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# Investigation of Origin and Growth of Fungi in Coastal Beach Sand

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Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

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Coastal Carolina University, 2019

## Abstract

In a previous study from this lab (Stevens, Evans, & Aguirre, 2012) yeast colonies were isolated and grown from sand collection at pristine, medium use and high use beaches along the grand strand. In that study greater yeast abundance and greater diversity both correlated with higher census of human use, suggesting that some yeast growth of sites was anthropogenic. However the sand matrix itself also varied from very fine, uniform, dark sand at the pristine beach to coarser, color varying, less tightly packed sand at residential and commercial beaches. This suggested an alternative hypothesis to differential colonization and growth depending on abiotic properties of sand itself.

This study aimed to distinguish between these two hypothesis and to further examine the likely origin and mechanism by which yeast are introduced onto the beaches of the grand strand. Growth of yeast seeded in control experiments in vitro showed equivalent growth patterns in all three types of sand, arguing against the abiotic hypothesis. A pattern of increasing abundance in high traffic areas than in lower traffic areas along a single commercial beach was observed from collected beach samples, suggesting that people and their pets are the likely source of greater abundance and diversity of yeast observed in beaches of the Grand Strand.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

### Introduction

Fungi are a unique group of eukaryotes that have over 140,000 known species that vary from simple to very complex (“About Microbiology – Fungi,” n.d.). They can be single or multicellular and are best known as rust, mildews, mushrooms and yeast (“About Microbiology – Fungi,” n.d.). This study focused on yeast which are a set of microorganisms that have approximately 1500 species known to man, are usually single cellular, and make up around 1% of the fungal kingdom (Botstein, Chervitz, & Cherry, 1997). The yeast that this study focused on would have been able to survive at high temperatures or in marine environments.

Marine yeast are a subset of yeast that have very unique qualities compared to regular yeast (Zaky, Tucker, Daw, & Du, 2014). There are marine yeast who are said to have evolved from terrestrial yeast that could survive in water, they are described as the yeast that can survive in marine or terrestrial environments (Zaky et al., 2014). Marine yeast that do not suggest evolution from terrestrial yeast are known as marine obligate yeast. Marine yeast are characterized by having traits that include higher osmotic pressure tolerance and higher chemical productivity than obligate terrestrial yeast. These traits are important in microorganisms because they can be isolated and manipulated to aid in metal detoxification, greenhouse gas reduction, nutrient cycling and many other industrial or environmental uses (Zaky et al., 2014). Marine yeast can also be isolated from sand and has been proven to grow subsequently on many different sand types (Kutty & Philip, 2008).

There are many organisms that live in and on the sand at the beach, these organisms can be broken into groups based off of their relative size or their location relative to the water

Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

(Whitman et al., 2014). Psammon is the group of organisms that inhabit the sand that is right above the water margin. Macropsammon are the large sized organisms found on the beach that are no strangers to beach goers and researchers. The meiopsammon of the beach refers to the subset of organisms that are visible to the human eye but not large in size. The last subset is known as the micropsammon which are the microbes that inhabit the sand (Whitman et al., 2014).

Yeast have been proven to inhabit the sand of beaches where the microbiome must be able to survive at a variety of temperatures that may be around or above 37 °C (Whitman et al., 2014). Most believe that sand is inhabitable but due to things like its water availability, isolation and bio film formation the sand environment is home to many organisms, microorganisms and can even support viruses. There are numerous yeast strains that are amongst the species capable of surviving and thriving in sand. Some of these yeast may be pathogenic and research suggest that they are brought on to the beach from runoff, humans and their animals, and simply from being carried by the air (Whitman et al., 2014).

It was important to this study to determine whether pathogenic yeast could survive at high temperatures on sterilized sand. Pathogenic yeast strains were purchased from the ARS and proven to grow on sterilized beach sand, at high temperatures (“ARS Home : USDA ARS,” n.d.). This led to alternative investigation to determine prevalence and origin of yeast in sand, initial protocols were similar to a previous study completed by this lab.

In the previous study a comparison was made for low use, residential and commercial beaches of whether the abundance and diversity of yeast species differed (Stevens et al., 2012).

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

It was suggested that multiple parameters, from oxygen content to human use, affected the diversity and abundance of yeast. Collection of sand was made from 9 beaches total in Horry County, South Carolina. Of the nine beaches that census was completed on 3 were low use beaches these beaches were defined by having keyed access and lack of development. Residential beaches were defined by being at least 500 meters away from the nearest commercial attraction and adjacent to private homes. Sand was collected from 3 residential beaches. Collection also took place at 3 commercial beaches which were defined as being adjacent to hotels, restaurants and businesses (Stevens et al., 2012).

The researchers of the previous study (Stevens et al., 2012) collected samples in 14 mL sterile plastic test tubes by inverting them into sand. From where sample collection was made 30 meter transects were used to census humans and collection spots were divided into 15 meters (Stevens et al., 2012). The collected sand was refrigerated to be kept until it could be processed.

This study used sand that was collected from commercial, residential and low use beaches. Initial sand collections were used to test the abiotic hypothesis, this hypothesis suggested that due to the fine particle size, nutrient content and low census of use private beaches would have less ability to harvest pathogenic yeast growth. Pathogenic strains of yeast were collected from the ARS and set to grow on sterilized sand. Penicillin-streptomycin was used to prevent bacterial growth in cell culture. Upon completion of determination of beach content and its effects on pathogenic growth this study diverged to study origin of potential pathogens on beach sand.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

On a commercial beach collection was made first from the swash of the beach to determine if yeast could be isolated. The swash is defined as the region of the beach where runoff water enters from the roads, yards and sidewalks, etc. Collection was also taken from the human ingress such that the first collection place was made as soon as one stepped onto the beach. The human ingress was defined as the region of beach where people entered, this area was blocked off on both sides forcing people to follow a specific path until they reached the open beach. Collection from the ingress continued in a horizontal pattern down the ingress and stopped before the tideline. Based off of previous research it was hypothesized that the runoff from the swash and human traffic were both plausible causes of yeast introduction on to the beach (Whitman et al., 2014).

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

## Materials and Methods

**Investigation of Pathogenic Growth on Sterile Sand**

Collection of sand was initially made from a commercial, residential or private beach. To complete collection a main entrance of the beach was used to enter and one would walk just below the dunes of the beach for a few minutes to get away from sand areas with high traffic. After walking for a few minutes the first collection of sand was made by placing a sterile tube labeled 1 into the sand where collection was to take place and twisting it down as far as needed for collection of correct sand amount. At the same location sterile tubes 2 and 3 were also filled with sand. Collection was made approximately every fifty feet and in triplicates such that at collection place 2 sterile tubes 4, 5 and 6 were filled. This was repeated until all nine sterile tubes were full of sand. Following collection sand was autoclaved for sterilization, in hopes of killing whatever pathogens were present in the sand. To complete sand preparation sand was sieved using a WS86 Tyler sieve.

Yeast strands were collected from the US Agriculture Research Service (“ARS Home : USDA ARS,” n.d.) and were as follows:

#	NRRL	Strain Name
1	Y-9549	<i>Rhodotorula mucilaginosa</i>
2	YB-516	<i>Rhodotorula mucilaginosa</i>
3	Y-12723	<i>Meyerozyma caribbica</i>
4	Y-17466	<i>Meyerozyma caribbica</i>
5	Y-27942	<i>Meyerozyma caribbica</i>
6	Y-17843	<i>Meyerozyma guilliermondii</i>
7	Y-27949	<i>Meyerozyma guilliermondii</i>
8	Y-2075	<i>Meyerozyma guilliermondii</i>

. Three

strains (YB-516: *R. mucilaginosa*, Y-17466: *M. caribbica* and Y-27949: *M. guilliermondii*) were initially picked to be grown. The strains came packaged in small glass tubes and were sat at



## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

room temperature in a cabinet for few days. The glass tubes were scored for efficient breaking, this was completed with gloves to free the yeast strains. The strains were suspended into sterile sea water creating 1:1, 1:10 or 1:100 dilutions and then plated on YPD agar medium. Plates were set out at room temperature for 48 to 72 hours to grow and then refrigerated. A control was used to ensure sterilization. Once discrete colonies could be collected they were re-suspended into 1 mL of YPD broth for further growth, penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO 63178) was also added to the broth. After 48 hours of growth another milliliter of YPD broth was added to each tube allowing yeast to grow comfortably.

The sterile, sieved sand was re-suspended into sterile sea water in 15 mL test tubes and yeast colonies were added to the test tube. Tubes were vortexed for 10 times a piece for 45 seconds each time they were vortexed to release yeast from sand. The tubes were kept on ice to keep solution from becoming too hot. The product was then plated on YPD agar and left for 48 to 72 hours to grow and then refrigerated.

### **Investigation of Potential Pathogens Growing in Sand**

A ruler was used to determine the 11.5 inch mark from the ground on the leg of a researcher. The researcher then entered the water at a commercial beach and walked out into the ocean until the water was consistently and constantly above the place that had been labeled for 11.5 inches. Water was collected by placing a tube labelled 1 into the water horizontally until it was full. Half a mile down from where the first collection was taken the tube labelled 2 was filled using the same technique and this was repeated once more to fill tube 3.

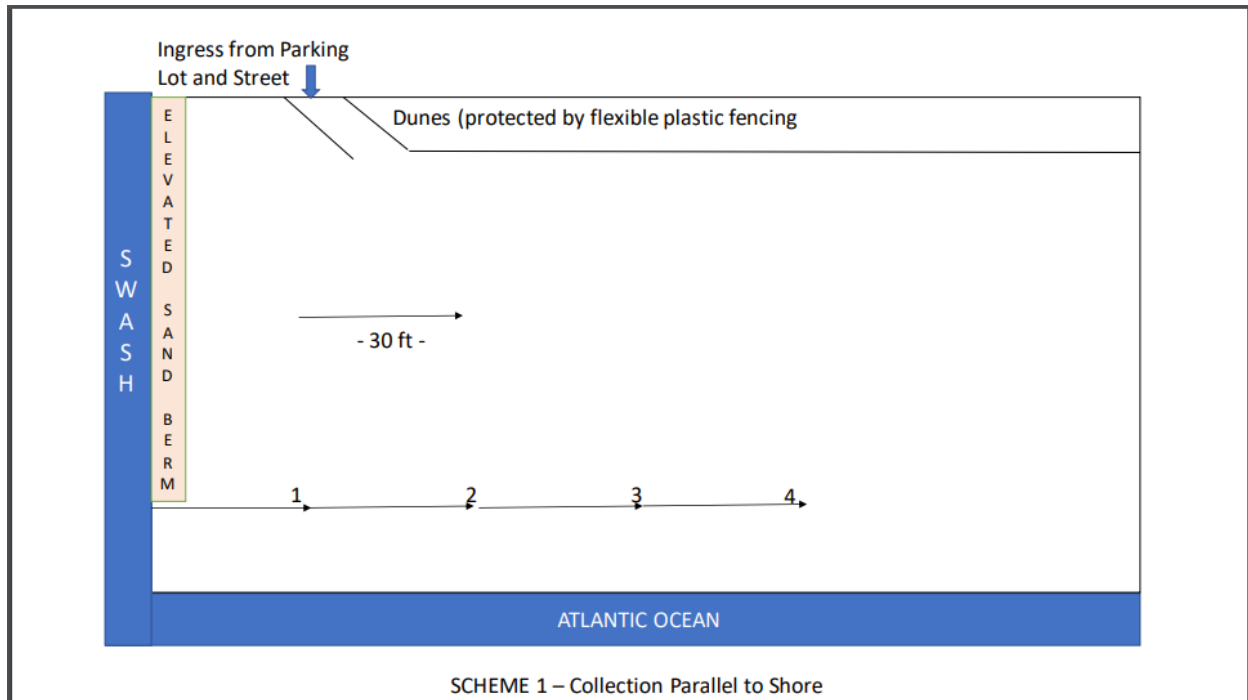
## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

Upon collection water was taken into the lab for investigation of pathogenic presence. This was done by streaking water samples on to YPD plates. Inconclusive results lead to next part.

Collection of sand on surfside beach began at the swash (Illustration 1.) where water runs into the beach from residential areas. The first collection was made just outside of the swash and about 5 meters away from where the first collection was made parallel to the water the second tube was filled with sand and, this was repeated until all 9 tubes were full. Tubes were labeled to correspond with their place of collection. Of the collected sand tube 1, 4 and 7 were chosen to be plated. To complete this 5 mL of each sand was placed into a sterile tube with 10 mL of sterile sea water. Each tube was vortexed 10 times for 30 seconds a piece and 1:1, 1:10 and 1:100 dilutions were made and plated on YPD broth. Each plate was incubated at 37 °C for 48 hours and then the incubation temperature was raised to 98 °C to allow for growth. This was repeated for tubes 2, 5 and 8 counts were made of colonies and their location on the plate. Of the colonies that grew some were picked and placed in 1 mL of YPD broth to

Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

grow another mL of YPD broth was added upon increased growth of selected colony.



**Illustration 1.** Schematic representation of start to finish collection from swash.

Sand was also collected coming from the human entrance ingress. The first collection was made immediately upon ones entrance onto the beach. Walking diagonally sand was collected approximately 5 meters from the first collection place and up until the water line. Tubes were labeled 0, 1, 2, 3 and 4 with 1 being the tube of sand that was collected closest to the entrance of the beach and 4 being the tube of sand that was collected closest to the water. Collection was taken from the swash and from the ingress two more times in the following semester and plated to verify data (Aguirre *et al*, 2017).

### Investigation of Potential Pathogenic Identity and Growth

Of the sand that was collected following the ingress sand was placed into sterile sea water vortexed five times per tube for 45 seconds and allowed to sit for 15 seconds between

Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

each vortex. The solutions were then streaked onto a small 6 well culture plate I still don't have the manufacturer info for the culture plates. Each 6-well agar plate was labeled to depict which tube sand that was plated came from. In a place where a discrete colony could be picked colonies were taken to run PCR for potential identification.

### **YPD Broth and Plate Production**

For a 2% YPD solution 200 mL of deionized water must be added to a flask no smaller than 400 ml. 4 grams of Dextrose, 2 grams of yeast extract and 2 grams of yeast nitrogen base with amino acids were added to the 200 mL of deionized water. The solution was swirled around then placed into an autoclave at the lowest liquid setting for sterilization. Upon completion of autoclave cycle the liquid was allowed to cool enough to be touched and a few mL of broth was added to sterile plates and allowed to solidify for future use.

## Results

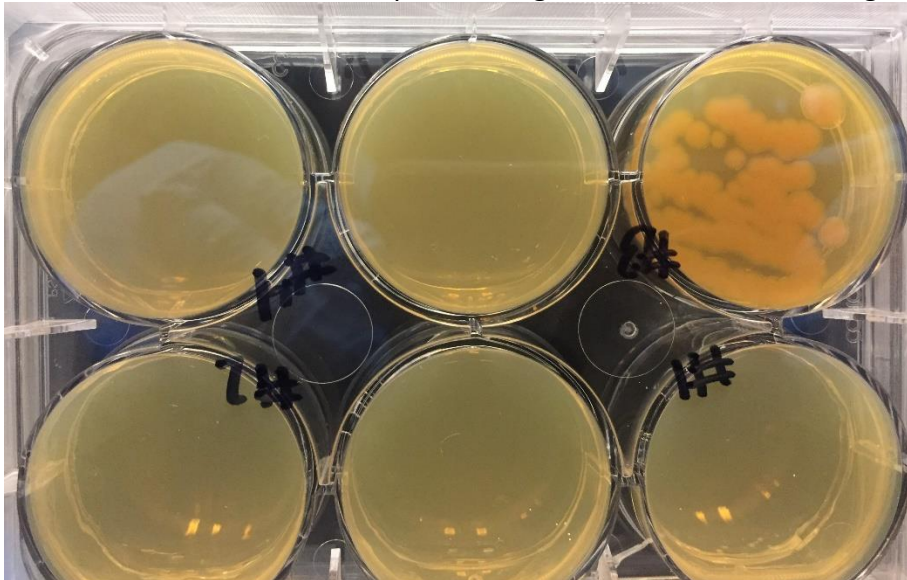
**Investigation of Pathogenic Growth on Sterile Sand**

*R. mucilaginosa* was observed to grow efficiently on commercial, residential and private beach sand regardless of sand size or nutrient composition. *M. caribbica* and *M. guilliermondii* grew equally as well on the private beach sand or the commercial beach sand with no growth observed on the residential beach.

**Investigation of Potential Pathogens Growing in Sand**

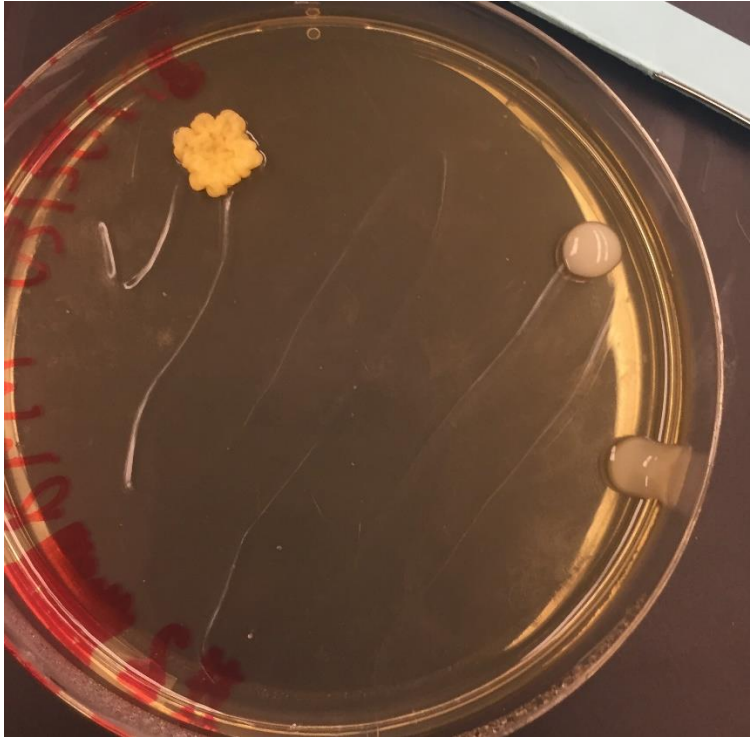
Samples that were taken from commercial beach water produced results that were inconclusive. Under our protocols pathogens in water were undetectable. This suggested that the yeast growth of water is too small to be isolated by small scale collection.

From the swash there was no pattern as growth seemed to be irregular.



**Figure 2.** Growth of yeast isolated from sand coming from swash on the initial collection. The blank had no growth suggesting that laboratory techniques were carried out correctly. Sand from collection places 1, 2 and 4 had no colonies present with 3 having too many colonies present to count. No pattern was found.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches



**Figure 3.** Picture of Collection place 2 from the swash on round 2 of sand collection from the swash. No pattern was observed for yeast growth.



**Figure 4.** Picture of Collection place 3 from the swash on round 2 of sand collection from swash. No pattern was observed for yeast growth.

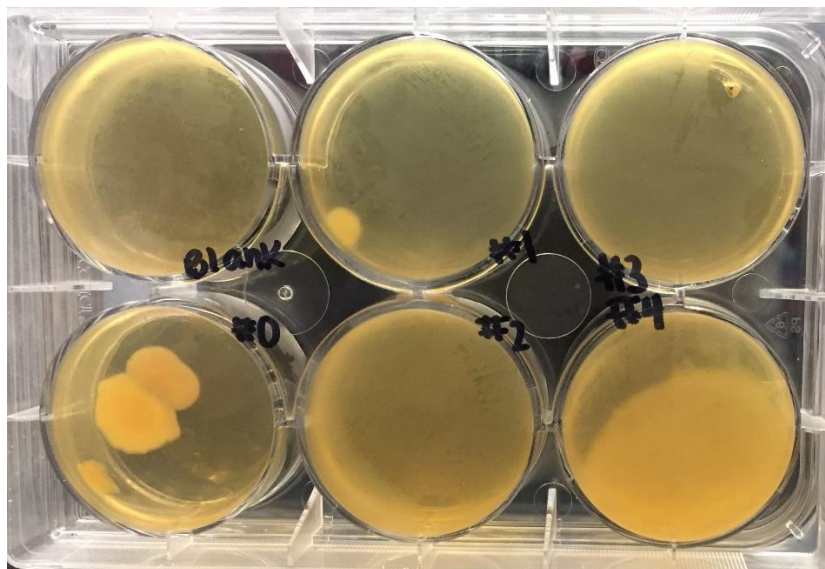
## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

<u>Collection 1</u>		<u>Collection 2</u>	
Collection Place	# of Colonies	Collection Place	# of Colonies
1	0	1	0
2	0	2	3
3 TMTc		3	2
4	0	4 n/a	

**Table 1.** Swash sand collection yeast colony count.

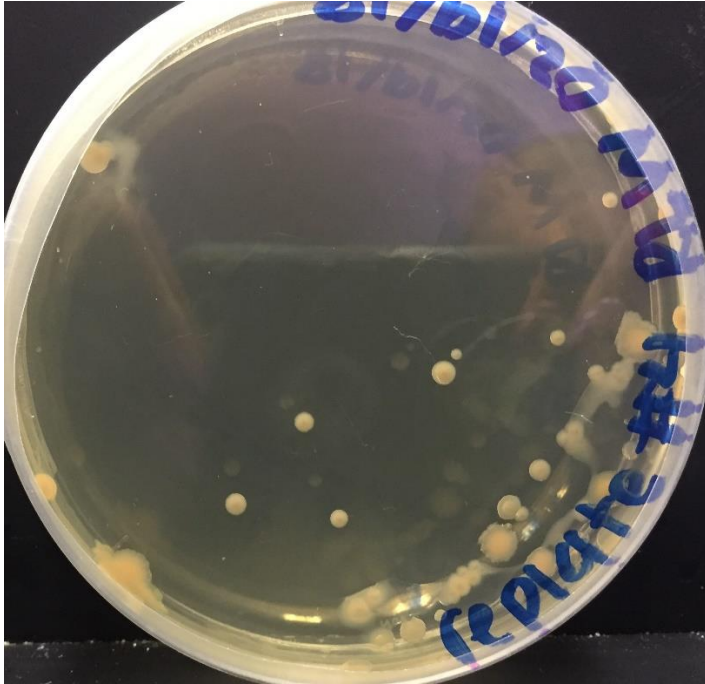
**Investigation of Potential Pathogenic Identity and Growth**

Coming from the human ingress onto the beach yeast growth was observed to be highest closest to where beach goers enter the beach and fade off as collection was taken closer to the water.



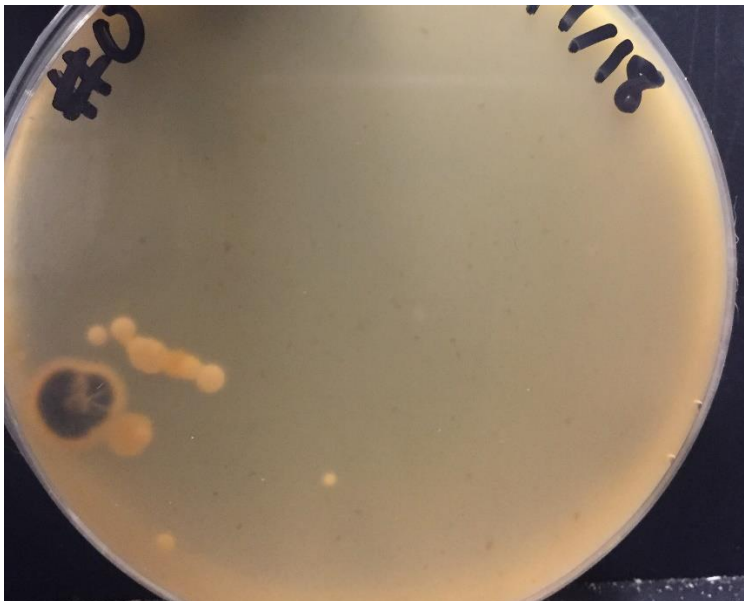
**Figure 5.** Picture of 6-hole agar plate showing no pattern of growth.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches



**Figure 6.** Picture of collection place 4 from ingress after being re-plated.

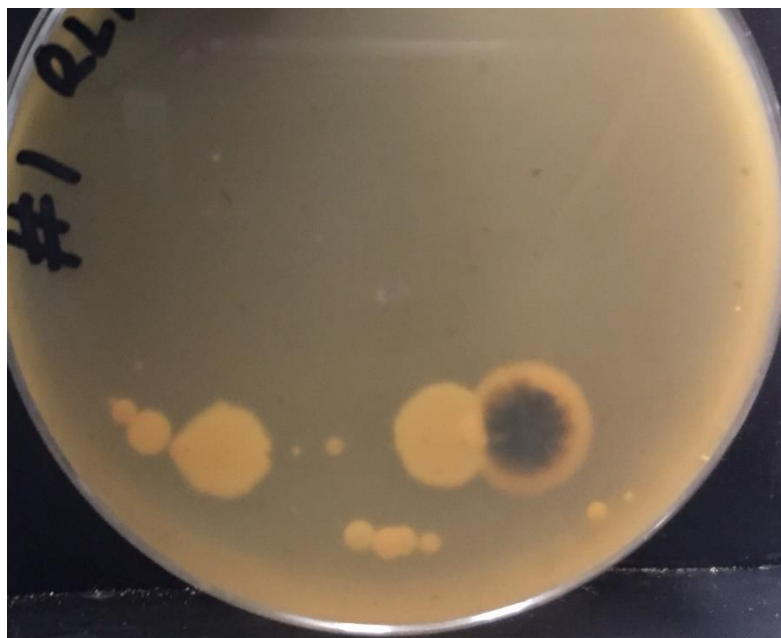
Upon collection from human ingress a second time a pattern was observed suggesting that yeast presence and growth was most abundant directly onto the beach and leveled off going towards the water.



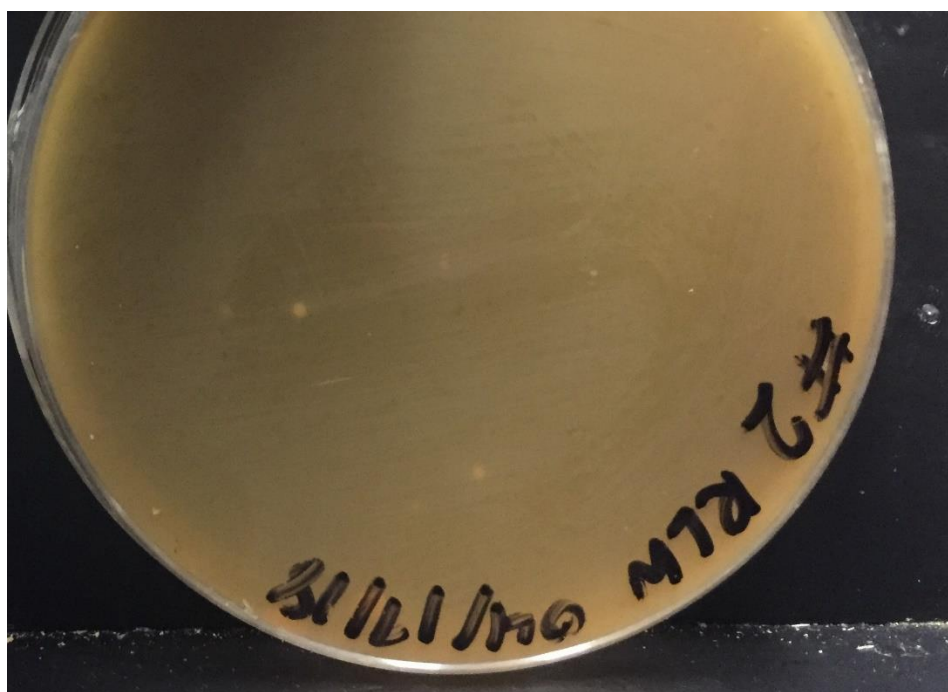
**Figure 7.** Collection from place zero on second ingress collection. Back of plate with 10 colonies observable. 13 colonies could be observed from the front.



## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

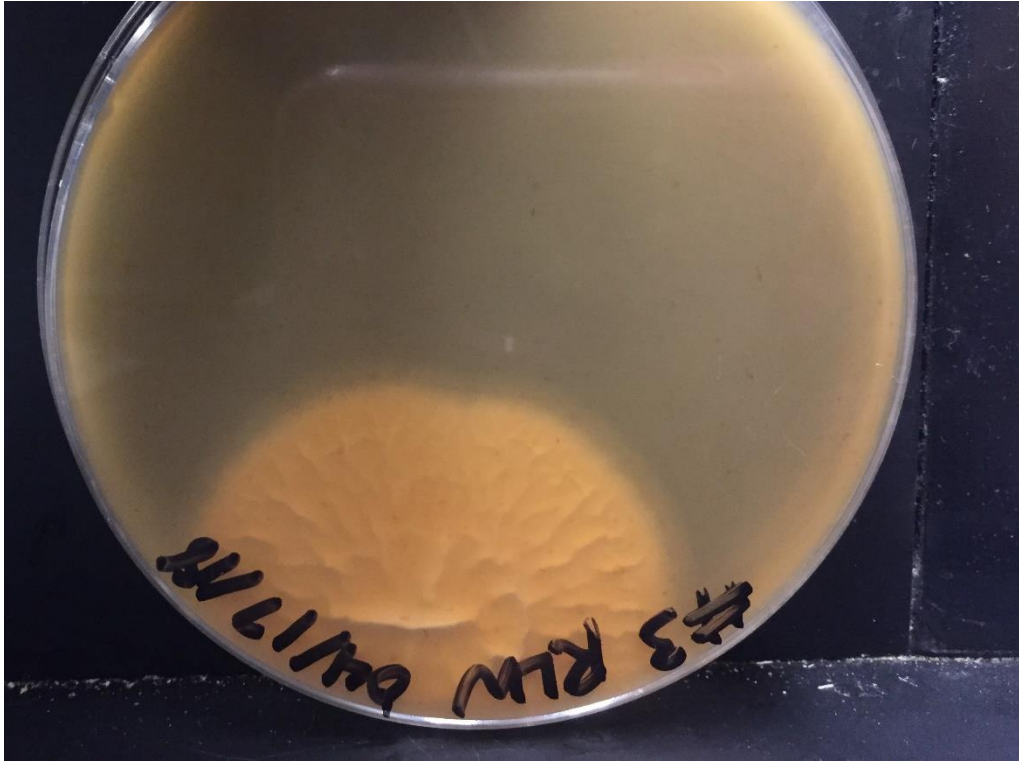


**Figure 8.** Collection from place 1 on second ingress collection. Back of plate with 14 colonies observable. 17 colonies could be observed from the front.

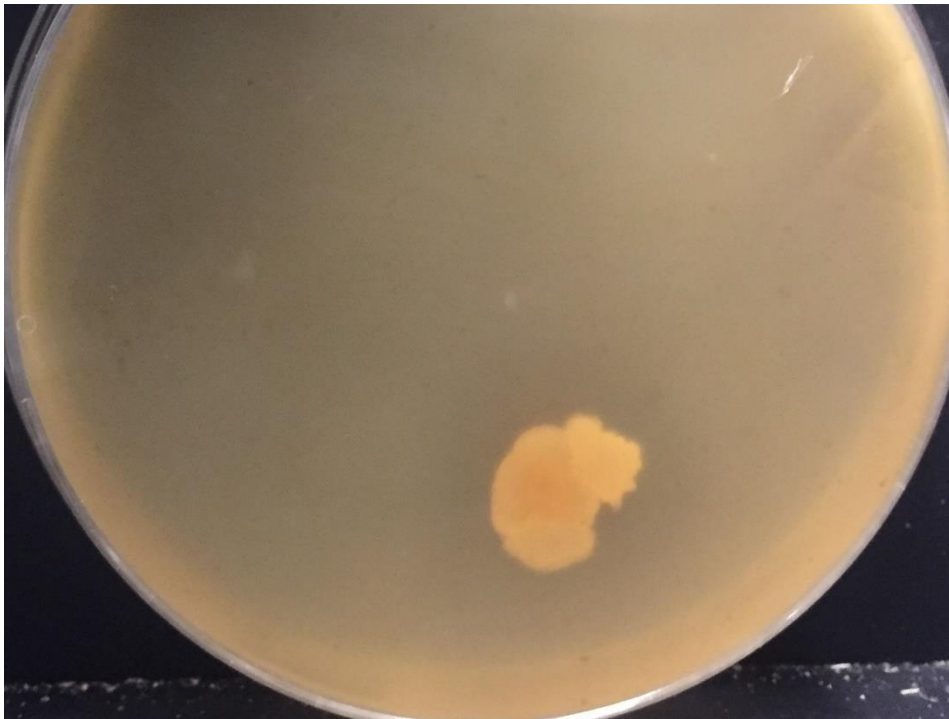


**Figure 9.** Collection from place 2 on second ingress collection. Back of plate with 6 colonies observable. 6 colonies could be observed from the front.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches



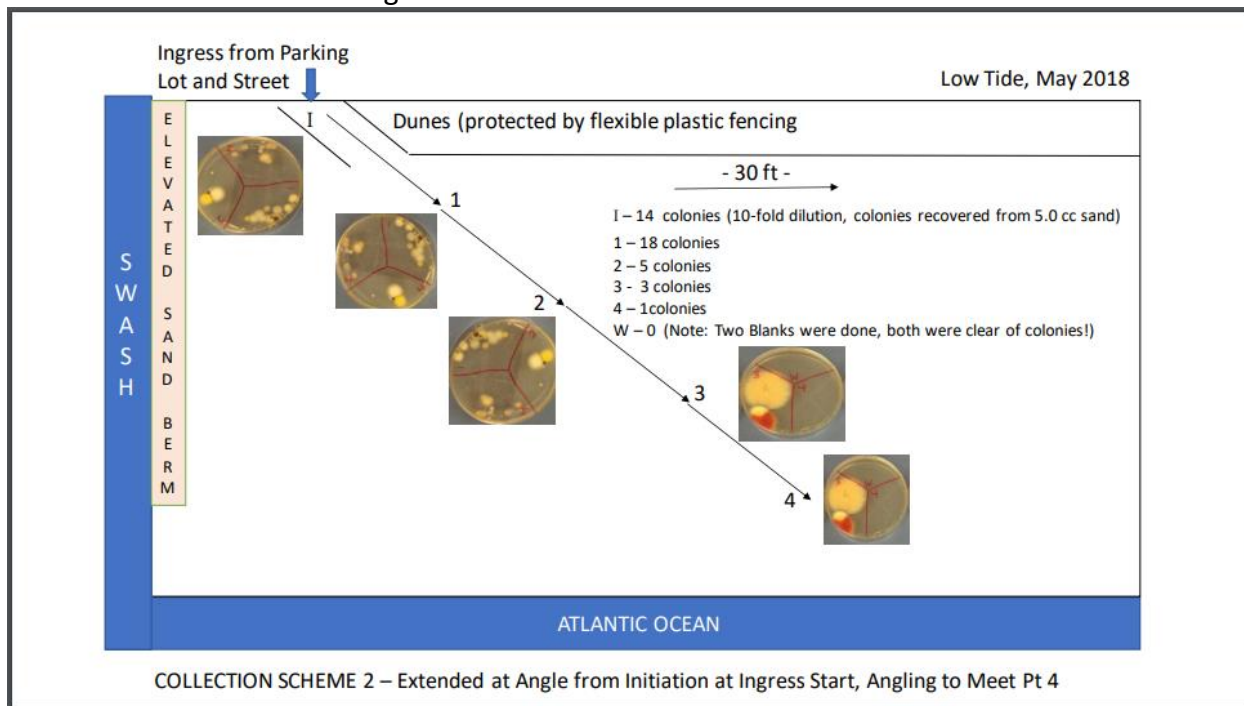
**Figure 10.** Collection from place 3 on second ingress collection. Back of plate with 1 colony observable. 1 colony could be observed from the front.



**Figure 11.** Collection from place 4 on second ingress collection. Back of plate with 2 colonies observable. 3 colonies could be observed from the front.

## Investigation of Yeast Origin, Support and Growth in Sand and on Coastal Beaches

The pattern that was seen with ingress collection 2 was observed on a third round of collection from the human ingress.



**Figure 12.** Depiction of ingress collection scheme, including pictures of plates from sand streaking and colony count.

<b>Table 2. Growth of Yeast in Sand coming from Human Ingress on Surfside Beach</b>						
<u>Collection 1</u>		<u>Collection 2</u>		<u>Collection 3</u>		
Collection Place	# of Colonies	Collection Place	# of Colonies	Collection Place	# of Colonies	
0	2	0	13	0	14	
1	1	1	17	1	18	
2	1	2	6	2	5	
3	0	3	1	3	3	
4	TMTC	4	3	4	1	

**Table 2.** Colony count of yeast from human ingress.

## Discussion

**Investigation of Pathogenic Growth on Sterile Sand**

Of the Pathogenic yeast strains that were collected from the ARS (“ARS Home : USDA ARS,” n.d.) no preference was observed for Y-17466: *M. caribbica* and Y-27949: *M. guilliermondii*. This observation suggested that for those strains there was no overall preference for sand characteristics, arguing against an abiotic cause for their growth in sterilized sand. For YB-516: *R. mucilaginosa* it grew relatively similarly on the low use and commercial beach suggesting against the abiotic hypothesis of growth. At the residential beach strain YB-516 hardly grew at all suggesting that there may be some kind of alternative treatment that would have prevented this strain from growing. More census of the area and treatment of the sand on residential beaches is required to verify these suggestions.

**Investigation of Potential Pathogens Growing in Sand**

Upon analysis of data from sand collection from the swash no pattern was observed. This finding was interesting because previous research suggested that a pattern would be observed to verify the hypothesis that runoff was a plausible cause of microbial origin on the beach (Whitman et al., 2014). Due to the findings of this study, it cannot be suggested that the swash has any reasonable effect on microbial introduction onto the beach which called for differential suggestions of how yeast were being brought onto the beach and harvested.

**Investigation of Potential Pathogenic Identity and Growth**

Based off of the work done in this study it could be suggested that yeast that were harvested from the beach had its origin from human beach goers. This could be suggested because where humans come on to the beach the presence of pathogens was most abundant and leveled off as one got closer to the water. This is also in line with the irregular pattern of swash growth. The areas of the ingress where all beach goers had to tread produced a greater number of colonies, this leveled off going further out. The work of this study could support a hypothesis that microbes were being introduced to the beach by humans, their belongings and their animals.

### Conclusion

Pathogenic yeast have been observed to grow in different types of sand regardless of the sand grain size, sand packaging, or nutrient content. The work of this study suggest that yeast are mainly carried on to the beach by humans and their animals that they bring onto the beach. The yeast isolated from the beach were not able to be identified but were very similar to yeast isolated in a previous study and the pathogenic yeast that were purchased from the ARS. This is important because children, immuno-deficient people and older people who spend most of their time in the sand while they are at the beach are very susceptible to coming into contact with yeast or other microbes that may be pathogenic. This would increase their possibility of getting sick if the microbes growing on the beach inhabited pathogens. Going forward sand could periodically be tested for presence of microbes and identification of pathogens. Guidelines would need to be set if pathogens were proven to grow and survive in beach sand. Further research of the sand/beach environment would be required to correctly assign preventative guidelines to keep those at higher risk from getting over-exposure and becoming sick.

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