayed low with no evidence of re-growth. Treatment 3 experienced a slight increase in *Enterococcus* counts on Day 29. This increase was due to only one column and therefore was most likely contamination and not actual re-growth of *Enterococcus* in the water column.

E. coli was able to survive longer in the water column (A) than *Enterococcus* (Fig. 23). Spikes in counts were evident in all three treatments and corresponded to the amount of influent added to each treatment. Treatment 1 counts were the first to drop on Day 7 where they remained low for the rest of sampling. Treatment 2 counts spiked on Day 7, dropped

dramatically by the next sampling day (Day 10) and maintained low with no evidence stabilization or re-growth. Treatment 3 counts spiked at Day 4 and steadily declined until Day 22, where no evidence of stabilization was evident.

Enterococcus counts in the sediment/water interface (B) also followed the introduction of influent (Fig. 21). Spikes in counts were evident for all three treatments on Day 1. With all three treatments, a sharp decline in *Enterococcus* occurred on Day 2. All three treatments spiked a second time on Day 4 and steadily declined until Day 22. This spike was mostly likely due to the addition of influent since all three treatments were still receiving influent. After Day 22, re-growth or stabilization was not evident in any of the three treatments.

E. coli in the sediment/water interface (B) behaved similarly to *E. coli* in the water column (A) (Fig. 23). Counts spiked in all three treatments on Day 4 and correlated to the amount of influent added to each treatment. Sharp declines occurred in both Treatments 1 and 2 from Day 4 to Day 10 where the counts more steadily declined until Day 22. Because influent was added in small doses to Treatment 3 for 15 days, counts steadily declined after the spike on Day 4 until Day 22. After Day 22, there was no evidence of re-growth or stabilization in any of the three treatments.

Unlike the large characteristic spikes in *Enterococcus* counts in the water column (A) and sediment/water interface (B) associated with the introduction of influent, those large spikes were not evident in the sediment (C) (Fig. 21). With one exception within all three treatments, *Enterococcus* counts were never higher than 300 CFU/100 mL. There was one large spike in *Enterococcus* counts in Treatment 2 on Day 4. This spike was due to one column only and therefore was most likely due to contamination. The counts in all three treatments remained low

throughout sampling and indicate that stabilization in sediment was evident but in very low numbers.

Lower counts were also evident for *E. coli* in the sediment (C) (Fig. 23). Counts within all three treatments spiked between Days 10 and 16. Treatment 3 count began higher than the other two treatments due to one column, but otherwise the spikes were associated with the time it took for the bacteria to settle out of the water column. During collection of this sample, contamination likely occurred since *E .coli* was not evident in either of the other two columns. After the spikes in *E. coli* counts, all three treatments experienced a steady decline that ended at Day 29. No re-growth or stabilization was evident after Day 29.

B.2. Re-circulated

Like with the Phase I columns, *Enterococcus* counts in the re-circulated columns were much lower than those in the non-circulated columns (Fig. 22). Figure 22 shows a close-up of the three sampling locations (A, B, and C). *Enterococcus* counts in the water column (A) closely followed the introduction of influent. Treatment 1 (968 mL over 5 days) counts spiked twice at the beginning of the sampling period (Day 1 and Day 4). By Day 7, counts in Treatment 1 had dropped off drastically. On Day 16, *Enterococcus* counts in Treatment 1 spiked for a third time. This increase in colony counts was found only in one column and was most likely due to contamination of the column because a spike in counts was found in that one column in the sediment/water interface (B) as well. Treatment 2 (484 mL over 10 days) counts also spiked twice in the water column—once on Day 2 and the other on Day 10. After the second spike, *Enterococcus* counts had declined by Day 16. The spike in *Enterococcus* counts in both Treatments 1 and 2 were associated with the introduction of influent and not re-growth. Treatment 3 (322.67 mL over 15 days) counts spiked one on Day 7. Counts showed a steady

increase up until that day, indicative of the low volume introduction of influent. After Day 10, counts began to fall until Day 16 where they never recovered. Therefore, stabilization was not evident in any of the treatments.

E. coli concentrations in the Phase II re-circulated columns were similar to those of *Enterococcus* (Fig. 23). Counts spiked between Days 2 and 4 for all three treatments. In the water column (A), Treatment 1 counts sharply declined on Day 7 after peaking and staying steady at Days 2 and 4. Treatment 2 counts also spiked at Day 2, but because influent was being added for a total of 10 days, the count slowly declined until Day 10. Treatment 3 counts spiked at Day 4 but decreased by 2.00×10^4 CFU/100 mL on Day 7. But on Day 10, a second spike in *E. coli* was detected. This was in accordance with the introduction of influent in the Treatment 3 columns for 15 days. By Day 16, *E. coli* counts were no longer detectable in the Treatment 3 columns. There was no indication of re-growth or stabilization of *E. coli* in any of the treatments.

In the sediment/water interface (B), Treatment 1 *Enterococcus* counts spiked twice (Days 1 and 4) before becoming undetectable by Day 10 (Fig. 22). Treatment 2 counts steadily increased until their peak at Day 10. By Day 16, *Enterococcus* was undetectable in the Treatment 2 columns. Treatment 3 counts also spiked twice—once at Day 2 and once at Day 7. Counts remained lower than the other two treatments, but maintained detectable until Day 16. Because the spikes occurred during the introduction of influent, no re-growth or stabilization was detected in any of the columns.

E. coli in the sediment water interface (B) showed predictable spikes in numbers (Fig. 23). Treatment 1 counts spiked on Day 2 and sharply decreased to undetectable levels by Day 10. Count in Treatment 2 columns also spiked (1.40×10^4 CFU/100 mL) on Day 2. *E. coli*

sharply decreased until counts were undetectable at Day 10. Treatment 3 counts spiked at two locations (Day 4 and Day 10) during the sampling period. *E. coli* counts were undetectable by Day 16. The spikes in *E. coli* counts were directly related to the introduction of influent within all three treatments. Therefore there was no evidence of re-growth or stabilization in any of the treatments.

Enterococcus counts in the sediment (C) were at much lower concentrations than the water column (A) or sediment/water interface (B) (Fig. 22). A large spike in *Enterococcus* counts occurred for both Treatments 1 and 2. In Treatment 1, the spike in *Enterococcus* occurred on Day 4 and immediately dropped back to very low counts by Day 7. The same trend held true for Treatment 2 counts except the spike occurred on Day 7. Treatment 3 counts also experienced a spike on Day 7, but at a lower concentration than Treatments 1 and 2. Even the control columns experienced a spike in *Enterococcus* counts on Day 7. The spikes in counts of the treatment columns indicate the settling of influent out of the water column. The spike in the controls was due to one column and therefore indicative of contamination of the column.

As with the *Enterococcus* counts, *E. coli* counts also spiked in all three of the treatments (Fig.23). In the sediment (C), Treatment 1 counts spiked on Day 2 and Day 7 but remained low throughout the sampling period until Day 10. Treatment 2 counts spiked on Day 7 and decreased upon the next sampling period. This spike was due only to one column and therefore is most likely a contaminant and not re-growth. Treatment 2 counts remained detectable until Day 16. Treatment 3 counts spiked on Day 4 decreased dramatically on Day 7 and were not detectable by Day 10. This spike on Day 7 was only caused by one column and not evident in all three. So the reason for the spike was most likely contamination and not re-growth. Like Treatment 2,

Treatment 3 counts were detectable until Day 16. There was no evidence of re-growth or stabilization in any of the columns.

C. Summary

Population stabilization was only evident in the Phase I (CSO event), non-circulated columns. It was only present in the water column (A) and sediment/water interface (B) and not in the sediment (C) (Fig. 17). It was also only evident with *Enterococcus* and not with *E. coli*. Re-growth was also only evident in the Phase I, non-circulated columns—only present with *Enterococcus* in the water column (A) and sediment/water interface (B) (Fig. 17). Stabilization or re-growth was not evident in any location in the Phase II (sewage leak) columns. With the Phase I columns, *Enterococcus* was able to survive longer than *E. coli*. In the Phase II columns, *E. coli* was able to survive better than *Enterococcus*.

III. Biolog Classification

Presumed *Enterococcus* isolates were collected from both Phases throughout the sampling period for the non-circulated and re-circulated columns. Once again, Phase I columns simulated a CSO event with large introductions of influent. Phase II columns simulated a low-volume sewage leak with small introductions of influent over a prolonged period of time. Before ARA was performed on each of these isolates, species were identified using Biolog. The purpose of the species identification was to observe the changes in distribution over time and also to observe the differences between species distribution between the non- and re-circulated columns. For the purposes of comparison, the sampling period was divided into three categories—beginning, middle, and end.

A. Phase I

A.1. Non-circulated

Enterococcus gallinarum was the dominant species found in the non-circulated Phase I columns (Fig. 24D). This was also the case (40%) in the beginning of sampling, followed by *Enterococcus faecium* (27%) (Fig. 24A). *Enterococcus faecalis* became the dominant species by the middle of sampling, followed by *E. gallinarum* (27%) (Fig. 24B). By the end of sampling, *Enterococcus avium* and *Enterococcus casseliflavus* made up 60% of the species distribution (Fig. 24C). As time progressed, more species were introduced but only two categories disappeared. *E. faecalis* was not present at the end of sampling. Also, there were no isolates that did not identify as *Enterococcus*. Isolates that were not identified as *Enterococcus* (Other[1]) were found at the beginning and middle of the sampling period (Fig. 24A & B).

A.2. Re-circulated

As with the non-circulated columns, *E. gallinarum* was the dominant species (46%) in the re-circulated columns (Fig. 25D). In fact, *E. gallinarum* was the dominant species at the beginning, middle, and end of sampling (Fig. 25A-C). But unlike the non-circulated columns, species diversity was greatest at the beginning of sampling (Fig. 25A). Six different *Enterococcus* species were present, with *E. gallinarum* taking up 46% of the species distribution. By the middle of the sampling period, three species of *Enterococcus* were not present and only one additional species was present (*Enterococcus flavescens*) (Fig. 25B). By the end of sampling, only two *Enterococcus* species were present (*E. gallinarum* and *E. flavescens*). *E. gallinarum*

[1] Other species included *Stretococcus gordonii, Pediococcus dextrnicus, Pediococcus acidilactici/parvulus, Alloiococcus otitis, Staphylococccus sciuri* and *Streptococcus infanterius ss coli*. Eight cultures in Phase I showed no identification and 1 colony in Phase II.

consisted of 72% of the species distribution, while *E. flavescens* made up 14%. The other 14% consisted of non-*Enterococcus* species.

B. Phase II

Unlike the Phase I isolates, *E. gallinarum* was not the dominant species in either the non- or re-circulated columns. Instead, *E. faecium* and *E. faecalis* were the two dominant species. Other species such as *Enterococcus mundtii*, *Enterococcus hirae* and *E. gallinarum* were present, just at lower percentages than in the Phase I columns.

B.1. Non-circulated

In the non-circulated columns, *E. faecium* (52%) was the dominant species present (Fig. 26D). *E. faecium* and *E. mundtii* made up 50% of the total distribution (25% and 25%) at the beginning of sampling (Fig. 26A). Twenty five percent of the isolates were identified as non-*Enterococcus* species. By the middle of sampling, *E. faecium* (59%) still dominated the total distribution (Fig. 26B). *E. faecalis* and *E. hirae* were still present, but at lower percentages than at the beginning and *E. gallinarum* was present at 6%. By the end of sampling, the diversity had diminished to just 3 species (Fig. 18C). *E. faecium* was again the dominant species making up 60% of the total distribution. *E. gallinarum* and *E. mundtii* were also present at 20% each.

B.2. Re-circulated

In the re-circulated columns, *E. faecalis* was the dominant species (45%) (Fig. 27D). At the beginning of sampling, only 3 *Enterococcus* species were present (Fig. 27A). *E. mundtii* made up 38%, E. *faecium* made up 37%, and *E. gallinarum* made up 25% of the distribution. By the middle of sampling, more species were introduced. *E. faecalis* became the dominant species (55%), with *E. faecium* coming in second with 25% (Fig. 27B). *E. hirae* was also introduced

with 5% of the species distribution. But by the end of sampling, only 2 *Enterococcus* species were present (Fig. 27C). *E. faecalis* made up 60% and *E. faecium* made up 10% of the species distribution. The remaining 30% were identified as non-*Enterococcus* species.

C. Summary

In Phase I there were many different species identified in both the non- and re-circulated columns. Because of all the variation, the species distribution would indicate mixed sources of fecal contamination. But in the Phase II isolates, identifications resembled mostly species associated with human pollution and were more closely related to the introduction of influent (Wheeler, 2002).

IV. Antibiotic Resistance Analysis

ARA was performed on all the isolates tested for identification using the Biolog system. The sampling time was again divided into three categories—beginning, middle, and end. Again, Phase I columns simulated a CSO event where large amounts of influent were added to the different treatments. Phase II columns simulated a low volume sewage leak where small amounts of influent were added over longer periods of time.

A. Phase I

A.1. Non-circulated

Source identification using ARA showed that the majority of isolates were from birds (44.44%) and wildlife (40.00%) (Table 22). Human sources were present in the non-circulated columns but only made up 8.88% of the total isolates. Both bird (60.00%) and human (20%) isolates were highest at the beginning of sampling and decreased as time progressed. Because the bay water was not sterilized beforehand, it is thought that the bird sources were already

present (from the presence of shore birds) in the bay water and decreased as those sources died off. The increased human isolates at the beginning of sampling, corresponds to the introduction of influent during that time. Wildlife isolates increased over time, which is indicative of the contamination issues seen in the FIB counts. Pet and livestock sources also increased slightly over time, this could just be due to the lack of isolates present at the end of sampling and not necessarily due to introduction of these sources.

A.2. Re-circulated

Pet (27.91%) and wildlife (39.53%) make up the majority of source isolates in the re-circulated columns (Table 23). Bird sources make up 25.58% of the total number of isolates. Wildlife sources increased over time, whereas pet, human and bird sources decreased over the sampling period. Livestock sources were evident at the beginning of sampling (6.67%) but were not detected throughout the rest of the sampling period.

B. Phase II

B.1. Non-circulated

In the non-circulated columns, bird (37.50%) and wildlife (22.50%) make up the majority of isolate sources (Table 24). Both wildlife and bird sources exhibited the same trends as those in the Phase I non-circulated columns. The percentage of bird isolates decreased over time and the wildlife sources increased over time. Human sources actually showed an increase (25.00% to 28.57%). Pet sources also showed an increase and by the end of sampling made up 42.86% of the total isolates tested. Livestock sources were only evident at the end of sampling (7.14%).

B.2. Re-circulated

As with the non-circulated columns, bird (53.49%) and wildlife (25.58%) made up the majority of isolates found in the re-circulated columns (Table 25). Bird sources increased from 37.50% at the beginning of sampling to 60.00% at the middle and decreased slightly to 53.33% by the end of sampling. Human sources were, again, highest in the beginning of sampling (37.50%) and decreased over time. As with the non-circulated columns, wildlife sources increased over time. Both pet and livestock sources were present at some point during the sampling period but relatively low (4.65% and 2.33 %, respectively).

C. Summary

ARA results did not indicate a large proportion of human fecal isolates. One of the disadvantages of ARA is the nature of the bacterial genome itself. Bacterial genomes are capable of mutating relatively easily and can lose or gain resistance to antibiotics over time (Simpson, 2002). Differences in antibiotic resistance patterns can also be due geographical variations (Ebdon, 2006). Because the library used in this ARA study was based largely out of northern Virginia isolates, this could account for the small percentages of human source identifications.

V. Bacteroides

The presence of *Bacteroides,* both the human-specific and non-specific types, was tested for throughout the sampling periods. The presence of *Bacteroides* was tested throughout each treatment and within each sampling location (A, B, and C).

A. Phase I

A.1. Non-circulated

The non-specific *Bacteroides* (using Bac32 primer) was detected on Day 0 throughout each treatment and control columns (Table 26). In treatment 1, the human-specific *Bacteroides* was present in the same capacity as the non-specific *Bacteroides*. In Treatment 2, the human-specific *Bacteroides* was present in 100% of the samples. That was not the case with the non-specific *Bacteroides*. Samples taken at the sediment/water interface revealed that only 2/3 were positive for the non-specific *Bacteroides.* In Treatment 3, human-specific *Bacteroides* was present in 100% of the water column and sediment/water interface samples. Again, only 2/3 of the samples taken at the sediment/water interface were positive for the non-specific *Bacteroides.* The control columns on Day 0 showed 1/3 of the samples taken at each location (A, B, and C) were positive for the non-specific *Bacteroides*. No control samples tested positive for the human-specific *Bacteroides.* By Day 7, the human-specific *Bacteroides* was no longer present in any of the columns. The non-specific *Bacteroides* was still present in the majority of samples taken on Day 7. By Day 30, the non-specific *Bacteroides* was still present in some of the sampling location in each treatment and even in the control columns. The human-specific *Bacteroides* was still absent from all samples.

The human-specific *Bacteroides* was present in the majority of samples that were also positive for the non-specific *Bacteroides*. One would hope that this would be true since the non-specific *Bacteroides* served as a positive control for the human-specific *Bacteroides.* The average rate of correct classification between the two was 95.12%. Using logistic regression, comparisons between both FIBs and the human-specific *Bacteroides* were made. Comparing the *Enterococcus* counts with the probability of human-specific *Bacteroides* being present indicates

a strong relationship between the two ($p < 0.0001$). The same was true when comparing *E. coli* counts with human-specific *Bacteroides* ($p < 0.0001$). In other words, as either FIB counts rise, the probability the presence of human-specific *Bacteroides* also rises.

A.2. Re-circulated

The presence of both the non-specific and human-specific *Bacteroides* were closely related in the samples taken on Day 0 (Table 27). In Treatment 1, water column only 2/3 of the samples tested positive for the non-specific while 100% tested positive for the human-specific *Bacteroides*. The same happened in the Treatment 3, water column samples. On Day 7, only the non-specific *Bacteroides* was present and by Day 21 only 1/3 of the samples taken from the sediment/water interface and sediment in the control columns were positive for the non-specific *Bacteroides*.

Comparing the presence of the non-specific *Bacteroides* and the human-specific *Bacteroides* revealed an average correct classification rate of 96.10%. Like the non-circulated columns, this also indicates a strong relationship between the non- and human-specific *Bacteroides*. Logistic regression revealed a strong positive relationship between both *Enterococcus* or *E. coli* and human-specific *Bacteroides* ($p = 0.014$ and $p < 0.0001$, respectively).

B. Phase II

B.1. Non-circulated

The non-specific *Bacteroides* was present throughout the sampling period for each of the three treatments (Table 28). It was even present in at least one location in the control columns. The human-specific *Bacteroides* was also present on Day 0, but in lower concentrations than the

Phase I columns. The human-specific *Bacteroides* was not present in any of the sediment (C) samples on Day 0. By Day 7, the non-specific *Bacteroides* was still present in all of the treatments. In the Treatment 1 columns, both the non-specific and human-specific *Bacteroides* shifted out of the water column (A) and were present in the sediment (C) in high percentages. In Treatment 2, Day 7, the non-specific *Bacteroides* was still present in all three sampling locations. Only 2/3 of the samples tested positive for the non-specific *Bacteroides* on Day 7 in comparison to 100% on Day 0. The human-specific *Bacteroides* (33.33%) was present only in the water column (A) on Day 7 in the Treatment 2 columns. Treatment 3 columns also contained both non-specific and human-specific *Bacteroides* on Day 7. In comparison to Day 0, the non-specific *Bacteroides* was no longer present in the sediment (C) and the human-specific *Bacteroides* was only found in 33/33% of the water columns samples (in comparison to 66.67% in Day 0 samples). By Day 22, the human-specific *Bacteroides* was no longer present in any of the samples taken. The non-specific *Bacteroides* was found throughout each location within location in every column (including the control columns).

The average correct classification rate between the non-specific and human-specific *Bacteroides* was 97.26%. Comparing the *Enterococcus* counts to the presence of the human-specific *Bacteroides* revealed no relationship between the two ($p = 0.294$). But comparing the human-specific *Bacteroides* to *E. coli* counts did prove that a strong relationship existed ($p = 0.004$). Because *Enterococcus* did not survive as long as *E. coli* in the non-circulated columns, low concentrations of *Enterococcus* were found at Day 7, whereas the human-specific *Bacteroides* was found in higher concentrations.

B.2. Re-circulated

Like the non-circulated columns, both the non-specific and human-specific *Bacteroides* were present on Day 0 samples within each of the three treatments (Table 29). The human-specific *Bacteroides* was found in both the water column (A) and sediment/water interface (B) in each of the three treatments. By Day 7, the non-specific *Bacteroides* was not present in the water column (A) or sediment/water interface (B) in Treatment 1 samples. The human-specific *Bacteroides* was not present in any of the locations in Treatment 1 samples on Day 7. Treatment 2 samples exhibited positive results for the non-specific *Bacteroides* for each of the three sampling locations on Day 7, as was the case with the human-specific *Bacteroides*. In the Treatment 3 samples on Day 7, both the non-specific and human-specific *Bacteroides* were present in the water column (A) and sediment/water interface (B). By Day 22, the human-specific *Bacteroides* was not found in any of the samples from any of the three treatments. However, the non-specific *Bacteroides* was present throughout all treatments and the control columns. This could possibly indicate contamination of the columns due to non-human sources such as wildlife.

Comparisons between the non-specific and human-specific *Bacteroides* revealed an average correct classification rate of 95.45%. Logistic regression between *Enterococcus* or *E. coli* and the human-specific *Bacteroides* revealed a strong relationship between the FIBs and the presence of the human-specific *Bacteroides* ($p < 0.0001$ and $p < 0.0001$, respectively).

VI. Fluorometry

Fluorometry readings were taken from all samples throughout Phase I and II sampling periods to detect the presence of OBs. Fluorometric readings over 100 FSU are considered to be positive for human fecal contamination (Hagedorn, 2003).

A. Phase I

A.1. Non-circulated

According to the fluorometry results, there was no indication of human fecal contamination within any of the columns (Table 30). All readings were below the recommended threshold of 100 FSU. Readings fluctuated throughout the sampling period, but overall there was an increase in FSUs within every treatment and control columns. This did not correlate with the introduction of influent. Comparing the each of the treatments (1, 2, and 3) with the control readings revealed no differences between them ($p = 0.204$, $p = 0.0194$, and $p = 0.070$, respectively).

A.2. Re-circulated

As with the non-circulated columns, there were no fluoromety readings over 100 FSU (Table 31). The same trend in fluctuation occurred throughout all columns. With the exception of the sediment in the control column revealed an increase in FSU over time. Although the control columns experienced the same variation throughout the sampling period, FSUs were lower than those in the treatments. Therefore, the controls were different from Treatments 1, 2, and 3 ($p < 0.001$, $p = 0.003$, and $p = 0.038$, respectively).

B. Phase II

B.1. Non-circulated

Like the Phase I columns, the fluorometry readings for the Phase II non-circulated columns fluctuated throughout the sampling period (Table 32). Unlike the Phase I columns, there was no trend or overall increase in FSUs. There was one instance of a reading over 100 FSU that occurred in the sediment in the control column on Day 7 (105.10 FSU). Because this

was the only reading and there is no correlation to the introduction of influent and the FSU readings, it is thought that this reading was not related to the high presence of OBs. Because of the high variation throughout all the columns (including the controls), comparisons between the controls and Treatments 1, 2, or 3 revealed a difference ($p = 0.991$, $p = 0.469$, and $p = 0.996$, respectively).

B.2. Re-circulated

There were several instances in the re-circulated columns where fluorometric readings were above 100 FSUs (Table 33). All occurred in the last two days of sampling (Days 16 and 22) and were evident in all three treatments. Because these readings occurred on the last two days of sampling and did not correlate with the introduction of influent, the readings are not related to the increased presence of OBs. Comparing Treatments 1, 2, and 3 with the control columns indicated differences ($p = 0.347$, $p = 0.426$, and $p = 0.591$, respectively).

C. Summary

Fluorometry results indicate that other compounds could be present in the sediment that fluoresce at the same wavelength as OBs. Algal growth was visibly apparent in most of the columns as time progressed. Algae contain compounds that can fluoresce under UV light (Amesz, 1967). This could be an explaination for the increasing FSU number observed throughout both Phases.

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Table 6. Average dissolved oxygen (% saturation) for Phase I, non-circulated columns.

	Day								
Treatment[1]	0	1	2	4	7	10	16	21	30
1	67.45[a2]	51.05[a]	48.20[a]	31.95[a]	42.30[a]	33.90[a]	51.10[a]	62.70[a]	71.05[a]
2	63.27[a]	54.07[a]	49.97[a]	33.13[a]	47.27[a]	34.33[a]	52.60[a]	56.10[a]	62.10[a]
3	63.00[a]	58.10[a]	50.47[a]	34.47[a]	40.30[a]	42.30[ab]	52.70[a]	58.20[a]	69.97[a]
Control	60.33[a]	57.20[a]	62.60[b]	54.57[b]	60.63[b]	58.93[b]	54.97[a]	54.67[a]	69.10[a]

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 7. Average dissolved oxygen (% saturation) for Phase I, re-circulated columns.

	Day							
Treatment[1]	0	1	2	4	7	10	16	21
1	65.23[a2]	66.73[a]	67.97[a]	68.97[a]	66.13[a]	70.60[a]	68.53[a]	63.77[a]
2	61.93[a]	61.83[b]	65.30[a]	72.43[a]	65.77[a]	66.77[a]	69.33[a]	62.83[a]
3	58.43[a]	61.57[a]	63.10[a]	68.53[a]	68.27[a]	73.40[ab]	69.53[a]	73.03[a]
Control	57.57[a]	60.23[a]	61.13[b]	60.27[b]	57.63[b]	72.73[b]	71.07[a]	72.63[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 9. Dissolved oxygen (% saturation) of Phase I, non-circulated (A) and re-circulated (B) columns for Treatment 1, 2, 3, and controls.

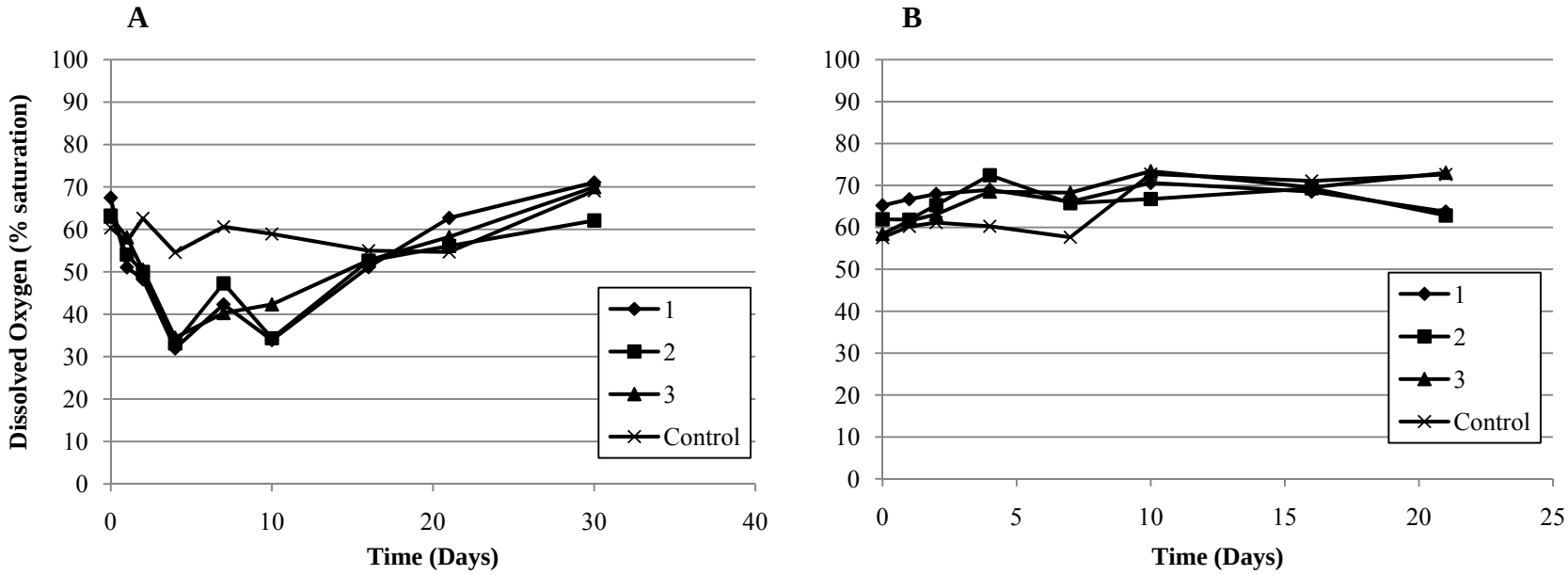

Table 8. Average dissolved oxygen (% saturation) for Phase II, non-circulated columns.

	Day									
Treatment[1]	0	1	2	4	7	10	16	22	29	36
1	64.90[a2]	62.20[a]	54.57[a]	35.43[a]	36.70[a]	29.80[a]	45.47[a]	45.63[a]	56.03[a]	56.53[ab]
2	61.60[a]	66.40[a]	55.17[a]	42.00[ab]	37.13[a]	22.67[a]	45.70[a]	48.37[a]	50.93[a]	54.03[ab]
3	68.27[a]	65.20[a]	62.27[a]	50.03[b]	42.70[a]	28.73[a]	53.30[ab]	54.83[a]	56.40[a]	37.63[b]
Control	64.10[a]	67.60[a]	66.15[a]	61.35[c]	57.40[b]	40.45[b]	58.40[b]	65.45[a]	65.30[a]	63.55[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 9. Dissolved oxygen (% saturation) of Phase II, re-circulated columns.

	Day							
Treatment[1]	0	1	2	4	7	10	16	22
1	79.33[a2]	86.07[a]	85.30[a]	90.57[a]	89.10[a]	88.07[a]	82.27[a]	83.60[a]
2	77.70[a]	88.23[ab]	89.00[ab]	90.33[a]	84.70[a]	87.70[a]	83.50[a]	83.73[a]
3	81.10[a]	92.47[b]	92.87[b]	93.70[a]	89.23[a]	95.40[b]	86.77[a]	89.47[a]
Control	80.80[a]	90.50[ab]	89.33[ab]	93.57[a]	90.33[a]	90.10[ab]	86.83[a]	89.73[a]

[1] Treatments consisted of 4.84 L introduced over varying time intervals. Treatment 1 was 968 mL of influent added over a total of 5 days. Treatment 2 involved an addition 484 mL of influent over 10 days, Treatment 3 was 322.67 mL of influent introduced over 15 days.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 10. Dissolved oxygen (% saturation) of Phase II, non-circulated (A) and re-circulated (B) columns for Treatment 1, 2, 3, and controls.

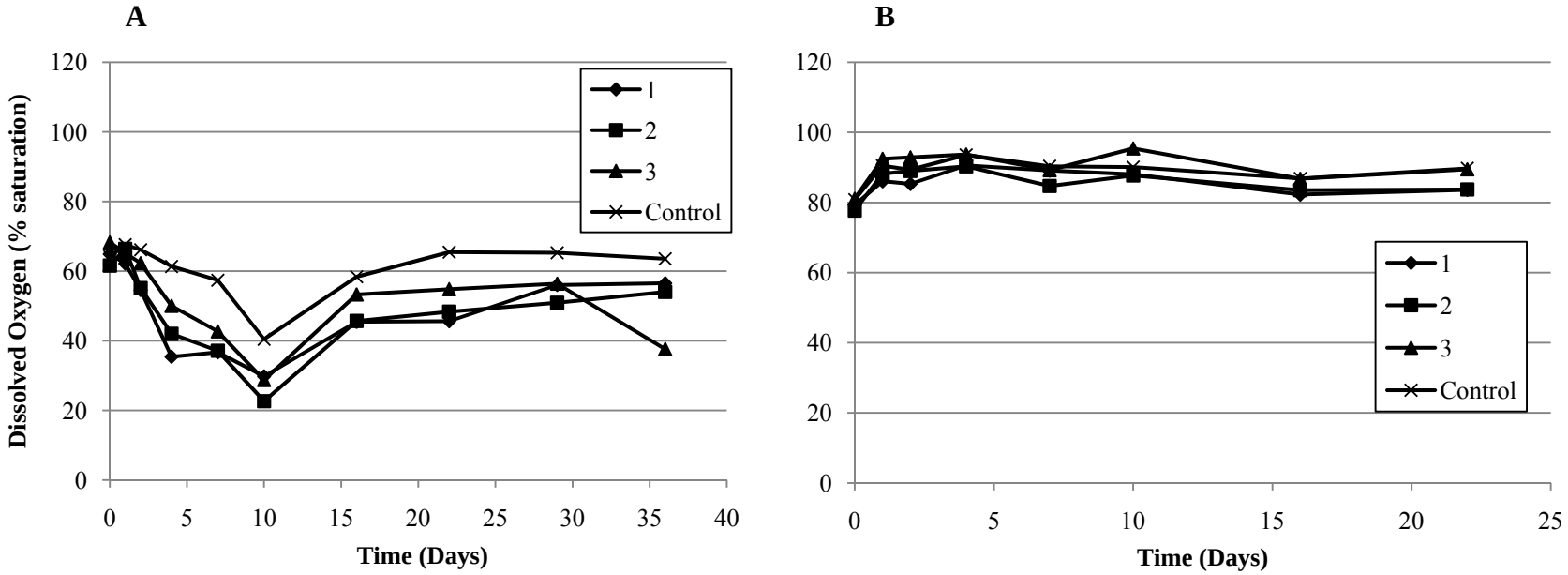

Table 10. Average temperature (°C) of Phase I, non-circulated columns.

	Day								
Treatment[1]	0	1	2	4	7	10	16	21	30
1	20.80[a2]	21.05[a]	22.60[a]	21.75[a]	19.25[a,b]	20.20[a]	21.55[a]	22.50[a]	21.65[a]
2	20.80[a]	20.60[b]	22.50[a]	21.73[a]	18.80[b]	20.60[a]	21.43[a]	21.83[a]	21.60[a]
3	21.67[a]	20.70[b]	22.30[a]	21.73[a]	19.63[a,b]	20.40[a]	21.60[a]	21.27[a]	21.63[a]
Control	20.83[a]	21.10[a]	22.77[a]	21.80[a]	19.90[a]	20.00[a]	21.63[a]	22.10[a]	21.73[a]

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 11. Average temperature (°C) of Phase I, re-circulated columns.

	Day							
Treatment[1]	0	1	2	4	7	10	16	21
1	29.00[a2]	26.23[a]	27.57[a]	23.33[a]	28.67[a]	27.57[a]	28.80[a]	26.00[a]
2	28.70[a]	26.27[a]	28.50[a]	24.17[a]	28.77[a]	27.43[a]	27.60[a]	24.67[a]
3	28.73[a]	25.57[a]	26.30[a]	23.30[a]	26.40[a]	29.23[a]	29.10[a]	23.60[a]
Control	27.73[a]	26.80[a]	28.30[a]	27.30[a]	29.10[a]	28.03[a]	28.87[a]	26.53[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 11. Average temperature (°C) for Phase I, non-circulated (A) and re-circulated (B) columns.

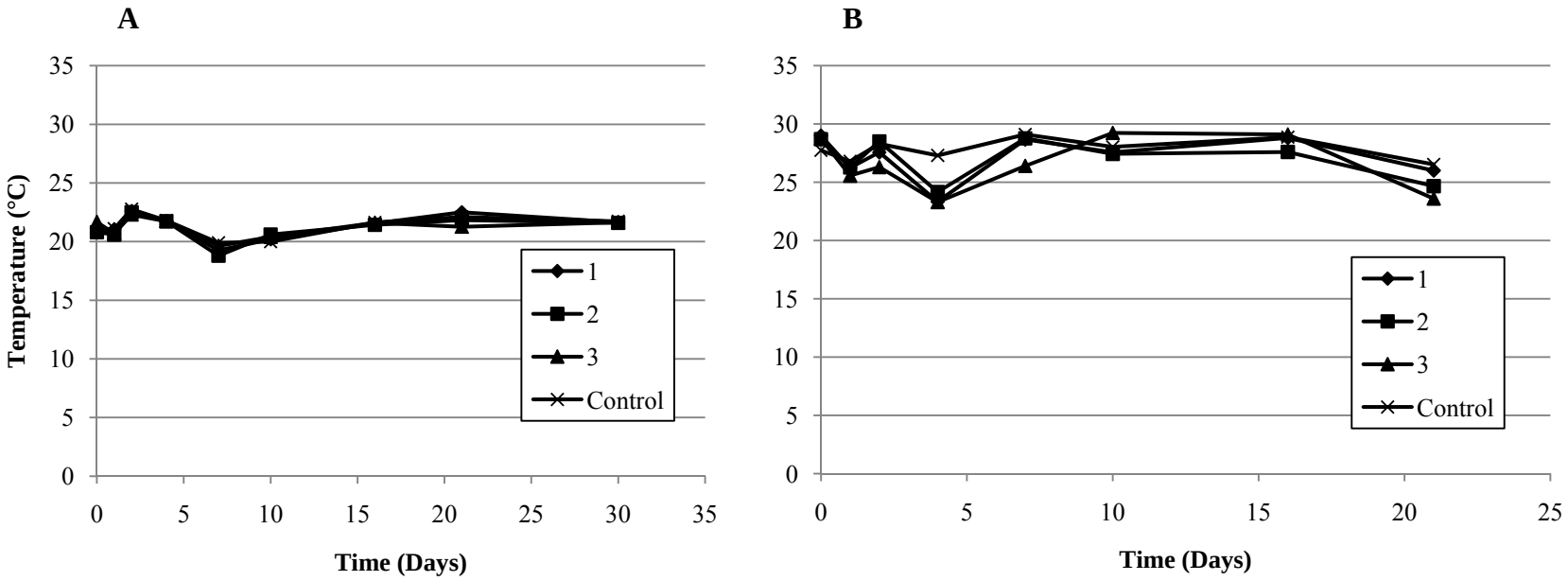

Table 12. Average temperature (°C) of Phase II, non-circulated columns.

	Day									
Treatment[1]	0	1	2	4	7	10	16	22	29	36
1	10.30[a2]	12.30[a]	12.73[ab]	17.93[a]	17.27[a]	13.90[ab]	18.47[a]	19.07[a]	18.00[a]	21.60[a]
2	10.70[a]	12.47[a]	12.87[a]	18.13[a]	17.10[a]	14.37[a]	18.43[a]	19.13[a]	17.67[a]	21.60[a]
3	10.63[a]	12.60[a]	12.77[ab]	18.07[a]	17.07[a]	13.77[b]	18.33[a]	19.27[a]	17.77[a]	21.50[a]
Control	10.30[a]	12.60[a]	12.50[b]	17.95[a]	17.40[a]	14.00[ab]	18.50[a]	19.00[a]	17.70[a]	21.45[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 13. Average temperature (°C) of Phase II, re-circulated columns.

	Day							
Treatment[1]	0	1	2	4	7	10	16	22
1	30.07[a2]	28.97[a]	27.80[a]	30.43[a]	31.00[a]	31.07[a]	29.83[a]	31.10[a]
2	30.73[a]	32.50[a]	30.03[a]	31.10[a]	30.83[a]	32.23[a]	31.43[a]	33.10[a]
3	30.10[a]	31.13[a]	27.67[a]	30.40[a]	30.43[a]	30.83[a]	29.57[a]	31.47[a]
Control	29.33[a]	31.07[a]	30.43[a]	30.70[a]	31.57[a]	31.63[a]	31.03[a]	30.93[a]

[1] Treatments consisted of 4.84 L introduced over varying time intervals. Treatment 1 was 968 mL of influent added over a total of 5 days. Treatment 2 involved an addition 484 mL of influent over 10 days, Treatment 3 was 322.67 mL of influent introduced over 15 days.
[2]Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 12. Average temperature (°C) for Phase II, non-circulated (A) and re-circulated (B) columns.

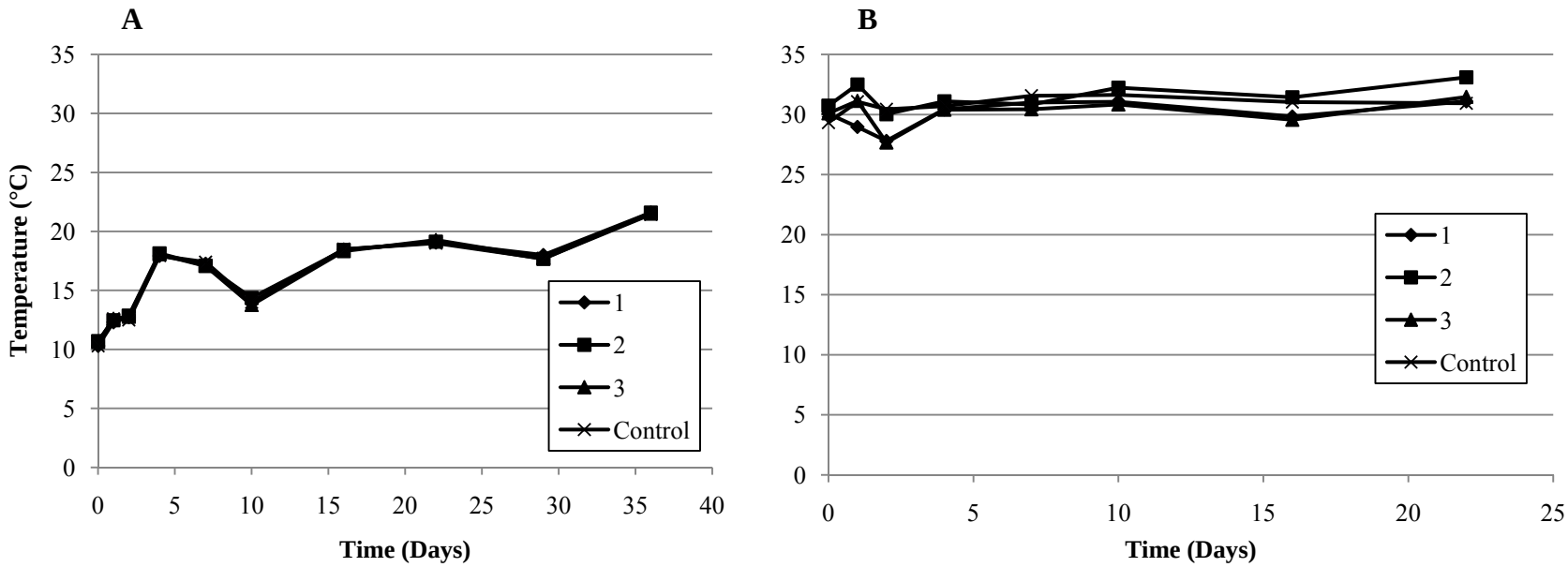

Table 14. Average electrical conductivity (mS/cm) for Phase I, non-circulated columns.

	Day								
Treatment[1]	0	1	2	4	7	10	16	21	30
1	23.20[a2]	23.30[a]	23.35[a]	23.45[a]	23.80[a]	23.70[a]	24.30[a]	24.80[a]	25.55[a]
2	24.37[b]	23.40[a]	23.40[a]	23.60[a]	23.97[ab]	23.30[a]	25.10[a]	25.07[a]	25.83[a]
3	25.00[c]	24.47[b]	23.97[b]	23.67[a]	24.03[b]	23.50[a]	25.13[a]	25.00[a]	25.83[a]
Control	25.60[d]	25.63[c]	25.60[c]	25.83[b]	26.17[c]	25.13[a]	26.43[a]	27.00[b]	27.87[b]

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.
[2] Numbers within the same column that are followed with the same letters are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 15. Average electrical conductivity (mS/cm) for Phase I re-circulated columns.

	Day							
Treatment[1]	0	1	2	4	7	10	16	21
1	25.60[a2]	25.70[a]	26.13[a]	26.97[a]	27.97[a]	29.27[ab]	32.13[a]	32.73[a]
2	27.20[b]	26.43[ab]	26.50[a]	27.33[a]	28.53[a]	30.00[ab]	32.73[a]	33.27[a]
3	27.57[b]	27.03[bc]	26.83[a]	26.80[a]	27.70[a]	28.93[a]	31.50[a]	31.80[a]
Control	28.23[c]	28.23[c]	28.70[b]	29.50[b]	30.47[b]	31.73[b]	34.30[a]	34.33[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 13. Average electrical conductivity (mS/cm) for Phase I, non-circulated (A) and re-circulated (B) columns.

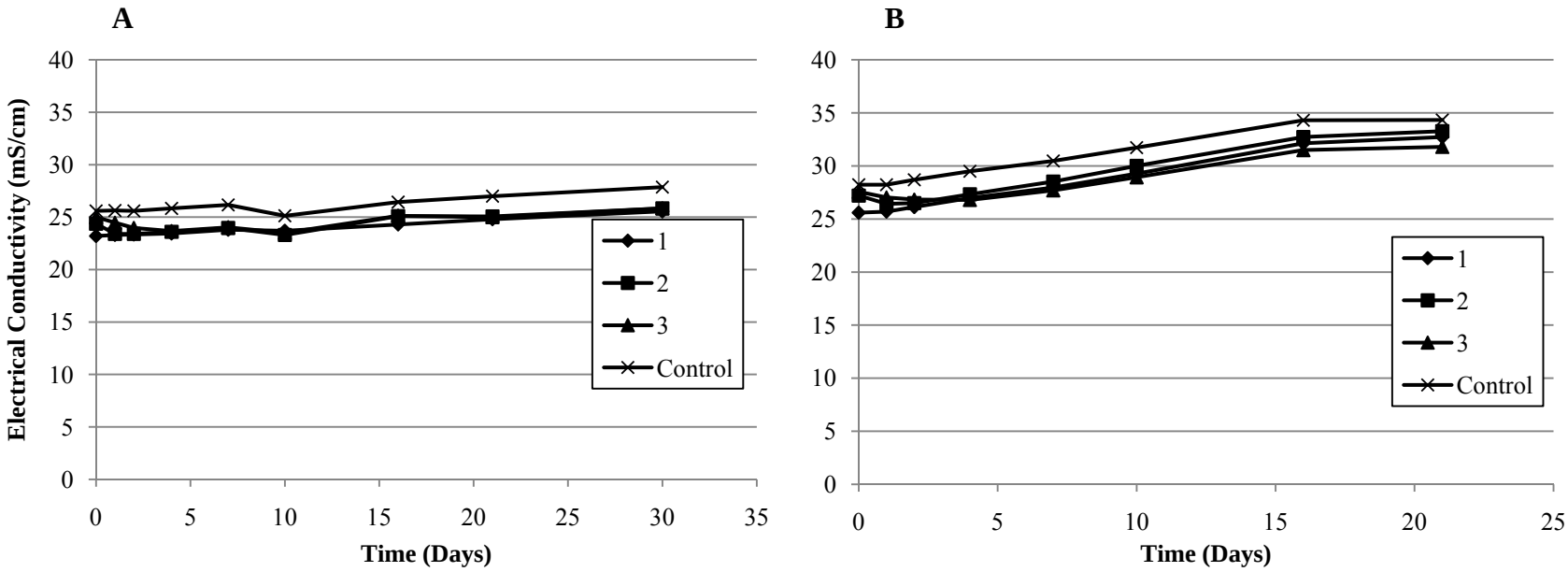

Table 16. Average electrical conductivity (mS/cm) for Phase II, non-circulated columns.

	Day									
Treatment[1]	0	1	2	4	7	10	16	22	29	36
1	21.23[a2]	20.17[a]	17.14[a]	13.31[a]	16.12[a]	12.00[a]	12.12[a]	14.51[a]	16.97[a]	17.87[a]
2	21.93[bc]	21.40[b]	18.64[b]	14.83[b]	16.88[a]	14.37[a]	12.39[a]	14.86[a]	17.21[a]	17.43[a]
3	21.80[b]	21.40[b]	18.75[b]	15.33[b]	16.70[a]	13.77[a]	11.65[a]	14.07[a]	16.36[a]	17.10[a]
Control	22.40[c]	22.10[c]	19.70[c]	16.51[c]	19.67[b]	14.00[b]	15.12[b]	18.14[b]	21.35[b]	22.15[b]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 17. Average electrical conductivity (mS/cm) for Phase II, re-circulated columns.

	Day						
Treatment[1]	1	2	4	7	10	16	22
1	22.13[a2]	19.20[a]	19.13[a]	22.83[a]	24.40[a]	27.63[a]	34.53[a]
2	22.77[a]	21.93[b]	22.63[b]	24.90[ab]	25.07[ab]	28.73[a]	31.00[a]
3	22.33[a]	21.63[ab]	23.17[b]	26.17[bc]	26.07[b]	27.93[a]	32.47[a]
Control	23.30[a]	23.40[b]	24.50[b]	28.30[c]	29.60[c]	33.47[b]	36.63[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 14. Average electrical conductivity (mS/cm) for Phase II, non-circulated (A) and re-circulated (B) columns.

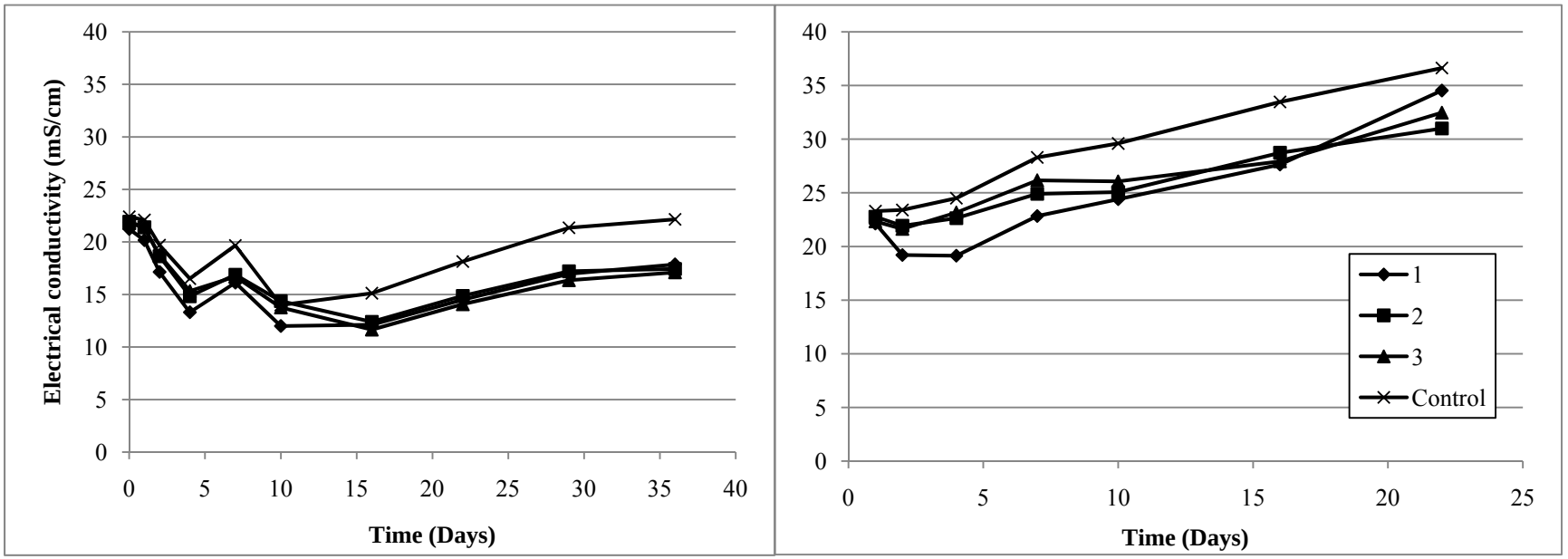

Table 18. Average pH for Phase I, non-circulated columns on the first (0) and last (30) day of sampling.

	Day	
Treatment[1]	0	30
1	6.10[a2]	7.80[a]
2	6.07[a]	7.70[a]
3	5.90[a]	7.27[a]
Control	6.47[a]	7.70[a]

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 19. Average pH for Phase I, re-circulated columns on the first (0) and last (21) day of sampling.

	Day	
Treatment[1]	0	21
1	8.03[a2]	8.43[a]
2	7.87[a]	8.30[a]
3	7.90[a]	8.40[a]
Control	8.00[a]	8.20[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 15. Average pH for Phase I, non-circulated (A) and re-circulated (B) columns.

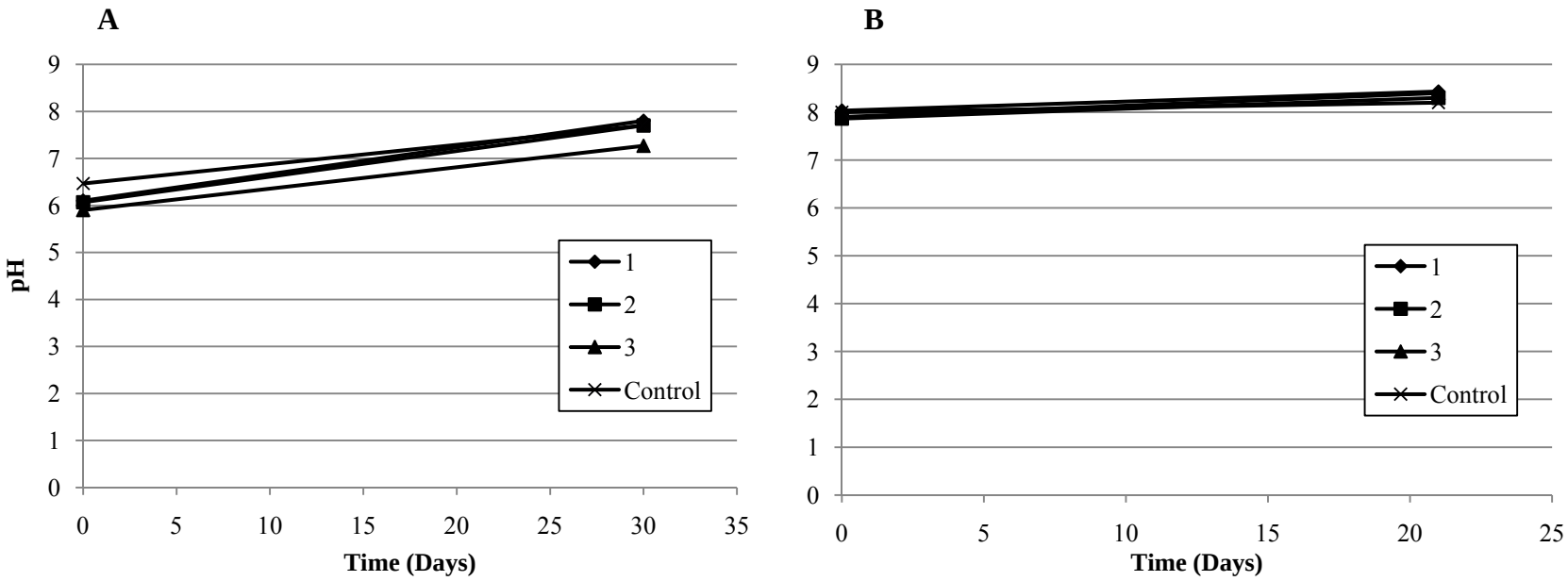

Table 20. Average pH for Phase II, non-circulated columns on the first (0) and last (36) day of sampling.

	Day	
Treatment[1]	0	36
1	6.57[a2]	8.67[a]
2	6.77[a]	8.63[a]
3	6.60[a]	8.53[a]
Control	6.65[a]	7.30[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Table 21. Average pH for Phase II, re-circulated columns on the first (0) and last (22) day of sampling.

	Day	
Treatment[1]	0	22
1	10.93[a2]	8.03[a]
2	11.37[ab]	8.07[a]
3	11.40[ab]	8.13[a]
Control	11.57[b]	8.10[a]

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.
[2] Numbers within the same column that are followed by the same letter are not statistically different from one another. Using Dunnett's multiple comparisons test at the 95% confidence level in JMP 8.0 (SAS Institute Inc.).

Figure 16. Average pH for Phase II, non-circulated (A) and re-circulated (B) columns.

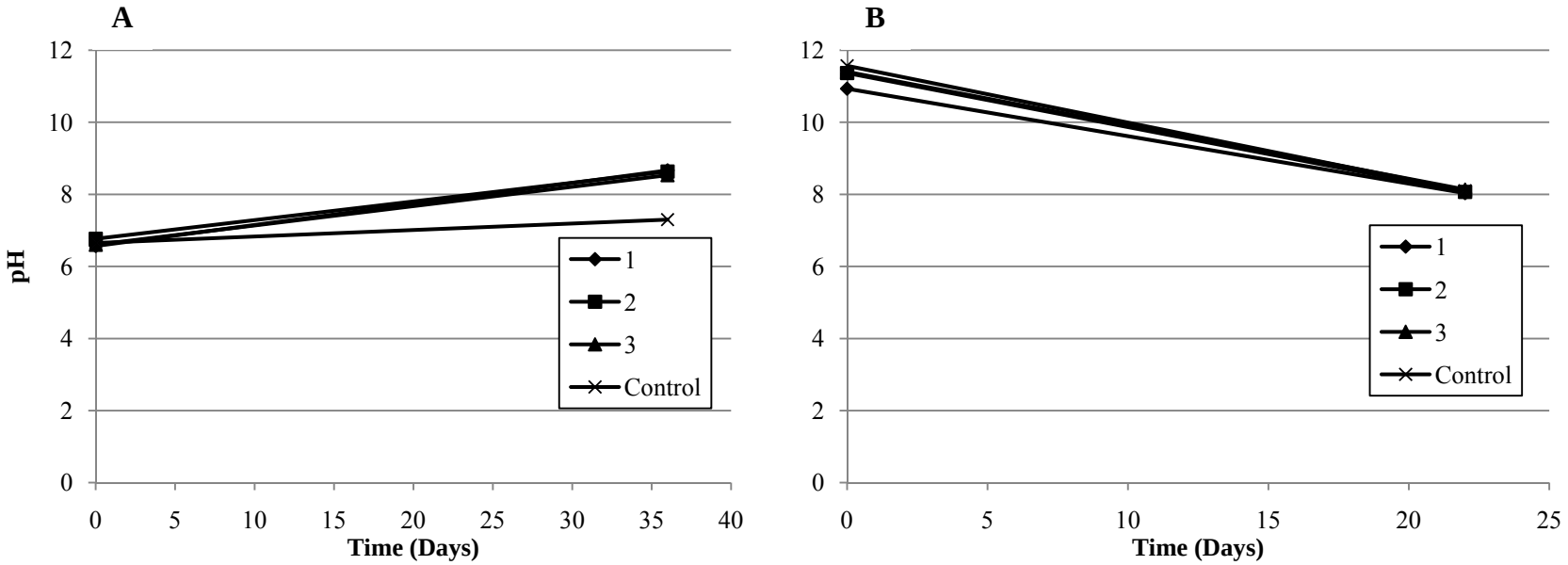

Figure 17. Average *Enterococcus* counts (CFU/100 mL) for Phase I, non-circulated and re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

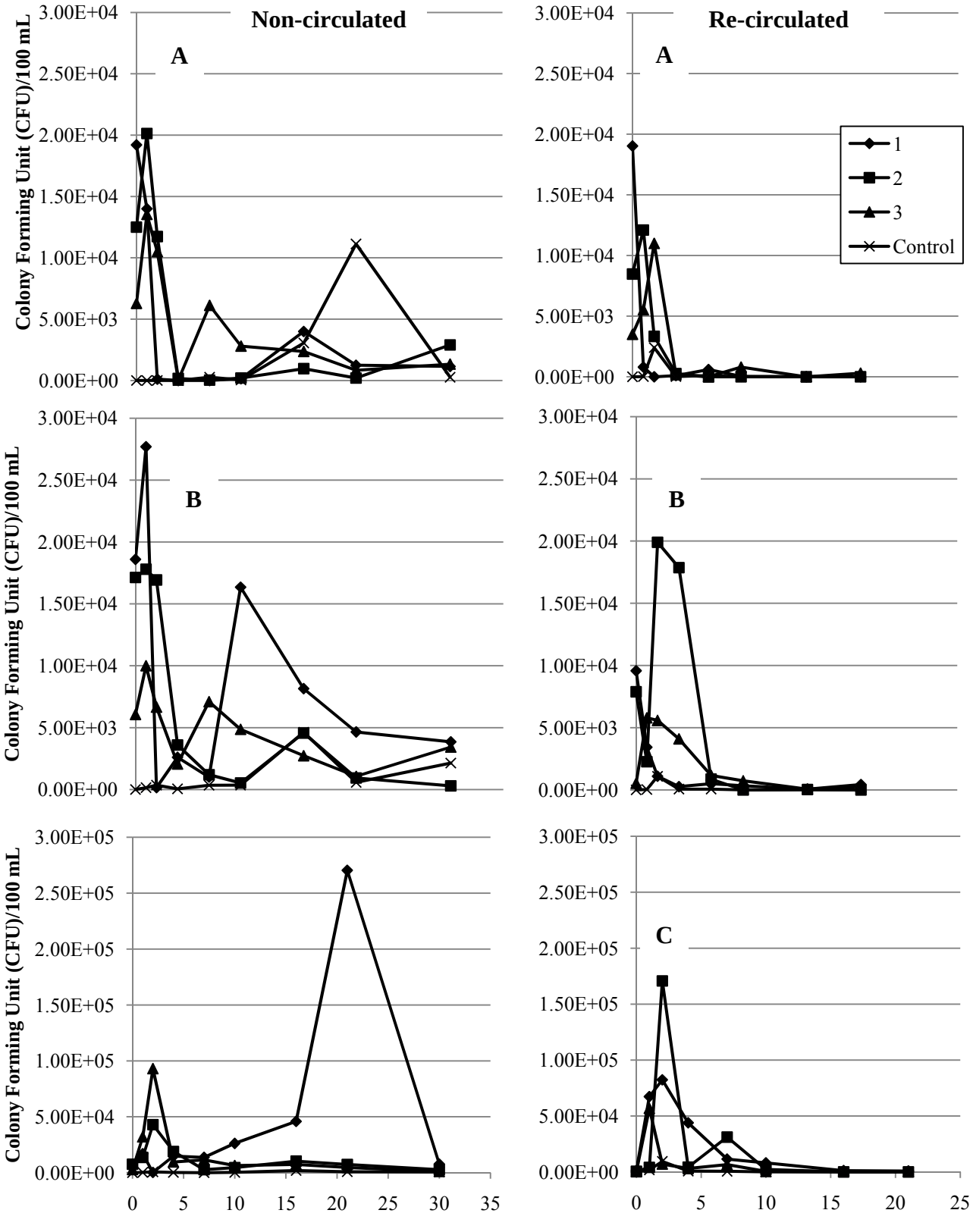

Figure 18. Close-up of average *Enterococcus* counts (CFU/100 mL) for Phase I, re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

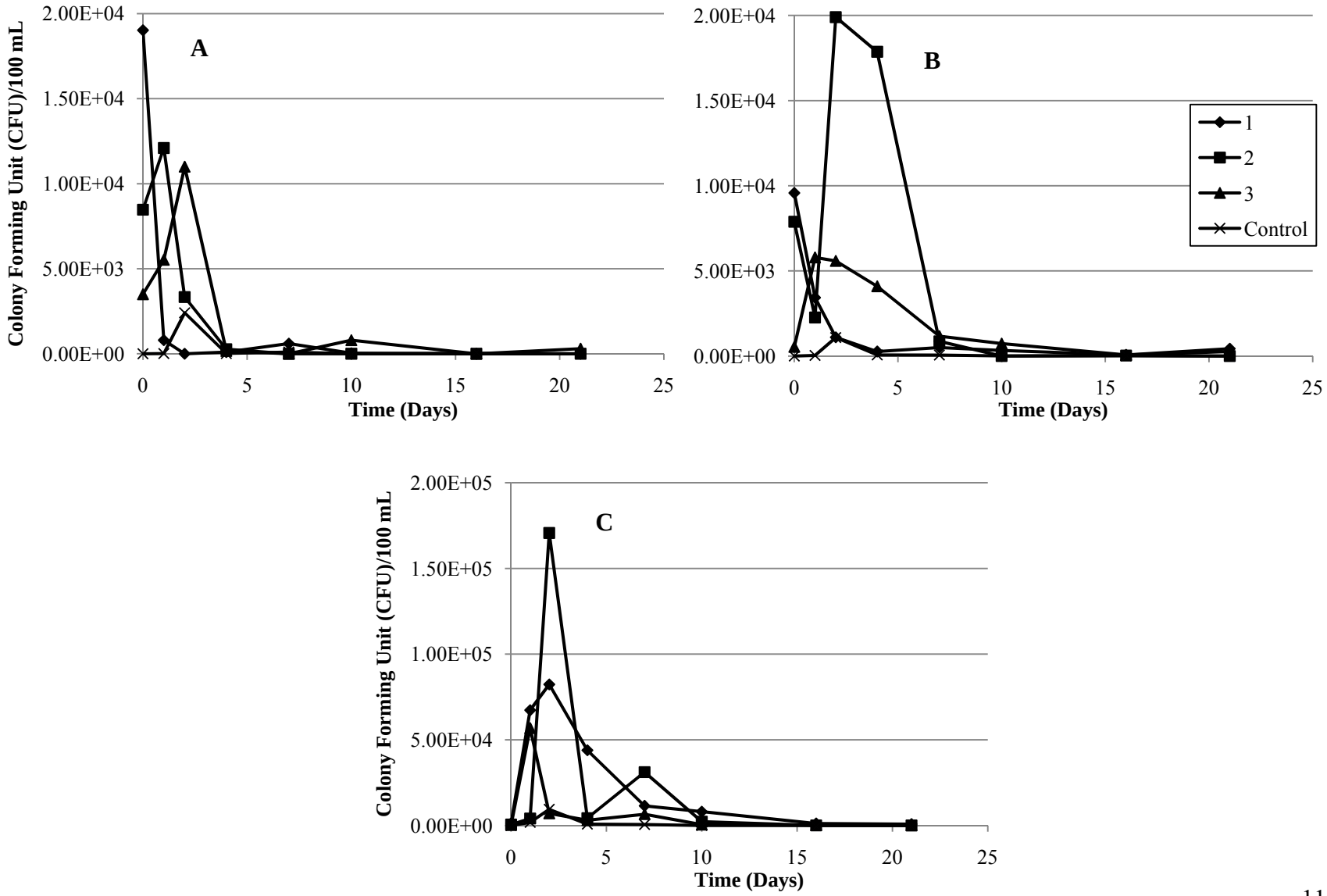

Figure 19. Average *E. coli* counts (CFU/100 mL) for Phase I, non-circulated and re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

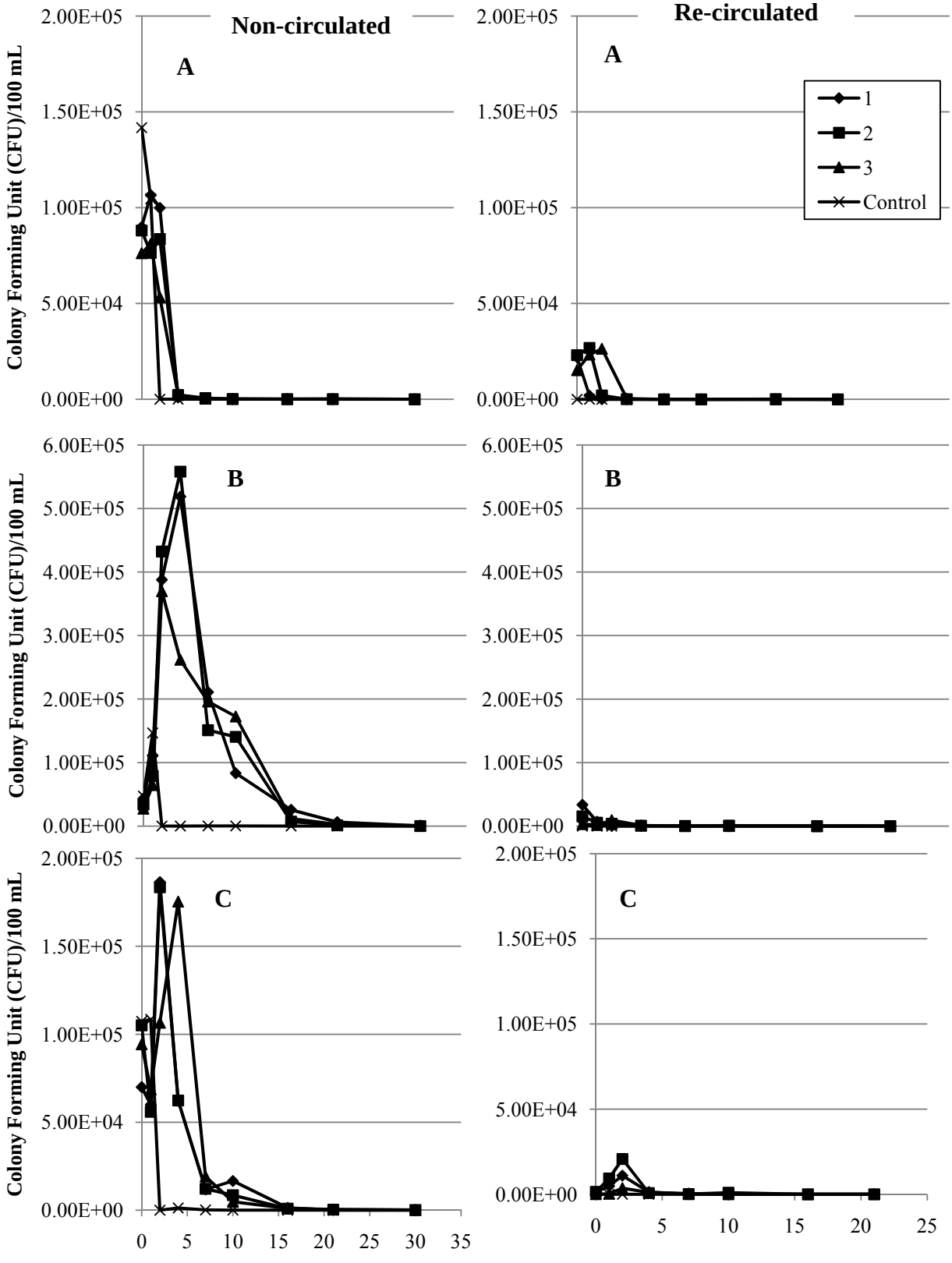

Figure 20. Close-up of average *E.coli* counts (CFU/100 mL) for Phase I, re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

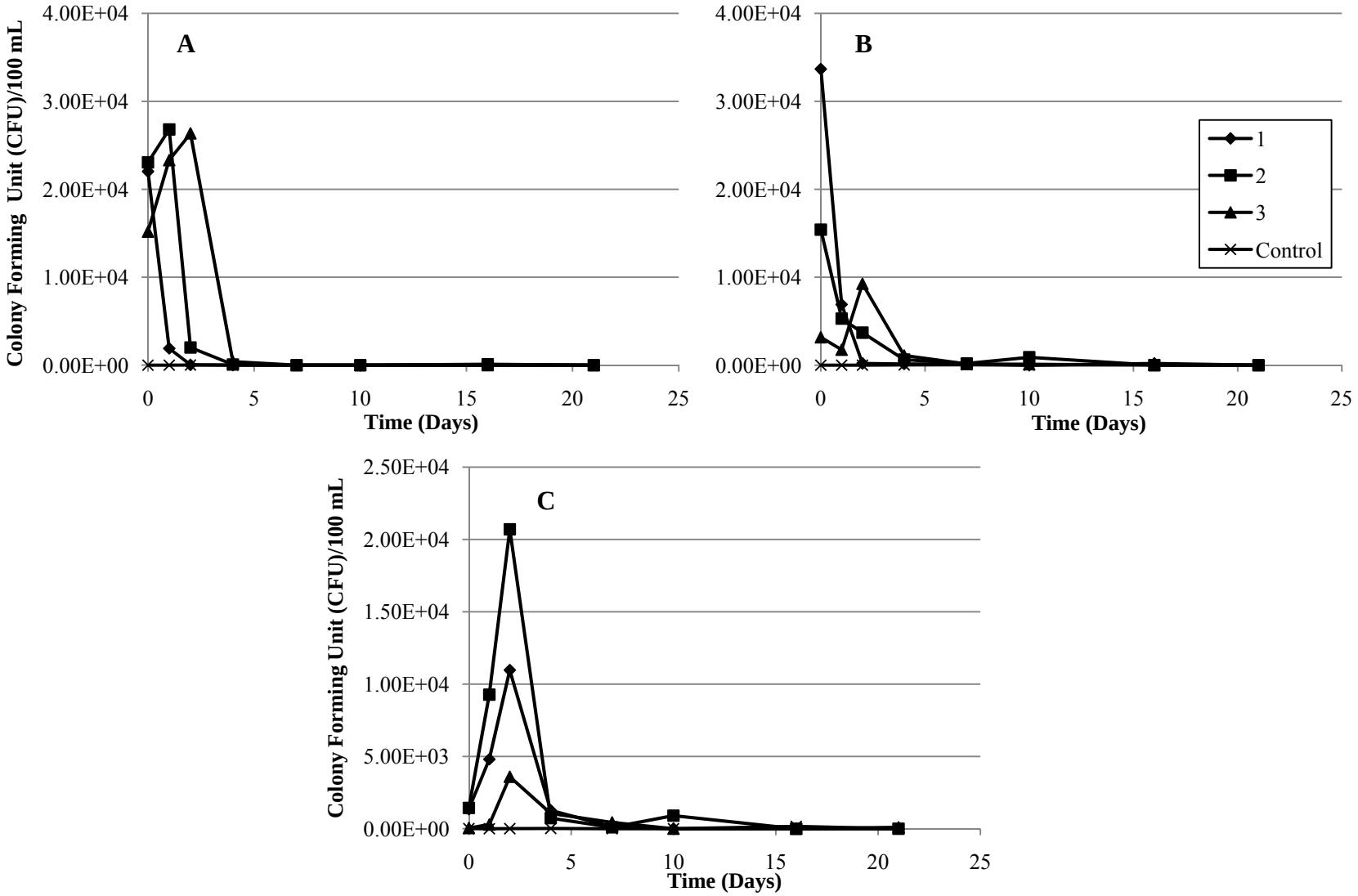

Figure 21. Average *Enterococcus* counts (CFU/100 mL) for Phase II, non-circulated and re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

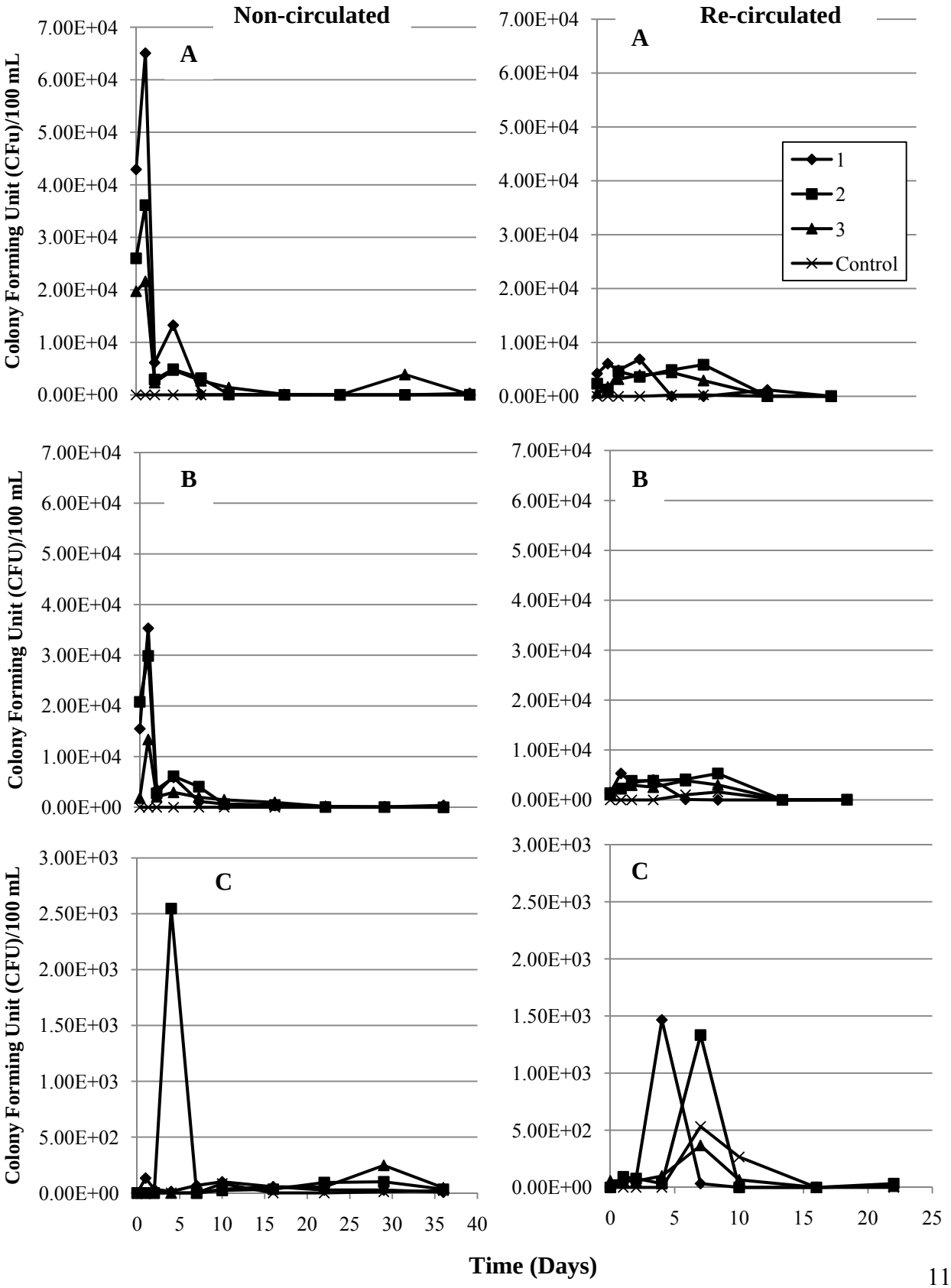

Figure 22. Close-up of average *Enterococcus* counts (CFU/100 mL) for PhaseII, re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

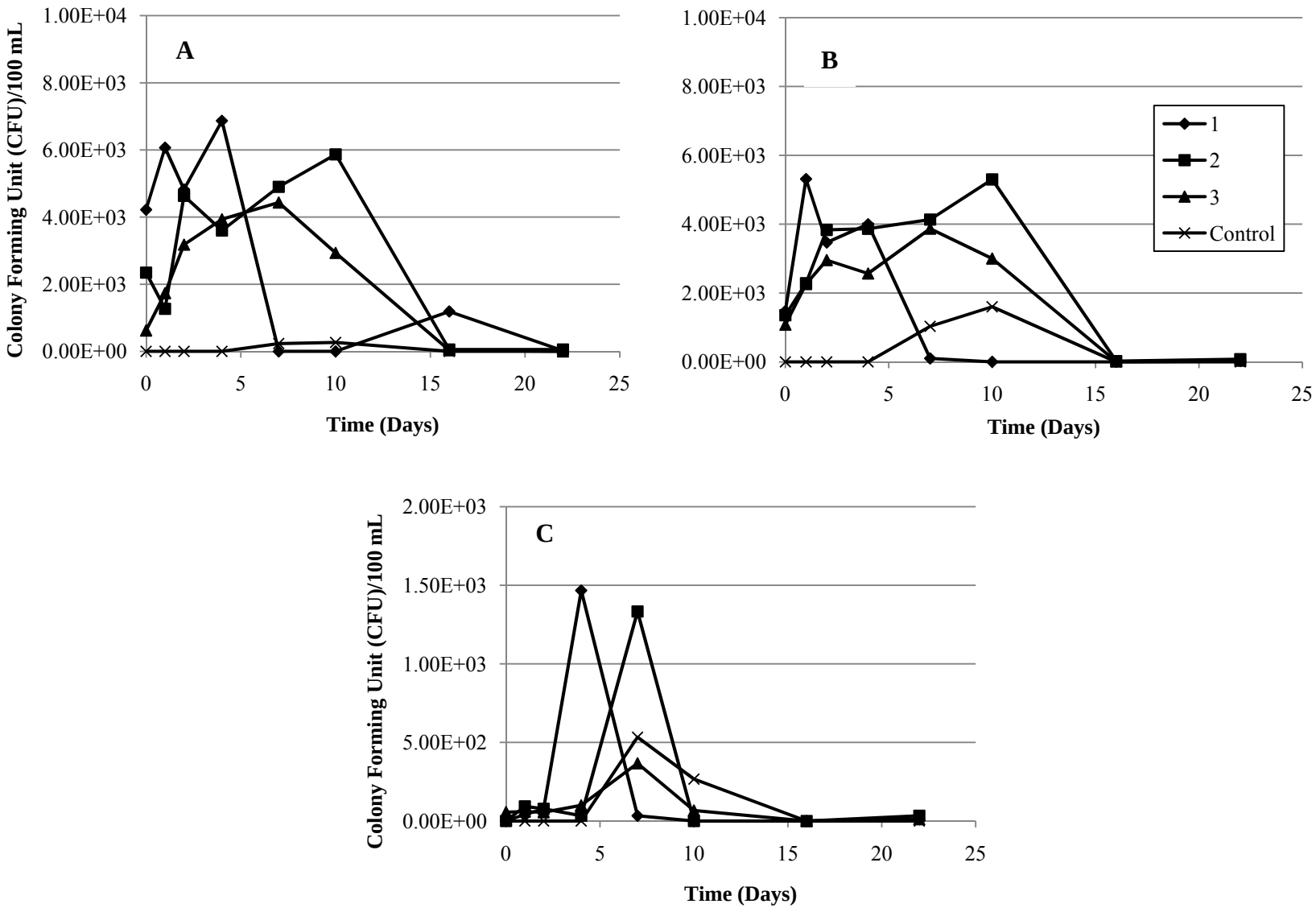

Figure 23. Average *E. coli* counts (CFU/100 mL) for Phase II, non-circulated and re-circulated columns in the three different sampling locations—water column (A), sediment/water interface (B), and sediment (C).

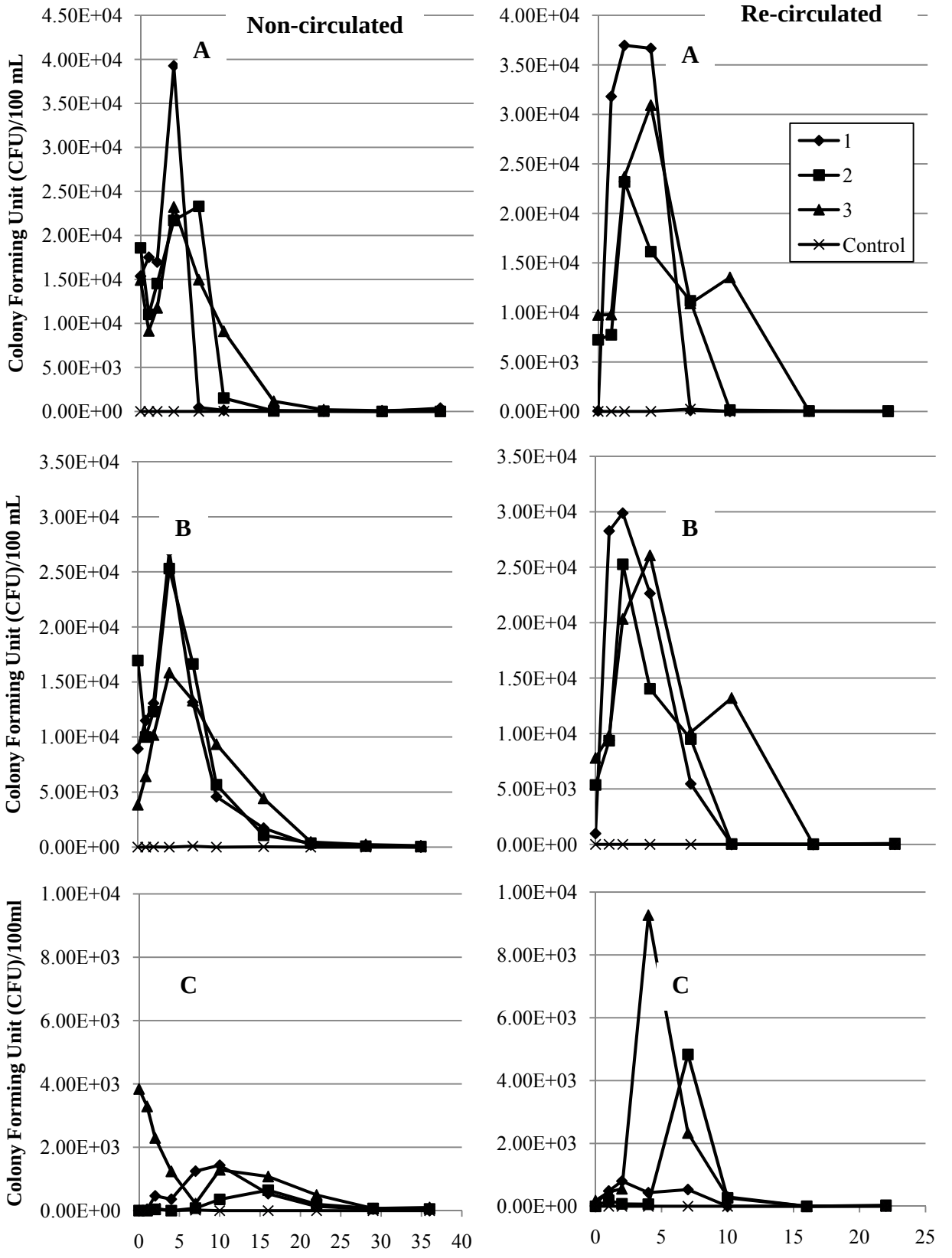

Figure 24. Species distribution of Phase I, non-circulated isolates throughout the sampling period—beginning (A), middle (B), end (C), and the total distribution (D).

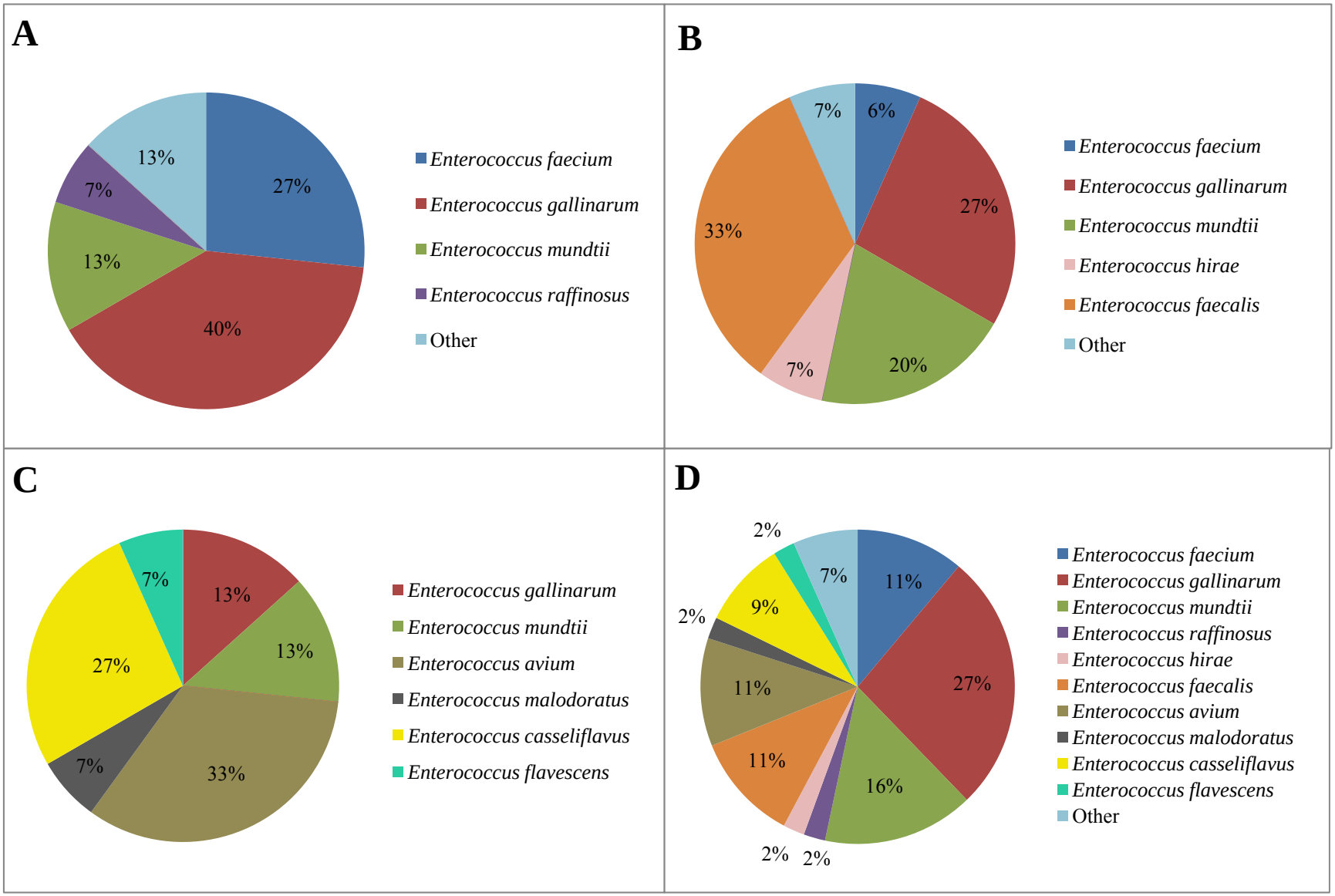

Figure 25. Species distribution of Phase I, re-circulated isolates throughout the sampling period—beginning (A), middle (B), end (C), and the total distribution (D).

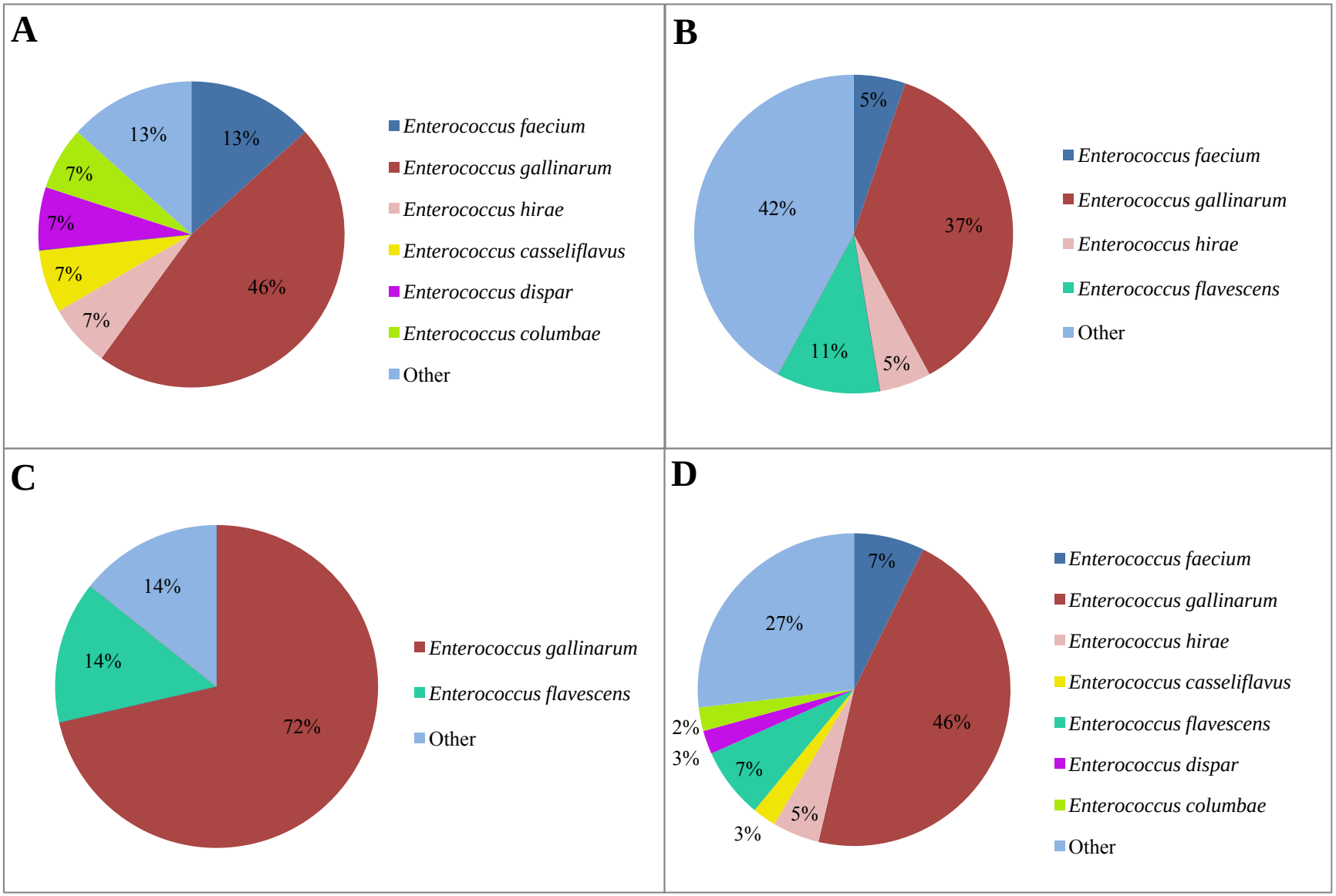

Figure 26. Species distribution of Phase II, non-circulated isolates throughout the sampling period—beginning (A), middle (B), end (C), and the total distribution (D).

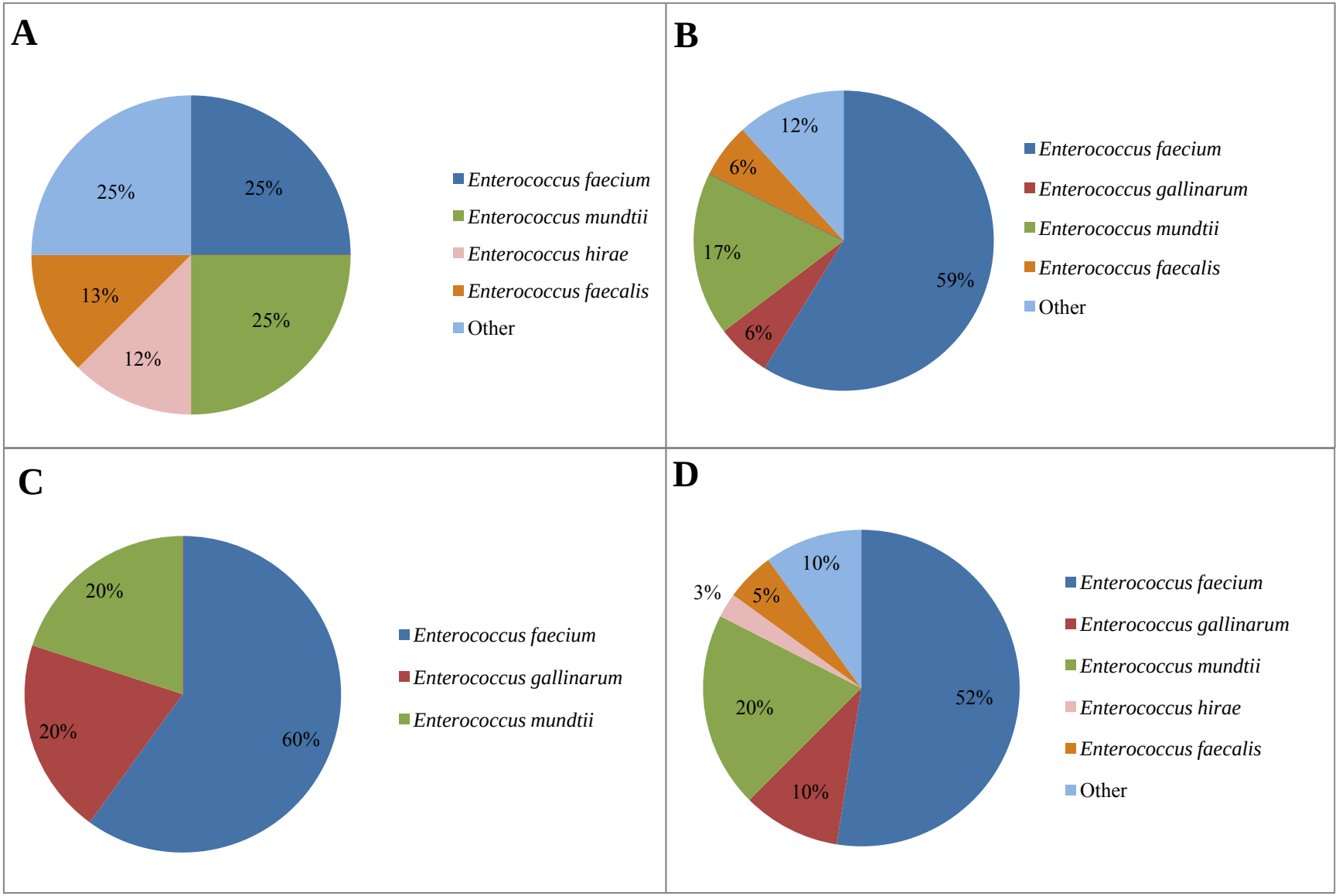

Figure 27. Species distribution of Phase II, re-circulated isolates throughout the sampling period—beginning (A), middle (B), end (C), and the total distribution (D).

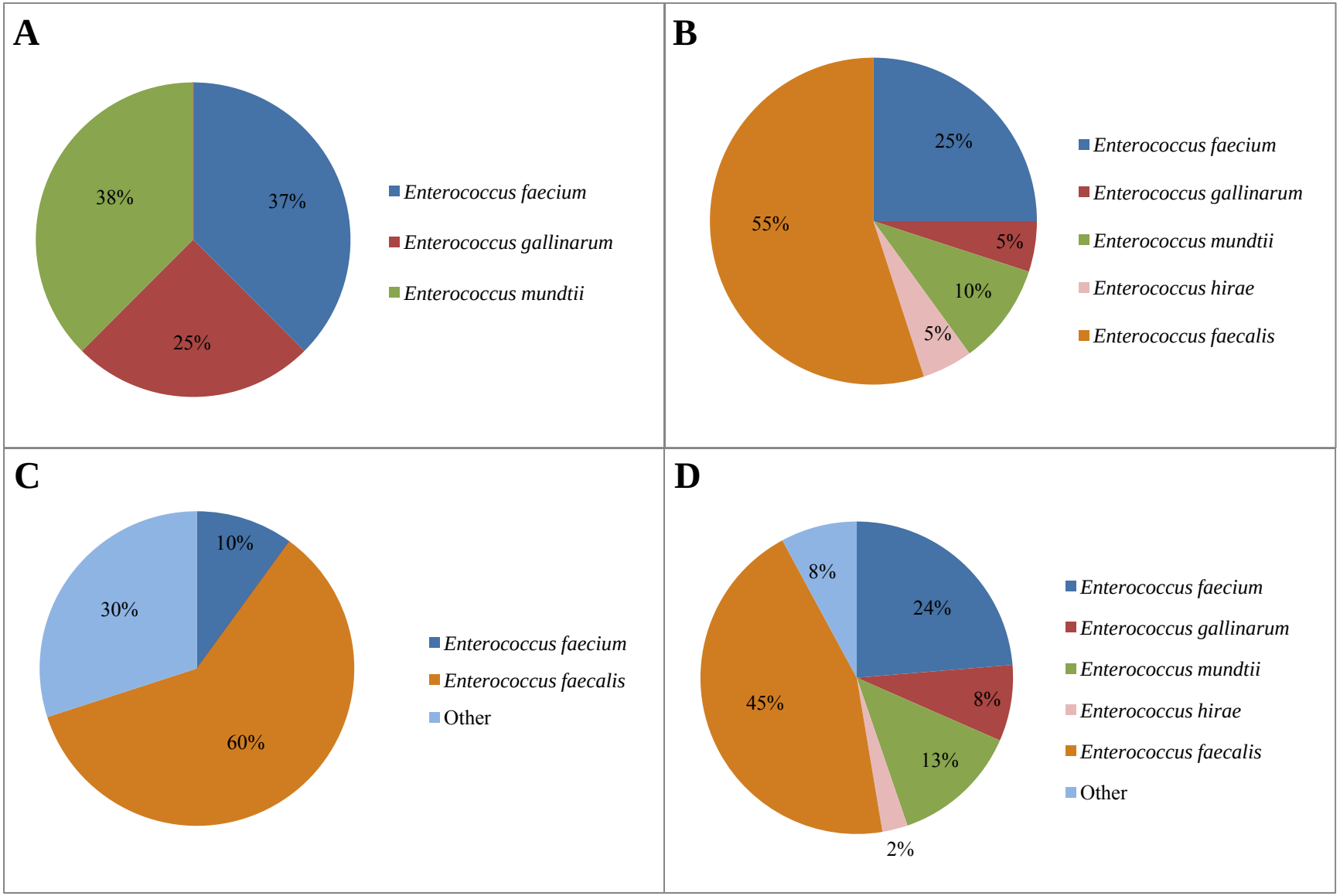

Table 22. Source identification (using ARA) of Phase I, non-circulated isolates.

	Number (%) of isolates based on predicted source[1]					
Sampling Time[2]	Bird	Human	Livestock	Pet	Wildlife	Total
Beginning	9[3] (60.00)	3 (20.00)	0 (0)	0 (0)	3 (20.00)	15 (100.00)
Middle	7 (46.67)	0 (0)	0 (0)	1 (6.67)	7 (46.67)	15 (100.00)
End	4 (26.67)	1 (6.67)	1 (6.67)	1 (6.67)	8 (53.33)	15 (100.00)
Total	20 (44.44)	4 (8.88)	1 (2.22)	2 (4.44)	18 (40.00)	45 (100.00)

[1] Isolates were compared to a library of known sources collected mostly in northern Virginia.
[2] Sampling time corresponds to the total sampling period (Day 0 to Day 30). This time was divided into three time periods (beginning, middle, and end).
[3] Sampling locations (A, B, and C) were lumped together and were not analyzed separately.

Table 23. Source identification (using ARA) of Phase I, re-circulated isolates.

	Number (%) of isolates based on predicted source[1]					
Sampling Time[2]	Bird	Human	Livestock	Pet	Wildlife	Total
Beginning	7[3] (46.67)	2 (13.33)	1 (6.67)	2 (13.33)	3 (20.00)	15 (100.00)
Middle	3 (14.29)	0 (0)	0 (0)	8 (38.10)	10 (47.62)	21 (100.00)
End	1 (14.29)	0 (0)	0 (0)	2 (28.57)	4 (57.14)	7 (100.00)
Total	11 (25.58)	2 (4.65)	1 (2.33)	12 (27.91)	17 (39.53)	43 (100.00)

[1] Isolates were compared to a library of known sources collected mostly in northern Virginia
[2] Sampling time corresponds to the total sampling period (Day 0 to Day 21). This time was divided into three time periods (beginning, middle, and end).
[3] Sampling locations (A, B, and C) were lumped together and were not analyzed separately.

Table 24. Source identification (using ARA) of Phase II, non-circulated isolates.

	Number (%) of isolates based on predicted source[1]					
Sampling Time[2]	Bird	Human	Livestock	Pet	Wildlife	Total
Beginning	6[3] (75.00)	2 (25.00)	0 (0)	0 (0)	0 (0)	8 (100.00)
Middle	8 (44.44)	2 (11.11)	0 (0)	1 (5.56)	7 (38.89)	18 (100.00)
End	1 (7.14)	4 (28.57)	1 (7.14)	6 (42.86)	2 (14.29)	14 (100.00)
Total	15 (37.50)	8 (20.00)	1 (2.50)	7 (17.50)	9 (22.50)	40 (100.00)

[1] Isolates were compared to a library of known sources collected mostly in northern Virginia
[2] Sampling time corresponds to the total sampling period (Day 0 to Day 36). This time was divided into three time periods (beginning, middle, and end).
[3] Sampling locations (A, B, and C) were lumped together and were not analyzed separately.

Table 25. Source identification (using ARA) of Phase II, re-circulated isolates.

	Number (%) of isolates based on predicted source[1]					
Sampling Time[2]	Bird	Human	Livestock	Pet	Wildlife	Total
Beginning	3[3] (37.50)	3 (37.50)	0 (0)	2 (25.00)	0 (0)	8 (100.00)
Middle	12 (60.00)	2 (10.00)	1 (5.00)	0 (0)	5 (25.00)	20 (100.00)
End	8 (53.33)	1 (6.67)	0 (0)	0 (0)	6 (40.00)	15 (100.00)
Total	23 (53.49)	6 (13.95)	1 (2.33)	2 (4.65)	11 (25.58)	43 (100.00)

[1] Isolates were compared to a library of known sources collected mostly in northern Virginia
[2] Sampling time corresponds to the total sampling period (Day 0 to Day 22). This time was divided into three time periods (beginning, middle, and end).
[3] Sampling locations (A, B, and C) were lumped together and were not analyzed separately.

Table 26. Percentage of positive results for the non-specific and human-specific *Bacteroides* (Bac32 and HF183) for Phase I, non-circulated columns by treatment and location in the column.

		Day					
		0		7		30	
Treatment[1]	Location	Bac32	HF183	Bac32	HF183	Bac32	HF183
1	A	100.00	100.00	0.00	0.00	50.00	0.00
1	B	100.00	100.00	100.00	0.00	0.00	0.00
1	C	50.00	50.00	100.00	0.00	50.00	0.00
2	A	100.00	100.00	0.00	0.00	0.00	0.00
2	B	66.67	100.00	100.00	0.00	66.67	0.00
2	C	100.00	100.00	100.00	0.00	33.33	0.00
3	A	100.00	100.00	0.00	0.00	33.33	0.00
3	B	66.67	100.00	100.00	0.00	33.33	0.00
3	C	66.67	33.33	100.00	0.00	66.67	0.00
Control	A	33.33	0.00	0.00	0.00	0.00	0.00
Control	B	33.33	0.00	0.00	0.00	0.00	0.00
Control	C	33.33	0.00	66.67	0.00	33.33	0.00

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.

Table 27. Percentage of positive results for the non-specific and human-specific *Bacteroides* (Bac32 and HF183) for Phase I, re-circulated columns by treatment and location in the column.

		Day					
		0		7		21	
Treatment[1]	Location	Bac32	HF183	Bac32	HF183	Bac32	HF183
1	A	66.67	100.00	33.33	0.00	0.00	0.00
1	B	100.00	100.00	0.00	0.00	0.00	0.00
1	C	66.67	33.33	100.00	0.00	0.00	0.00
2	A	100.00	100.00	66.67	0.00	0.00	0.00
2	B	66.67	33.33	33.33	0.00	0.00	0.00
2	C	33.33	33.33	0.00	0.00	0.00	0.00
3	A	66.67	100.00	33.33	0.00	0.00	0.00
3	B	66.67	66.67	0.00	0.00	0.00	0.00
3	C	33.33	0.00	0.00	0.00	0.00	0.00
Control	A	0.00	0.00	66.67	0.00	0.00	0.00
Control	B	0.00	0.00	0.00	0.00	33.33	0.00
Control	C	33.33	0.00	0.00	0.00	33.33	0.00

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

Table 28. Percentage of positive results for the non-specific and human-specific *Bacteroides* (Bac32 and HF183) for Phase II, non-circulated columns by treatment and location in the column.

		Day					
		0		7		22	
Treatment[1]	Location	Bac32	HF183	Bac32	HF183	Bac32	HF183
1	A	100.00	33.33	33.33	0.00	33.33	0.00
1	B	100.00	33.33	100.00	33.33	100.00	0.00
1	C	33.33	0.00	66.67	33.33	100.00	0.00
2	A	100.00	0.00	66.67	33.33	33.33	0.00
2	B	33.33	33.33	66.67	0.00	100.00	0.00
2	C	33.33	0.00	33.33	0.00	66.67	0.00
3	A	66.67	66.67	66.67	33.33	33.33	0.00
3	B	66.67	33.33	33.33	33.33	66.67	0.00
3	C	33.33	0.00	0.00	0.00	66.67	0.00
Control	A	66.67	0.00	0.00	0.00	33.33	0.00
Control	B	33.33	0.00	0.00	0.00	33.33	0.00
Control	C	0.00	0.00	33.33	0.00	66.67	0.00

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.

Table 29. Percentage of positive results for the non-specific and human-specific *Bacteroides* (Bac32 and HF183) for Phase II, re-circulated columns by treatment and location in the column.

		Day					
		0		7		22	
Treatment[1]	Location	Bac32	HF183	Bac32	HF183	Bac32	HF183
1	A	66.67	33.33	0.00	0.00	100.00	0.00
1	B	100.00	66.67	0.00	0.00	66.67	0.00
1	C	33.33	0.00	33.33	0.00	100.00	0.00
2	A	66.67	66.67	66.67	66.67	100.00	0.00
2	B	66.67	33.33	33.33	33.33	100.00	0.00
2	C	66.67	0.00	66.67	33.33	100.00	0.00
3	A	100.00	100.00	66.67	100.00	100.00	0.00
3	B	66.67	66.67	66.67	100.00	100.00	0.00
3	C	33.33	0.00	0.00	0.00	100.00	0.00
Control	A	0.00	0.00	0.00	0.00	100.00	0.00
Control	B	0.00	0.00	0.00	0.00	100.00	0.00
Control	C	33.33	0.00	0.00	0.00	100.00	0.00

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.

Table 30. Average Fluorometry results (FSU) for Phase I, non-circulated columns by treatment and location within the columns.

		Day								
Treatment[1]	Location	0	1	2	4	7	10	16	21	30
1	A	12.35	13.60	9.70	10.60	9.05	16.05	24.55	15.55	14.95
1	B	12.00	9.50	8.05	-1.10	4.30	19.35	24.60	12.45	16.50
1	C	0.00	34.00	18.50	5.00	-34.70	28.90	66.00	40.15	45.35
2	A	14.23	25.13	10.00	-7.70	-13.37	40.27	76.73	46.00	42.57
2	B	8.40	13.90	10.03	10.80	8.93	19.23	27.30	15.87	18.50
2	C	13.03	13.77	10.47	7.00	7.60	18.63	25.67	16.17	38.03
3	A	12.37	15.57	11.93	6.57	6.03	16.77	24.40	14.17	15.33
3	B	12.33	32.53	2.67	-0.67	-27.47	38.90	86.53	58.37	57.23
3	C	7.33	11.00	10.60	12.17	11.70	19.13	27.33	17.00	19.13
Control	A	9.60	13.50	9.83	10.33	8.70	18.43	26.67	16.50	19.07
Control	B	14.80	12.43	9.40	1.50	4.47	17.53	24.10	12.93	16.70
Control	C	-4.00	31.13	10.67	-4.00	-2.77	32.97	68.57	38.30	37.50

[1] Treatments consisted of 1 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 500 mL of influent on Day 0 and Day 1, and Treatment 3 was 250 mL of influent introduced on Days 0, 1, 2, and 3.

Table 31. Average Fluorometry results (FSU) for Phase I, re-circulated columns by treatment and location within the columns.

		Day							
Treatment[1]	Location	0	1	2	4	7	10	16	21
1	A	43.53	25.47	20.27	19.87	55.57	29.00	45.17	58.53
1	B	25.17	14.57	18.27	24.37	56.83	39.70	60.70	66.57
1	C	20.23	14.23	18.97	21.70	53.37	42.30	59.17	47.47
2	A	31.00	16.27	22.10	23.83	54.20	37.90	66.97	63.13
2	B	32.33	24.23	21.17	12.47	30.67	5.93	27.53	79.70
2	C	17.80	19.07	22.03	25.87	61.70	46.30	71.20	60.60
3	A	22.17	21.43	27.13	18.57	73.40	54.07	81.97	34.10
3	B	25.57	16.87	17.67	22.80	54.83	39.30	61.47	41.87
3	C	35.53	20.70	36.50	27.37	55.90	29.87	57.40	39.47
Control	A	12.40	14.03	14.23	19.80	33.80	40.00	61.87	41.93
Control	B	34.20	17.50	24.60	25.93	55.63	34.47	62.30	43.17
Control	C	45.73	30.73	23.13	15.87	38.80	17.90	36.07	31.57

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 2.2L of influent on Day 0, Treatment 2 involved an addition of 1.1 L of influent on Day 0 and Day 1, and Treatment 3 was 550 mL of influent introduced on Days 0, 1, 2, and 3.

Table 32. Average Fluorometry results (FSU) for Phase II, non-circulated columns by treatment and location within the columns.

		Day									
Treatment[1]	Location	0	1	2	4	7	10	16	22	29	36
1	A	13.27	16.47	23.67	35.20	34.43	17.84	35.13	33.13	51.33	19.93
1	B	24.83	22.93	28.80	34.03	34.10	19.73	31.30	17.00	46.37	30.37
1	C	43.83	44.60	55.27	63.67	70.63	44.57	53.40	-45.40	10.63	-8.03
2	A	17.03	17.73	21.97	29.43	33.00	27.00	40.33	38.27	55.17	32.23
2	B	17.43	18.53	19.60	30.33	32.13	23.93	37.40	37.07	53.87	30.70
2	C	49.03	48.27	57.87	66.20	64.80	44.03	47.40	20.80	61.87	7.03
3	A	9.50	10.33	13.90	19.50	26.00	22.87	36.27	36.50	52.50	30.30
3	B	14.70	13.37	16.47	22.67	28.00	22.80	33.60	31.10	45.73	28.73
3	C	37.30	37.33	43.63	51.50	53.43	42.33	76.90	26.47	16.87	-62.57
Control	A	8.30	7.85	9.60	11.15	14.45	10.55	16.75	17.10	29.05	18.50
Control	B	8.35	8.30	10.50	11.55	14.45	10.90	16.85	16.35	24.90	20.15
Control	C	67.05	70.50	81.75	96.50	105.10	60.75	73.60	-6.70	65.15	-32.95

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.

Table 33. Average Fluorometry results (FSU) for Phase II, re-circulated columns by treatment and location within the columns.

		Day							
Treatment[1]	Location	0	1	2	4	7	10	16	22
1	A	17.40	21.13	26.53	39.63	69.20	75.27	90.60	107.23
1	B	41.97	22.80	28.17	23.30	60.77	69.40	85.27	94.93
1	C	53.07	46.60	45.93	-20.17	61.87	84.13	100.03	101.67
2	A	15.23	22.97	24.70	3.67	70.03	87.67	110.80	121.67
2	B	14.83	21.17	27.80	2.23	69.63	83.70	106.10	129.57
2	C	58.47	31.07	46.13	-7.57	38.17	65.73	84.37	87.67
3	A	15.77	19.60	18.60	34.10	53.90	74.60	116.20	125.20
3	B	19.50	21.80	22.90	33.40	52.83	70.37	104.03	124.57
3	C	40.90	36.53	35.57	13.83	39.57	46.00	57.20	81.67
Control	A	8.37	10.33	13.77	19.57	30.37	41.83	59.93	73.47
Control	B	28.93	24.83	22.70	24.70	33.50	40.40	54.07	65.47
Control	C	49.13	50.43	50.83	36.57	52.53	61.23	80.80	83.40

[1] Treatments consisted of 2.2 L introduced over varying time intervals. Treatment 1 was 440 mL of influent added over a total of 5 days. Treatment 2 involved an addition of 220 mL of influent over 10 days, Treatment 3 was 146.67 mL of influent introduced over 15 days.

Chapter 5. Conclusions

The goals of this project were to determine at what point in time, under different amendment rates, would FIB populations stabilize and possibly re-grow in water and/or sediment. Specifically to (1) determine under what concentration of raw sewage amendments would FIB populations stabilize; (2) determine how long stabilization would last after the last sewage amendment; (3) determine if re-growth could be detected in the sediment populations after the last sewage amendment; and (4) compare the three microbial source tracking (MST) methods; fluorometry, *Bacteroides* human primer test, and antibiotic resistance analysis (ARA) with one another and with both FIBs (*E. coli* and *Enterococcus)* to determine possible correlations between the methods and/or between the methods and the FIB populations.

The nature of this project involved many different variables that had to be analyzed. The two Phases (CSO and small sewage leak) each contained 12 non-circulated and 12 re-circulated columns. Within each of those 12 columns, there were three different treatments plus control columns. Phase I, non-circulated columns contained three treatments where 1 L of influent was added all at once (Treatment 1), 500 mL per day was added over 2 days (Treatment 2), or where 250 mL per day was added over 4 days. Phase I, re-circulated columns consisted of three treatments where 2.2 L of influent was added on 1 day (Treatment 1), 1.1L per day was added over 2 days (Treatment 2), or where 550 mL per day was added over 4 days (Treatment 3). Phase II, non-circulated columns contained three treatments where 440 mL per day of influent was added over 5 days (Treatment 1), 220 mL per day was added over 10 days (Treatment 2), or where 146.67 mL per day was added over 15 days (Treatment 3). Phase II, re-circulated columns consisted of three treatments where 968 mL per day of influent was added over 5 days, 484 mL per day was added over 10 days, or where 322.67 mL per day was added over 15 days.

Within each of these treatments and controls, three different locations were sampled—water column (A), sediment/water interface (B), and sediment (C). From the samples taken at every location, two FIBs (*Enterococcus* and *E. coli*) were counted, and four MST methods were evaluated. Having so many variables can be daunting when trying to determine which comparisons were the most important.

One goal of this project was to determine if re-growth was evident. Re-growth indicates that FIBs were capable of reproducing within the columns. This re-growth would be evident by an increase in CFU/100 mL that would be present throughout each of the triplicate columns. It would also only be re-growth if it occurs after all the treatments were administered. Re-growth was evident only in the Phase I, non-circulated columns pertaining only to *Enterococcus*. Re-growth occurred on Day 16 within all three treatments in the water column. It was also evident in the sediment/water interface for Treatment 1 on Day 10, Treatment 2 on Day 16, and for Treatment 3 on Days 7 and 30. Because Phase I columns simulated a large CSO event, it's obvious that this large introduction of fecal pollution is more conducive to re-growth of FIBs.

Along with determining if re-growth was evident, another goal was to determine if stabilization of the FIB populations was evident. Stabilization of bacterial populations indicates a trend of sustained CFU/100 mL counts after all treatments have been administered. This signifies that ideal environmental conditions exist and can sustain bacterial populations for an extended period of time. This is more likely to be associated with re-growth, but re-growth does not necessarily mean that stabilization occurred. Stabilization was evident only in the Phase I, non-circulated columns—again, only pertaining to *Enterococcus.* Stabilization was evident in both the water column and sediment/water interface. While it can occur without evidence of significant re-growth, re-growth was evident throughout the stabilization period. This population

fluctuation began on Day 16 for Treatments 1 and 2. Treatment 3 stabilization began on Day 21 without any re-growth evident within the water column. The treatment in the sediment/water interface all saw periods of re-growth associated with population stabilization. Stabilization began with re-growth for Treatment 1 on Day 10, Treatment 2 on Day 16, and for Treatment 3 on Day 7. Along with determining if stabilization occurred, another objective was to determine how long stabilization lasted. After the initial re-growth, stabilization remained evident until the end of sampling. As with the evidence for re-growth, large doses of fecal pollution simulating a CSO event are more conducive to FIB stabilization.

The final goal of this project was to compare the three MST methods with one another and with the FIB counts. Comparisons between the three different MST methods (ARA, human-specific *Bacteroides* primer test, and fluorometry) identified strong correlations between ARA and the human-specific *Bacteroides* test. Correlations between these two methods and fluorometry were not evident. In fact, the fluorometry results indicated the inverse of the other two methods with an increase of FSU over time. Comparisons between FIBs and the three MST methods yielded the same results. Strong correlations were present between both ARA and the human-specific *Bacteroides* and FIB counts. Increasing fluorometry measurements did not correlate with the decreasing FIB counts.

Recreational water monitoring utilizes FIBs for detecting fecal pollution. These FIBs, in the past, have proven reliable organisms but now have been called into question. The variable survival between Phase I and II indicate that using only one organism may not be reliable. This also raises the question of what is happening to the pathogenic organisms in the water/sediment when one FIB is below standards but the other is above. More studies need to investigate the

differences in survival of FIBs in water and sediment and how it relates to the survival of pathogenic organisms.

Even though re-growth and stabilization were evident in the Phase I non-circulated columns, re-growth and/or stabilization of the re-circulated columns was not seen. This re-circulation is characteristic of many recreational waters and therefore is an important characteristic to understand. Longer studies need to be performed to find the point in time that FIBs stabilize or re-growth in the sediment. In the future, non-circulated environments would not necessarily need to be evaluated alongside re-circulated environments. Evaluation of one or the other in more depth would provide better understanding.

The unusual results found when testing for the presence of OBs, indicate that other compounds could be present in the sediment that have the same wavelength as OBs. In order to successfully use fluorometry in detecting human pollution, knowing what those other compounds are would be important. If using fluorometry as a preliminary test, the presence of those compounds could provide false results and invalidate any subsequent results using other MST methods.

This project was one of the first to simulate two types of pollution events while determining what happens to the pollution as it settles out of the water and into the sediment. This has huge implications in the field of water quality as well as MST. Studies have looked at FIB survival in sand and sediment both in a laboratory setting (columns/mesocosms) or monitoring recreational areas (Haller, 2009; Lee, 2006; Pote, 2009). Understanding how these FIBs behave in sediment would shed light on the ability of pathogenic organisms to survive and whether or not exposure to sediment can cause disease.

Although others have used multiple methods to identify human fecal pollution, comparisons between ARA, human-specific *Bacteroides*, and fluorometry were unique because it evaluated three different types of MST methods—biological, molecular, and chemical (Boehm, 2003; Harwood, 2009; Myoda, 2003). It also compared an older, library-dependent method with a newer, library-independent molecular method with success. This is another indication that using multiple MST methods provides the proper validation needed to determine the correct source of pollution. The failure of fluorometry to correlate with either ARA or human-specific *Bacteroides* provides more evidence on how MST methods should be utilized.

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