JOURNAL OF
EMERGING INVESTIGATORS

Article

An analysis of soil microhabitats in Revolutionary War, Civil War, and modern graveyards on Long Island, NY

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SUMMARY

Previously established data indicate that cemeteries have contributed to groundwater and soil pollution. This study aimed to determine whether there was a variation in the microbes in cemeteries due to embalming techniques and to identify primary microbial communities in each cemetery. Different embalming techniques were used during different eras in recent history. These fluids can impact both epinecrotic and thanatomicrobiomes, the microbiomes that exist in decomposing remains. Additionally, these fluids can seep into the microbial communities, affecting the microbiome composition, and continue to leach into the larger surrounding environment, contributing to water source and aquifer pollution. We evaluated the 16S rDNA microbial gene followed by QIIME analyses using the Jupyter Notebooks application. We hypothesized that microbial variation would be high between cemeteries of different eras due to dissimilarities between embalming techniques employed, and furthermore, that specific microbes would act as an indication for certain contaminants. The results indicate that the following phyla were present: Proteobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, Chloroflexi, Verrucomicrobia, Acidobacteria, and AD3. These taxonomic classifications were present in all sites with the exception of AD3 which was absent in the Civil War Cemetery. Overall, cemetery sites were clustered together based on location due to variations in the concentrations of the phyla and their more specific taxa.

INTRODUCTION

A microbiome is a collection of microorganisms that reside in a particular environment, and microbiomes are the basis for all ecosystems. Microorganisms are partially responsible for the intake of carbon into an organism, as well as contributing to tissue growth or the decomposition and recycling of biomatter (1). The biodiversity within microbiomes is important to study because it can indicate the health of an environment and whether or not pollution is present (2). Microbiomes can metabolize or respond to many different chemicals, and different microbes can succeed in different environments due to their metabolisms. Additionally, microorganisms are responsible for carbon uptake, oxygen release, and nitrogen conversion (3).

Cemeteries contain unique microbiomes that require special attention due to the risks of soil or water contamination (4). The type of embalming fluids used may contribute to pollutants in aquifers. Revolutionary War cemeteries used no embalming techniques, Civil War cemeteries used arsenic, and modern cemeteries use formaldehyde (5). Approximately 827,060 gallons of embalming fluid is also buried, which primarily consists of formaldehyde (6).

Leachate is water that has percolated through the soil and retains some of the pollutants within the soil. Leachate can contribute to the spreading of harmful substances such as the components of embalming fluids. If not properly contained, leachate can have noticeable implications to the surrounding ecosystem health, as contaminated leachate may contain pathogenic bacteria and viruses that can contaminate drinking water (4).

Certain microbes have been identified as able to metabolize either arsenic or formaldehyde, which are used by these microbes to perform functions such as anaerobic respiration, methylation, detoxification, and assimilation (7). Previous research suggests the possibility for heavy metal contamination from embalming fluids at gravesite locations. Copper, lead, zinc, and iron have been found in increased concentrations at gravesites as well was a dramatic increase, as opposed to cemeteries not using embalming fluids, in arsenic levels indicating graveyard contaminates (8). Heavy metal pollution from sources such as iron, copper, and zinc has been shown to have carcinogenic and non-carcinogenic negative health implications, especially for children and people living on or near contaminated areas (9).

Three cemeteries were selected in Long Island, NY in this study based on the time period when burials were taking place. The Manor of St. George, New York contains a cemetery that houses bodies from 1775-1783, the Revolutionary War era. The Union Cemetery in Middle Island contains bodies from 1861-1865, the Civil War era. The Holy Sepulcher Cemetery in Coram contains bodies which have been buried in the past 50 years, roughly.

The purpose of the study is to determine if the different burial techniques utilized have affected the surrounding areas' soil microbiomes. We hypothesized that there will be a high variation in microbiomes between the three cemeteries, due to the dissimilarities in embalming techniques. Triplicate samples were collected from three different cemeteries and the 16S gene was isolated and amplified. The samples were

then sequenced and processed through a data informatics pipeline to create graphical representations of the data. There was variation between all three locations; however, the distance was the greatest between the Civil War cemetery relative to the other two cemeteries.

RESULTS

Phyla Present Across All Cemeteries

To reiterate, this research was conducted in order to determine if there were varying concentrations of microbiomes, due to dissimilarities in embalming technique. This research was conducted by analyzing the 16S rRNA gene which yields data on microbial taxonomic presence and approximate population counts. Post sequencing, the data was analyzed through a bioinformatics pipeline (QIIME) and generated graphical representations of microbial data.

Each sample taken from the various cemeteries contained similar phyla. The predominant phyla present included: Proteobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, Chloroflexi, Verrucomicrobia, Acidobacteria. The phylum AD3 is present in both the modern and Revolutionary cemeteries but appears to be absent within the Civil War cemeteries (**Figures 1 and 2**).

Alpha Diversity Between the Soil of Different Cemeteries

Alpha diversity is a measure of microbial diversity within a specific location, which indicate species richness. Again, the aims of this project were to determine if microbiomes varied due to different embalming techniques. The sediments of the Civil War cemetery appear to have the highest microbial species richness while the Revolutionary War cemetery contains the lowest (**Figure 3**).

Variations Between the Civil War, Modern, and Revolutionary War Cemeteries

The Civil War cemetery contained many phyla that are similar to the other cemeteries but are present in different concentrations (**Figure 1**). The Civil War site contained more taxa that appeared to be evenly distributed (**Figure 3 and 5**). The Principal Component Analysis (PCoA) demonstrated the increased variation between the microbial Civil War community in comparison to the Revolutionary War and modern cemeteries (**Figure 4**). This indicates that there are differences in the microbial communities, with the Civil War cemetery demonstrating the largest difference as compared to the other two sites.

The Civil War cemetery contained greater Proteobacteria concentrations, which varied from 29% – 35% (**Figure 1**). In contrast, the other cemetery samples had lower representations with the highest being 26% and the lowest being 18%. Another aspect contributing to this variation in beta diversity, is the Civil War cemetery consisted of greater Bacteroidetes proportions, averaging 9.22% in contrast to modern Cemeteries, which contained approximately 2.16% and Revolutionary which contained 1.38% relative amount, which would heavily impact species richness. The two phyla in which the Civil War cemetery contained lower representations were: Acidobacteria and Verrucomicrobia. The Civil War Cemetery contained the least Acidobacteria representation, averaging 10.26% compared to modern Cemeteries which is shown to have approximately 40.91% and Revolutionary which contained 23.56% Acidobacteria representation. The Civil War cemetery contained the lowest Verrucomicrobia representation, averaging 4.36% as opposed to modern cemeteries which contained approximately 8.23%, and Revolutionary cemeteries, which contained 10.46% Verrucomicrobia representation.

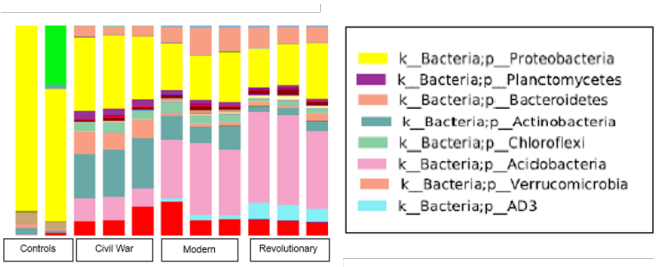

Figure 1. Phyla concentrations in different cemeteries. Shown above is a taxonomic plot that represents the phyla concentrations in different cemeteries. Most notably, AD3 was present in all sites, with the exception of the Civil War cemetery.

DISCUSSION

The research question posed was: Is there a variation in microbiomes in Revolutionary, Civil War, and modern cemeteries? It was hypothesized that microbial variation would be high between different era cemeteries due to dissimilarities between embalming techniques employed. The data indicates the microbial communities within each of the cemeteries are clearly distinct but consist of many of the same bacterial phyla. Phyla such as Proteobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, Chloroflexi, Verrucomicrobia, and Acidobacteria were present across all locations (**Figures 1 and 2**). Proteobacteria and Acidobacteria are a diverse set of heterotrophic microorganisms that are

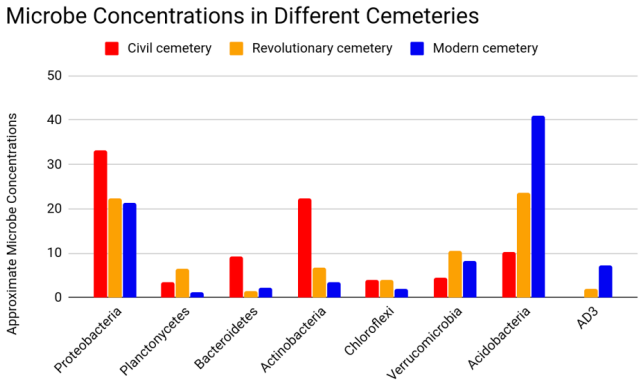

Figure 2. Microbe concentrations in different cemeteries. Results from the bar graph further support the lack of AD3 within the Civil War Cemetery. Additionally, of the primary phyla present, Proteobacteria, Bacteroidetes, and Actinobacteria are at notably higher concentrations at the Civil War site.

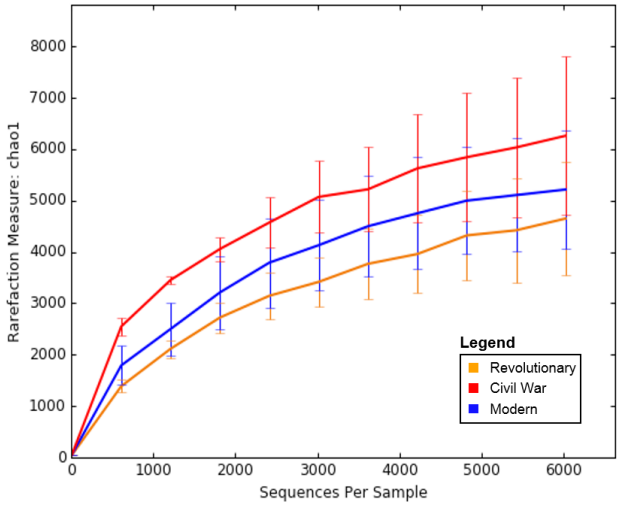

Figure 3. Microbial species richness of sediments from different era cemeteries. The rarefaction chart data indicates that the Civil War cemetery had the highest alpha diversity while the Revolutionary War contained the lowest.

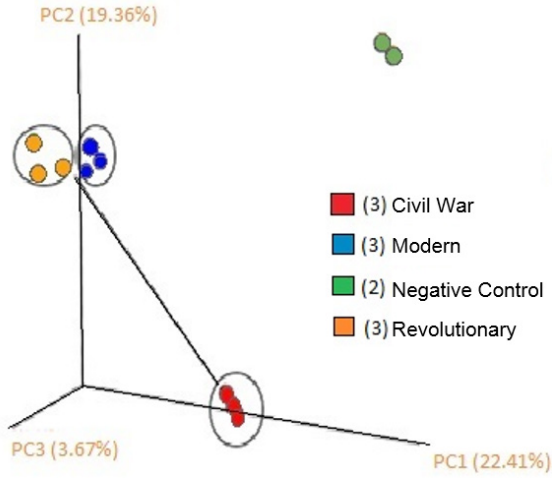

Figure 4. Principle component clusters of microbes from Revolutionary, Civil War, and Modern eras. The large distance between the three sample sites on the principle component analysis (PCoA) plot is indicative of high beta diversity between the Civil War microbial community and the other two cemeteries. The modern and Revolutionary War cemeteries are clustered more closely together suggesting similarities between microbes present in those two communities while still maintaining distinct microbiomes. Additionally, it is important to note that the lab controls are distinctly separate from all samples, supporting the validity of the results.

expected to reside within soil microbial communities. Previous research indicates that cultured Acidobacteria are capable of utilizing carbohydrates, fixating nitrites, and can respond to both macronutrients and micronutrients within acidic soil (10). Additionally, Verrucomicrobia and α-Proteobacteria are associated with the soil microbial communities near forested regions (11). The only phyla that was highest in the Revolutionary War cemetery was Verrucomicrobia (**Figure 2**). This is relevant as this cemetery was more heavily wooded than the other two sites. The Revolutionary site also had the highest concentration of Planctomycetes. This phylum is metabolically diverse but is capable of sulfur and sulfide reduction in both aerobic and anaerobic conditions (12). Throughout all sample sites, the Revolutionary War site had the lowest concentrations of Chloroflexi, a phylum that is strongly associated with the metabolism in ecosystems containing high nitrogen and organic matter concentrations (13). This may indicate that the Revolutionary War site contained the lowest concentrations of organic decaying matter.

The rarefaction plot in suggests that the Civil War cemetery had the highest microbial species richness while the Revolutionary War cemetery contained the lowest (**Figure 3**). This may be attributed to the variations between microbial presence between the different sites.

Each community is clearly clustered together based on location, as seen in the PCoA plot (**Figure 4**). Out of the sites analyzed, the most distinct microbiome was found at the site containing bodies from the Civil War time period. This was evident as it had the highest distance in relation to the other sample sites (**Figure 4**). For example, although Proteobacteria, Bacteroidetes, and Actinobacteria were present in all sites, they are present in notably higher concentrations at the Civil War site (**Figure 2**). Additionally, this site also contained the highest species richness (**Figure 3**). These distinctions may be attributed to numerous abiotic and biotic factors including but not limited to: soil pH, biomatter presence, different rates of decaying bodies, embalming techniques, and different ecosystems in close proximity to the cemeteries (14).

High concentrations of Koribacteraceae and Ellin 6513 bacteria are present within the modern and Civil War cemeteries and are decreased or negligible within the Revolutionary War cemetery. This may be attributed to decomposing biomatter present in modern and Civil War cemeteries, but not necessarily present in Revolutionary War cemeteries due to increased time elapsed for bodies to decompose (15). Another variation evident between communities was that increased Acidobacteria was present in

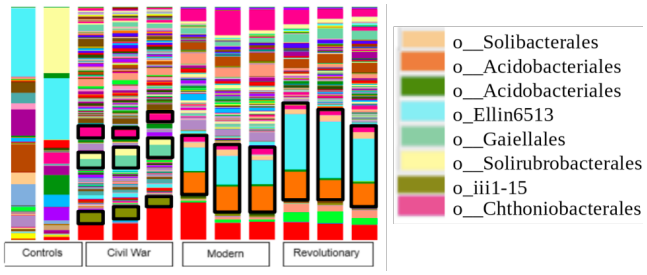

Figure 5. Distribution of taxa. The taxonomic plot depicts the microbial orders and distribution of taxa present. Bolded boxes highlight the orders on the legend to the right. This depicts that the Civil War era cemetery has a microbiome that is greatly different than the other two. The iii1-15 microbes were somewhat successful in the Civil War era cemetery while present in near negligible amounts in the Modern and Revolutionary War cemeteries. The Ellin6513 microbes are the opposite of iii1-15, where they are successful in the Modern and Revolutionary war era cemeteries while present in small amounts in the Civil war era cemeteries.

the Revolutionary War cemetery (**Figure 2**). This is indicative of low soil pH; however, that cannot be confirmed (14).

The PCoA plot also demonstrates that the distance was the least between the Revolutionary War cemetery and the modern cemetery (**Figure 4**). They also had similar microbe concentrations (**Figure 2**) and were closer in species richness (**Figure 3**). This may be attributed to caskets used in modern cemeteries and increased decomposition of bodies present in the Revolutionary War cemetery. Conversely, the Civil War cemetery was the furthest from the other sample sites on the PCoA plot (**Figure 4**). This variation may be due to the lack of the AD3 or containing sediment that can support high species richness (**Figures 2 and 3**). This could also be due to consisting of notably higher concentrations of Proteobacteria, Bacteroidetes, and Actinobacteria or, containing notably lower concentrations of Verrucomicrobia and Acidobacteria (**Figures 1 and 2**). It could also be due to different abiotic and biotic factors influencing microbial diversity.

Overall, according to the data presented, there is a variation in the microbiomes of the different eras' cemeteries. Data indicates the following: Proteobacteria, Bacteriodetes and, Actinobacteria concentration is increased in the Civil War cemetery. Verrucomicrobia and Planctomycetes concentration is increased in the Revolutionary War cemetery. While AD3 is only present in increased concentration in the modern cemetery, and Chloroflexi is present in decreased concentration in the modern cemetery. These variations are believed to be due to a number of factors such as pH level, biomatter variation, caskets utilized, and dissimilarities in burial techniques.

MATERIALS AND METHODS

At each graveyard, samples were collected 12 inches beneath the organic layer in a corner that is not directly above a gravesite. At each sample site, approximately 2.0 grams of soil was collected in triplicate. The 2014 Detailed MO BIO protocol (Powersoil DNA isolation kit, Qiagen) was used for DNA extraction and isolation (16). 0.25 grams of soil was added into MO BIO PowerBead Tubes alongside negative controls, which were comprised of empty microfuge tubes. Following this, the tubes were mixed using a vortex. Then, 60 µl of Solution C1, a type of cell membrane lysis solution, was added. Afterwards, the tubes were vortexed to mix the contents. The tubes were mixed for 10 minutes using the vortex at maximum speed. The tubes were then centrifuged at 10,000 x g for 30 seconds at room temperature (16).

Then, 250 µl of Solution C2, a solution that aids in the precipitation of non-DNA organic and inorganic material such as humic substances, cell debris, and proteins, was added and the tubes were centrifuged again for 5 seconds, then incubated at 4°C for 5 minutes. After incubation, the tubes were centrifuged again for 1 minute at 10,000 x g. Then, avoiding the pellet, 600 µl of the supernatant was transferred to a clean tube (16).

Then, 200 µl of Solution C3, another solution that aids in the precipitation of non-DNA material, was added, vortexed briefly, and incubated at 4℃ for 5 minutes. The tubes were then centrifuged for 1 minute at 10,000 x g, and 750 µl of the supernatant was transferred to a clean tube. The solution was mixed before adding 1.2 ml of the Solution C4, a high-concentration salt solution, to the supernatant and vortexed for 5 minutes. Then, 675 µl of supernatant was loaded onto a spin filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow-through was discarded, and an additional 675 µl of supernatant was added to the spin filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow-through was discarded, and the remaining supernatant was loaded and centrifuged at 10,000 x g for 1 minute at room temperature (16).

Next, 500 µl of Solution C5, an ethanol-based wash solution used to further clean the DNA, was added and centrifuged at room temperature for 30 seconds at 10,000 x g. The flow-through was discarded from the 2ml Collection Tube. The remainder was centrifuged at room temperature for 1 minute at 10,000 x g. The spin filter was placed into a clean 2ml Collection Tube (16).

Then, 100 µl of Solution C6, an elution buffer, was added to the center of the white filter membrane. Then the tube was centrifuged for 30 seconds at 10,000 x g at room temperature. The spin filter was discarded. The DNA in the tube was then ready for further downstream applications (16).

After DNA extraction, the 16S gene was amplified using the 16S primer and a thermocycler. Gel electrophoresis was used in order to confirm that the 16S gene was amplified. The samples that successfully amplified were sent to Cold Spring Harbor Laboratory for indexing, and from there, to be sequenced using Illumina's MiSeq system. The resulting collection of sequences from each sample were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) analyses conducted through the Jupyter Notebook web application, which allowed for the generation of the PCoA, rarefaction, and taxonomy plots.

Received: April 11, 2018
Accepted: October 29, 2019
Published: May 5, 2019

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