FLUCONAZOL RESISTANCE IN YEAST POPULATIONS FROM COASTAL HABITATS IMPACTED BY SEWAGE AND WASTEWATER DISCHARGES

By

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Abstract

Coastal areas, rivers and other waterbodies are constantly impacted by microbial pollutants originating from wastewater discharges. The objective of this research was directed to study fluconazol resistance in yeast populations from coastal habitats with different exposure levels to human influence. A total of 76 samples (16% from soil, 59% from water, 8% from mangrove rhizosphere, 8% from mangrove sediment and 9% from sand) were evaluated from rural and urban wastewater treatment plants, Guajataca and Susua Forests, Cupeyes River (Sabana Grande), Ensenada Dakity beach (Culebra), La Boca estuary (Barceloneta), La Parguera (Lajas), Peña Blanca beach (Aguadilla), Pico de Piedra estuary (Aguada) and Sewage pipeline discharge (Isabela). All isolates were screened to determine their resistance to fluconazol, using a broth microdilution method according to standard methods. A total of 86 yeasts were isolated and 48% of them showed resistance to fluconazol (MIC ≥64 ug/ml). Phylogenetic analyses revealed the presence of genotypes related to *Acremonium strictum* (4.9%), *Aureobasidium pullulans* (17.1%), *Candida diddensiae* (2.4%), *C.* thaimueangesis (2.4%), C. *tropicalis (*17.1%), *Cryptococcus* spp. (7.3%), *C. flavescens* (2.4%), *Exophiala jeanselmei* (2.4%), *Hanseniaspora opuntiae* (2.4%), *H. thailandica* (4.9%), *Hortaea werneckii* (4.9%), *Pichia anomala* (4.9%), *Pseudozyma* sp. (9.8%), *Rhodotorula glutinis* (2.4%), *R. graminis* (4.9%), *Simpodiomycopsis paphiopedili* (2.4%) and unknown strains (7.3%). Isolates of *Aureobasidium pullulans* were predominant from samples of the urban wastewater treatment plant, while *C. tropicalis* were predominant from samples of the rural wastewater treatment plant. These species have proven to be opportunistic pathogens causing fungal infections in immunocompromised patients. All the resistant strains showed a high Minimal Inhibitory Concentration (MIC) of ≥64 ug/ml to fluconazol in the *in vitro* resistance tests. This study provides the first survey and data about the distribution of fluconazole-resistant yeasts associated with coastal habitats in Puerto Rico.

Results show the need to include a monitoring method for clinically important yeasts in the current protocols used in Puerto Rico and other countries of the world to evaluate water quality.

Resumen

Las áreas costeras, ríos y otros cuerpos de agua constantemente están siendo impactados por contaminantes microbianos procedentes de plantas de tratamientos. El objetivo principal de esta investigación está dirigido al estudio de la resistencia al antifúngico fluconazol en levaduras de hábitats costeros expuestas a diferentes grados de influencia antropogénica. Un total de 76 muestras (16% del suelo, 59% del agua, 8% de la rizosfera de mangle, 8% del sedimento de mangle y 9% de la arena) fueron procesadas de las plantas de tratamiento rural y urbana, Bosques de Guajataca y Susua, Río Cupeyes (Sabana Grande), Ensenada Dakity (Culebra), estuario La Boca (Barceloneta), La Parguera (Lajas), playa Peña Blanca (Aguadilla), estuario Pico de Piedra (Aguada) y línea de desagüe (Isabela). Todos los aislados fueron analizados para determinar su resistencia al antifúngico fluconazol utilizando el método estándar de microdilución. Un total de 86 levaduras fueron aisladas y un 48% de éstas mostraron resistencia al fluconazol (MIC ≥64 ug/ml). Análisis filogenéticos revelaron la presencia de genotipos relacionados a *Acremonium strictum* (4.9%), *Aureobasidium pullulans* (17.1%), *Candida diddensiae* (2.4%), *C.* thaimueangesis (2.4%), *C. tropicalis (*17.1%), *Cryptococcus* spp. (7.3%), *C. flavescens* (2.4%), *Exophiala jeanselmei* (2.4%), *Hanseniaspora opuntiae* (2.4%), *H. thailandica* (4.9%), *Hortaea werneckii* (4.9%), *Pichia anomala* (4.9%), *Pseudozyma* sp. (9.8%), *Rhodotorula glutinis* (2.4%), *R. graminis* (4.9%), *Simpodiomycopsis paphiopedili* (2.4%) y cepas desconocidas (7.3%). Los aislados de *Aureobasidium pullulans* fueron predominantes en muestras de la planta de tratamiento urbana mientras que los aislados de *Candida tropicalis* predominaron en muestras de la planta de tratamiento rural. Estas especies han demostrado ser patógenos oportunistas causando infecciones fúngicas en pacientes inmunocomprometidos. Todos los aislados resistentes mostraron alta resistencia en la Concentración Mínima Inhibitoria (CMI) de ≥64 ug/ml en las pruebas de resistencia a fluconazol *in vitro*. Este estudio provee el primer catastro y datos sobre la distribución de cepas levaduriformes resistentes asociadas a

hábitats costeros en Puerto Rico. Los resultados de este estudio sugieren la necesidad de incluir procedimientos de monitoreo para las especies levaduriformes de importancia clínica en el protocolo utilizado en Puerto Rico y otros países del mundo para evaluar la calidad de agua.

Dedicatory

I want to dedicate my research project to both my parents, Mayda Juliá Oliveras and Alberto O. Román Martínez. Thanks to my mom for always being available to listen and guide me to the right path. Dad, thanks for believe in the possibilities to reach new challenges during my academic preparation, where you always have been present. Finally, I want to thank Andy for always be together with me, for the perseverance and for remembering me that all is possible 100% of the time.

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1. Introduction

Puerto Rico is a Caribbean island located in a subtropical region (18º15'N 66º30'W) and the demand for the use of beach and coastal areas by the population is high. Coastal areas, rivers and other waterbodies are constantly impacted by microbial pollutants as a result of anthropogenic activities. Sources of microbial pollution include sewage from urban communities, wastewater discharges, runoff from agricultural activities and the recreational use of boats. The quality of the waters across all recreational beaches has been affected as a result of human influence. This poses potential health risks for the population due to exposure to which may include opportunistic microorganisms resistant against antimycotic agents used for the treatment of infection in humans.

Yeasts are unicellular fungi of diverse forms (oval, spherical, or cylindrical) usually about 3 to 5 μm in diameter. They can reproduce asexually, by budding or by binary fission. Yeasts can provide diverse benefits when consumed as probiotic food. Some of the benefits include the generation of fatty acids and the production of vitamins and other nutrients that improve our body defenses. However, few have been described as pathogens of plants, animals and humans.

For several years, clinical institutions have reported an increase in the rate of nosocomial infections caused by fungal pathogens resistant against antimycotic agents (Fridkin and Jarvies, 1996; Maebashi et al. 2002). Common yeast pathogens include: *Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei* and *Cryptococcus neoformans* (Pfaller and Diekema, 2007). Moreover, antifungal agents are being detected among the most common organic contaminants in water supplies through the United States (Kolpin et al. 2002). Coastal ecosystems are frequently impacted by wastewater discharges. A study by Papadakis et al.

(1997) suggests that sand is an important reservoir for yeasts that may represent potential risks to beach users. Contamination of the coastal marine environment by sewage and wastewater discharges increase the number of diseases linked to recreational activities (GESAMP, 2001). Research about fluconazol-resistant strains has been increasing during recent years by clinical programs due to the high MIC found between isolates from different people including cancer patients. (Boschman et al. 1998; Maebashi et al. 2002). So far, Puerto Rico lacks a study providing information on the occurrence of fluconazol-resistant yeasts in our coastal habitats. Due to the geographic characteristics of Puerto Rico, most wastewater treatment plants effluents are discharges into coastal settings, which constitute important locations for recreational uses and commercial fishing, such as beaches and mangrove ecosystems. This research provides the first survey and information of the distribution of fluconazol-resistant yeasts associated with coastal habitats in Puerto Rico. The objective of this research is directed towards the study of fluconazol-resistant yeast populations from coastal habitats in Puerto Rico.

2. Literature Review

2.1 Wastewater Treatment

Domestic wastewater is a combination of physiological waste created by humans with sewage from washing and food preparation. Wastewater treatment plants can receive sewage from industrial activity and agricultural runoff. The principal function of wastewater treatment plants in Puerto Rico is the removal of suspended solids, organic materials, bacteria and nutrients from wastewater to decrease the effect of these discharges on the environment and on public health. This is achieved with the application of primary and secondary treatments in the management of wastewater.

The primary treatment consist on separating the solids suspended in the influents using grids, a system of sand removal and a clarifier tank to remove solids by sedimentation. The process starts with the entry of wastewater to grids. Once separated from large solids, water pass to a sedimentation tank. The sediment gets through a drying process and is eventually used as fertilizer. After this process the water is chlorinated to eliminate microorganisms and is discharged in rivers, lakes or sea (Britton, 2005; Maier et al., 2009).

A secondary treatment plant uses a bioreactor that allows biological degradation through microorganisms using activated sludge. These aerobic bacteria are responsible for the oxidation of organic matter into CO_2, H_2O and NH_4. Once the water is directed to the bioreactor, is injected with air or pure oxygen. This allows the growth of aerobic bacteria and the decomposition of the organic matter suspended. The water is moved to a secondary clarifier where the biomass is retained and settles at the bottom of the tank to be remove later (Britton, 2005; Maier et al., 2009).

The efficiency of a wastewater treatment plant depends on the composition and activity of its microbial community (Wagner et al., 2002). The effectively of the above procedures in microbial communities has been studied by dehydrogenase assay catalase activity measurements, redox potential, whole cell biosensors and fluorescence monitoring (Holtmann and Sell, 2002). In all wastewater treatments plants, prokaryotic microorganisms dominate and are responsible for the microbial activity. Otherwise, there is little information regarding the presence of yeast in this environment. Baldi et al., (1990) have reported yeasts resistant to chromium compounds. These compounds have been the cause of carcinogenic and mutagenic activities in animals and bacteria. Also, Vallini et al., (1997) reported a new species of *Candida* from a wastewater treatment plant in Italy.

In our study, we obtained samples from rural and urban wastewater treatment plants that apply secondary treatment to wastewaters in Puerto Rico. Both wastewater treatment plants are managed by Puerto Rico Aqueduct and Sewer Authority (PRASA). The rural wastewater treatment plant has a capacity of 0.31 million gallons per day (mgd), because it is consider a municipal plant. The plant only receives domestic wastewater and runoff from the municipality. The urban wastewater treatment plant has a greater capacity of 10.7 mgd because it receives wastewater from the neighboring municipalities. This plant discharges the effluent into a coastal site through a submarine tube while the rural wastewater treatment plant discharges the effluent to adjacent rivers that eventually get to other lakes, which supplies the metropolitan area of San Juan. Both wastewater treatment plants have the permission of PRASA to discharge their effluents into its adjacent areas.

2.2 Sampling Areas and Anthropogenic Impact

Natural environments that receive discharges from agricultural facilities have reported a significant increase in the presence of resistant microorganisms (Chee-Sanford et al. 2001; Nandi et al. 2004). Coastal environments impacted by treatment plants and wastewater from industrial process have reported similar increases on resistant microorganisms (Andersen and Sandaa, 1994). The presence and activity of yeasts in beaches, rivers, and freshwater environments is primarily related to their role in the decomposition of organic substrates, nutrient recycling, biodegradation of hydrocarbons, and as prey for a variety of marine organisms. Usually, yeast populations are less in marine water than in fresh water, and their abundance decrease with depth and distance from land (Uden and Fell, 1968).

Yeasts populations examined in this study represent two beaches from Peña Blanca (Aguadilla) and sewage pipeline discharge (Isabela), two mangroves from La Parguera (Lajas), Ensenada Dakity (Culebra), two estuaries from Pico de Piedra (Aguada) and La Boca (Barceloneta), one river from Cupeyes (Sabana Grande). According to information provided by the US Environmental Protection Agency (EPA) waterbodies in Puerto Rico are constantly being impacted by a variety of pollutants. The principal cause of pollution in all waterbodies is the presence of arsenic, a toxic element for humans. Fecal coliforms are documented as second type of contamination in waterbodies of Puerto Rico. Recent reports by EPA (2008) indicate that on-site treatment systems (septic and decentralized systems), urban runoff and recreational boating are the most probable pollution sources in Puerto Rico. This information implies that sampling sites from this study may be directly or indirectly impacted by different types of anthropogenic pollution.

Table 2.1 Summary of the waterbodies affected in Puerto Rico according EPA (2008).

Location Assessed	Water Size Affected (Miles)	Percent of Water Affected
Bays and Estuaries	775.1	**59 %**
Coastal Shorelines	44.9	**16 %**
Lakes and Reservoirs	7,323	**100%**
Rivers and Streams	4, 711	**97 %**

Pico de Piedra (18º23'0.62"N 67º12'50.74"W) is a recreational beach located in Aguada. Through the year the beach is visited by a large number of people. The beach is impacted by a stream located around 180 m to the left of the beach. In the period of high rainfall the stream discharges significant amounts of organic material to the beach. In addition, about 450 m from the beach a bridge construction can be observed. For this reason, the stream is now transporting noxious waste from sewage and road construction. The Government of Puerto Rico has a beach monitoring and public notice program directed by the *Environmental Quality Board* (EQB) dedicated to monitor waterbodies each month. Pico de Piedra is included in the list of beaches monitored by this agency.

Peña Blanca (Aguadilla) beach area (18º28'43.77"N 67º10'7.79"W) is located at the northwest of Puerto Rico and receives smaller amount of visitors due to its location. It is a rocky beach with minimal extension of sand. Residential areas are located approximately half a mile from the beach. The beach has no point pollution sources in the vicinity and there are no linked waterbodies. The impact on this beach is considered indirect because of the proximity to Crash Boat Beach (1.46 miles) which is highly impacted by humans and other sources of contamination. According to EPA (2008), Crash Boat Beach shows high levels of fecal coliforms across the year.

Ensenada Dakity (18º17’44.52”N 65º17’50.42”W) is located in the Southwest of Culebra municipality. This area is impacted by boating activities at all times. Around Ensenada Dakity, the construction of hotels, guesthouses and new marinas is evident. Culebra residents used septic system tanks for wastewater disposal. Inadequate maintenance of these septic tanks and porous sandy soil may contribute to the contamination of the coastal region of Culebra. Investigations about anthropogenic impact are scarce according to EPA and EQB reports. Molina (2005) reported presence of fecal coliforms after rainfall events as part of a research about water quality assessment of habitats in Culebra. However, the sampling site was a location with low levels of anthropogenic impact.

La Parguera (17º58’31.76”N 97º3’44.67”W) is a recreational and fishing area located in the Lajas municipality. For several decades this location has been use for boating activities and tourism attractions among other water activities. Surface sediments originate mainly from mangrove and salt marshes. Hertler et al. (2009) confirms the presence of watershed, streams and sewage directly impacting the coastal shoreline of La Parguera. They reported high concentrations of metals in sediments from runoff and streams. The pressure of this anthropogenic impact may be a factor of variability in the microbiological communities present across coastal sites in La Parguera. The sample location of this study is directly associated with a treatment plant that discharges organic materials to the sea.

A sewage pipeline discharge at Isabela (18º30’37.91N 67º1’0.54”W) located in the northern coast was included in this study. The sewage discharge is located approximately 100 m from building constructions and discharges directly to the shore.

Cupeyes River (18º7’32.37”N 66º58’54.62”W) is a tributary of the Guanajibo River. According to Whitmire et al. (2010) the river showed low anthropogenic impact, though there is

evidence of past agricultural use. Their study documented low concentration of nutrients (Whitmire et al. 2010).

Estuary La Boca , (18º28'50.43"N 66º32'3.71"W) is a section of Río Grande de Manatí. Historically, the Manatí River has suffered high anthropogenic impacts by different sources of pollution which include industrial activities by pharmaceuticals in the area. Manatí River is the nearest body of water to pharmaceuticals located 3 miles east of the facility (EPA, 2010). There are no documented impacts to surface water or sediment as a result of pharmaceutical activities conducted at the site (Negrón and Hany, 2007).

2.3 Yeast Diversity Associated to Coastal Impacted Sites

A study by Nagahama (2006) showed that most yeast species isolated from freshwater environments impacted by human activity belong to the genera *Aureobasidium, Candida, Cryptococcus, Debaryomyces, Pichia,* and *Rhodotorula*. Nagahama's study was supported by Medeiros et al. (2008) who also found that 79% of isolates from sites impacted by contaminated water in Brazil showed resistance to fluconazol and most likely, originated from terrestrial settings.

A recent work on the diversity of yeasts from coastal habitats in tropical environments suggests the use of *Candida albicans* and *Candida tropicalis* as bioindicators of water quality in recreational beaches (Vogel et al. 2007). These species were most abundant in their sample from three bathing beaches in South Florida. Also, different species of *Candida*, *Rhodotorula*, and *Cryptococcus* were found from samples in recreational beaches.

2.4 Fungicides

Fungicides are chemical compounds that kill or inhibit the grow of filamentous fungi and yeast. These chemicals are crucial as medication in the treatment of infections in humans, animals, and plants. Fungal infections, in general, are treated with drugs that target DNA and/or RNA synthesis and ergosterol biosynthetic pathway, or the biosynthesis of cell wall components.

Flucytosine is an example of an antifungal that targets the DNA and RNA synthesis. This agent is mostly used for infections caused by genus *Candida* sp. across the United States. *C. tropicalis* and *C. parapsilosis* are yeasts that have shown susceptibility to flucytosine. The presence of resistance is based in the loss or mutation of some of the enzymes responsible of its uptake, metabolism, or incorporation into the RNA (Vanden Bossche et al. 1994). To avoid or decrease the incidence of resistance, flucytosine is being administered in combination with another antifungal, amphotericine B (Polak and Hartman, 1991).

Polyenes are antifungals that interfere with membrane functions. *Candida albicans, C. tropicalis, C. glabrata* and *Cryptococcus neoformans* have been reported to show high levels of resistance to polyenes (Georgopapadakou and Walsh, 1996). According to Dick et al. (1980) the composition of ergosterol in the membrane of resistant yeast has decreased as part of the resistance mechanism. This is because less ergosterol means a decrease in the interaction with the antifungal.

The largest and most widely used classes of antifungals are the azoles, characterized by the presence of a ring of five atoms connected with other aromatics rings. The first azole used as antifungal agent was ketoconazole, recently replaced by fluconazol. Fluconazol has some advantages in the mechanism of action, toxicity and pharmacology placing it in the top of the most effective antifungals.

2.5 Fluconazol

Fluconazol was approved by the Food and Drugs Administration (FDA) in January, 1990 for the treatment in clinical conditions know as cryptococcosis and candidiasis in the United States. In the 80's, when the first case of HIV was reported, cases of these fungal infection increased.

Fluconazol is a water-soluble antifungal with a favorable pharmacokinetic profile and a low incidence of side effects. It's mode of action involves the inhibition of cytochrome P450 enzymes, particularly the 14 α-demethylase involved in the synthesis of fungal ergosterol, and provides a common mechanism of antifungal action for the whole azole-derivates series (Odds, 1995) (Figure 2.1). Among the advantages of fluconazol, studies suggest that the oral absorption is better in comparison with other antifungals. Also, an intravenous preparation is available for patients who cannot take oral treatments (Odds, 1995).

Azole antifungal drugs, like fluconazol, are frequently used for effective treatment of yeast infections in most areas of the body. This drug is commonly obtained without prescription and its indiscriminate use has been associated with increased drug resistance in clinical isolates (Goodman et al. 1992).

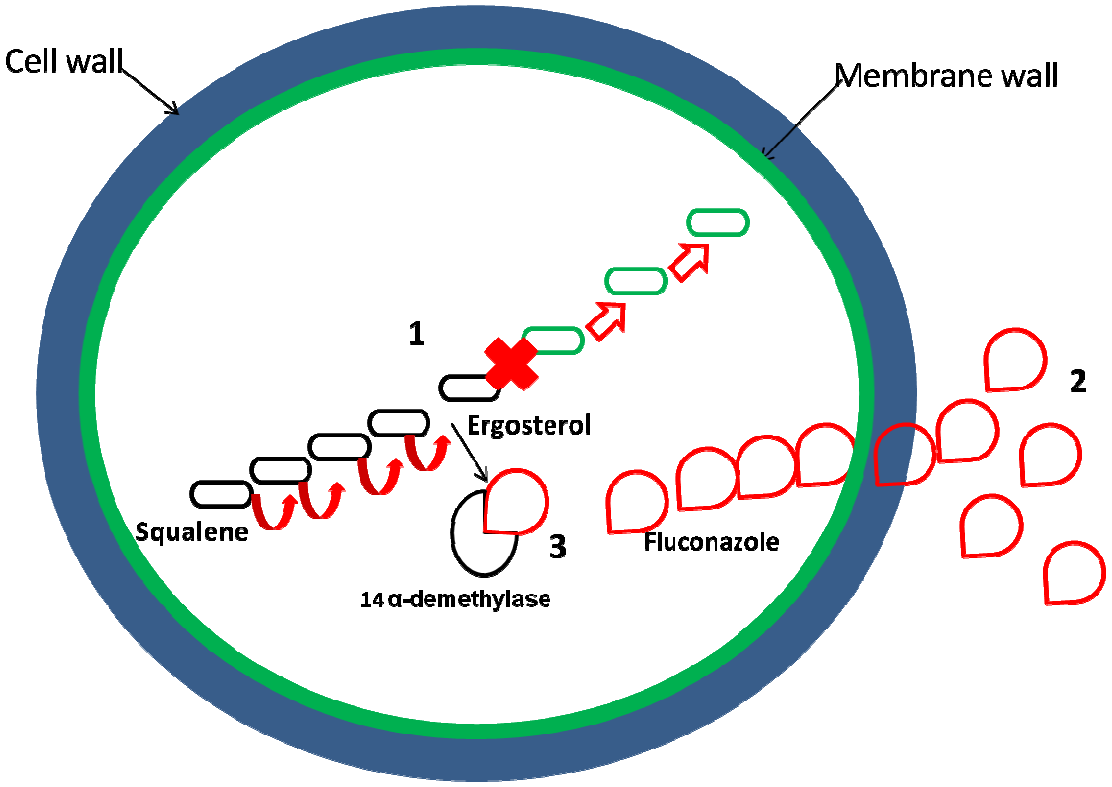

Figure 2.1 Mechanism of action of the antifungal fluconazol. Ergosterol synthesis pathway in fungal cell membrane (1). Fluconazol passes across the cell to inhibit the production of ergosterol (2). Inhibition of 14 α-demethylase in the ergosterol pathway by fluconazol (3).

2.6 Fluconazol Resistance

Antifungal resistance may be defined as a stable, inheritable adjustment to the inhibitory of toxic effects of an antifungal agent. Traditionally, the resistance has been classified as primary (intrinsic) and secondary (extrinsic). In primary resistance the fungus has not been exposed to the drug, however, it is resistant. Secondary resistance implies a prior exposure to the antifungal agent. Yeasts have three different mechanisms against fluconazole. These consist of reduced accumulation by active efflux, alterations in the binding sites of the enzyme 14 α-demethylase, and expressing mutations in genes of ergosterol pathway (Sanglard and Odds, 2002).

3. Objectives

Our main objective was to provide the first survey of fluconazol-resistant yeasts from coastal habitats impacted by sewage and wastewater discharges in Puerto Rico. Secondary objectives in this study included:

- To assess fluconazol resistance among environmental yeast isolates according to the M27-A3 method of the Clinical and Laboratory Standards Institute.
- To characterize fluconazol-resistant yeasts by sequencing the ITS region of rRNA genes.
- To evaluate the presence of resistant isolates across impacted and relatively undisturbed coastal ecosystems.

4. Materials and Methods

4.1 Sampling Areas

As a practical setting to obtain our data, we selected seven sampling sites representing beaches, beach mangroves, estuaries and river-related habitats with history of anthropogenic impact. In addition, we selected two areas with high anthropogenic impact and two areas with low anthropogenic impact as reference sites.

Wastewater treatment plant discharges from urban and rural areas were selected as sites of high anthropogenic impact for being sites that receive wastewater from diverse locations. Forest soils from Sabana Grande and Guajataca were selected as sites with low anthropogenic impact. This research included other coastal areas, rivers and sewage discharges as part of the experimental protocol (Table and Figure 4.1).

Table 4.1 Study sites sampled to isolate fluconazol-resistant yeasts.

Study Area	Municipality	Sample Type	Coordinates
Pico de Piedra estuary	Aguada	Water, Sand	18º23'0.62"N 67º12'50.74"W
Peña Blanca	Aguadilla	Water, Sand	18º28'43.77"N 67º10'7.79"W
Ensenada Dakity Beach	Culebra	Sediment, Rhizosphere	18º17'44.52"N 65º17'50.42"W
La Parguera	Lajas	Sediment, Rhizosphere	17º58'31.76"N 97º3'44.67"W
Cupeyes River	Sabana Grande	Water	18º7'32.37"N 66º58'54.62"W
La Boca estuary	Barceloneta	Water	18º28'50.43"N 66º32'3.71"W
Sewage pipeline discharge	Isabela	Water	18º30'37.91N 67º1'0.54"W

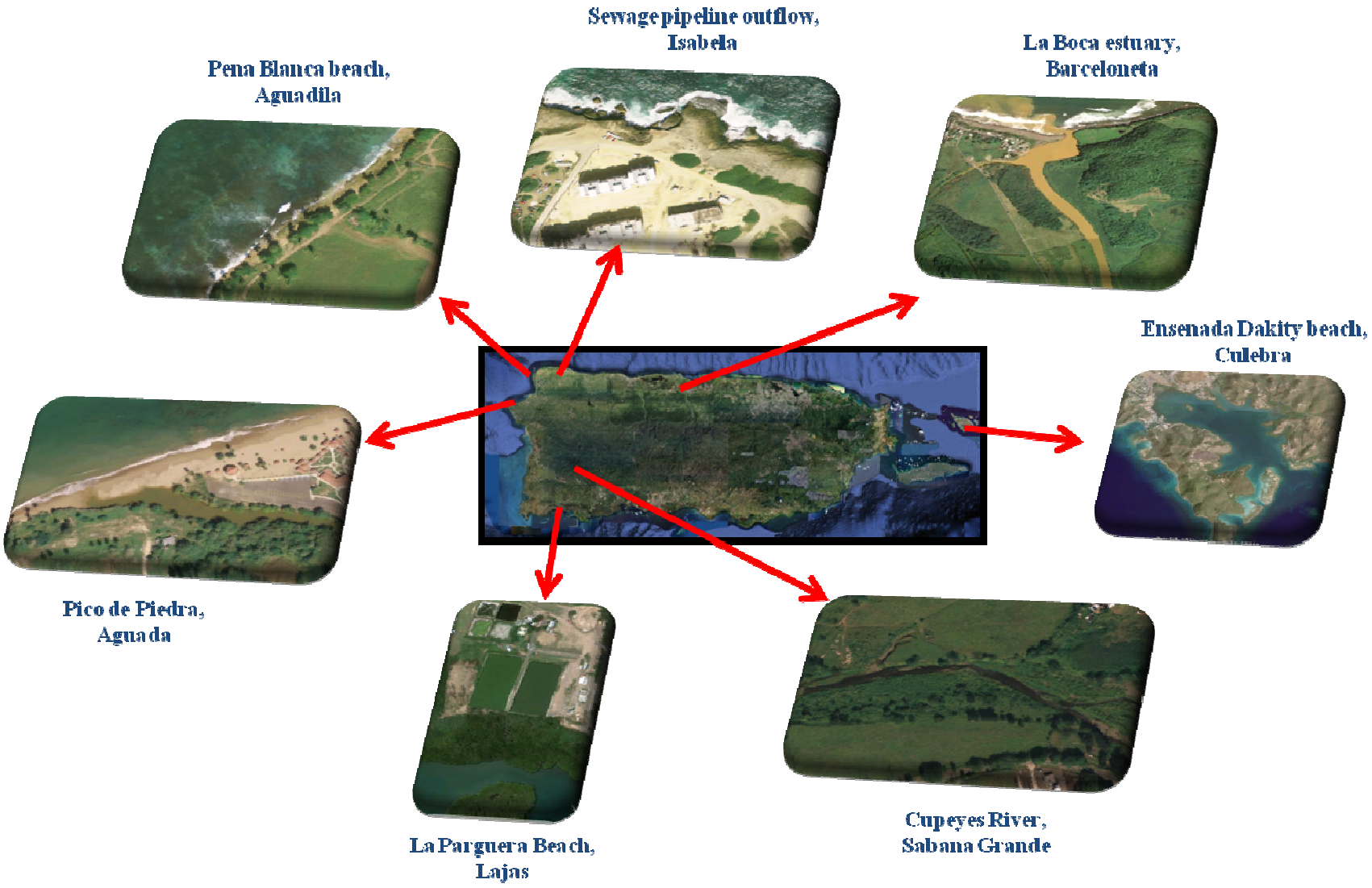

Figure 4.1 Location of the study sites analyzed for this research.

4.2 Sample Collection

Samples were collected from soil, sand, rhizosphere, wet sediments, and the water column, depending on the nature of the sampling site. Six samples of soil and four samples of sand from each location were collected using sterile spoons after discarding the material corresponding to the top five inches. To obtain samples of the soil in both forests we selected three transects (10 x 10m). In each transect the sample was randomly selected from two sites. At each beach, one transect (2m apart) running perpendicular to the water line were selected.

Sediments and rhizosphere samples were collected by scraping the roots submerged of four mangroves trees using a sterile razor. All samples were processed within a 24 hours period after collection. Four samples of the water column were transported in sterile 250 ml plastic bottles to the laboratory the same day for processing, while individual samples of sand, soil, rhizosphere and wet sediments were transported in Whirl-Pack® bags.

4.3 Yeast Isolation

Samples of soil, sediments, and rhizosphere were processed individually by adding 1 gram of the sample to 99 ml of sterile phosphate buffer (7.2 pH) and then homogenized for a period of 5 minutes in a Stomacher® apparatus. Samples were serially diluted from 10^{-1} to 10^{-4}. A volume of 0.1 ml from each dilution was spread in Petri dishes containing Czapeck Yeast Agar (CYA) with 100µg/ml chloramphenicol to inhibit bacterial growth. Plates were incubated at 25ºC ± 2ºC in duplicate for a period of 7 to 30 days. After the incubation period, colonies were counted and purified in Saboraud Dextrose Agar (SDA) Petri dishes.

Sand samples were processed by adding 20 grams to 180 ml of sterile phosphate buffer (7.2 pH) and then homogenized for a period of 30 minutes by macrocentrifugation. A volume of

0.1 ml of the supernatant was spread directly in Petri dishes of CYA. The remaining supernatant was filtered through a 0.45 μm sterile nitrocellulose membrane and placed on CYA supplemented with 100μg/ml chloramphenicol and incubated at 25ºC ± 2ºC for a period of 7 to 30 days. After the incubation period, filters showing yeast-like growth were transferred in to a CYA Petri dish and incubated at 25ºC ± 2ºC for 2 days. This replication facilitated the isolation of yeasts colonies from the filters. Finally, colonies in the membrane and Petri dishes were counted and purified in Petri dishes containing SDA. The water samples were processed in the same way but without the centrifugation step.

4.4 Storage and Preservation of Isolates

Isolates showing yeast-like growth were transferred in Yeast Extract Peptone and Dextrose (YPD) broth. This broth contained a solution of penicillin-streptomycin (1mg/ml) to inhibit bacterial growth. Cultures were incubated at 25ºC ± 2ºC for a period of 48hrs. After the incubation period, 1 ml of each individual yeast culture was mixed with 0.75 μl of sterile glycerol 60% in 2 ml cryovials and stored at -80ºC. Pure isolates were deposited in the Yeast Culture Collection (YCC) located in an ultra freezer at B-218A at the Biology Building.

4.5 Antifungal Drug Resistance Tests and DNA Characterization of Yeast Isolates

All isolates were screened to determine their resistance to fluconazol (Pfizer Pharmaceuticals Group, New York), using the broth microdilution method according to the Clinical and Laboratory Standards Institute M27-A3 (2008). Fluconazol resistance was evaluated at concentrations ranging from 0.125 to 64 μg/ml. Each yeast was grown at 35ºC ± 2ºC for a period of 48 hrs to determine their respective minimal inhibitory concentrations (MIC).

The MIC is the lowest concentration of an antifungal agent that causes a specified reduction in visible growth in an agar or broth dilution susceptibility test. The MIC was determined measuring the growth of the yeast at each concentration with an spectrophotometer set at a wavelength of 530nm.

All organisms were subcultured in Petri dishes containing SDA to ensure purity and viability. The inoculum was prepared from five colonies of ~1 mm in diameter from 24 hours old cultures. Colonies were suspended in 5 ml of sterile 0.145 mol/L saline solution. The resulting suspension was vortexed for 15 seconds and the cell density adjusted in an spectrophotometer to an equivalent of 0.5 McFarland Standard at 530 nm wavelength. This procedure provided a yeast stock suspension equivalent to 1×10^6 to 5×10^6 cells per ml. A working suspension was made by a 1:20 dilution of the stock suspension in Roswell Park Memorial Institute (RPMI 1640) broth medium, which provides 5.0×10^2 to 2.5×10^3 cells/ml. The RPMI 1640 medium was inoculated individually with 0.1 ml of the antifungal concentration tubes. A growth control containing 0.1 ml of drug diluents without antifungal agent was used for comparison. Within 15 minutes after the inoculum were standardized 0.9 ml of the adjusted inoculum was added to each tube in the dilution series and mixed.

The concentration of fluconazol that reduces the 50% of the growth was considered as the MIC for that isolate. Each concentration of fluconazol was tested in the spectrophotometer at 530 nm wavelength to determine the MIC. If the MIC was < 8 ug/ml the yeast was considered susceptible, 16-32 ug/ml susceptible-dose dependent, and ≥ 64 ug/ml resistant (M27-A3, 2008).

4.6 DNA Extraction

DNA was extracted from yeasts cultures showing resistance to fluconazol using the FastDNA Spin Kit for Soil (MPBiomedicals, Solon, OH) with the following modification to the manufacturer's protocol. Yeast cultures were grown in 2 ml microcentrifuge tubes for 24-48 hr in liquid YPD medium (10g Yeast Extract, 20g Peptone, and 20g Dextrose in 1L distilled water) and incubated with agitation at 25ºC. After incubation, the extraction protocol was performed.

4.7 PCR Amplification and Gel Electrophoresis

The Internal Transcribed Spacer (ITS) regions from nuclear rRNA genes of all isolates were amplified by PCR with primers sets ITS1 and ITS4 as described by White et al. (1990). PCR reactions were performed using a Green Master Mix 2X (Promega Inc., Wisconsin, USA). PCR reactions consisted of 12.5 µl Go Taq Green Master Mix 2X, 2.0 µl ITS-1 (50 pmol), 2.0 µl ITS-4 (50 pmol), 2.5 µl yeast DNA (15 ng) and 6.0 µl sterile deionized water. Amplification involved 30 cycles of denaturing 94ºC for 30 s, anneling 55ºC for 30 s, and extension at 72ºC for 1 min. The cycles were preceded by 4 min of denaturation at 94ºC. PCR primers (ITS-1 CCGTAGGTGAACCTGCGG, 290pb; ITS-4 TCCTCCGCTTATTGATATGC, 330pb) are targeted at conserved regions of the 18S, 5.8S and 28S rRNA genes to amplify the noncoding regions between them.

Electrophoresis of PCR-amplified products was performed in 1.5% agarose gels after staining with ethidium bromide (10µg/mL). Prior to sequence analysis, PCR products were purified using Wizard SV Gel and PCR Clean-Up System Kit (Promega Inc., Wisconsin, USA) according to manufacturer's protocol.

4.8 Sequence Analysis

PCR products were sent to the UW-Htseq Sequencing Facility (Washington University at St. Louis, MO) for sequence analysis. DNA sequences were matched with available data in GenBank using the BLAST program to identify their closest relatives based on sequence identity. Gene identity values were calculated by pairwise comparison of the sequences within the alignment using BioEdit program. Values represent the percentage of 2,000 bootstrap replications. The relationship among yeast groups was also analyzed, creating a tree diagram that depicted the relationship of fluconazol-resistant yeasts isolates with respect to database matches. The tree was created using the Neighbor-joining tools method by MEGA version 4.

5. Results

5.1 Isolation of Fluconazol Resistant Yeast

A total of 76 samples (16% from soil, 59% from water, 8% from the rhizosphere, 8% from sediment and 9% from sand) were processed from all locations. A total of 86 yeasts were isolated, with 48% showing resistance to fluconazol. To obtain pure cultures, yeast-like colonies emerging from nitrocellulose filters were inoculated in CYA plates at 25ºC of 24-48 hours of incubation.

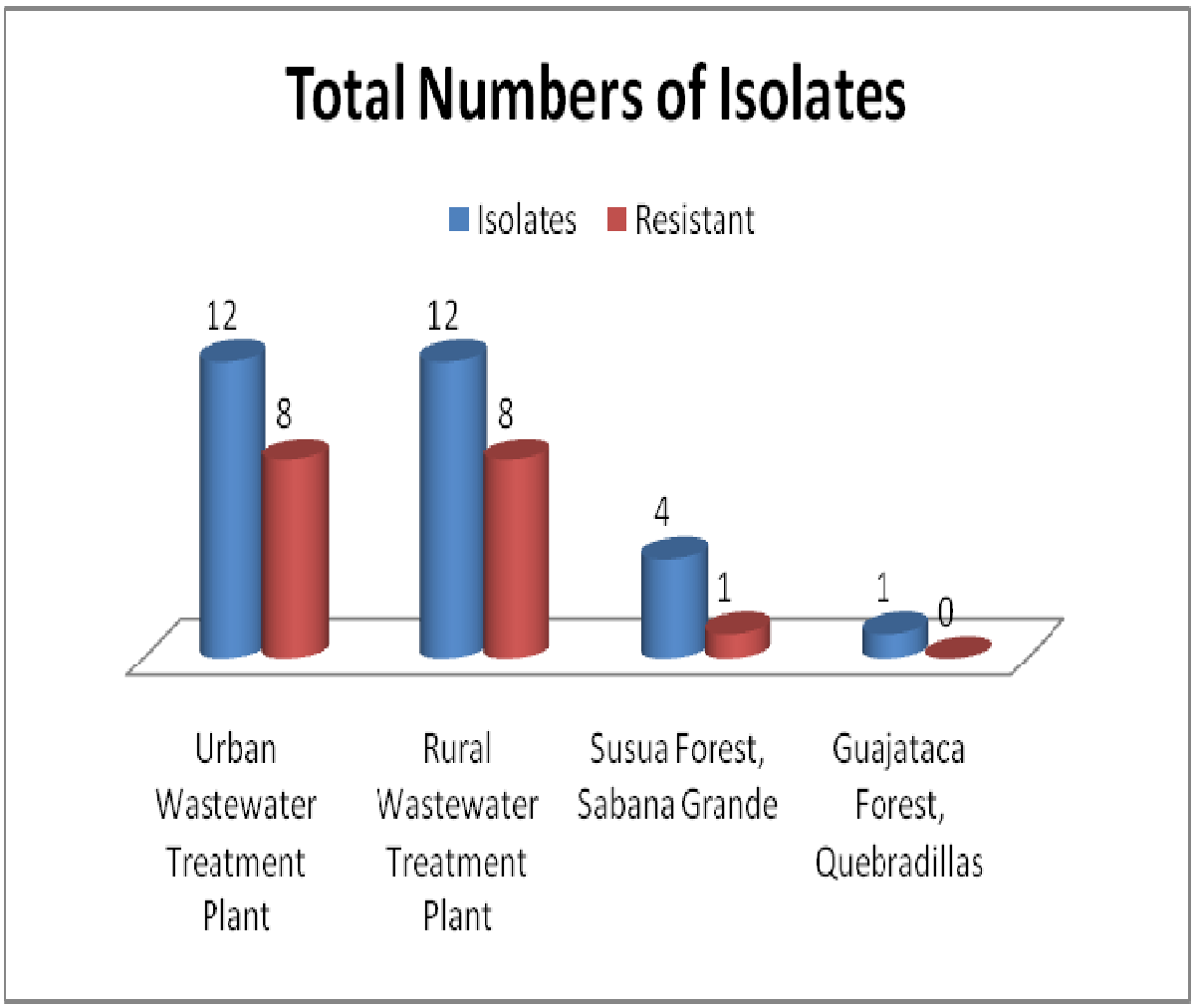

Figure 5.1 Total numbers of isolates and fluconazol resistant yeasts per reference sites ranging from high to low levels of anthropogenic impact.

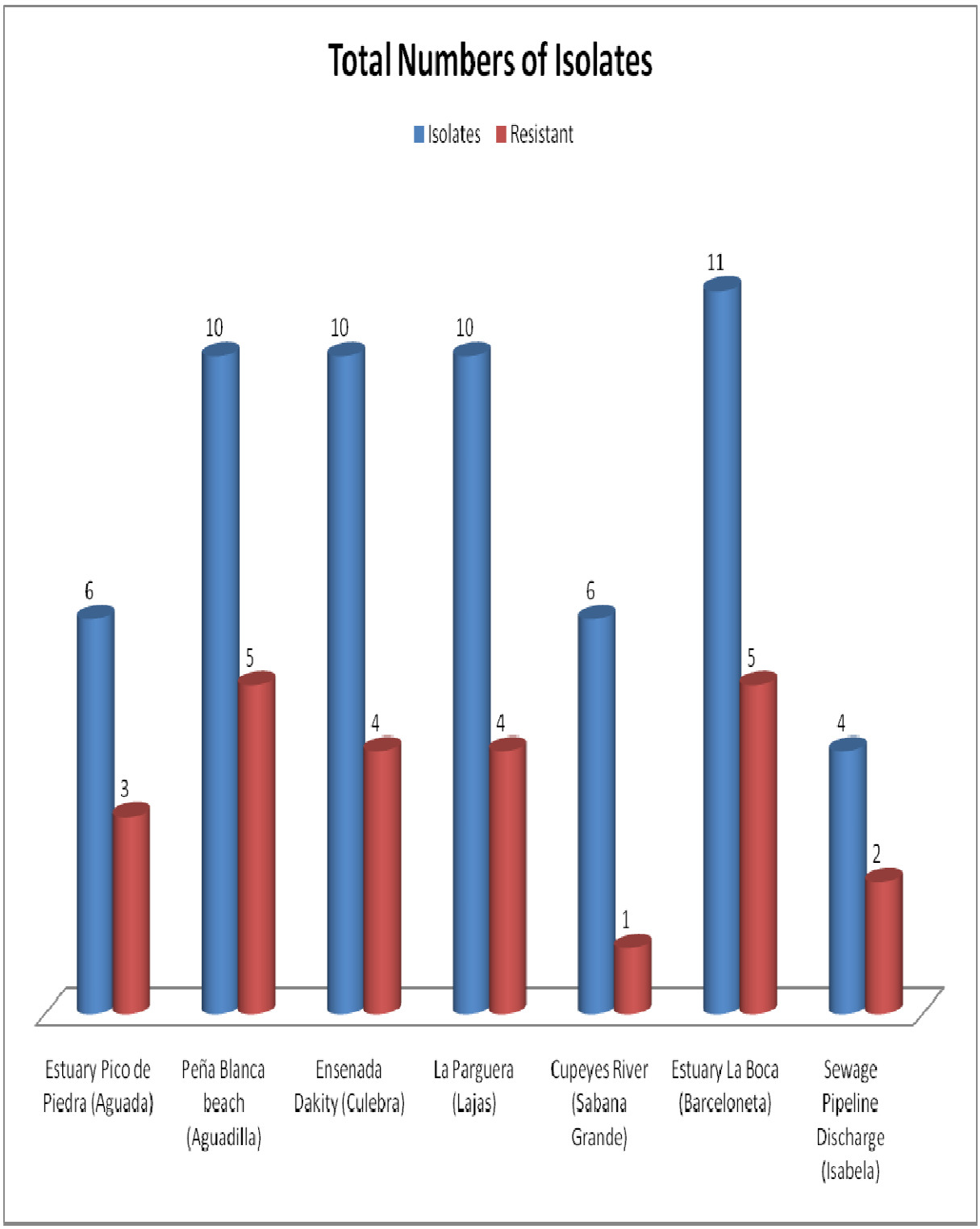

Figure 5.2 Total numbers of isolates and fluconazol resistant yeasts per site from coastal areas, rivers and sewage.

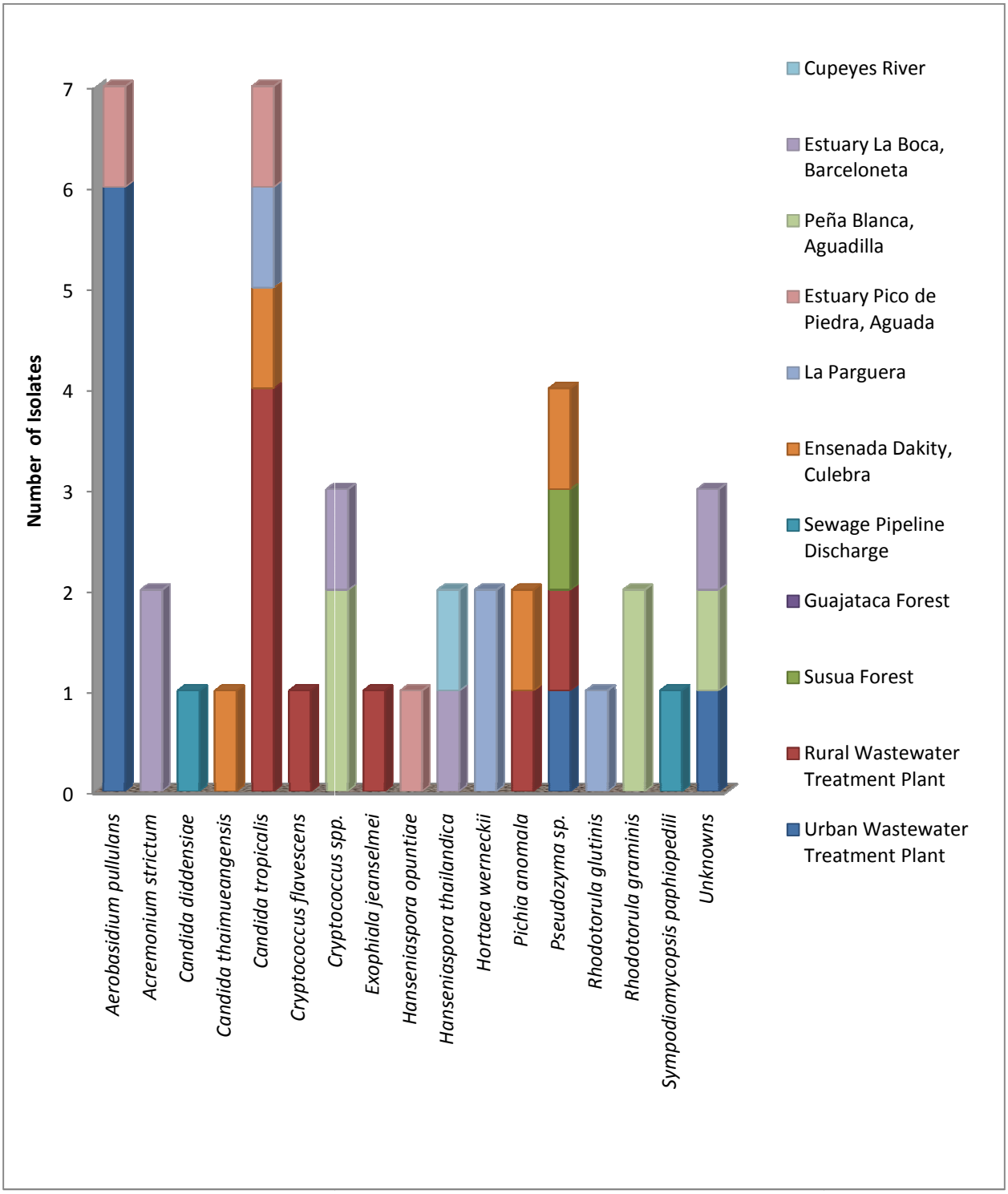

Figure 5.3 Genus affiliation and number of isolates of fluconazol resistant yeasts isolated from each sampling site.

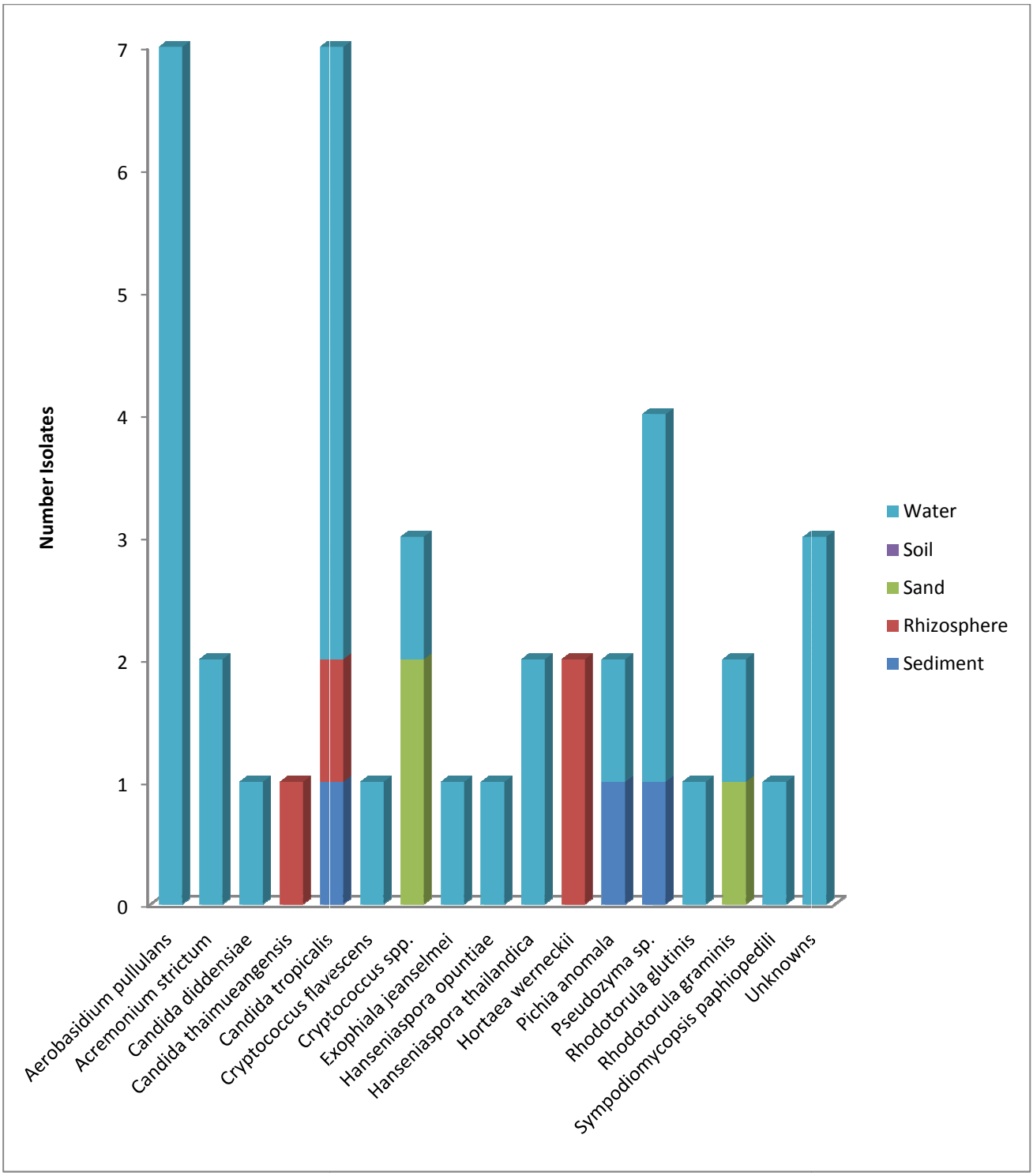

Figure 5.4 Genus affiliation and number of isolates of fluconazol resistant yeasts from different types of samples analyzed.

5.2 DNA Extraction and PCR of Pure Cultures

After purification of each isolate, each strain was further characterized using molecular tools. In Figure 5.5 (A) the quality of the genomic DNA from representative strains is documented. Figure 5.5 (B and C) shows the amplification of the ITS1 and ITS4 rDNA regions.

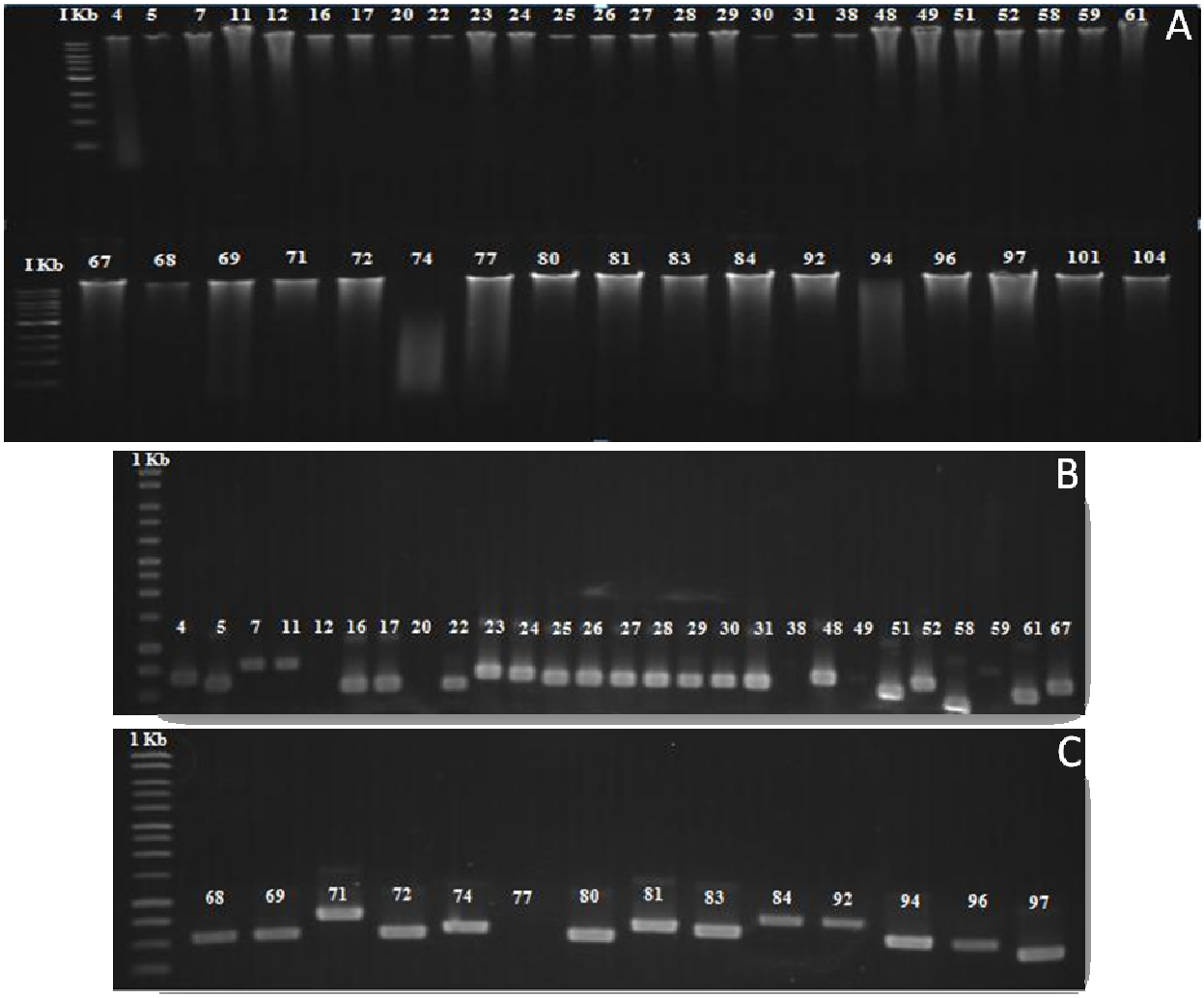

Figure 5.5 (A) Genomic DNA extractions of fluconazol resistant strains isolated from all sampling sites showing high concentration yields after the extraction protocol, amplification of ITS rRNA genes using ITS-1 and ITS-4 primers from representative strains. (B and C) Molecular marker corresponds to 1Kb ladder DNA marker.

5.3 Identification of Resistant Yeast Isolates

5.3.1 Acremonium strictum (W. Gams)

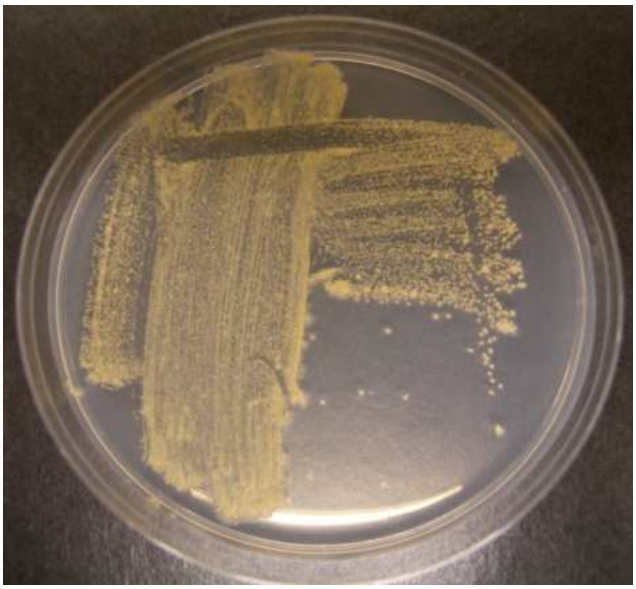

Figure 5.6 Pale pink colonies of *Acremonium strictum* on SDA.

The genus *Acremonium*, (synonym *Cephalosporium)*, comprises opportunistic, environmentally widespread soil saprophytes that can occasionally be pathogenic in humans. Colonies are usually slow growing, often compact and moist at first, becoming powdery when mature (Barnett et al. 1983). Some species are recognized as opportunistic pathogens of human and animals, causing mycetoma, bone infections, endocarditis, and subcutaneous mycosis (Fincher et al. 1991).

The infections caused by this "yeast-like" fungus have been increasing in recent years according to Guarro et al. (1997). Infections mainly occurred in patients with predisposing conditions such as Addison's disease, neutropenia, immune suppression and intravenous drug abuse. Yalaz et al. 2003 reported a fatal disseminated mycotic infection in a newborn, caused by this fungus.

5.3.2 Aureobasidium pullulans (De Bary)

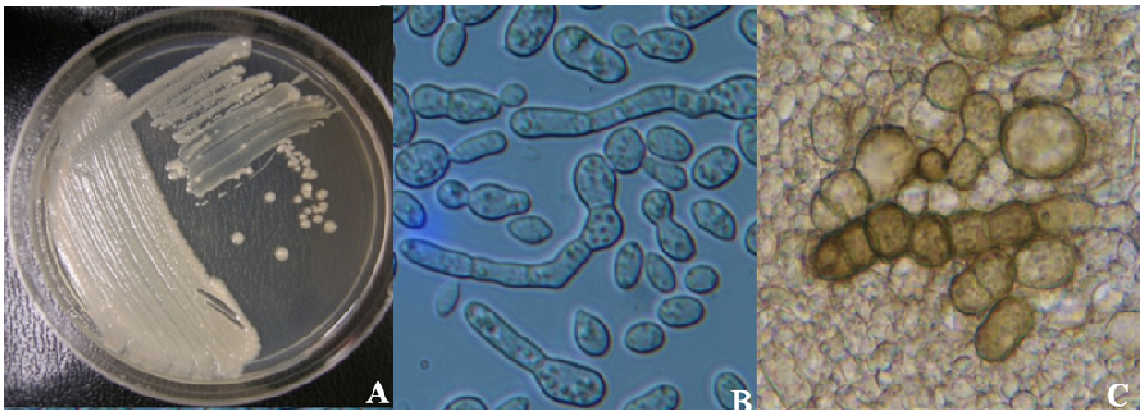

Figure 5.7 (A) Macroscopical characteristics of *Aureobasidium pullulans* on SDA. (B) Unicellular blastoconidia and germ tube. (C) Pigmented arthroconidia produced in old, mature cultures. Viewed on Nomarsky.

The morphology of *Aureobasidium pullulans* is rare. In early stages the growth behaves as a yeast with pink coloration. At maturity, the colony produced a black pigment called melanin (Barnett et al. 1983). This fungus is commonly known as a black yeast-like species, and produce the biodegradable extracellular polysaccharide pullulan (Rekha and Sharma 2007). This material is currently used for packaging of food and drugs. *A. pullulans* is a ubiquitous and widespread oligotrophe that can be found in environments with fluctuating water activities, bathrooms, rocks and monuments (Samson et al. 2004). It can also be found in osmotically stressed environments as hypersaline waters in salterns (Gunde-Cimerman et al. 2000). In recent studies it was also found in Antartic soil and Siberian permafrost (Zalar et al. 2008).

5.3.3 Candida diddensiae (Phaff, Mrak & O.B. Williams) Fell & S.A. Meyer

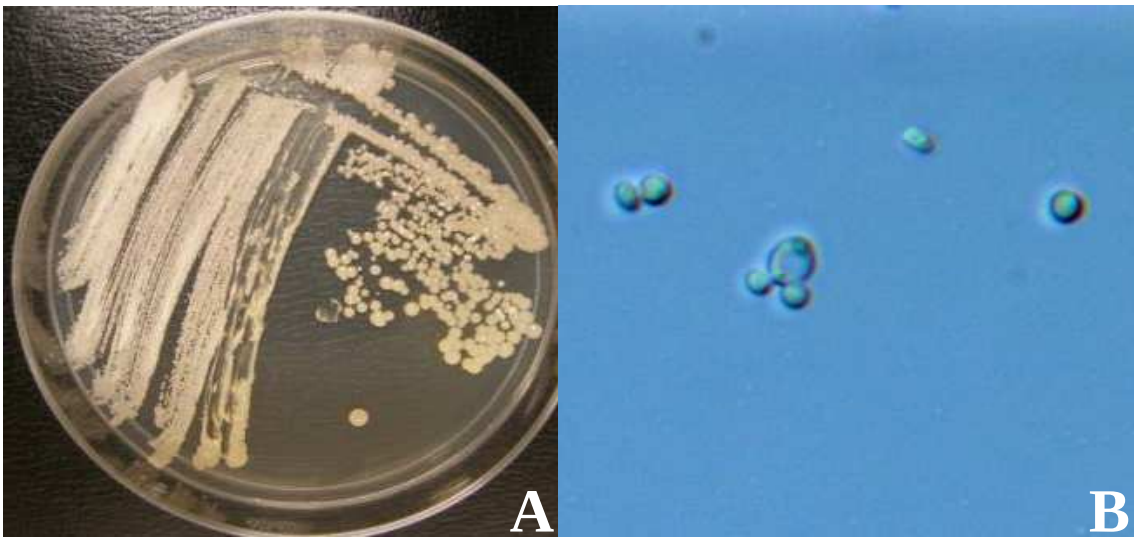

Figure 5.8 (A) Macroscopical characteristics of *Candida diddensiae* on SDA. (B) Unicellular spherical blastoconidia. Viewed on Nomarsky.

C. diddensiae is commonly found in olives, shrimp and skin lesion. It growth forming white, smooth and flat colonies. Microscopically, blastoconidia are subglobose, ellipsoidal to cylindrical with a single or paired, multilateral budding. After 7 days at 25ºC pseudohyphae are visible (Meyer, 1983).

The literature has not reported opportunistic or pathogenic cases of *C. diddensiae* in humans. Hovewer, the most recent study by Zullo et al. (2010) found this yeast in olive oil. In our study, we isolated *C. diddensiae* from water and sediment samples from the rural wastewater treatment plant and Ensenada Dakity, respectively. Isolates were resistant for all fluconazol concentrations tested. There are no previous reports about fluconazol-resistant strains of *C. diddensiae*.

5.3.4 Candida thaimueangensis (Limtong, Yongman., H. Kawas. & Tats. Seki)

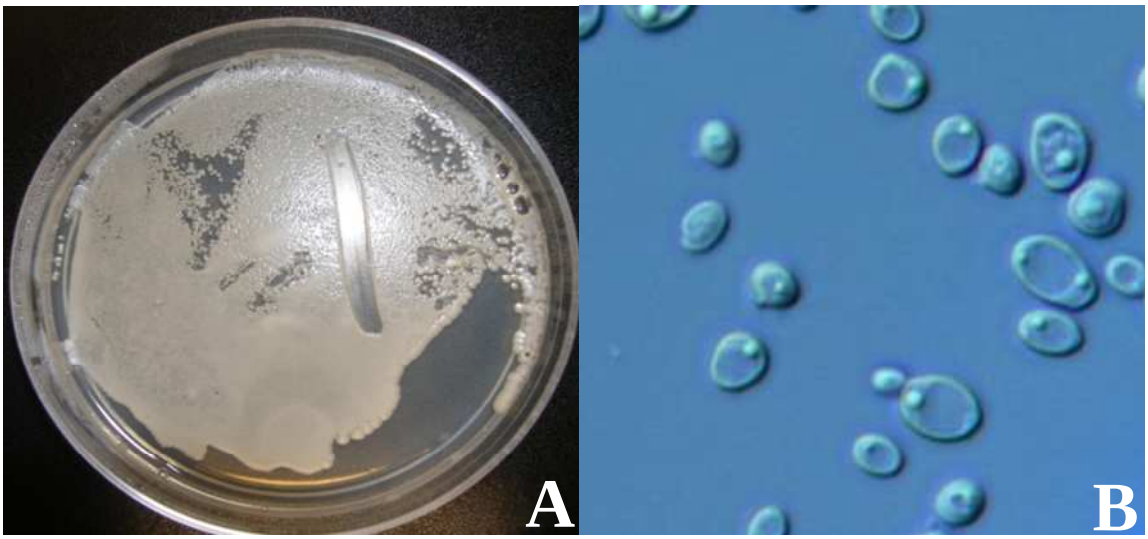

Figure 5.9 (A) Macroscopical characteristics of *Candida thaimueangensis* on SDA. (B) Unicellular spherical to elongate blastoconidia. Viewed on Nomarsky.

C. thaimueangensis is commonly found in seawater environments. Colonies are cream-coloured with a smooth surface. Under the microscope blastoconidia are spherical, ellipsoidal to elongate with a single or paired chains and multilateral budding. After 7 days at 25ºC pseudohyphae are visible (Limtong et al. 2007).

The literature has not reported opportunistic or pathogenic cases of *C. thaimueangensis* in humans. This genus was reported by Limtong et al. (2007) for first time from estuarine water in a mangrove forest in Thailand. In our study, we isolated *C. thaimueangensis* from rhizosphere samples in a mangrove ecosystem in Ensenada Dakity, Culebra. The isolates were tested with fluconazol, resulting resistant with MIC of 64ug/mL. There no previous reports about fluconazol-resistant strains in *C. thaimueangensis*.

5.3.5 Candida tropicalis (Castell)

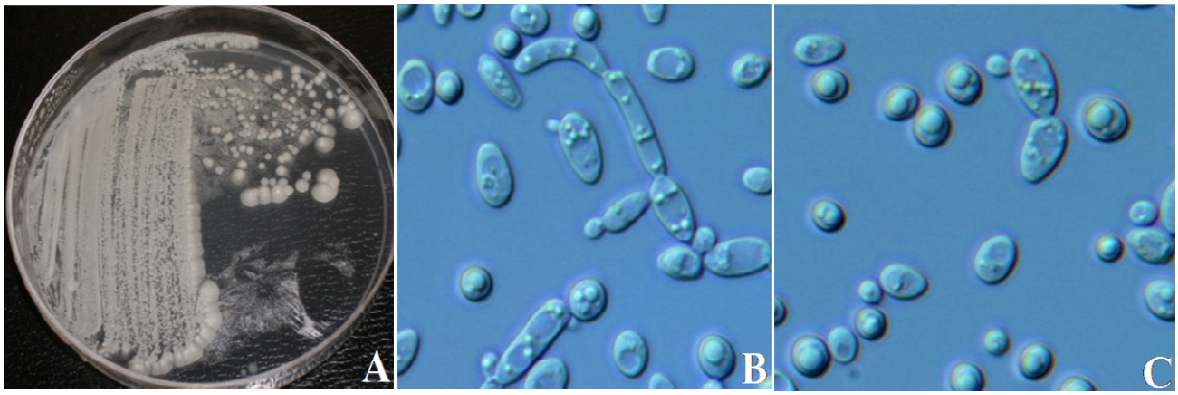

Figure 5.10 (A) White colonies of *Candida tropicalis* on SDA. (B and C) Oval blastoconidia located along the long pseudohyphae. Viewed on Nomarsky.

C. tropicalis shows white or cream, smooth and yeast-like in appearance. Microscopic morphology showed spherical budding blastoconidia (Barnett et al. 1983). This organism is one of the causal agents of candidiasis, the most common opportunistic fungal infection. Because the genus *Candida* sp. is part of our normal flora, infections vary according to our immune system. The yeast frequently colonizes the skin, mucous membranes, oropharynx, lower respiratory, gastrointestinal and genitourinary tracts (Hedayati and Ghazal, 2010). According to Pappas et al. (2009) *Candida* is the fourth most common cause of nosocomial bloodstream infections and invasive candidiasis in the United States. Some statistics showed by the International and United States Epidemiology Centers, indicates that *C. tropicalis* caused 48.4% of the infections reported, followed by C. albicans with 29.7%. An important data about invasive candidiasis in United States is the mortality rate of 40-50% (Hedayati and Ghazal, 2010). Today, the constant increase of infection makes this organism the principal cause of mycosis by species of *Candida* sp. in Puerto Rico (Ruiz, 2001).

Figure 5.11 Isolates of *Candida tropicalis* showing blue pigment in Chromagar.

5.3.6 Cryptococcus spp. (Kufferath & C.E. Skinner)

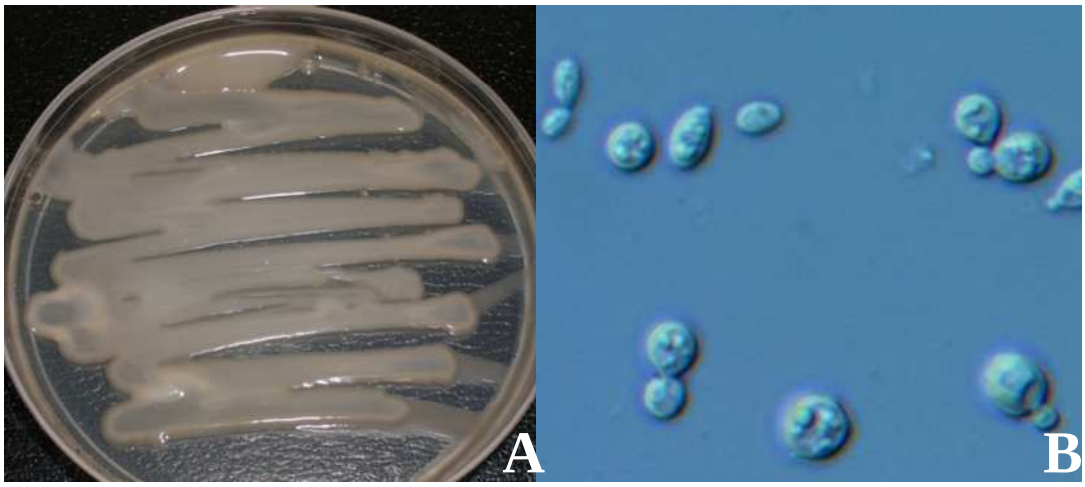

Figure 5.12 Mucoid and cream colonies of *Cryptococcus flavescens* on SDA. (B) Round budding yeast's blastoconidia. Viewed on Nomarsky

Colonies of *Cryptococcus* spp. are fast growing, smooth, mucoid and cream, pink or brown in color (Barnett et al. 1983). The capsule is best visible in India ink preparation. This genus has the capacity to grow at 37ºC.

The genus *Cryptococcus* includes approximately 37 species, some of them with the potential to cause cryptococcosis. Cryptococcosis is an infection of neurotropic nature and the principal condition associated with it is meningitis. Cryptococcosis is a general condition that may include a disseminate infection around the body (Bailly et al. 1991). The principal representative of this genera is *C. neoformans* due to its clinical relevance. *C. neoformans* is commonly found in soil contaminated with pigeon droppings or eucalyptus trees (Callejas et al. 1998). Species of *Cryptococcus* spp. usually respond efficiently to the antifungal fluconazol. However, authors have reported resistant strains for species of *Cryptococcus* spp. during patient's treatments (Jessup et al. 1998).

5.3.7 Exophiala jeanselmei (Langeron)

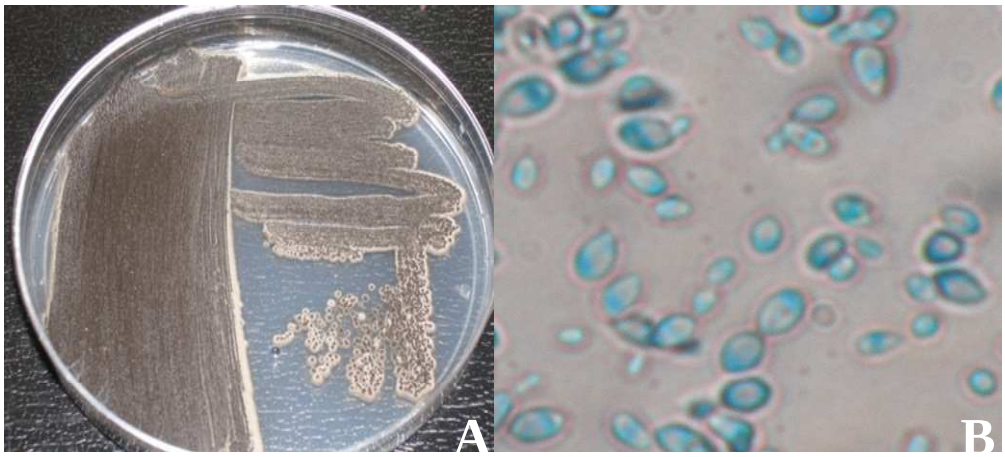

Figure 5.13 (A) Yeast-like brownish to greenish colonies of *Exophiala jeanselmei* on SDA. (B) Subspherical budding blastoconidia on light microscopy.

Exophiala jeanselmei belongs to the group of black yeasts along with *Hortaea werneckii*. *E. jeanselmei* is usually considered a pathogenic yeast in immunocompromised hosts. Colonies are slow growing, dark-brown, greenish or black, often slimy when young, but later becoming downy (Barnett et al. 1983).

E. jeanselmei is commonly found in oligotrophic water sources and preservative-treated wood. Three species have been commonly implicated in human infections: *E. jeanselmei, E. spinifera* and *E. dermatitidis*. According to Hoog et al. (2003) *E. jeanselmei* has been responsible for serious, even fatal human infections. Among the infections found in human are keratitis, olecranon bursitis, human mycetoma, and fatal cerebral infection in a healthy woman. Authors suggest there is an apparent link between waterborne contamination by this species and opportunistic infection in humans.

5.3.8 Hanseniaspora opuntiae (Cadez)
Hanseniaspora thailandica (Jindam)

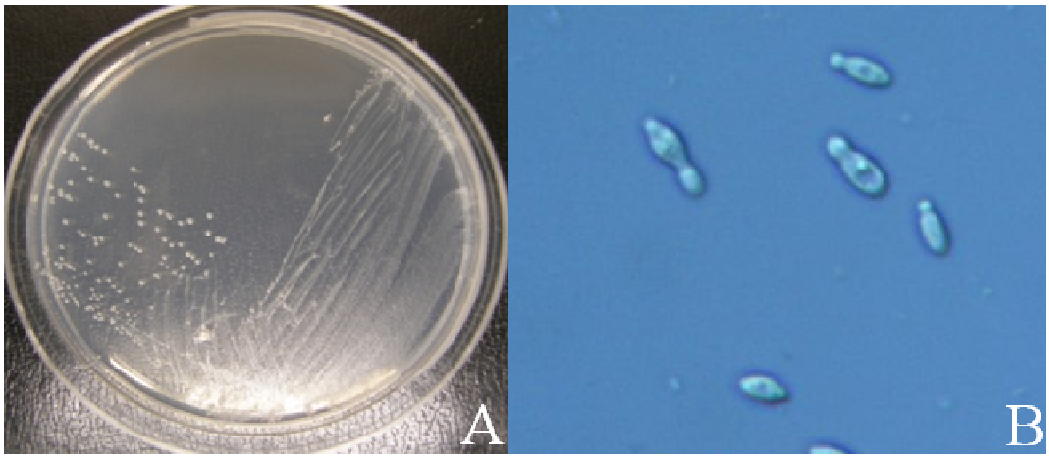

Figure 5.14 (A) White colonies of *Hanseniaspora opuntiae* on SDA. (B) Oval blastoconidia showing polar budding growth. Viewed on Nomarsky.

H. opuntiae and *H. thailandica* are yeasts related to infection on wine production grapes. White colonies with a polar budding pattern were observed in SDA. Filaments or pseudohypha were absent. Commonly, these yeasts has been found in bottled tomatoes, grape juice and must, fruits and berries, soil and baboon (Barnett et al. 1983). Human infections caused by these yeasts are rare, but the Russian Collection of Microorganisms (VKM) reported strains of *Hanseniaspora* sp. from a diseased nail in South Africa (VKM's Yeast Catalogue, 1928).

In this study *H. opuntiae* was isolated from the estuary of Aguada. Also, *H. thailandica* was isolated from Cupeyes and Manatí rivers. *Hanseniaspora* sp. has been found in the intestine of fish (*Scomber scombrus).* Previous studies suggest that other species of this genus have been report as opportunistic yeasts. According to García-Martos, et al. (1999) *H. uvarum,* a related species has been reported from nail infections in three patients from Spain. This is the first report of fluconazol-resistant strains in genera *Hanseniaspora* spp.

5.3.9 Hortaea werneckii (Nishim. & Miyaji)

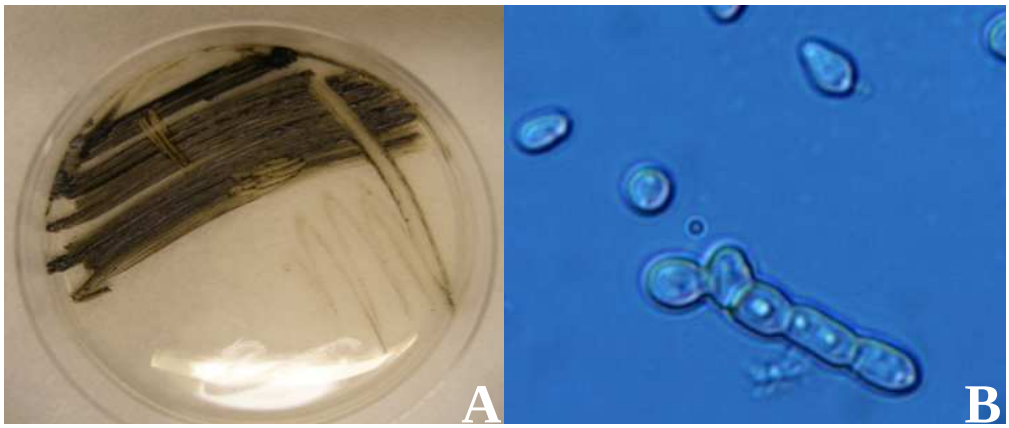

Figure 5.15 (A) Olive black colonies of *Hortaea werneckii* on SDA. (B) Septate hyphae and "yeast-like" blastoconidia (B). Viewed on Nomarsky.

Hortaea werneckii is a black yeast well-known as are opportunistic fungus. Colonies are slow growing, initially mucoid and then shiny black. Microscopically, colonies consist of dark septate hyphal elements and numerous 2-celled, cylindrical to spindle-shaped yeast –like cells (Barnett et al. 1983). It is a common saprophytic fungus in soil, compost, humus and on wood of humid tropical regions. However, *H. werneckii* has been identified as the dominant fungal species in hypersaline waters. According to Gunde-Cimerman and Ana (2006) this yeast represent a new model organism for studying the mechanism of salt tolerance in eukaryotes. *H. werneckii* has been isolated from three different continents exclusively from hypersaline water. Díaz-Muñoz and Montalvo (2005) made the first report of this yeast in an extreme environment in Puerto Rico, located in the Cabo Rojo salterns. Also, Cantrell et al. (2006) reported this yeast as part of the microbial mats in hypersaline waters of Cabo Rojo.

H. werneckii is classified as pathogenic yeast due to its ability to cause tinea nigra in humans. Tinea nigra is a superficial skin mycosis characterized by brown to black regions which usually occur on the palm of the hands and occasionally on other surfaces of the skin. According to Ng et al. (2005) *H. werneckii* was isolated from blood and splenic abscess in acute myeloid leukaemia patients causing serious fungal infections. Most of the time these infections were treated with itraconazole, voriconazole and amphotericin B.

5.3.10 Pichia anomala (E.C. Hansen)

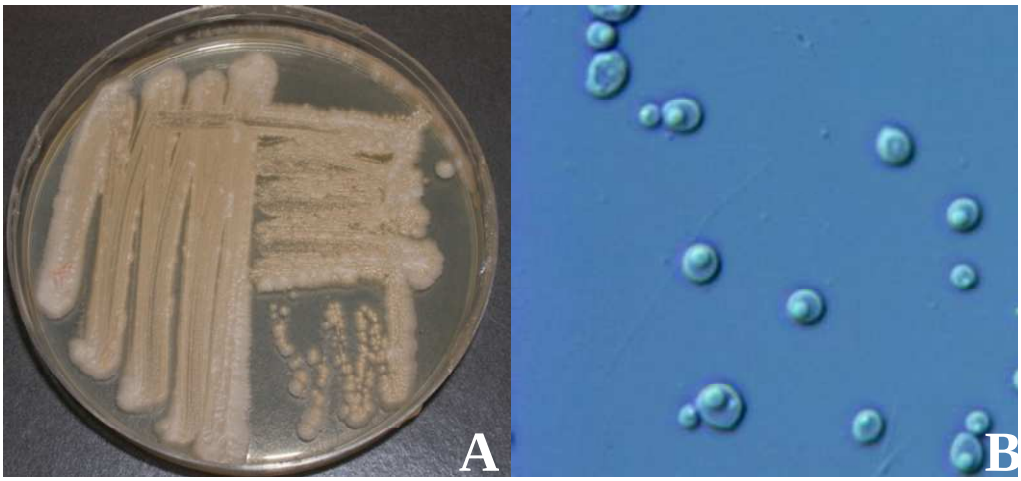

Figure 5.16 (A) Macroscopical characteristics of *Pichia anomala* on SDA. (B) Unicellular spherical blastoconidia. Viewed on Nomarsky

Pichia sp. is a teleomorph of various *Candida* species. Colonies of *P. anomala* are white or cream colored, smooth and yeast-like in appearance. Microscopic morphology showed spherical budding blastoconidia (Barnett et al. 1983). Clinically, *Pichia* sp. has generally been considered to be opportunistic pathogen. Some of the species has been reported as causal agents of premature, low birth weight and other nosocomial infections from surgical intensive care unit (Chakrabarti et al. 2001). According to Choy et al. 2000 *P. anomala* has achieved clinical success with fluconazol. However, the strains isolated from rural wastewater treatment plant in and sediment in Culebra showed fluconazol-resistance.

5.3.11 Pseudozyma sp. (S. Goto, Sugiyama & Iizuka)

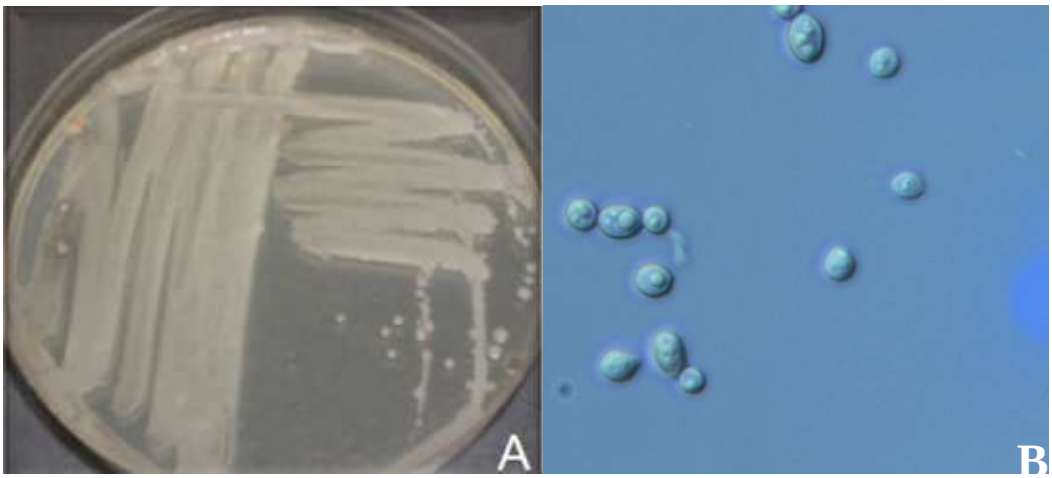

Figure 5.17 (A) Cream moist colonies of *Pseudozyma* sp. on SDA. (B) Round budding blastoconidia. Viewed on Nomarsky.

Pseudozyma sp. are economically important fungi. *Pseudozyma* species are unusual yeast-like fungi most frequently isolated from plant materials, such as leaves, flowers, and fruits (Boekhout and Fell, 1998), but is infrequently found causing human infections. However, Sugita et al. (2003) isolated *Pseudozyma* strains from patient's blood in Thailand and named two new species, *P. parantarctica* and *P. thailandica*.

This research reports the isolation of *Pseudozyma* sp. for the first time in Puerto Rico from Isabela sewage, Susua forest (Sabana Grande), Ensenada Dakity (Culebra) and rural wastewater treatment plant. We are also reporting for first time the resistance of *Pseudozyma* spp. to fluconazol.

5.3.12 Rhodotorula glutinis (S.Y. Newell & Fell)
Rhodotorula graminis (di Menna)

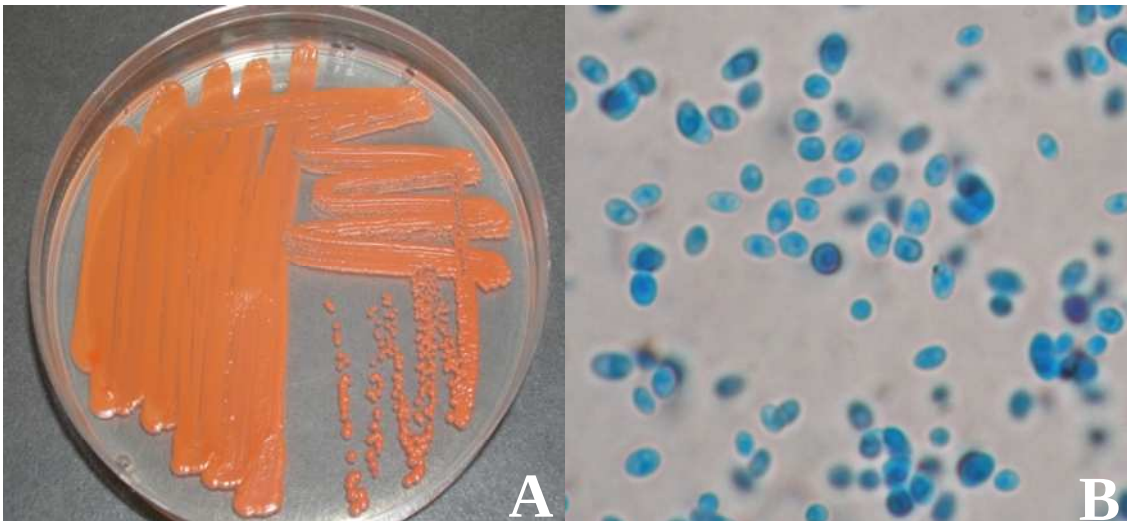

Figure 5.18 (A) Coral red smooth and mucoid colonies of *Rhodotorula glutinis* on SDA. (B) Unicellular and globose blastoconidia on light microscopy.

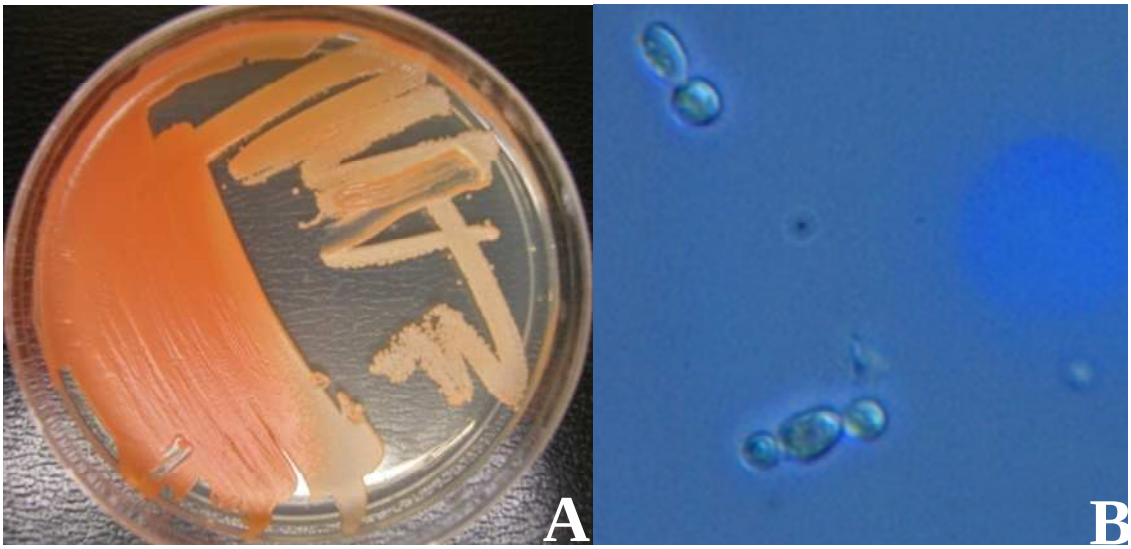

Figure 5.19 (A) Pink to red, smooth colonies of *Rhodotorula graminis* on SDA. (B) Globose blastoconidia. Viewed on Nomarsky.

R*hodotorula* species are common saprophytes in the environment and are usually found in water, air, soil, boracic lotions, leaves, fruits and mammals. Some applications of this yeast are the production and accumulation of glycerol (Yagi and Ashibe, 1994). This yeast produces a characteristic pink or red colored colonies in the medium, and was considered non-virulent until recent times. *Rhodotorula* spp. has been linked to endocarditis, peritonitis, meningitis, keratitis and central venous catheter infections (Pamidimukkala et al. 2007). *R. glutinis* is also emerging as human pathogen with the increasing number of immunocompetent patients in the last few decades. Riedel et al. (2007) published the first case of fungemia related to *R. glutinis* in a liver-kidney transplant patient.

5.3.13 Sympodiomycopsis paphiopedili (Sugiy., Tokuoka & Komag)

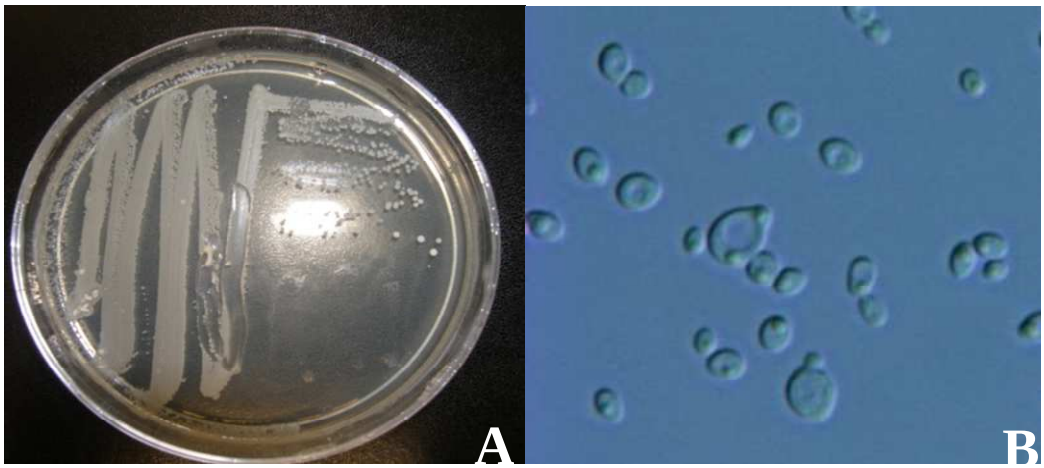

Figure 5.20 (A) Cream-colored, smooth colonies of *Sympodiomycopsis pahiopedili* on SDA. (B) Globose blastoconidia. Viewed on Nomarsky.

S. paphiopedili is commonly found from orchid's nectar. Colonies are cream-colored with a smooth surface. Blastoconidia are spherical to elongate with a single or paired chains and multilateral budding (Sugiyama et al. 1991).

The literature has not reported opportunistic or pathogenic cases of *S. paphiopedili* in humans. This genus was reported by Sugiyama et al. 1991 from *P. primurinum* (orchid flower) in Japan. Also, the yeast has been reported as a precursor of an extracellular glycolypid with antifungal activity for some pathogenic species (Golubev et al. 2004). In our study, we isolated *S. paphiopedili* from sewage pipeline discharge (Isabela). The isolates were tested against fluconazol, resulting resistant with MIC of 64ug/mL. There are no previous reports about fluconazol-resistant strains of *S. paphiopedili*.

6. Discussion

6.1 Fluconazol Resistant-Yeast Survey

Puerto Rico lacks studies about the incidence of pathogenic and opportunistic yeasts in different waterbodies across the Island. However, according to EPA most waterbodies have been impacted directly and/or indirectly by different anthropogenic activities (Table 2.1). Possible sources of contamination include: agriculture, constructions, hydromodification, industrial activities, municipal discharges/sewage, recreational boating and urban runoff (Puerto Rico Water Quality Assesment Report, 2008). This type of information indicates that waterbodies across the Island has been impacted by diverse contaminant agents that may cause changes in the indigenous microbiological communities.

Urban and rural wastewater treatment plants are secondary plants that receive thousands of gallons per day of sewage to be treated by physical and chemical processes. Samples from the influents and the effluents of each plant were analyzed. Seven resistant isolates were recovered from the rural plant's influents. Among these, *Candida tropicalis* was the most abundant yeast with 4 isolates in this location, followed by *Exophiala jeanselmei*, *Pichia anomala*, and *Pseudozyma* sp. A single fluconazol resistant yeast identified as *Cryptococcus flavescens* was retrieved from the effluents. Four fluconazol-resistant yeasts were recovered from the influents and effluents of the urban plant, respectively. *Aureobasidium pullulans* was the most abundant yeast in this location with six isolates, followed by *Pseudozyma* spp. and unknown strain. This suggests that *A. pullulans* and *C. flavescens* have the capacity to resist the water treatment process and the need to improve this process, because some of them remain after the disinfection processes and may cause serious health problems to inmunocompromised patients.

Susua and Guajataca forests were used as reference environments with low anthropogenic impact. Susua Forest yielded only 1 resistant yeast (*Pseudozyma* spp.). From comparison of DNA sequences, a similar isolate was reported for the rural wastewater treatment plant. However, the isolate from Susua Forest was from a soil sample and the rural wastewater treatment plant isolate from a water sample. Guajataca Forest was the only site not yielding any fluconazol resistant yeast. This suggests that anthropogenic activities from this area are less or different from those in the Guajataca forest sampling area.

Considering the rural and urban wastewater treatment plants as highly impacted areas, for comparison purposes we also studied coastal areas and rivers impacted by different sources of contamination according to the EPA reports (Figure 5.1). All these studied sites revealed the presence of different clinically important yeasts such as *Candida tropicalis, Aureobasidium pullulans, Cryptococcus flavescens and Exophiala jeanselmei, (*Figure 5.3).

Coastal areas, rivers and wastewater treatment plants showed high abundance of fluconazol-resistant yeasts. Water (73%) and soil (3%) samples showed the most and least affiliation of fluconazol resistant yeasts, respectively. Samples from La Parguera exhibited presence of *Candida tropicalis* (25%), *Rhodotorula glutinis* (25%) and two isolates corresponding to a pathogenic yeast responsible for tinea negra, *Hortaea werneckii* (50%). Peña Blanca beach (Aguadilla), despite being a beach located far from homes, rivers, and industrial zones, the presence of 5 fluconazol-resistant yeasts was detected in both water and sand samples. Isolates from sand samples were identified as *Cryptococcus* spp. (40%), *R. graminis* (20%) which was also found in a water sample (20%) and an unknown strain (20%). From Pico de Piedra estuary in Aguada we recovered isolates of *A. pullulans* (33.3%), *C. tropicalis* (33.3%) and *Hanseniaspora opuntiae* (33.3%). According to the DNA sequences, the strains of *A.*

pullulans and *C. tropicalis* isolated from this location are similar to the ones isolated from samples of both wastewater treatment plants. This may reflected inefficient processes by wastewater treatment plants in coastal areas that are commonly used for recreational activities.

Ensenada Dakity in Culebra reported *C. tropicalis* (25%), *Pichia anomala* (25%) and *Pseudozyma* spp. (25%) from sediment samples. A rhizosphere isolated was identified as *C. thaimueangensis* (25%). Sewage pipeline discharge (Isabela) was the least impacted coastal area in this study. Only two resistant yeasts were reported, one of the strains belongs to *Sympodiomycopsis paphiopedili* (50%) and the other to *C. diddensiae* (50%). *Sympodiomycopsis paphiopedili* is not considered pathogenic, however it is a yeast of clinical importance because it has been reported as a precursor of an extracellular glycolypid with antifungal activity for some pathogenic species (Golubev et al. 2004).

The river most impacted as revealed by this study was the estuary La Boca (Barceloneta). From this location, we reported isolates of *A. strictum* (40%), *Cryptococcus* spp. (20%), *H. thailandica* (20%) and an unknown strain (20%). According to EPA, this location has been directly impacted by the collection system, onsite of the wastewater systems, urban runoff and industrial activities. In contrast, Cupeyes River is a low impacted anthropogenic location, consistent with our findings. We only found 1 fluconazol-resistant yeast, *H. thailandica* (100%) similar to the strain isolated in La Boca, estuary according to the DNA sequences.

6.2 *Fluconazole-Resistant Yeast Isolates*

After following the Standard Protocol M27-A3 *Reference method for broth dilution antifungal susceptibility test for yeasts* (2008) we conclude that A. *strictum, A. pullulans, C. diddensiae, C. thaimueangensi, C. tropicalis, Cryptococcus* spp., *C. flavescens, Exophiala jeanselmei, H. opuntiae, H. thailandica, Hortaea werneckii, Pichia anomala, Pseudozyma* spp.,

R. glutinis, R.graminis and *Sympodiomycopsis paphiopedili* are resistant to fluconazol. It is important to notice that all these yeasts have the ability to be opportunistic pathogenic agents and reported a high MIC (≥64ug/ml) in the *in vitro* test.

Acremonium strictum showed low MIC *in vitro* activity to some azoles (Guarro et al. 1997). However, in our study *A. strictum* showed resistance to fluconazol in all concentrations tested. This finding agrees the previous study by Novicki et al. (2003) where they reported resistance of *A. strictum* to fluconazol. This could evidence the effect of pharmaceutical and other industrial activities on the microbial community of La Boca, estuary (Barceloneta). According to the US Environmental Protection Agency (EPA) in the 2010 Cycle 303(d) List Report called Evaluation and Strategic Planning Area Puerto Rico Environmental Quality Board, pollution sources of water sampled were identified. Agriculture land development, collection system failure, confined animal feeding operations, minor industrial point sources, onsite wastewater systems and urban runoff/storm sewers were identified as potential sources. Also, the report establishes the presence of arsenic, turbidity and fecal coliforms as cause of impairment in the river.

A. pullulans is an opportunistic yeast that could cause infections in immunocompromised patients. This organism can be the causal agent of phaehyphomycosis or nervous system infections (Rinaldi, 1996), as well as keratomycosis, meningitis, splenic abscess, jaw abscess, pulmonary mycosis, sepsis and other opportunistic infections (Michael et al. 2005). *A. pullulans* strain was the most abundant fluconazol-resistant yeast isolated from our samples of the urban wastewater treatment plant. Isolates strains from Pico de Piedra in Aguada, were also resistant. Recently, it was documented for first time by Mershon et al. (2011).

The strains of *C. tropicalis* isolated in our study showed high MIC (64ug/ml) for the *in vitro* test, allowing us to classify this strain as resistant from Puerto Rico. Schwab et al. (1997) also report some strains of *C. tropicalis* as resistant to the antifungal fluconazol. *C. tropicalis* was the most frequent yeast present in four of the eleven locations sampled for this study (Pico de Piedra estuary (Aguada), La Parguera (Lajas), Ensenada Dakity (Culebra) and Rural Wastewater Treatment Plant. All strains isolated of *C. tropicalis* in this research showed a high MIC (≥64ug/mL).

C. flavescens (synonym *Cryptococcus laurentii*), has been reported as an unusual cause of pulmonary and cutaneous infections associated to peritonitis in humans and fungemia in premature neonates (Cheng et al. 2001). In our study we reported *C. flavescens* from water samples obtained from the rural wastewater treatment plant after disinfection.

E. jeanselmei was also isolated from rural wastewater treatment plant. The isolate tested for fluconazol susceptibility resulting resistant for the antifungal in all the concentrations tested, consistent with a study realized by Carrillo-Muñoz et al. (2000). Previous studies report susceptibility of *E. jeanselmei* for other drugs such as flucytosine, itraconazole, voriconazole, and amphotericin B (Bossler et al. 2003).

H. werneckii was reported in this study from La Parguera samples, from rhizosphere samples impacted by sewage from a treatment plant. Previous studies for *H. werneckii* always reported low MIC's (susceptible) for the *in vitro* tests. However, our strain isolated from La Parguera showed high MIC for the *in vitro* test. This could evidence of the effect of inefficient processes at wastewater treatment plants in the coastal areas that are commonly use for recreational and fishing activities. Our study is the first report of fluconazol resistant strains of *H. werneckii*.

According to Diekema et al. (2005) most species of *Rhodotorula* sp. in their research showed resistance to fluconazol. Our results confirmed that behavior because all *Rhodotorula* spp. isolates showed a high MIC (≥64ug/mL) for fluconazol.

R. glutinis has been isolated previously from sea water of Japan and the Antarctic Sea (Yagi and Ashibe, 1994). In the present study the yeast was isolated from sea water in La Parguera. In contrast, *R. graminis* was isolated from sand and water in Peña Blanca beach. *R. graminis* has not been reported as an opportunistic or pathogenic yeast previously.

Reported strains of *C. diddensiae, C. thaimueangensis, H. opuntiae, H. thailandica, Pseudozyma* spp. and *S. paphiopedili* have little or none clinical relevancy. However, our data revealed that our isolates are fluconazol-resistant strains. Due to the recurrent impact of different anthropogenic sources that affect our coasts, these yeasts might show a change in their physiology. Due to the fact that resistance to antifungals posses a health risk a monitoring program should be put in place for these species as a proactive behavior in case they become of clinical relevance.

6.3 ***Phylogenetic Analysis of Fluconazole-Resistant Yeast Isolates***

A total of 39 operational taxonomic unit (OTU's) of fluconazol-resistant yeasts isolated were obtained from our study. Sequences were identified using the BLAST program to search the GenBank database. Phylogenetic and molecular analyses were made, through the alignment of the ITS regions amplified with ITS-1 and ITS-4 primers from each resistant strain with respect to that of their closest database match (Figure and Table 6.1).

Fluconazol-resistant strains of *C. tropicalis* were obtained from four different locations: rural wastewater treatment plant, Ensenada Dakity, estuary Pico de Piedra and La Parguera. All

strains were 99% identical to *C. tropicalis* (GenBank accession number AY939810). This strain detected using DNA microarray by Leinberger et al. (2005) from invasive fungal infections. Finding these fluconazol-resistant strains in our coastal areas represents a new hazard, especially to individuals with immunocompromised systems.

Only two fluconazol-resistant strains of *A. strictum* were obtained from the most impacted stream, La Boca estuary (Barceloneta). Both strains were 99% identical to *A. strictum* (GenBank accession number AY138848). This strain was isolated by Novicki et al. (2003) during a research of a fatal mycosis with *A. strictum.* La Boca estuary, spread its water directly to coastal areas affecting water quality across the north coast of Puerto Rico. Alarming to us, fluconazol-resistant yeasts that could cause fatal infections could be spreading through our coastal waters.

Other strains of our study showed close relationship between pathogenic strains of *H. werneckii* and *E. jeanselmei*. Two strains from La Parguera, Lajas were 99% identical to *H. wernecki* (GenBank accession number AY213656). This strain was used for rapidly identification of pathogenic fungi by Rakeman et al. (2005) in their molecular research. Both strains were isolated from the rhizosphere of mangrove plants, suggesting that mangrove in that areas are being impacted with fluconazol-resistant yeast commonly known as responsible of tinea palmaris infection. Also, a strain from rural wastewater treatment plant was 99% identical to *E. jeanselmei* (GenBank accession number AJ866273). This strain was reported as a causal infection agent from catheters (Ahmad, 2004).

A phylogenetic analysis of our isolates representing by *A. pullulans, C. diddensiae, C. thaimueangensis, C. flavescens, H. opuntiae, H. thailandica, P. anomala, Pseudozyma sp., R. glutinis, R. graminis* and *S. paphiopedili* did not show a close relationship with opportunistic or

pathogenic agents according GenBank database (Figure 6.2). Due to the recurrent impact of different anthropogenic sources that affect our coasts, these yeasts might show a change in their physiology. We suggest the implementation of a monitoring program for these species as a proactive measure in case they become of clinical relevancy.

Currently, the enumeration of coliform and enterococci are used as standard test for measuring water quality. The results of this study show the need to incorporate a protocol to detect the presence of clinically important yeasts in our water and other countries of the world.

Table 6.1 Fluconazole-resistant strains associate with human infections according to GenBank database.

Strains no.	Organism ITS identified	Accession no. GenBank	Infection reported[a]	% ITS Identity[b]
94,97	*Acremonium strictum*	AY138848	Fatal mycosis	99%
12,16,17, 20,51,61, 72	*Candida tropicalis*	AY939810	Candidiasis, nosocomial infections	99%
23	*Exophiala jeanselmei*	AJ866273	Fatal cerebral and catheter infections	99%
68, 69	*Hortaea werneckii*	AY213656	Tinea nigra palmaris	99%

[a] Infections reported in the literature.
[b] Percent of identity between sequences obtained from comparison using GenBank database.

Table 6.2 Fluconazole-resistant strains associate with other sources according to GenBank database.

Strains no.	Organism ITS identified	Accession no. GenBank	Infection reported[a]	% ITS Identity[b]
25,26,27 29,30,31, 74	*Aureobasidium pullulans*	EF197817	Marine yeast	99%
48	*Candida diddensiae*	AM117818	Marine yeast	99%
58	*Candida thaimueangensis*	FN428902	Sugar cane	99%
71	*Hanseniaspora opuntiae*	FM199955	Cocoa bean	99%
92,96	*Hanseniaspora thailandica*	AB501145	Mangrove	99%
24,52	*Pichia anomala*	EU380207	Hami melon	94%
7,11,38, 59	*Pseudozyma* sp.	EF643577	Marine yeast	90-99%
77,84	*Rhodotorula graminis*	AF444492	Molecular research	99%
67	*Rhodotorula glutinis*	AF444499	Molecular research	99%
49	*Sympodiomycopsis paphiopedili*	GU319998	Orchid nectar	99%

[a] Infections reported in the literature.
[b] Percent of identity between sequences obtained from comparison using GenBank database.

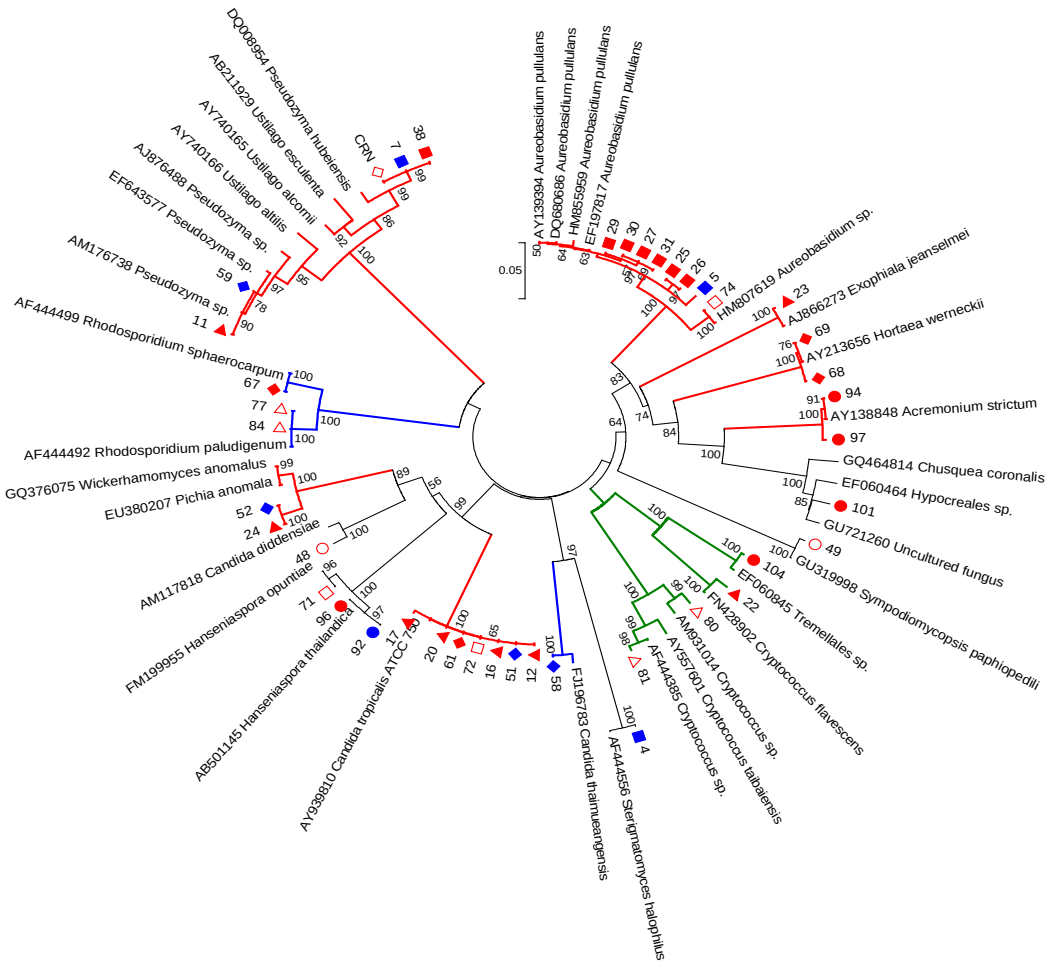

Figure 6.1 Phylogenetic tree depicting the relationship of fluconazol-resistant (MIC≥64ug/mL) yeasts isolated from all sampling sites. Red branches illustrate the clustering of isolates related to species reported as opportunistic or common human pathogens. Green and blue branches indicate the position of isolates that grouped with commensal species described in marine or plant–related niches. Cultures retrieved from impacted and relatively undisturbed settings are highlighted with red and blue labels respectively, as follows: ■ = Urban wastewater treatment plant; ▲= Rural wastewater treatment plant; ● = La Boca, estuary (Barceloneta); ♦ = mangrove ecosystem at La Parguera (Lajas); Δ = Peña Blanca beach (Aguadilla); □ = Pico de Piedra estuary (Aguada); ○ = wastewater pipeline discharge (Isabela); ◊ = Vega Baja Beach; ♦ = mangrove ecosystem at Ensenada Dakity, Culebra; ● = Río Cupeyes, Sabana Grande; ■ = soil from Susa Forest Reserve.

7. Conclusions

- This study reported the presence of fluconazol-resistant yeasts such as *Acremonium strictum, Aureobasidium pullulans, Candida diddensiae, C. thaimueangensis, C. tropicalis, Cryptococcus flavescens, Exophiala jeanselmei, Hanseniaspora opuntiae, H. thailandica, Hortaea werneckii, Pichia anomala, Pseudozyma spp., Rhodotorula glutinis, R. graminis* and *Sympodiomycopsis paphiopedili* among all locations sampled in Puerto Rico.
- According to standard method M27-A3 all the fluconazol-resistant yeasts reported MIC of >64 ug/ml.
- *Candida tropicalis* and *Aureobasidium pullulans* were the most abundant species from both wastewater treatment plants, respectively. Also, these strains were reported in Ensenada Dakity (Culebra), La Parguera (Lajas) and Balneario Pico de Piedra (Aguada).
- Guajataca Forest was the only site that did not show the presence of any fluconazol-resistant yeast.
- This research was the first attempt to monitor the presence of clinical importance yeasts isolated in coastal habitats from Puerto Rico.

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APPENDICES

Appendices 1 – 12 contain information regarding samples that yielded yeast isolates when processed.
* Indicates that no fluconazol-resistant yeasts were isolates.

Appendix 1. Water Samples from Rural Wastewater Plant (n=10)

Source	Sample	Dilution	Colony Count	CFU/ml	Fluconazol-resistant identified
Influent	1	10^{-1}	2	20	*Pseudozyma spp.* *Candida tropicalis*
	2	10^{-2}	1	100	*
		10^{-3}	1	1000	*Exophiala jeanselmei*
		10^{-4}	1	10000	*
	3	10^{-2}	1	100	*
		10^{-3}	2	2000	*C. tropicalis*
	4	10^{-3}	1	1000	*
	5	10^{-2}	1	100	*Pichia anomala*
		10^{-4}	1	10000	*
Effluent	1	10^{-3}	1	1000	*Cryptococcus flavescens*

Appendix 2. Water Samples from Urban Wastewater Plant (n=10)

Source	Sample	Dilution	Colony Count	CFU/ml	Fluconazol-resistant identified
Influent	1	10^{-2}	1	100	*
	2	10^{-1}	1	10	*Aureobasidium pullulans*
		10^{-2}	1	100	*A. pullulans*
		10^{-3}	2	2000	*A. pullulans*
		10^{-4}	2	20000	*A. pullulans*
	3	10^{-3}	1	1000	*
	4	10^{-2}	1	100	Unknown
		10^{-3}	1	1000	*A. pullulans*
		10^{-4}	2	20000	*A. pullulans*
	5	10^{-3}	1	1000	*Pseudozyma* spp.
Effluent	2	10^{-1}	1	10	*A. pullulans*
		10^{-2}	2	200	*A. pullulans*
	3	10^{-1}	2	20	*A. pullulans*
		10^{-2}	2	200	*A. pullulans*
		10^{-3}	2	2000	*A. pullulans*
		10^{-4}	2	20000	*A. pullulans*
	4	10^{-1}	2	20	*A. pullulans*
		10^{-2}	2	200	*A. pullulans*
		10^{-4}	2	20000	*A. pullulans*

Appendix 3. Soil Samples from Susua Forest, Sabana Grande (n=6)

Source	Sample	Dilution	Colony Count	CFU/g	Fluconazol-resistant identified
Soil	1A	10^{-2}	1	100	*
	1B	10^{-2}	1	100	*
	2A	10^{-3}	1	1000	*Pseudozyma* spp.
	2B	10^{-2}	1	100	*
	3A	10^{-2}	1	100	*

Appendix 4. Soil Samples from Guajataca Forest, Quebradillas (n=6)

Source	Sample	Dilution	Colony Count	CFU/g	Fluconazol-resistant identified
Soil	2B	10^{-2}	1	100	*
	3B	10^{-2}	1	100	*

Appendix 5. Water Samples from Pipeline Sewage, Isabela

Source	Sample	Dilution	Colony Count	CFU/ml	Fluconazol-resistant identified
Tube	1	10^{-1}	1	10	*
		10^{-2}	1	100	*
		10^{-4}	1	10000	*
	2	10^{-1}	1	10	*Candida diddensiae*
		10^{-4}	1	10000	*
	3	10^{-4}	1	10000	*S. paphiopedili*

Appendix 6. Rhizosphere and Sediment Samples from Ensenada Dakity, Culebra (n=3 each)

Source	Sample	Dilution	Colony Count	CFU/g	Fluconazol-resistant identified
Sediment	1	10^{-1}	1	10	*
	2	10^{-1}	2	20	*
		10^{-3}	2	2000	*C. tropicalis/Psudozyma* sp.
	3	10^{-1}	2	20	*P. anomala*
Rhizosphere	1	10^{-1}	2	20	*
		10^{-2}	2	200	*
		10^{-3}	1	1000	*
	2	10^{-1}	2	20	*
		10^{-2}	2	200	*
		10^{-3}	2	2000	*C. thaimuagensis*
	3	10^{-1}	2	20	*
		10^{-3}	2	2000	*

Appendix 7. Rhizosphere and Sediment Samples from La Parguera, Lajas (n=3 each)

Source	Sample	Dilution	Colony Count	CFU/g	Fluconazol-resistant identified
Sediment	1	10^{-1}	8	80	*
		10^{-2}	1	100	*
	2	10^{-1}	6	60	*
	3	10^{-1}	2	20	*
		10^{-2}	10	1000	*
		10^{-3}	2	2000	*
Rhizosphere	1	10^{-1}	3	30	*
	2	10^{-2}	2	200	*Hortaea werneckii*

Appendix 8. Water Samples from Pico de Piedra, Aguada (n=5)

Source	Sample	Dilution	Colony Count	CFU/ml	Fluconazol-resistant identified
Estuary	1	10^{-1}	19	190	*
		10^{-2}	2	200	*Hanseniaspora opuntiae*
	2	10^{-1}	3	30	*
		10^{-2}	1	100	*C. tropicalis*
	3	10^{-1}	10	100	*
		10^{-2}	1	100	*A. pullulans*

Appendix 9. Water Samples from La Boca Estuary, Barceloneta (n=4)

Source	Sample	Dilution	Colony Count	CFU/ml	Fluconazol-resistant identified
Estuary	1	10^{-1}	12	120	*A. strictum/H. thailandica*
	2	10^{-1}	3	30	*
		10^{-2}	5	500	*
	3	10^{-1}	7	70	*
		10^{-2}	2	200	*
		10^{-3}	1	1000	*
		10^{-4}	1	10000	Unknown
	4	10^{-1}	4	40	*Cryptococcus* sp.
		10^{-2}	1	100	*

Appendix 10. Water Samples from Cupeyes River, Sabana Grande processed by filtration (n=4)

Source	Sample	Filtration	Colony Count	Fluconazol-resistant identified
River water	1	C	1	*
	3	A	1	*
		B	1	*
		C	2	*
	4	B	1	*H. thailandica*

Appendix 11. Water and Sand Samples from Peña Blanca beach, Aguadilla processed by filtration (n=4)

Source	Sample	Colony Count	Fluconazol-resistant identified
Sand	1	1	*
	2	4	*
	3	1	*Rhodotorula graminis*
	4	2	*
Sea water	1	0	*R. graminis*
	2	3	*
	3	3	Unknown
	4	7	*

Appendix 12. Rhizosphere and Water Samples from La Parguera, Lajas processed by filtration (n=4)

Source	Sample	Colony Count	Fluconazol-resistant identified
Rhizosphere	1	26	*
	2	30	*
Water	1	29	*
	2	43	*

Appendix 13. Fluconazol Resistance Tests Data

Yeast	Genus	Location	Control	64 ug/ml	32 ug/ml	16 ug/ml	8 ug/ml	4 ug/ml	2 ug/ml	1 ug/ml	0.5 ug/ml	0.25 ug/ml	0.125 ug/ml
7	*Pseudozyma spp.*	Susua Forest	**0.352**	**0.148**	**0.172**	0.190	0.200	0.213	0.221	0.226	0.229	0.247	0.296
11	*Pseudozyma spp.*	Rural	**0.382**	0.346	0.332	0.358	0.363	0.387	0.376	0.366	0.458	0.602	0.812
12	*Candida tropicalis*	Rural	**0.631**	0.319	0.330	0.340	0.352	0.387	0.365	0.384	0.281	0.379	).486
16	*Candida tropicalis*	Rural	**0.597**	0.350	0.113	0.350	0.356	0.387	0.344	0.366	0.952	0.919	0.505
17	*Candida tropicalis*	Rural	**0.451**	0.434	0.908	0.878	0.886	0.902	0.943	0.923	**0.168**	0.317	0.323
20	*Candida tropicalis*	Rural	**0.634**	0.415	0.384	0.496	0.346	0.378	0.369	0.363	0.630	0.492	0.537
22	*Cryptococcus flavescens*	Rural	**0.111**	0.107	0.111	0.111	0.112	0.114	0.122	0.487	0.129	0.136	0.159
24	*Pichia anomala*	Rural	**0.167**	0.137	0.154	0.191	0.400	0.205	0.239	0.272	0.474	0.446	0.520
25	*Aureobasidium pullulans*	Urban	**0.172**	0.131	0.128	0.176	0.127	0.144	0.422	0.552	0.166	0.192	0.118
26	*Aureobasidium pullulans*	Urban	**0.205**	**0.103**	0.107	0.109	0.110	0.110	0.111	0.126	0.161	0.276	0.244
27	*Aureobasidium pullulans*	Urban	**0.121**	0.120	0.116	0.125	0.126	0.126	0.130	0.337	0.415	0.217	0.317
28	Unknown	Urban	**0.250**	0.196	0.252	0.258	0.259	0.263	0.300	0.310	0.321	0.329	0.191
29	*Aureobasidium pullulans*	Urban	**0.127**	0.117	0.116	0.118	0.123	0.115	0.110	0.114	0.320	0.117	0.118
30	*Aureobasidium pullulans*	Urban	**0.185**	0.109	0.213	0.117	0.118	0.117	0.117	0.219	0.130	0.132	0.147
31	*Aureobasidium pullulans*	Urban	**0.330**	0.307	0.328	0.329	0.331	0.325	**0.116**	0.320	**0.118**	**0.122**	0.603
38	*Pseudozyma spp.*	Urban	**0.361**	0.363	0.364	0.37	0.346	0.334	0.346	0.378	0.265	0.305	0.34
48	*Candida diddensiae*	Isabela Sewage	**0.409**	0.358	0.367	0.390	0.437	0.431	0.415	0.395	0.390	0.361	0.352
49	*Sympodiomycopsis sp.*	Isabela Sewage	**0.230**	**0.105**	0.205	0.208	0.227	0.209	0.210	0.211	**0.102**	**0.104**	**0.105**
51	*Candida tropicalis*	Dakity, Culebra	**0.405**	0.347	0.386	0.341	0.312	0.415	0.408	0.397	**0.144**	0.346	0.465

Appendix 13. Fluconazole Resistance Tests Data (Continue)

Yeast	Genus	Location	Control	64 ug/ml	32 ug/ml	16 ug/ml	8 ug/ml	4 ug/ml	2 ug/ml	1 ug/ml	0.5 ug/ml	0.25 ug/ml	0.125 ug/ml
52	*Pichia anomala*	Dakity, Culebra	**0.127**	0.099	0.100	0.106	0.226	0.101	0.102	0.102	0.123	0.123	0.119
58	*Candida thaimueangensis*	Dakity, Culebra	**0.330**	0.120	**0.126**	0.264	0.260	0.266	0.252	**0.120**	0.239	0.247	0.285
59	*Pseudozyma spp.*	Dakity, Culebra	**0.263**	0.148	0.150	0.168	0.173	0.167	0.175	0.189	0.236	0.245	0.224
61	*Candida tropicalis*	La Parguera	**0.480**	0.375	0.393	0.359	0.370	0.377	0.390	0.390	0.305	0.299	0.317
67	*R. sphaerocarpum*	La Parguera	**0.140**	0.110	0.114	0.118	0.119	0.124	0.130	0.130	0.915	0.115	0.320
68	*Hortaea werneckii*	La Parguera	**0.178**	0.110	0.155	0.113	0.117	0.170	0.114	0.113	0.108	0.108	0.107
69	*Hortaea werneckii*	La Parguera	**0.173**	0.123	0.103	0.154	0.829	0.187	0.292	0.166	0.324	0.443	0.628
71	*Hanseniaspora opuntiae*	Pico de Piedra	**0.104**	.106	0.105	0.105	0.105	0.106	0.108	0.110	0.111	0.107	0.112
72	*Candida tropicalis*	Pico de Piedra	**0.223**	**0.110**	0.113	**0.111**	0.112	**0.111**	0.117	0.192	0.115	0.137	0.174
74	*Aureobasidium pullulans*	Pico de Piedra	**0.109**	0.103	0.11	0.108	0.126	0.114	0.112	0.113	0.153	0.135	0.139
77	*R. graminis*	Peña Blanca	**0.140**	0.107	0.104	0.105	0.106	0.105	0.105	0.105	0.215	0.226	0.200
80	*Cryptococcus spp.*	Peña Blanca	**0.113**	0.108	0.112	0.111	0.112	0.116	0.112	0.113	0.113	0.132	0.126
81	*Cryptococcus spp.*	Peña Blanca	**0.126**	0.644	0.669	0.110	0.111	0.226	0.167	0.112	0.190	0.195	0.196
83	Unknown	Peña Blanca	**0.163**	0.120	0.118	0.131	0.114	0.112	0.130	0.361	0.116	0.116	0.161
84	*R. graminis*	Peña Blanca	**0.112**	0.106	0.540	0.114	0.117	0.112	0.110	0.112	0.118	0.118	0.178
92	*Hanseniaspora thailandica*	Cupeyes River	**0.151**	0.102	0.108	0.107	0.194	0.108	0.108	0.109	0.107	0.110	0.107
94	*Acremonium strictum*	La Boca	**0.184**	0.332	0.135	0.167	0.173	0.164	0.246	0.193	0.116	0.268	0.211
96	*Hanseniaspora thailandica*	La Boca	**0.103**	0.100	0.101	0.103	0.211	0.102	0.102	0.103	0.101	0.100	0.100

Appendix 13. Fluconazole Resistance Tests Data (Continue)

Yeast	Genus	Location	Control	64 ug/ml	32 ug/ml	16 ug/ml	8 ug/ml	4 ug/ml	2 ug/ml	1 ug/ml	0.5 ug/ml	0.25 ug/ml	0.125 ug/ml
97	*Acremonium strictum*	La Boca	**0.300**	0.150	0.153	0.226	0.273	0.298	0.288	0.271	0.262	0.230	0.246
101	Unknown	La Boca	**0.218**	0.182	0.150	0.147	0.210	0.165	0.157	0.158	0.163	0.169	0.154
104	*Cryptococcus spp.*	La Boca	**0.455**	0.275	0.276	0.383	**0.165**	0.395	0.376	0.403	0.460	0.400	0.415