Proceedings of The National Conference
On Undergraduate Research (NCUR) 2017
University of Memphis TN
Memphis, Tennessee
April 6-8, 2017

Investigating the Microbiome of the Eastern Oyster (*Crassostrea virginica*) along a Latitudinal Gradient

Dupah Gobin
Department of Biology
City University of New York, York College
New York, New York

Faculty Advisor: Dr. Elizabeth Alter

Abstract

Oysters are ecosystem engineers that provide a habitat for native species and are important nitrogen transformers in estuaries. Oysters also perform ecosystem services such as water filtration, nitrogen sequestration and lead to the improvement of water quality. Past studies have shown that gut microbiomes in oysters have high denitrification action thus producing N2O and N2 as the final product. Surprisingly, little information is currently known about the gut microbiome diversity of oysters or how the gut microbiota varies across spatial scales. The purpose of this study is to use next-generation sequencing and metagenomic analyses of the 16S rRNA gene to characterize the gut microbiota of oysters living along a latitudinal gradient along the Atlantic Coast of the United States to determine whether these communities shift predictably with changes in water temperature. 16S data were used to determine community composition, alpha and beta diversity, and important clades of bacteria found in the oyster gut. Selected oysters from several locations, (Cape May, Blue Point, and Gerrish Island) were obtained, and their stomachs were dissected out. Microbial DNA was extracted from oyster guts using the PowerSoil DNA Isolation kit. Collected oysters from different geographically distinct sites, shows similar bacteria within the gut microbiomes. The phyla *Spirochaetes* and *Tenericutes* were the most overrepresented in the oyster gut. Variations and differences between different sites within the gut microbiomes suggest that microbiome composition might respond to local factors, and perhaps to genetic differences among oysters.

Keywords: 16S rRNA gene, *Spirochaetes*, *Tenericutes*.

1. Introduction

Microbiotas are a microbial community living in humans and other species. They usually refer to the whole population of microorganisms that inhabits a particular location.[1] Microbiota is essential regarding the function of the human body, whether they help with the digestion of food products or synthesis of vitamins. They are useful regarding the integrity of the gut mucosa as well as the maturation of the immune system. Over the years significant interest has evolved on the gut microbiota within the scientific community where the gut microbiota has been related to several human diseases.[2]

Eastern oysters, *Crassostrea virginica,* are well-known for their role as important ecosystem engineers along the Atlantic coast.[3] Oysters play a crucial role in the environment by performing essential tasks such as nitrogen transformation in estuaries, water filtration, nitrogen sequestration and are involved in the improvement of water quality. However, the microbial communities within the oyster gut are still relatively unknown. From an ecological perspective, the gut microbiota is considered a unique microbial habitat in which microbial diversity and community structure can be determined by several factors. Some includes island biography, community ecology theory and the historic process of the community assembly.[4] Gut Microbial communities are imperative for the ecosystem function because they can help determine whether bacteria in a

community is functional. Also it can consequently assess their functions, interactions with other bacteria and its environment it lives in .[5]

To better understand and appreciate oyster gut microbial communities, we analyzed oysters from three different locations in this study. With recent technology, we are better equipped and have found ways to harvest marine organisms. One of these methods is known as farming, but in this setting, its oyster farming. Organisms that are being farmed from the sea or any aquatic organisms are known as mariculture and have become economically and ecologically beneficial.[6] Oyster farming in Louisiana consists of six important steps. The first is site selection, algae selection, broodstock, spawning, larval rearing, and lastly setting.[6] The site selection is one of the most important stages because the location that is being selected needs to have suitable water quality. This includes focusing on the water temperature. The water temperature is crucial because one may want to maintain constant temperature throughout the entire process.[6] The temperature is vital for understanding the oyster's impact in a changing environment.[7]

Our first set of oysters came from Cape May, New Jersey. These oysters were obtained from the Salt Oyster Co. farm. The oysters from this location are grown in traditional regions of the lower Delaware Bay. Water temperature in Delaware Bay is less than 5 °C for less than 13 weeks according to a past study.[8] The second set of oysters came from Long Island Blue Point, New York. This oyster farm is located towards the Atlantic, Fire Island inlet in the Great South Bay. The farm that provided these oysters are from the Long Island South. Which is a tidal estuary of the Atlantic Ocean that lies between the eastern shores of Bronx County, New York City, southern Westchester County, and Connecticut to the north, and the North Shore of Long Island, to the south. The third set of oysters came from Gerrish Island in Maine. These oysters were obtained from Mid-Atlantic waters and were placed in tanks of sterilized Maine seawater at Spinney Creek Shellfish, Inc.

2. Materials and Methods

2.1 Locations:

Nine oysters altogether were collected from three different locations. There was no way of telling whether these oysters were male or female. Three oysters were collected from Cape May New Jersey, three from Blue Point Long Island New York, one from Blue Point Suffolk County New York, one from Cape May New Jersey and one from Gerrish Island Maine. The oysters that were collected were all medium in sizes (fig. 1). Collected oysters were looked under the microscope to identify the main parts of the oysters such as the heart, gills pericardium, adductor muscle and intestine.

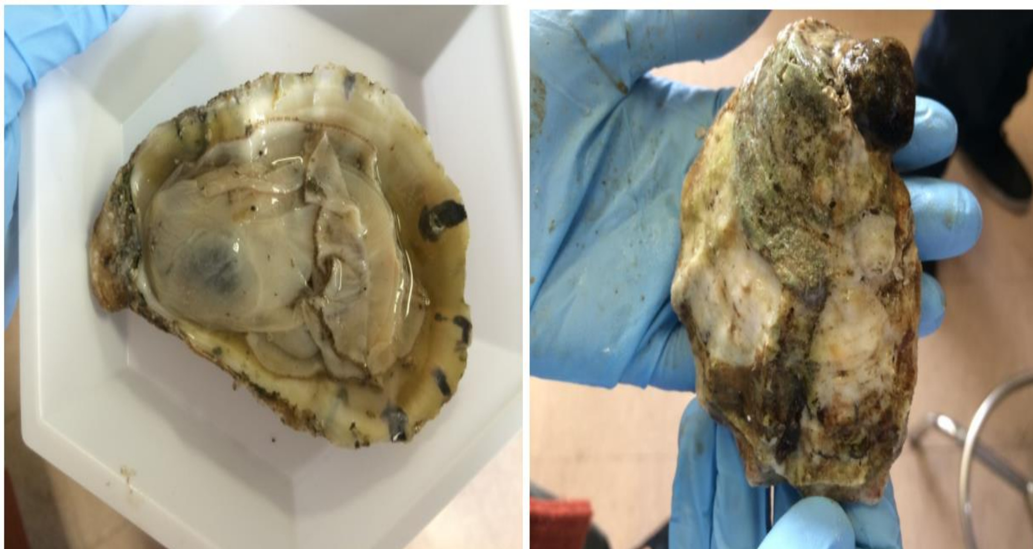

Figure 1: Collected oysters from New Jersey, Cape Neddick showing its beautiful color and size.

2.2 DNA Extraction:

DNA extraction was carried out on all nine oyster samples using the Power soil DNA isolation kit. Approximately 0.25g of dissected oyster gut was added to the PowerBead tubes and it was gently vortexed to mix the components in the PowerBead. Solution C1 contains SDS detergent and other disruption agents that are important for cell lysis. 60 µL of solution C1 was added and vortex. Samples were then centrifuged at 10,000 x g for 30 seconds. The supernatant was transferred to the 2-mL collection tube. Solution C2 is a patented inhibitor removal technology, where it contains a specific reagent to precipitate non-DNA organic and inorganic material. Samples were then vortex for 5 seconds after the addition of 250 µL of solution C2. Samples were centrifuged for a minute at 10,000 x g. After that was completed, 600 µL of the supernatant was transferred to a 2-mL collection tube. At this step, the pellet contains non-DNA organic and inorganic material such as proteins. Solution C3 is also a patented inhibitor removal technology. This is a second reagent and its main purpose is to precipitate additional non-DNA organic and inorganic material. For 5 minutes the samples were vortex briefly after 200 µL of solution C3 was placed into the tube. Samples were centrifuged for a minute at 10,000 x g. Then, 750 µL of supernatant was transferred to a 2-mL collection tube. At this step, the pellet still contains non-DNA organic and inorganic material. Solution C4 is known as a high concentration salt solution. This solution helped adjust the DNA solution salt concentrations to allow the binding of DNA. Approximately 1.2 mL of solution C4 was added to the supernatant and it was vortex for 5 seconds. After that was completed, 675 µL supernatant was loaded onto a spin filter and centrifuged at 10,000 x g for one minute. The flow through was discarded and an additional 675 µL of the supernatant was added and then centrifuged for 1 minute. Solution C5 is an ethanol based wash solution which is used to clean the DNA that is bound to the silica filter membrane in the spin filter. 500 µL of solution C5 was then added and centrifuged for 30 seconds at 10,000 x g. The flow through was then discarded, and contained non-DNA organic and inorganic waste that was removed from the silica spin filter membrane by the ethanol wash solution. The remaining solution was centrifuged for 1 minute at 10,000 x g. This spin was considered the second spin and its main purpose was to remove residual solution C5. The spin filter was placed into a clean 2 mL collection tube. Then 100 µL of solution C6 was added to the center of the small white membrane of the spin filter. Solution C6 serves to make sure the entire membrane is wet and as a result, there will be a more efficient and complete release of the DNA from the silica spin filter membrane. After that was completed it was centrifuged for 30 seconds at 10,000 x g. The spin filter was discarded, and the DNA was ready for downstream analysis.

2.3 NanoDrop^{TM} Spectrophotometer:

We collected 0.2 µL of DNA from each sample. This method was used to measure the concentration and purity of DNA. DNA purity is measure by using the absorbance from 230nm to 320nm to detect possible contaminants. The most widespread calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm.

2.4 MR DNA:

The nine samples were sent to MR DNA for sequencing and comprehensive taxonomic analysis. The MR DNA analysis pipeline included a single step 30 cycle PCR that used HotStarTaq Plus Master Mix Kit. There were several conditions used. First, for 3 minutes at 94^{0}C, followed by 28 cycles of 94^{0}C for 30 seconds then at 53^{0}C for 40 seconds and 72^{0}C for 1 minute. The next step that follows was the elongation step which was at 72C for 5 minutes. Agencourt Ampure beads were used for the following PCR where amplicon products from different samples were mixed in equal concentrations and purified. After that was completed, the library samples were sequenced using Roche 454 FLX titanium instruments and reagents. The data that was provided from MR DNA and was imported into Excel and R studio for further analysis.

2.5 Data Analysis:

R Studio was used in loading, manipulating, visualizing, and reporting the data. R studio was used to create the non- metric multidimensional scaling (NMDS) plot showing clustering of microbial communities from the three different oyster locations. The oysters were sorted by source and location using R Studio where the *P*-values

were then used to determine statistical significance. Taxonomy is used primarily to identify different organisms and thus to categorize them into groups to give them names. Phylum was used chiefly because it better helps divide organism based on their specific traits for us to examine its genetic relationship. Excel was used to create a graph showing the diversity of oyster microbiota from the three different locations at the phylum level of taxonomy.

3. Results

A total of nice samples had its DNA absorbance and purity of isolated nucleic acids measured by utilizing the NanoDrop™ spectropotometer. The ratio between the readings at 260nm and 280nm ($OD_{260}:OD_{280}$) provides an estimate of the purity for the nucleic acid. Pure preparations of DNA A260/280 and A260/230 values greater than 1.8 are typically suitable for analysis. Almost all our samples were greater than 1.8 (Table 1). Only one of our samples was 1.75 however if rounded that would be a 1.8 thereby it's safe to say that all our samples were greater than 1.8. However, any samples lower than A260/280 values may indicate protein contamination while lower A260/230 values would indicate contamination with salts or some solvents such as phenol.

Analysis of phylum and classes between the three different locations for oysters resulted in the classification of several different operational taxonomic unit (OTUs). OUT was used primarily to conduct a diversity analysis. There were several similarities between the nine different oysters based on the OTU composition. The phyla *Spirochaetes*, and *Tenericutes* were the most overrepresented in the oyster gut, while other phyla were underrepresented (Figure 2).

Variations between the oysters were captured by the cluster analysis. Cluster analysis is a fundamental tool use in taxonomy. Results from the cluster analysis (Figure 3) showed that gut contents between the nine oysters collected from three different locations were not very similar to each other. The oysters that were collected from the same location were somewhat similar to each other than the oysters collected in a different location.

We were curious to know whether the location (where the oysters originated from) or the source (where we got the oysters from) play any important role in our experiment. We looked at the *P*-values to determine statistical significance. There was a 0.03 difference in the *P*-value for its source and a difference of 0.05 for its location. There were not many differences between these two-data analyses. However, sorting by source was significant while the location was close to being statistically significant. *P*-value 0.03, means that there is a 3% chance that we would get these results (or more extreme) by chance if the null hypothesis were true. The null hypothesis states that there will be no significant difference between the sources of the oyster. While the alternative hypothesis states that there will be significant differences among the sources of the oysters.

Table 1: NanoDrop^{TM} Spectropotometer

Location	Concentration ng/ µL	A260	A280	260/280	260/230
Blue Point A	54.7	1.033	0.48	2.12	-2.79
Cape May A	92.7	1.854	1.021	1.82	3.23
Gerrish Island Maine	54.0	1.08	0.616	1.75	3.25
Blue Point A Sample 1	36.1	0.723	0.392	1.84	3.07
Blue Point A Sample 2	36.5	0.729	0.404	1.8	2.58
Blue Point A Sample 3	50.3	1.006	0.57	1.77	2.36
Cape May B Sample 1	85.3	1.666	0.926	1.8	2.73
Cape May B Sample 2	158.9	3.179	1.771	1.8	2.38
Cape May B Sample 3	99.5	1.99	1.09	1.83	3.84

The NanoDrop^{TM} Spectropotometer is used to measure the absorbance of DNA or is used to measure the purity of isolated nucleic acids. When quantitating the amount of DNA, readings are taken at the wavelength of 260 nm and 280 nm. The reading 260nm allows calculation of the concentration of nucleic acid in the samples. The ratio between the readings at 260nm and 280 nm (OD_{260}:OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA A260/280 and A260/230 values greater than 1.8 are typically suitable for analysis. Lower A260/280 values may indicate protein contamination. Lower A260/230 values indicate contamination with salts or some solvents (e.g., phenol).

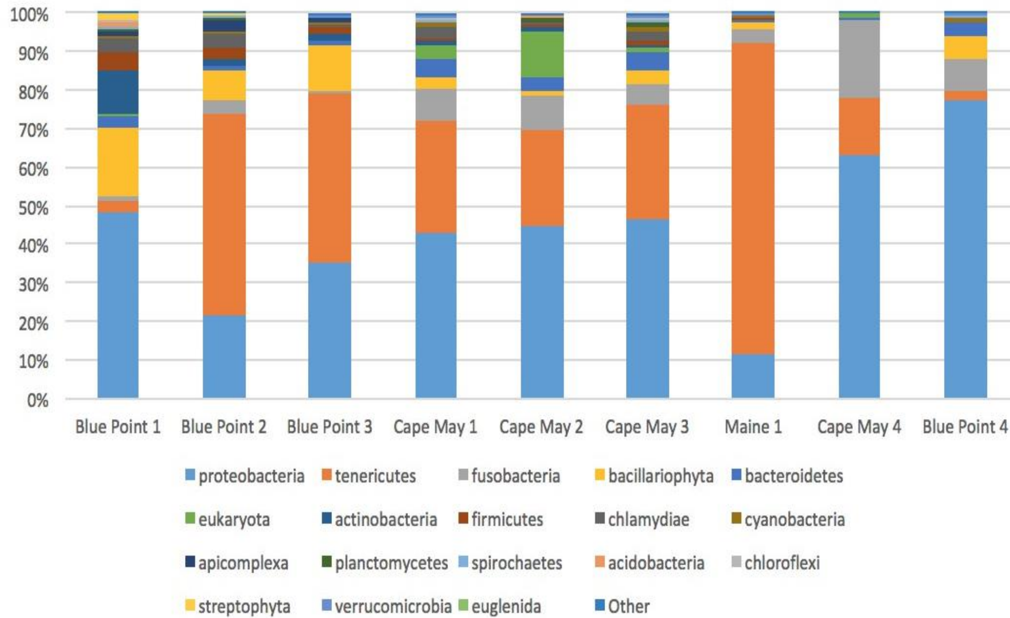

Figure 2: Graph showing the highest percentage of bacteria phylum found in the different location.

The graph shows the diversity of oyster microbiota from the three different locations at the phylum level of taxonomy. Bacterial phyla are indicated by the color. This statistical analysis of diversity helps us to see the differences of bacteria phylum in the different locations. The high percentage of bacteria indicated on the chart in blue represents the phylum *Spirochaetes*, and in orange represents *Tenericutes*, which are most prevalent in the oyster guts.

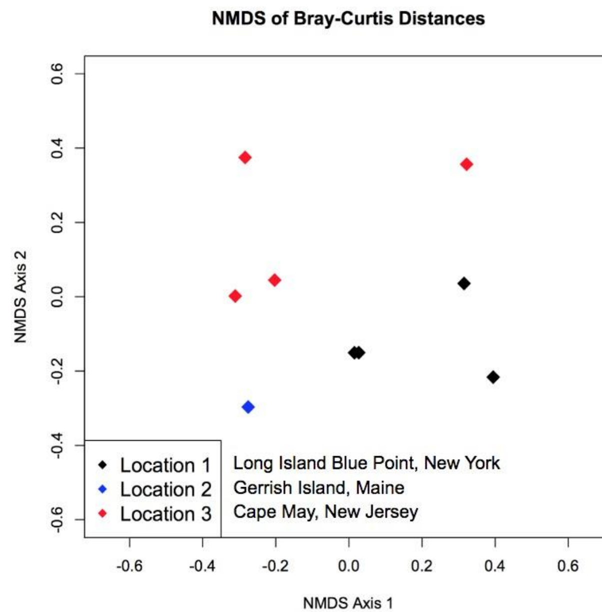

Figure 3: Non-metric multidimensional scaling (NMDS) plot showing clustering of microbial communities from the three different oyster locations.

NMDS plot based on Bray-Curtis dissimilarity. Microbial communities of each location are indicated by different colors: black diamonds- Long Island Blue Point New York; blue diamonds- Gerrish Island in Maine; and red diamonds- Cape May New Jersey. The distance linking two oyster sample is shorter, indicating higher similarity between these

Table 2: Oyster were sorted by source (A) and location (B) using R Studio. *P*-values were used to determine statistical significance.

A

```
Call:
adonis(formula = oyster_distances ~ Source, data = oyster_mapping,      permutations = 999)

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

          Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)  
Source     2   0.73158 0.36579  1.6432 0.35389  0.033 *
Residuals  6   1.33567 0.22261         0.64611         
Total      8   2.06724                 1.00000         
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

B

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Call:
adonis(formula = oyster_distances ~ Location, data = oyster_mapping,      permutations = 999)

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

          Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)  
Location   2   0.73158 0.36579  1.6432 0.35389  0.054 .
Residuals  6   1.33567 0.22261         0.64611         
Total      8   2.06724                 1.00000         
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

As a result, there were a 0.03 difference in the *P*-value for its source and a difference of 0.05 for its location. They were not much differences between these two-data analysis, however sorting by source were significant while the location was close to being statistically significant. *P*-value 0.03, means that there is a 3% chance that we would get these results (or more extreme) by chance if the null hypothesis were true. The null hypothesis states that there will be no significant difference between the sources of the oyster while the alternative hypothesis states that there will be significant differences among the sources of the oysters.

4. Discussion

The 16s rRNA gene sequence analysis reveals the differences in oyster microbiome across multiple taxonomic levels, including phylum and operational taxonomic unit (OTU) percentage. OUT's are advantageous in classifying groups of closely related individuals. After sequencing and analyzing the gut microbiota of oysters from three different locations along the Atlantic Coast, we determined that the oyster guts had very similar microbial compositions. The highest percentage of bacteria phyla (fig. 2) in each oyster was between *Spirochaetes* and *Tenericutes*. *Spirochaetes* are known as spiral microorganisms in which its individual cell occupies one or more complete spiral turns.[9] Previous research shows that *Spirochaetes* were found in the digestive tract of ninety percent of the oysters sold in the markets of Baltimore.[9] Researchers found that *Spirochaetes* are parasites in the gut of the oyster. *Spirochaetes* can survive in the digestive tract of the oyster

if there is liquid present.[9] On the other hand, *Tenericutes* are a distinctive class of bacteria that lack a cell wall. This includes many lineages known as parasites[10] and known as commensals of eukaryotic hosts.[11] Maine had the highest number of *Tenericutes* in its collected oysters (fig. 2). Reasons why Maine had a high number in *Tenericutes* could be due to environment factors such as water movements, salinity, temperature and food. A recent study utilized metagenomic 16S rRNA sequencing analysis of Pacific Oyster microbiota from the Puget Sound Region in the United States concluded that *Tenericutes* is one of the most abundant phyla.[12] Even though this study didn't focus on eastern oysters, they both had a high number of *Tenericutes.*

Although the oysters were obtained from different locations, the gut contents were primarily composed of these two phyla with some variations in each. A previous study analyzed the stomach and gut microbiome of Eastern oysters resulting in the identification of 5 OUT's, 3 phyla's in the stomach microbiome, 44 OTU's, and 12 phyla's in the gut microbiome.[1] In this research, we found 18 phylum's that were of significant. Analysis of the phylum composition revealed some similarities between gut and stomach microbiome between two locations, Hackberry Bay and Lake Caillou.[1] After sequencing the data, it determined that cyanobacteria and eukaryotes dominated the data set.[1] The composition of the gut was composed of Chloroflexi, Firmicutes, α-Proteobacteria, Planctomyces, and Verrucomicrobia.[1] However, the relative abundance of OTU percentage substantially differed. In comparison to our data, the differences in the gut composition is likely due to differences in the environment such as water temperature that the oysters were raised in or even seasonal changes.

The NMDS plot analysis (fig. 3) suggests that the gut microbiota composition is very similar to the microbial communities from the three different oyster locations. The varying oyster's location could have played a part in its similarities or variances. The plots for Blue Point and Cape May oysters are dispersed on the graph and show prominent difference. However, the interval between some clusters are relatively close in distance showing similarities in microbial community composition. Similarly, in the original study, the gut contents of oysters from Lake Caillou and Hackberry Bay were relatively close in distance on the plot analysis showing similarities between these two locations.

The *P*-values were obtained to determine the statistical significance of differences in community composition between location and its source. The location is where the oysters came from. Whether it is New York, New Jersey, or Maine. While the source is where the oysters were collected from such whole foods market. The *P*-value obtained from the oyster source was substantial because its value was < 0.05 (table 2). The null hypothesis states that there will be no significant difference between the sources of the oyster while the alternative hypothesis states that there will be significant differences among the sources of the oysters. Our *P*-value (0.033) indicates that we can reject the null hypothesis. This suggests that the source and the location of the oysters does play a role in shaping the gut microbial community.

When comparing one location with another, it was not the same in diversity but similar. By obtaining oysters from geographically distinct sites, the results reveal similar bacteria within the gut microbiomes of oysters. Variations and differences between varying sites within the gut microbiomes may suggest that microbiome composition might respond to local environmental factors, and perhaps to genetic differences among oysters. We know that oysters harbor a large bacterial diversity and by understanding a little more about these communities, we can determine whether it could potentially shift in a predictable way with changes in water temperature in the future.

Future research aims to look at oysters from the Gulf of Mexico to compare to the oyster from the Atlantic Coast. Our goal is to look at the different geographic signals and compare them to our first batch of samples. We will also attempt to look at oysters from the west coast.

5. Acknowledgements

I would like to express great appreciation to my mentor Dr. Elizabeth Alter for providing me with this opportunity to work in the Biology Laboratory at CUNY York College and for providing me with valuable insight towards my research. I am particularly grateful for the assistance given by Nathan Morris, PhD student, Kimberly Sukhnandan, undergraduate student and Shivendra Balgobin, undergraduate student.

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