

9-1-2017

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Recommended Citation

Calderón, Olga; Porter-Morgan, Holly; Jacob, Joby; and Elkins, Willis, "Bacterial diversity impacts as a result of combined sewer overflow in a polluted waterway" (2017). *CUNY Academic Works*.

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CASE STUDY

Bacterial diversity impacts as a result of combined sewer overflow in a polluted waterway

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Received 8 May 2016; revised 16 August 2017; accepted 26 August 2017; available online 1 September 2017

ABSTRACT: Newtown Creek is an industrial waterway and former tidal wetland in New York City. It is one of the most polluted water bodies in the United States and was designated as a superfund site in 2010. For over a century, organic compounds, heavy metals, and other forms of industrial pollution have disrupted the creek's environment. The creek is also impacted by discharges from twenty combined sewer overflow pipes, which may deposit raw sewage or partially treated wastewater directly into the creek during heavy or sustained rain events. Combined sewer overflow events and associated nutrient over-enrichment at the creek drive eutrophication and subsequent hypoxia. At the current study, three sites were sampled one week apart during a dry period and a wet period, where indication of a combined sewage overflow event could be detected. 16s rRNA high-throughput sequencing from these three sites collectively yielded over 1000 species of bacteria belonging to twenty-two classes. Based on these data, it is hypothesized that differences identified in the microbiome on wet versus dry days are as a result of combined sewage overflow, street runoff, and additional fluctuations in the creek's environment associated with rain. It was found that after a combined sewer overflows event, the levels of Gamma Proteobacteria increased while the levels of Actinobacteria decreased. However, levels of bacteria stayed relatively unchanged at a site further away from combined sewer overflows discharge. Species found in Newtown Creek include pelagic, marine, human and animal pathogens, hydrocarbonoclastic, and other environmental microbes.

KEYWORDS: *Bioremediation; Combined sewer overflows (CSO); Hydrocarbonoclastic bacteria; Microbiome; Rickettsia.*

INTRODUCTION

Prior to the introduction of rail roads and highways in the United States, waterways were used to transport both people and goods, including industrial products. As such, many industrial and manufacturing facilities were sited near waterways. The decline in heavy industry and the passage of the Clean Water Act has

improved water quality, but many rivers and canals still contain appreciable levels of contaminants in their sediments (Shanahan, 1994, Frignani *et al.*, 2001). A continuing impact on urban waterways is the presence of combined sewer systems, which collect rainwater runoff, domestic sewage, and industrial wastewater into a single pipe. Typically, the wastewater flows to a sewage treatment plant where it undergoes treatment, before being discharged to a local waterway. However, the volume of wastewater can exceed the

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Note: Discussion period for this manuscript open until December 1, 2017 on GJESM website at the "Show Article".

capacity of the treatment plant especially under heavy or sustained rains or heavy snow melt. This may result in a combined sewer overflow (CSO) event, wherein untreated water discharges directly into water bodies (Suter et al., 2011; Rouff et al., 2013). CSO effluent can contain untreated or partially treated human organic and industrial waste, toxic materials, and pathogens (Gasperi et al., 2008), increasing the potential for waterborne diseases. A direct association has been made between levels of *Enterococcus faecalis* and sewage loading in waterways impacted by CSOs (Suter et al., 2011). In fact, as the sewage introduced during a CSO is only partially treated, if at all, these events provide a supply of bacteria laden effluent to local water bodies. Studies conducted using bioindicators such as *Enterococcus* on urban waterways indicate the presence of human microbiota and antibiotic resistant bacteria (Young et al., 2013). The tremendous metabolic flexibility of bacteria allow them to inhabit extremely unfavorable environments (Robbins, 2007) the organisms which inhabit polluted aquatic urban environments are subject to unique selection pressures. Microorganisms are ubiquitous and numerous in living environments, but it is theorized that anthropogenic contributions to the environment may greatly increase their numbers (Lighthart, 2000). In an age of increased bacterial infection and rising antibiotic resistance we need to better understand the microbes and their interaction with our environment. In addition, we now know that bacteria can play a role as bioremediators to

help clean up oil and chemical spills. Bacterial species identification and characterization has been one of the main goals of scientists for the last two hundred years. However, while differential staining and enzymatic tests provide important and specific information about the organisms being identified, they are not the optimal techniques to process organisms that cannot be cultured. New microbial identification techniques using 16S rRNA (a phylogenetic marker) offers information about what microbial taxa are present in a given sample (Pace, 1997). Metagenomics on the other hand, provide insight into all of the genes present in a given community (Konstantinidis and Tiedje, 2005). These new technologies have proven to be extremely useful in the identification of organisms that otherwise are not discernible with traditional microbiological methods. The objectives of this research were to utilize the aforementioned molecular techniques of bacterial identification to survey and better understand A) the composition of the marine bacterial community in the creek's surface water ecosystem and B) how that community can change after a CSO event. This study has been carried out in Newtown Creek, Brooklyn, New York in 2016.

MATERIALS AND METHODS

Study area

Newtown Creek is 6.1 km long and flows into the East River. In the early 19th century, it was transformed from a waterway meandering through



Fig. 1: Map of water sampling locations along Newtown Creek, New York, USA

salt marshes into a widened, deepened, bulkheaded canal. A long history of industrial pollution has left the creek's sediments packed with chemical contaminants (Newtown Creek Alliance, 2010). A number of chemical plumes, as well as two large oil spills - the Greenpoint Oil Spill (GOS) and the 'Blissville Seep'(BLS) surround the creek (USDOJ, 2012; CNYDEP, 2016). These issues contributed to the designation of Newtown Creek as a federal Superfund site in 2010 (Newtown Creek Alliance, 2010). In addition, to industrial pollution, the creek is impacted by twenty CSO pipes, which can deposit up to 5.6 billion liters annually of sewage, wastewater, and street runoff to the creek (USEPA, 2004; CNYDEP, 2011).

This research examined microorganisms inhabiting the surface water at three sites in Newtown Creek (Fig. 1). The Turning Basin (TRB) sample site lies near two CSOs, (one of which is the largest one in the creek) and is subject to more wind and sunlight exposure than the other sites (CNYDEP, 2011). The Apollo Street (AST) site is located on the main channel of Newtown Creek near the epicenter of the GOS (Exxon Mobil Corporation, 2012) and the BLS (NYS DEC 2014). In addition to a CSO outfall, numerous storm water drains located in close proximity to this site transport upland contamination from industries and streets into the creek. The Dutch Kills Mouth (DKM) site is close to the Newtown Creek Wastewater Treatment Plant, which treats sewage for over one million city residents. During dry weather it releases 1.173 billion liters of treated wastewater into the creek, but during wet weather it can release up to 2.65 billion liters of partially treated wastewater into the Whale Creek tributary, just a few hundred meters from DKM (CNYDEP, 2017).

Metagenomics of Newtown Creek

One-100ml sample was collected at each site (TRB, AST and DKM) on September 8th, 2016 and

another 100-ml sample was collected at each site (TRB, AST and DKM) on September 15th. If an abnormal coloration in surface waters was present, it was noted as a field observation and qualified as a bloom. The most common discolorations present in Newtown Creek are brown, green and white. Dissolved oxygen, temperature, and salinity readings were taken *in situ* at surface level using a YSI Pro 2030 multi-parameter sensor/probe. Samples were placed in coolers, transported to the laboratory and stored at 4°C, dark conditions. Bacterial analysis for *Enterococcus* was conducted within four hours using the IDEXX Enterolert method (Fisher and Phillips, 2009) protocol. A reagent was added to the samples, which were diluted, and they were then sealed in a Quanti-tray and incubated at 41° Celsius. After 24 hours, samples were enumerated under ultraviolet (UV) light. Water samples were filtered using a 0.22 micrometers (µm) filter membrane and deoxyribonucleic acid (DNA) was extracted using a PowerWater® DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). Purified DNA samples were stored in a -20°C freezer until shipped for sequencing. Sequencing and data mining were conducted at Molecular Research DNA Laboratory (MR DNA), Shallowater, TX, USA. The PCR primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') (Osborne *et al.*, 2005) and 519R (5'-GWATTACCG-CGGCKGCTG-3') (Engelbrektson *et al.*, 2010) were used with barcode on the forward primer to amplify a section of the 16S rRNA. PCR utilized the HotStarTaq Plus Master Mix Kit (Qiagen, USA).

Table 1 indicates the levels of *Enterococcus*, dissolved oxygen, temperature and salinity were measured at each site during a 2-day period without and with rainfall, respectively. Newtown Creek is connected to the East River Newtown Creek is connected to the East River, a tidal strait running between Long Island sound and Upper New York

Table 1: Levels of *Enterococcus*, dissolved oxygen, temperature and salinity were measured at each site during a 2-day period without and with rainfall

	Without Rainfall (09/08/16)			With Rainfall (09/15/2016)		
	AST	DKM	TRB	AST	DKM	TRB
<i>Enterococcus</i> (MPN)	83	20	203	8164	>24196*	>24196*
Dissolved Oxygen (%)	66.5	63	53.4	43.7	36.9	25.1
Temperature (°C)	24.1	24.2	24.1	22.7	24.2	22.7
Salinity (ppt)	23.4	24	23.2	20	21.2	18.1

* indicates test limit

Bay. There is very little natural fresh water input into Newtown Creek, thus the higher salinity levels. In regards to the terminology of the creek, Newtown Creek is more of a sheltered waterway as opposed to association with freshwater or river tributary.

The polymerase chain reaction (PCR) program began with 94°C for 3 minutes, followed by 28 cycles of: 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 min and ended with a final elongation at 72°C for 5 minutes. PCR amplification product was checked on a 2% agarose gel. An Illumina DNA library was prepared using the PCR product. Samples sequencing was performed at MR DNA on a MiSeq by manufacturer’s instructions (Shallowater, TX, USA). Data sequence processing was done using MR DNA analysis pipeline (MR DNA, Shallowater, TX). Resulting sequences were joined, depleted of barcodes, and sequences that were less than 150bp were removed, along with those sequences that contained ambiguous bases. In addition, sequences were denoised and Operational Taxonomic Units (OTU’s) and chimeras removed. OTUs were delineated by similarity (97%) and divergence (3%) factors. Ultimately, the obtained OTUs were taxonomically classified using Basic Local Alignment Search Tool Nucleotide (BLASTn) against a curated database derived from the National Center for Biotechnology Information (NCBI) and ultrahigh quality daylight-type Iso 100 color reversal film (RDPIII).

RESULTS AND DISCUSSION

Enterococcus levels, an indicator of CSO overflow (Fisher and Phillips, 2009) were lower on 09/08 than on 09/15 (Table 1). In addition, 2-day rain totals prior to sample collection were 0.0 and 1.42 cm respectively (NCEI, 2016). Thus, these dates can give a broad overview of changes in the creek microbiome after rain. High levels of *Enterococcus* bacteria are an indication of CSOs after a rain episode. Although this study is looking at the overall diversity of Newtown Creek’s Microbiome, Enterococcus - bacteria found in the intestines of humans and other animals, is used as an indicator of the changes in bacterial population when anthropogenic sources of bacteria are discharged into the creek.

Creek microbiome changes after CSO overflow

DNA sequencing of the 16S rRNA gene was used to compare the taxonomic composition of the microbiome from samples collected during a dry period and after a CSO event. Over 1000 species of bacteria belonging to 22 classes were identified. Of these, *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria*, and *Actinobacteria* constituted over 80% of the bacteria found (Fig. 1). Additionally, levels of *Actinobacteria* and *Gammaproteobacteria* changed most markedly on wet and dry days.

In Fig. 2, species abundance on dry and wet days at Newtown Creek based on 16s rRNA analysis, OTUs

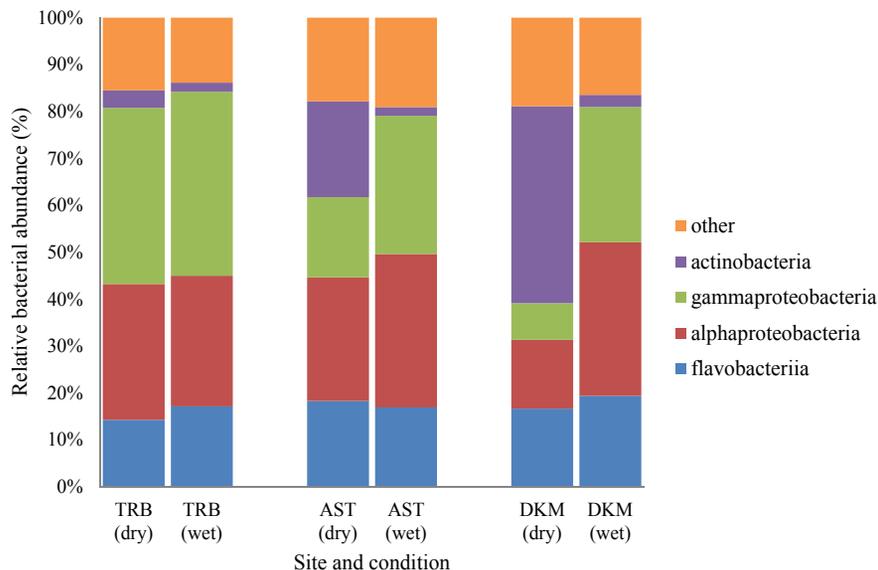


Fig. 2: Relative bacterial abundance (by class) of samples taken from Newtown Creek on dry (09/08/2016) and wet (09/15/2016) days from TRB, AST, and DKM sites

for these species were combined at the class level and used to create this graph. The clusters represent the four classes that yielded the greatest number of species. Classes which constituted relatively lower numbers are combined into “other” in Fig. 2.

The relative abundance of Gammaproteobacteria increased after rain (Fig. 1). Gammaproteobacteria have varied lifestyles and metabolisms. In fact, they are so numerous and diverse that it is the 16S rRNA sequence alone that unites these organisms into a single class. This group also includes the pathogenic bacteria, *Yersinia*, and *Salmonella* (R). 264 species of Gammaproteobacteria were detected across the three sites and two days. Within Gammaproteobacteria, the most notable changes occurred in the families Alteromonadaceae, Oceanospirillaceae and Vibrionaceae, whose relative abundance rose in all three locations (Fig. 3), however the Oceanospirillaceae noticeably increased at DKM. Most of the rise in the Alteromonadaceae can be attributed to changes in levels of the marine bacteria *Halieta* (Suzuki *et al.*, 2012) and copiotrophic sewage associated bacterium *Marinobacterium* (Merino *et al.*, 1995). Most of the rise in Oceanospirillaceae can be attributed to *Neptunomonas* which are associated with marine sediments (Hedlund *et al.* 1999, Miyazaki *et al.*, 2008); *Marinomonas* which are associated with coastal water (Simó 2001); and the hydrocarbonoclastic *Thalassolituus* (McKew

et al., 2007) and *Pseudosprillum* (Satomi and Fujii, 2014). Most of the rise in the Vibrionaceae can be attributed to *Vibrio natrigens* a bacteria associated with coastal and littoral sediment (Wang *et al.*, 2013), and *Vibrio diabolicus* a species previously isolated from deep sea vent worms (Raguénès *et al.*, 1997). Interestingly, the relative abundance of the *Oleiphilus* a hydrocarbonoclastic organism (Satomi and Fujii, 2014) and *Coxiella* a human pathogen (Voth and Heinzen, 2007), decreased after rain.

Conversely, the relative abundance of the Actinobacteria was found to decline after rain (Fig. 1). These bacteria are found in both terrestrial and aquatic environments where they are most abundant in soils, as well as sediments. This class includes bacteria that are human, plant, and animal pathogens, such as *Mycobacterium tuberculosis*. Other actinobacteria function as symbionts or endosymbionts with plant or animals, including the nitrogen-fixing genus *Frankia* (R). 71 species of Actinobacteria were detected across the three sites and two days. Within the Actinobacteria, the families with the biggest changes were microbacteriaceae and actinomycetaceae (Fig. 4). The relative abundance of both taxa dropped, particularly at AST and DKM. Most of the decline in the relative abundance of microbacteriaceae can be attributed to levels of *Candidatus aquiluna*, a freshwater bacterioplankton (Hahn 2009). Similarly, the relative abundance of actinomycetes decreases

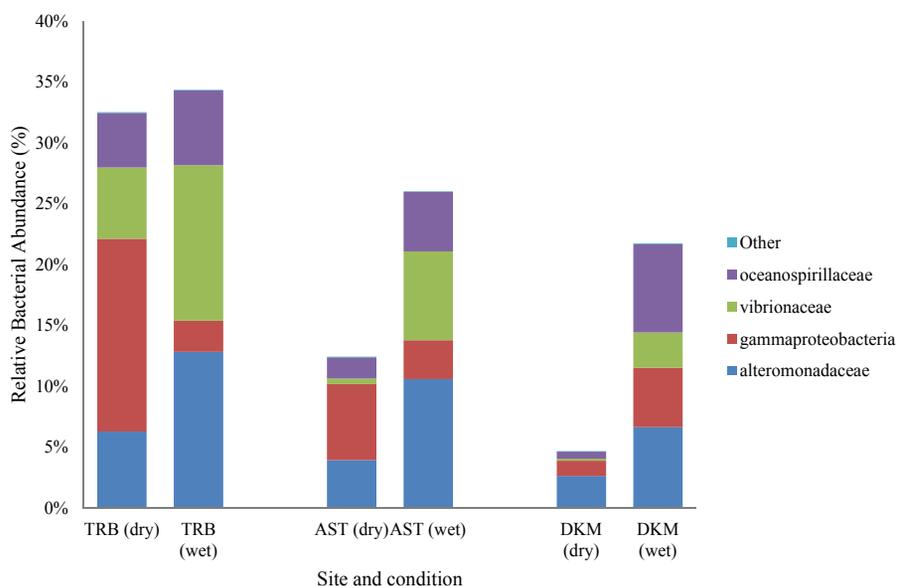


Fig. 3: Relative bacterial abundance of gammaproteobacteria families of samples taken from Newtown Creek on dry (09/08/16) and wet (09/15/17) days from TRB, AST and DKM sites.

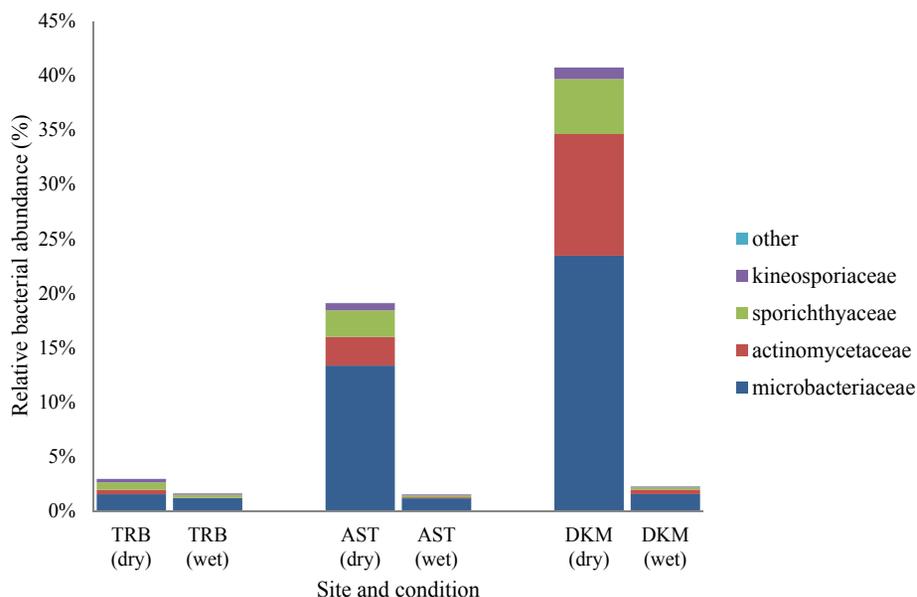


Fig. 4: Relative abundance of Actinobacteria (by family) of samples taken from Newtown Creek on dry (09/08/16) and wet (09/15/17) days from TRB, AST and DKM sites

due to the decline in levels of *Actinomyces* bacteria, which can be part of the normal microbiota of animals and plants, as well as pathogens (Ventura *et al.*, 2007). Interestingly, some *Actinomyces* are known to produce antimicrobial compounds (Mustafa Oskay *et al.*, 2004). Several prototypical patterns in bacterial population emerged as a result of the research comparing a dry to a wet sample day at three sites on Newtown Creek.

Transient microbiota

Some bacteria populations rise at DKM versus the other sites (e.g. *N. italica*, *Spongibacter*, *Candidatus Ruthia magnifica*, *Candidatus Thiobios Zoothamr*, *Gaiella*, *Amycolatopsis lexingtonensis* and *Phaeobacter*). During rain storms partially treated sewage is released near here (CNYDEP, 2017). Further study will be required to determine whether these organisms are part of the microbiome of the creek or transients released into the creek as part of the partially treated sewage from the wastewater treatment plant.

Proximity effect of wastewater treatment plant

There are other bacteria whose levels rise at both DKM and AST after rainfall, but not at TRB (e.g. *Rhizobium*, *Rhodobium*, *Marinomonas*,

Neptunomonas naphthovorans). We hypothesize this may be due to a proximity effect. During periods of high or sustained precipitation, sewage, wastewater, and street runoff is discharged into the creek (CNYDEP, 2011). These organisms may be best equipped to utilize nutrients in the effluent. Bacteria are regulated by these allochthonous resources, which trigger growth and reproductive responses.

A microbiome rich in hydrocarbonoclasts

After rain, populations of many hydrocarbonoclastic bacteria rose at the three sites, increasing the most at DKM and the least at TRB. These results are expected, as TRB is the furthest sample site from the East River and therefore the site where the tidal effect is the weakest. Hydrocarbonoclasts species identified at Newtown Creek included bacteria from the genera: *Alteromonas* (Math *et al.*, 2012), *Citricella* (Wang *et al.*, 2014), *Flavobacterium* (Al-Mailem *et al.*, 2015; Olapade 2013), *Gaetbulibacter* (Al-Mailem *et al.*, 2015), *Haliaea* (Suzuki *et al.*, 2012), *Mycobacterium* (Wang *et al.*, 2014), *Oleispira* (Dong *et al.*, 2014), *Owenweeksia* (Al-Mailem *et al.*, 2015), *Thalassolituus* (McKew *et al.*, 2007), *Marinobacter* (Wang *et al.*, 2014), *Neptunomonas* (Hedlund *et al.*, 1999), and *Mycobacterium* (Wang *et al.*, 2014). Higher tides, street runoff and CSO overflow may

cause turnover within the creek. The turnover may mix oil-containing bottom sediments into the water column making hydrocarbons available for these bacteria.

Human and animal pathogens in the Creek

Klebsiella pneumoniae, is an important human opportunistic pathogen associated with nosocomial infections (Podschun and Ullmann, 1998). As part of the intestinal microbiota, it is unsurprising that levels of *K. pneumoniae* increased after a potential CSO event. The presence of *V. parahaemolyticus* and *V. vulnificus* in the creek on the dry sample day and the rise in their levels on the wet sample day may be a result of the inflow of human waste, as both of these organisms are associated with diarrhea. While, human pathogens are to be expected in Newtown Creek as a result of CSO effluent loading, the detection of the obligate intracellular parasites Rickettsiales was surprising. Several species of ticks that carry *R. parkeri* can be borne by migrating birds (Mukherjee *et al.*, 2014). Levels of the pathogen are high at the TRB prior to the rain. *E. chaffeensis* requires reservoir organisms including white tailed deer, goats, dogs, foxes, birds and coyotes (Kocan *et al.*, 2000; Ganguly and Mukhopadhyay 2008). The fact that levels of *E. chaffeensis* and *Rickettsia spp.* rose after rainfall at both AST and DKM appears anomalous since sewage is unlikely to bear these intracellular parasites.

CONCLUSION

Molecular identification methods enabled us to identify organisms from Newtown Creek that are not discernible through traditional microbiological methods. Our results indicate that the creek supports a diverse bacterial population of over 1000 species including pathogenic, benthic, environmental, mutualistic and hydrocarbonoclastic bacteria. The microbial data presented here provide insight into the ecology of the creek. The plethora of microbes plays an essential role in nutrient cycling of complex industrial compounds and pollutants, affecting the entire ecosystem. Some of the organisms found in this survey are endemic to extreme environments, yet appear to have adapted to this heavily modified waterway. Newtown Creek has been subject to two centuries of industrial pollution and more recently to wastewater pollution. The process of ridding the

creek of chemicals, pollution sources, and employing green infrastructure has already made significant progress (NYDEC, 2016). Estuaries polluted by oil can be remediated by hydrocarbonoclastic bacterial communities (McKew *et al.*, 2007). The presence of hydrocarbonoclastic bacteria in the creek suggests future remediation efforts should consider utilizing bioremediation. Additionally, levels of these bacteria might be used as indicators of pollution levels at the site. Our results suggest that many pathogenic bacteria inhabit the creek. Nosocomial infectious pathogens, intracellular bacterial parasites, and other bacteria known to be pathogenic to humans, animals and other organisms inhabit the creek at different levels depending on weather and nutrient conditions. Monitoring levels of these bacteria as well as their genetics may be useful for development and implementation of health alert programs and community education. As a polluted body of water subject to combined sewage overflows, algae blooms and the resultant eutrophication cycle, the relative abundance of several essential nutrients may play a large role in changes in the microbiome of Newtown Creek. To our knowledge this has not yet been studied, however the data presented here suggests this is an area for further study. Levels of dissolved oxygen dropped at all three sites after rain. This may be due to algal blooms as phytoplankton utilize nutrients present in CSO effluent and this may affect the creek's ecosystem (Pinhassi *et al.*, 1999). As a result, correlating plankton blooms and CSO events may warrant further study.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Natural Sciences Department at LaGuardia Community College, Greenpoint Community Environmental Fund, Office of the New York State Attorney General, as well as the professional staff Congress and CUNY Research Scholars Program of the City University of New York for providing research funds. The Newtown Creek Alliance and our research students L. Akther, Y. Izumiyama, J. Miller, A. Nugent, Z. Rebong, M. Tzanidakis, who helped with collecting and processing samples and Dr. S. Durand for providing mentorship.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest regarding the publication of this manuscript.

ABBREVIATIONS

<i>16S rRNA</i>	16 subunit ribosomal ribonucleic acid
<i>AST</i>	Apollo street
<i>BLASTn</i>	Basic local assignment search tool nucleotide
<i>BLS</i>	Blissville seep
<i>CNYDEP</i>	City of New York Department of Environmental Protection
<i>CSOs</i>	Combined sewer overflows
<i>CSS</i>	Combined sewer system
<i>DKM</i>	Dutch kills mouth
<i>DNA</i>	Deoxyribonucleic acid
<i>EPA</i>	Environmental Protection Agency
<i>GOS</i>	Greenpoint oil spill
<i>ISO</i>	Isomeric
<i>km</i>	Kilometer
<i>min</i>	Minimum
<i>ml</i>	Mililiters
<i>MPN</i>	Most probable number
<i>n.d.</i>	Not dated
<i>NCBI</i>	National Center for Biotechnology Information
<i>NCEI</i>	National Centers for Environmental Information
<i>OTUs</i>	Operational taxonomic units
<i>PCR</i>	Polymerase chain reaction
<i>ppt</i>	Parts per thousand
<i>TRB</i>	Turning basin
<i>UEA</i>	Urban aquatic environments
<i>um</i>	Micrometers
<i>USDOI</i>	United States Department of the Interior
<i>USEPA</i>	United States Environmental Protection Agency
<i>UV</i>	Ultraviolet

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HOW TO CITE THIS ARTICLE

Calderón, O.; Porter-Morgan, H.; Jacob, J.; Elkins, W., (2017). Bacterial diversity impacts as a result of combined sewer overflow in a polluted waterway. *Global J. Environ. Sci. Manage.*, 3(4): 437-446.

DOI: [10.22034/gjesm.2017.03.04.009](https://doi.org/10.22034/gjesm.2017.03.04.009)

url: http://gjesm.net/article_27147.html

