Multiple Antibiotic Resistance of *Enterococcus* spp. in Waters and Sediments of Barceloneta–Manatí, Puerto Rico

by

Nydia J. Rodríguez Rodríguez

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Approved by:

_____	_____
Ernesto Otero, Ph.D. Member, Graduate Committee	Date
_____	_____
Dallas E. Alston, Ph.D. Member, Graduate Committee	Date
_____	_____
Bor- Sen Chiou, Ph.D. Member, Graduate Committee	Date
_____	_____
Baqar R. Zaidi, Ph.D. President, Graduate Committee	Date
_____	_____
Carlos Ríos Velázquez, Ph.D. Representative, Graduate Studies	Date
_____	_____
Nilda E. Aponte, Ph.D. Chairperson of the Department	Date

ABSTRACT

Recently the number of antibiotic-resistant bacteria in the environment has increased, creating a worldwide human health problem. Antibiotic resistance of *Enterococcus* spp. in coastal areas of Mayagüez, Guayanilla, Guánica, and Barceloneta, Puerto Rico was studied. The results indicated that Barceloneta was the area with highest numbers of antibiotic resistant microorganisms, so a detailed study of this area was undertaken. Samples were taken from Barceloneta-Manatí area from river, estuarine, and coastal waters, and from sediments. The samples were evaluated for the ability of *Enterococcus* species to resist penicillin, tetracycline, and vancomycin antibiotics. The results showed that during the dry period, the highest percentage of *Enterococcus* species were resistant to both penicillin and tetracycline. These results can be linked with the uses of these antibiotics in cattle for prophylaxis and treatment of infections in the dairy industry found in this area. Although the coastal area has the lowest numbers of *Enterococcus* that are multiple antibiotic resistance (MAR), it has the highest diversity of species. The distribution of species in Barceloneta zone indicated the predominance *of E. faecalis, E. hirae,* and *E. seriolicida*. *E. faecalis* was also the species with highest antibiotic resistance followed by *E. hirae*, *E. durans,* and *E. faecium*. The change in the population of Barceloneta coastal sediments after treatment of samples with antibiotics was evaluated with the terminal restriction fragment length polymorphism (T-RFLP). A decline in the microbial community of Barceloneta-Manatí sediments was observed in the samples exposed to antibiotics. Libraries of 16S rRNA were constructed to characterize multiple antibiotic resistant strains. In Barceloneta samples treated with multiple antibiotics, Proteobacteria dominates, which indicated resistance to multiple antibiotics. The resistance of Proteobacteria to multiple antibiotics and survival of multiple antibiotic-resistant *Enterococcus* in the coastal environment of

Barceloneta may indicate that transfer of antibiotic resistance is possible from terrestrial to marine microorganisms.

RESUMEN

Recientemente, el número de bacterias resistentes a antibióticos en el ambiente ha aumentado, creando un problema en la salud humana a nivel mundial. La resistencia de *Enterococcus* a antibióticos fue estudiada en áreas costeras de Mayagüez, Guayanilla, Guánica y Barceloneta. Los resultados indicaron que Barceloneta fue el área con mayor número de organismos resistentes a antibióticos. Estudios detallados de esta área fueron realizados. Muestras de agua y sedimentos del río, estuario y la zona costera fueron tomados del área de Barceloneta-Manatí. Las muestras fueron evaluadas respecto a la habilidad de las especies de *Enterococcus* a resistir los antibióticos de penicilina, tetraciclina y vancomicina. Los resultados mostraron que durante el período de sequía un alto porcentaje de *Enterococcus* fueron resistentes a la penicilina y tetraciclina. Los resultados podrían estar correlacionados con el uso de antibióticos por la industria lechera de esta área, usados en el ganado para propósitos de profilaxis y tratamiento. Aunque en el área costera se obtuvo el menor número de organismos resistentes a antibióticos múltiples, esta área fue la que presentó mayor diversidad de especies. La distribución de especies en la zona de Barceloneta indicó la predominancia de *E. faecalis, E. hirae* y *E. seriolicida*. *Enterococcus faecalis* fue la especie con mayor resistencia a antibióticos seguidos por *E. hirae, E. durans* y *E. faecium*. Los cambios de las poblaciones resistentes a antibióticos en los sedimentos costeros de Barceloneta fueron evaluados con la técnica de polimorfismo de longitud del fragmento terminal por restricción (T-RFLP). Disminución en las comunidades microbianas en los sedimentos de Barceloneta-Manatí fue observada en las muestras expuestas a antibióticos. Una genoteca del 16S rRNA fue construida para caracterizar cepas resistentes a antibióticos múltiples. En Barceloneta las muestras tratadas con antibióticos múltiples presentan dominancia de Proteobacteria, indicando resistencia a antibióticos múltiples. La resistencia de Proteobacteria a antibióticos múltiples y sobrevivencia de *Enterococcus*

a múltiples antibióticos en ambientes costeros de Barceloneta podrían indicar que la transferencia de resistencia a antibióticos puede ser posible de microorganismos terrestres a microorganismos marinos.

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Signed: Nydia J. Rodriguez

DEDICATED

TO MY BELOVED MOM (NYDIA RODRIGUEZ TORRES) WHO MADE ME A BETTER PERSON. YOU ARE ALWAYS IN MY HEART.

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ABBREVIATION LIST

DNA-Deoxyribonucleic acid

ES-*Enterococcus* spp.

GPS-Global Position System

MIC-Minimum Inhibitory Concentration

MAR-Multiple Antibiotic Resistance

OTU-Operational Taxonomic Unit

PCR-Polymerase Chain Reaction

Pen-Penicillin

rRNA-ribosomal ribonucleic acid

TRFLP-terminal restriction fragments length polymorphism

Tet-Tetracycline

Van-Vancomycin

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INTRODUCTION

The problem of antibiotic resistance in microorganism is of worldwide concern. The use of antibiotics in human, veterinary medicine, poultry, dairy, and aquaculture industries have exerted a strong selective pressure among environmental bacteria, leading to the emergence and dissemination of antibiotic resistance genes (Hinton *et al.*, 1986; Young, 1993; Nordmann and Poirel, 2005). Bacteria with high levels of resistance have been isolated from environments such as hospital, sewage, effluents, and wastewater which have been contaminated with antimicrobial agents (Olayemi and Opaleye, 2005; Toranzo *et al.,* 1984). However resistant bacteria also have been isolated from nonselective environments (Kobori *et al.,* 1984).

The principal mechanism of dissemination in the environment of resistance genes is the horizontal transfer of plasmid encoded genes, although there are other mechanisms like transformation and natural mutations (Spratt, 1994). These mechanisms have been shown to occur even between ecologically and evolutionarily disparate organisms (Toranzo *et al.,* 1984; Trevor *et al.,* 1987).

The selective pressure of antimicrobial agents in streams may be creating environments where high levels of antimicrobial resistance will be developed. Large volume effluents containing antibiotic resistance bacteria are discharged into the marine ecosystem. Besides, rain waters and flooding displace several antibiotic-resistant bacteria into marine waters. In these situations, the organism introduced into the marine environment could enter to a viable, but not culturable state, and could maintain its metabolic potential which could transfer the resistance plasmids (Chandrasekaran, 1998).

Potential transfer of resistant bacteria and resistance genes from aquaculture environments to humans may occur through direct consumption of antimicrobial-resistant bacteria present in fish and associated products (Petersen *et al.,* 2002). Similarly, dairy and poultry industries where the products can be vector to gene resistances (Aarestrup, 1999). Considering the above, it is hypothesized that antibiotic resistant enterococci will be higher near areas subjected to antibiotic contamination. Consequently, areas closer to intensive use of antibiotics such as upstream river waters in close proximity to dairy and pharmaceutical industries (input sources) may have higher

numbers of antibiotic resistant enterococci than area farther downstream, such as estuarine and coastal waters.

The main objectives of this research were to:

1. Compare the number of *Enterococcus* species in river near dairy industry, estuarine and coastal waters, and sediments during dry and rainy periods. To determine seasonal variation in *Enterococcus* species in the sampling areas and evaluate physical parameters such as pH, temperature, dissolved oxygen, and nutrients.
2. Determine the percentage of populations of *Enterococcus* in river, estuarine and coastal waters, and in sediments that are multiple antibiotic resistant (MAR) and compare their numbers from rivers near dairy and pharmaceutical industries (input sources) with those from estuarine and coastal environments.
3. Use culture independent techniques to ascertain MAR bacteria in coastal water sediments.

Literature Cited

Aarestrup F.M. 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. International Journal of Antimicrobial Agents. 12: 279-285.

Chandrasekaran S.,Venkatesh B., and D. Lalithakumary. 1998. Transfer and expression of multiple antibiotic resistance plasmids in marine bacteria. Current Microbiology. 37: 347-351

Hinton M., Kaukas A., and A.H. Lipton. 1986. The ecology of drug resistance in enteric bacteria. Journal of Applied Bacteriology. 61: 77-92.

Kobori H., Sullivan C.W., and H. Shizu. 1984. Bacterial plasmid in Antarctic natural microbial assemblages. Applied and Environmental Microbiology. 48: 515-518.

Nordmann P., and L. Poirel. 2005. Emergence of plasmid –mediated resistance to quinolones in Enterobacteriaceae. Journal of Antimicrobial Agents and Chemotherapy. 56: 463–469.

Olayemi A.B., and F.I. Opaleye. 2005. Antibiotic resistance among coliform bacteria isolated from hospital and urban wastewaters. World Journal of Microbiology and Biotechnology. 6(3): 285-288.

Spratt B.G.1994.Resistance to antibiotics mediated by target alteration. Sciences. 264: 388-393.

Petersen A., Andersen J.S., Kaewmak T., Somsiri T., and A. Dalsgaard. 2002. Impact of integrated fish farming on antimicrobial resistance in pond environment. Applied and Environmental Microbiology. 68(12): 6036-6042.

Toranzo A.E., Combarro P., Lemos M., and J.L. Barja. 1984. Plasmid coding for transferable drug resistance in bacteria isolated from culturable rainbow trout. Applied and Environmental Microbiology. 48: 872-877.

Trevors J.T., Barkay T., and A.W. Borquin. 1987. Gene transfer among bacteria in soil and aquatics environments: a review. Canadian Journal of Microbiology. 33: 191-198.

Young H.K. 1993. Antimicrobial resistance spread in aquatic environment. Journal of Antimicrobial Agents and Chemotherapy. 31: 627-635.

REVIEW OF LITERATURE

History of antibiotics

Since the 1929 discovery of penicillin by Alexander Fleming, the evolution of antibiotics has been well documented (Rosen and Barkin, 1992; Levy, 2005). Antibiotics are substances that can harm or destroy other microorganisms. These products can be produced naturally or synthetically by pharmaceutical industries by large-scale fermentation processes. The discovery of these products has been of great importance because in many clinical settings the antibiotics are the only way to kill bacteria that cause diseases in animals and humans.

During 1950-60, many classes of antibiotics were discovered, but it was not until the 1980's and 1990's that those scientists were able to make improvements within classes. The main classes of antibiotics are aminoglycoside, cephalosporines, fluoroquinolones, macrolides, penicillins, and tetracyclines. These can be classified by their chemical structure, their microbial origin, or mode of action. Most antibiotics have two names, the trade or brand name (capitalized), which are used by drug companies, and a generic name, based on the antibiotic's chemical structure or class (not capitalized).

Aminoglycosides: are used to treat infections by gram-negative bacteria, although some aminoglycosides, e.g. the streptomycin group is also effective against some gram-positive bacteria. Streptomycin is the first aminoglycoside used for the treatment of tuberculosis (Burman and Jaresko, 2001). These antibiotics work by binding to the bacterial 30S ribosomal subunit, leaving the bacterium unable to synthesize proteins vital to its growth (Van Bambeke, 1999). Some work by binding to the 50S subunit, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA. The aminoglycoside most commonly prescribed are gentamycin (Garamycin®), kanamycin, neomycin, streptomycin, and tobramycin (Tobrex®).

Cephalosporines: This type is grouped into generations of cephalosporines according to the antimicrobial properties (Pegler and Healy, 2007). Each newer generation of cephalosporine has greater antimicrobial properties than the preceding generation. The first generations of antibiotics are effective against gram-positive, and next generations are use against gram-negative although with reduced activity. These antibiotics are used to treat pneumonia, tonsillitis, *Staphylococcus*

infections, bronchitis, otitis media, and gonorrhea. This group of antibiotics acts by disrupting the synthesis of the peptidoglycan layer of bacterial cell walls. The most common are first generation (cephazoli, cefadroxil); second generation (cefuroxime, cefprozil); third generation (cefotaxime, ceftazidine), and fourth generation (cefepime, cefpirome).

Fluoroquinolones: These are broad spectrum antibiotics i.e. they are effective against many different bacteria (Norris and Mandell, 1988). These are used to treat common urinary, skin, and respiratory infections such as sinusitis and pneumonia. These antibiotics interfere with the DNA synthesis of the bacterium. The most common are ciprofloxacin (Cipro®), gemifloxacin, norfloxacin, trovafloxacin, levofloxacin (Levaquin®), and ofloxacin (Floxin®). Resistance to quinolones can evolve rapidly. Numerous pathogens, including *Staphylococcus aureus*, enterococci, and *Streptococcus pyogenes* now exhibit worldwide resistance (Jacobs, 2005).

Macrolides: The erythromycin [azithromycin (Zithromax®)] and clarithromycin (Biaxin®) antibiotics are known as macrolides. These are effective against gram-positive bacteria. These are used to treat respiratory tract infection, genital, gastrointestinal tract, and soft tissue infections (Schultz, 2004). These antibiotics act by inhibiting bacterial protein synthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl tRNA. This action is mainly bacteriostatic, but can also be bactericidal in high concentrations.

Penicillin: Sometimes penicillin is combined with beta-lactamase inhibitors, which protect from bacterial enzymes that may destroy it before it can do its work. Penicillin is used to treat skin, dental, ear, respiratory, and urinary tract infections. People allergic to penicillin can be safely treated by cephalosporin (Pichichero, 2006). This antibiotic acts by preventing the synthesis of bacterial cell walls. The most commonly prescribed antibiotics in this group are: amoxicillin, ampicillin, oxacillin, and penicillin.

Tetracycline: Discovered in the 1940's, exhibited activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, Chlamydia, mycoplasmas, rickettsiae, and protozoan parasites. From the beginning, this group of antibiotics was popular because of the broad-spectrum action. These are used to treat mild acne, Rocky Mountain spotted fever, Lyme disease, upper respiratory tract infections, urinary infections, sexually transmitted disease, and typhus. Tetracycline works by binding the 30S ribosomal subunit and through an

interaction with 16S rRNA, prevents the docking of amino-acylated tRNA (Chopra and Roberts, 2001). The most commonly prescribed tetracyclines are (Sumycin), doxycycline (Vibramycin®), and minocycline.

Antibiotic use and development of resistance

Animal Agriculture

Antibiotics in animal agriculture are administered for therapeutic purposes to treat infections and non-therapeutic purposes to enhance growth rates and improve feed efficiency (Greko, 1999; Wegener 2003). Antibiotics are also routinely used in feed and waters to prevent diseases. Glycopeptides like avoparcin and streptogramine have become important drugs used for growth promotion. Association between the use of antimicrobial growth promoters and occurrence of bacterial resistance in food animals has been well documented (Kruse and Sorum, 2004; Silbergeld *et al*., 2008). The association for the use of avoparcin and the glycopeptides resistant enterococci has been investigated, but it has also been shown for other antimicrobial growth promoters belonging to other classes, such as macrolides (tylosin, streptogramins) and bacitracin (Aerestrup, 2000)**.** The use of antibiotica as growth promoters was first banned in Denmark and then in the European Union. In Europe after the ban, there was decline in resistant bacteria in farm animals, in retail meat and poultry, and within the general human population (Smith *et al.,* 2002). In United States, the avoparcin was banned in 1997 and most other antibiotics used as growth promoters in 1999. However, in 2006, some antibiotics like Salinomycin Sodium and avilamycin were still in use. While the use of non-therapeutic levels of antibiotics in animal feed is approved and regulated by FDA, it has been shown that this practice has resulted in selection of antibiotic resistance in commensal and pathogenic bacteria in the animals themselves (Wegener 2003), in subsequent animal products (Hayes *et al.,* 2003; White *et al.,* 2001) and in water and soil samples collected around large-scale animal feeding operations (Jensen *et al.,* 2002, Gibbs *et al.,* 2006). Schwalbe *et al.* (1999) tested poultry feeds and isolated *E. faecium* that was resistant to vancomycin, gentamicin, streptomycin and ampicillin. In a study of cattle feed, Dargatz *et al.* (2005) found that 38.7% of 514 *E. coli* isolates were resistant to cephalosporin, 24.7% resistant to ampicillin and 16.6 % resistant to cefoxitin. In the same study the author detected multiple antibiotic resistant *E. coli* and *Salmonella spp.*

It has been suggested that transmission of antibiotic resistance bacteria from agriculture can have greater impact in the human population than that of hospital transmission (Smith *et al.,* 2005). A comparison of pattern of colonization of vancomycin resistant *Enterococcus* (VRE) in Europe and the United States, which had different patterns of agricultural and hospital antibiotic use suggest that agricultural antibiotic use can have important quantitative effects on the spread of resistance.

Wegener in 2003 suggested that the resistance can spread from animals to humans. In this study, the data shows that although levels of bacterial antibiotic resistance in animals and foods has been markedly reduced after the termination of use, the effects on animal health and productivity have been considerable during short time periods. The swine industry alone uses an estimated 10.3 million pounds of antibiotics annually for non-therapeutic purposes. Among the antibiotics used are ampicillin, bacitracin, erythromycin, lincomycin, virginiamycin, and tetracycline, some of which are important in human clinical medicine. The selection of resistant bacteria in food animals by antimicrobial growth promotion and subsequent spread between animals in the farm environment are important factors in the propagation of resistant bacteria in the animal reservoir (Wegener, 2003). The rate of spread of resistant bacteria from animals to the environment, and more importantly, in the food production, are keys to the spread to humans.

Aquaculture

In Europe and United States, the use of some antibiotics in aquaculture has been banned, but in countries of South America like Chile and some areas of Asia, there is no restriction in use of antibiotics. There are five drugs legally used in aquaculture in the USA. These include three antibiotics: oxytetracycline HCL (Terramycin 10), sulfamerazine, and a combination drug containing sulfadimethozine and ormetoprim (Romet-30) (Benbrook, 2002). In some countries antibiotics are available without doctor's prescription. This combined with overly prescribed antibiotics in the USA and elsewhere ensures that large amounts of antibiotics remain in the aquatic environment. This could increase transfer of these determinants to bacteria of land animals and to human pathogens. Also the introduction of these compounds may alter bacterial flora in sediments and the water column and in the organisms as well. Antibiotic resistance was monitored in sediments under fish farm cages and in sediments 200 meters from the fish farm used as control (Chelosssi *et al.,* 2003). In this study the antibiotic sensitivity tests showed a high percentage of resistant strains in both fish farm sediment and in the control, which indicated a widespread

antibiotic resistance within bacterial populations in areas surrounding fish farms. A high frequency of antibiotic resistance was observed for ampicillin in impacted sediments. Gram-negative bacteria displayed the highest resistance to ampicillin and streptomycin, and the shift in the structure of microbial assemblage was apparently related to the presence of gram-negative resistant strains in fish-farm sediments (Chelossi *et al.,* 2003).

The use of large amounts of antibiotics that have to be mixed with fish food also increases the opportunities for the presence of residual antibiotics in fish meat and fish products (Cabello, 2006). The use of antimicrobial agents in ornamental fish, particularly in some exporting countries, is significant, and evidence exists that multiple antibiotic-resistant bacteria may be frequent in such fishes. Although ornamental fish are not eaten, they can be in close contact with humans (Alderman and Hasting 1998).

Veterinary

It is estimated that more than one million tons of antibiotic have been released into the biosphere during the last 50 years from animal agriculture and veterinary applications. The veterinary uses of antibiotics include the use in pets, farm animals, and animals in aquatic environments. The main infections treated are enteric, pulmonary, skin, organ abscessed, and mastitis. Quinolones are the most frequently used antibiotic in veterinary practice (Teube, 2001). Widespread veterinary usage of quinolones, particularly in Europe, has been implicated to the development of resistance in bacteria to this type of antibiotic. Plasmid mediated quinolones resistance were first identified in a *Klebsiella pneumonia* clinical isolates from the United States (Martinez *et al.,* 1998). Bacteria that develop antibiotic resistance in animals are also found in opportunistic pathogens, food-borne pathogens and commensal bacteria. Antibiotics resistant in zoonotic bacteria constitute a public health hazard especially to zoonotic bacteria like *Salmonella*, *Campylobacter* and entero-hemorragic *E. coli* (Wegener *et al.,* 1999). For this reason the veterinary industry recommended wise use of antibiotics. Research by the British Society for Antimicrobial Chemotherapy pointed out how the use of antimicrobial agents in veterinary medicine and animal husbandry cause antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy (Piddock, 1996).

Human health effects

During the last decade the number of bacteria resistant to antibiotic has increased, thereby resulting in one of the world's most critical public health problem. The biomedical association has established that decreasing inappropriate antibiotic use is the best way to control resistance. Studies published by the American Medical Association has established that spread of antibiotic resistance in *Streptococcus pneumonia* in United States communities is due in part to the excessive use of antibiotics for acute respiratory tract infections. The excessive use of antibiotics in ambulatory practices has contributed to the emergence and spread of antibiotic-resistant bacteria in the communities. Retrospective and prospective studies have documented the strong association between prior antibiotic use and the risk of carriage and invasive infection with resistant *S. pneumonia* (Dowell and Schwartz, 1997). Special attention to antibiotic resistance in *S. pneumonia* is necessary since this pathogen is the leading cause of community-acquired bacterial pneumonia, meningitis, and otitis media in the United States (Gonzalez*,* 1999).

Research in day care centers in Iceland and Finland indicated a decrease in the antibiotic resistance bacteria after decrease in use of the antibiotics within the community. For example in Iceland penicillin resistant *S. pneumonia* isolates carried by children in day care centers decreased by 25% over a 3 year period. In Finland a 40% reduction in community macrolides use was associated with a 48% decrease in the erythromycin resistance among group A streptococcal isolates over a 4-year period (Stephenson, 1996; Seppala *et al.,* 1997).

There is strong evidence that resistant strains of three specific organisms that cause illness in humans, *Salmonella*, *Campylobacter,* and *E. coli* are linked to the use of antibiotics in animals (Phillips *et al.,* 2004). The real danger is the transfer of resistance genes from animal to human intestinal bacteria and eventually to serious human pathogens (Marvick, 1999). Transient colonization by vancomycin-resistant enterococci of animal origin has been documented and the study showed that transfer of the *vanA* gene from an *E. faecium* isolate of animal origin to an *E. faecium* isolate of human origin can occur in the intestines of humans. In the same research the transfers of quinupristin-dalfopristin resistance was also observed. Intestinal colonization by enterococci carrying mobile elements with resistance genes represents a risk for spread of resistance genes to other enterococci that are part of the human indigenous flora, which can be responsible for infections in immune-compromised patients (Lester *et al.,* 2006).

Antibiotics in the environment

Antibiotics are currently widely used, not just for the treatment of human infections, but also in agriculture and animal/fish farming with the possibility that high amounts of such compounds may find their way into natural habitats (Smith, 2005). In the environment, there are natural antibiotic products synthesized by bacteria actinomycetes and some types of algae (Klein and Alexander, 1986: Lemos *et a*l., 1985). The antibiotic substances secreted by various organisms are phenazines, bacteriocins, glycolipids, and bromopyrrolic compounds (Barja *et al.,* 1989; Lemos *et al.*, 1991). Beside autochthonous sources, the antibiotics are also introduced by anthropogenic sources. For example, the antibiotics used in fish farms to control diseases may enter the environment (Herwig *et al*., 1997). Also the antibiotics used in animal farms as growth promoters or as therapeutics may sometimes be released into water and sediments by means of water runoff. Thus, selection of resistant organisms in nature may result from natural production of antibiotics by soil organisms, runoff from animal feed or crops, or waste products from treated animals or humans (Davies, 1994; Witte, 1998).

Development of resistance bacterial strains in the environment

Resistance to antibiotics poses a serious and growing problem, because some infectious diseases are becoming more difficult to treat. Resistant bacteria do not respond to antibiotics and continue to cause infection. Some of these resistant bacteria can be treated with more powerful medicines, but there are some infections difficult to cure even with new or experimental drugs (Seveno *et al.,* 2002)

Many studies indicate the occurrence and distribution of antibiotic-resistant bacteria in the environment (Baya *et al.,* 1986, Herwig *et al.*, 1999) including fresh water basin (Young, 1993) estuarine ecosystems (Mudryk, 2002) and marine environments (Mudryk and Skorczewski, 1998 and Mudryk 2005). Culturable bacteria resistant to high levels of ß-lactams ampicillin, cefotaxime, and ceftazidime were widespread in non-concentrated water samples from many U.S. rivers (Ash *et al.,* 2002). Strains of *Clostridium perfringens*, a gram-positive spore-forming anaerobe that is widely distributed in soil, sewage, and food can be a reservoir for macrolide resistance genes and the *tet*(M) gene (Soge *et al.*, 2008). Some findings indicated that the *qnr* genes (the genes that encodes resistance) identified in members of Enterobacteriaceae originated from environmental

gram-negative bacterial species, such as *Shewanella algae* and *Vibrio splendidus*. Contamination by industrial pollution may also select for antibiotic resistance in nature. For example, heavy metal pollution can select for antibiotic resistance, this has the potential to increase recombination and horizontal gene transfer in a way that favors widely spreading of antibiotic resistance genes in the environment (McArthur and Tuckfield, 2000). One effect of antibiotic contamination is the quinolones resistance gene (*qnr)*, which is present in the chromosomes of waterborne bacteria. This gene contributes to low-level resistance of its new bacterial host to quinolones (Martinez *et al.,* 1998). Research has shown that contamination of river waters by quinolones enriches for plasmid-encoded (*qnr*) and this may be first step in the transfer of this gene to human pathogens (Cattoir *et al.,* 2008*).*

Mechanism of transfer resistance genes

Acquisition of genetic material among microorganisms has been known for many years. It is also known that element-like phages and transposons are able to transfer antibiotic resistance to many bacterial strains. Antibiotic resistance may be acquired by mutation and selection, with passage of the trait vertically to daughter cells. More commonly, resistance is acquired by horizontal transfer of resistance determinants by transduction, transformation, or conjugation (Zahid *et al.,* 2009). The intraspecies and interspecies exchanges of genetic information play an important role in the evolution of the bacteria (Cruz and Davies, 2000; Jain *et al.,* 1999). Resistance that is acquired by horizontal transfer can become rapidly and widely disseminated either by clonal propagation of the resistant strain or by further genetic transfer from the resistant strain to other susceptible strains. The horizontal transfer of the integrons is considered as the most efficient means for the emergence and dissemination of resistance genes and multiple antibiotic resistant strains (Chandler and Claverys, 2001). Zahid *et al.,* 2009 demonstrated that the tetracycline resistant gene in combination with sulphamethoxazole-trimethoprim (SXT), streptomycin and erythromycin can be spread horizontally by the conjugation process between bacteria in surface water of Bangladesh. They concluded that mobility of antibiotic resistance determinants may further contribute to the development and spread of antibiotic resistance among pathogenic bacteria, particularly since antibiotics are indiscriminately used in Bangladesh. There are four classic mechanisms of resistance in aquatic environments that are specified by plasmids: inactivation, impermeability, bypasses, and altered target site (Davies and Smith, 1978). Also, intracellular binding seems to be a valid

mechanism for immobilizing an inhibitor (Foster, 1983). Resistance can also be associated with the production of enzymes that modify and inactivate antibiotics (Koch, 1981) According to Hermansson *et al.* (1987), some strains of bacteria resistant to antibiotics do not contain any plasmid, and in this case bacterial resistance to antibiotics depends on the other mobile genetic elements.

Enterococci and antibiotics resistance

Because of their tolerance to high salt concentrations, enterococci species have been isolated from different aquatic environments and used as indicators of the occurrence and transfer of antimicrobial resistance. *Enteroccocu*s spp. are member of the normal flora of the gastrointestinal tract in human and animals and have emerged as the leading cause of nosocomial infection. This genus is mainly related to the streptococci of fecal origin but the unique characteristics give rise to a separate genus. The two major pathogenic species in humans are *E. faecium* and *E. faecalis* but species like *durans, gallinarum, casseliflavus, hirae,* and *mundtii* are occasionally pathogens (Devriese *et al.,* 1993). At recreational beaches, the abundance of enterococci in bathing waters is correlated with the incidence of swimming-related gastroenteritis (Cabelli *et al.,* 1982). To protect human health during water recreation, enterococci have been used as an indicator of fecal contamination in the United States coastal waters. There are two parts of the swimming standard: the water should have an average (geometric mean) of *Enterococcus* counts less than 35 colonies per 100 ml and there should be no more than 104 *Enterococcus* colonies/100 ml in a single sample. Enterococci are known to acquire and spread antibiotic resistances to other species with relative ease. Resistant *Enterococcus* has been commonly isolated from humans, sewage, aquatic habitats, agricultural runoff and animal sources (Peterson *et al.*, 2002; Olayemi and Opaleye, 2005). The mechanisms of resistance to antimicrobial agents in enterococci are alterations of the targets or enzymatic production of methylating and modifying enzymes (Facklan *et al.,* 2002).

Literature Cited

Aarestrup F.M., Agerso Y., Gerner-Smidt P., Madsen M., and L.B. Jensen. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagnostic Microbiology Infection Disease. 37: 127-137.

Alderman D. J., and T.S Hastings. 1998. Antibiotic use in aquaculture: development of antibiotic resistance potential for consumer health risks. International Journal of Food Science and Technology. 33(2): 139 –155.

Ash R.J., Mauck B., and M. Morgan. 2002. Antibiotic resistance of gram-negative bacteria in rivers, United States. Emerging Infectious Disease. 8(7): 713-716.

Barja J.L., Lemos M.L., and A. Toranzo. 1989. Purification and characterization of an antibacterial substance produced by a marine Alteromonas species. Journal of Antimicrobial Agents of Chemotherapy. 33:1674-1679.

Baya A.M., Barayton P.R., Brown V.L., Grimes D.J., Russek-C E., and R.R. Colwell. 1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Applied and Environmental Microbiology. 51(6): 1285-1292.

Bogaard A.E., and E.E. Stobbering. 2000. Epidemiology of resistance to antibiotics links between animals and humans. International Journal of Antimicrobial Agents.14: 327-335.

Burman M., and W.J. Jaresko. 2001. "Population pharmacokinetics of intravenous and intramuscular streptomycin in patients with tuberculosis". Pharmacotherapy. 21 (9): 1037–1045.

Cabelli V.J., A. P. Dufour., McCabe L.J., and M. A. Levin. 1982. Swimming associated gastroenteritis and water quality. American Journal of Epidemiology. 115: 606-616.

Cabello F. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Mini Review of Environmental Microbiology. 8(7): 1137-1144.

Cattoir V., Poirel L., Aubert C., Soussy C-J., and P. Nordman. 2008. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. Emerging Infectious Disease. 14 (2):1345-1349.

Chandler M., and J.P. Claverys. 2001. Genome diversity: sources and forces. Current Opinion of Microbiology. 4: 547-549.

Chelossi E., Vezzulli L., Milano A., Branzoni M., Fabiano M, Riccardi G., and I.M Banat. 2003. Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. Aquaculture. 219(1): 83-9.

Chopra I., and M. Roberts. 2001. "Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance" Microbiology and Molecular Biology Reviews 65(2): 232-260.

Cruz D.L.F., and J. Davies. 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. Trends on Microbiology. 8: 128-133.

Dargatz D.A., Strohmeyer R.A., Morley P.S., Hyatt D.R,. and M.D. Salman. 2005. Characterization of *Escherichia coli* and *Salmonella* enterica from cattle feed ingredients. Foodborne Pathogenic Diseases. 2:341–347.

Davies J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science. 264:375–82.

Davies J., and D.J. Smith. 1978. Plasmid determined resistance to antibacterial agents. Annual Review of Microbiology. 32:469.

Devriese L. A., Pot B., and M.D. Collins. 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. Journal of Applied Bacteriology. 75: 399-408.

Dowell S.F., and B. Schwartz. 1997. Resistant pneumococci: protecting patients through judicious use of antibiotics. American Family Physician. 55: 1647-1654.

Facklam R. R, Carvalho M.S., and L.M. Texeira. 2002. The enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. Edited by Michael S. Gilmore. History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci. 1-54

Foster T.J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiology Reviews. 47(3): 361-409.

Gibbs S.G., Green C.F., Tarwater P.M., Mota L.C., Mena K.D., and P.V. Scarpino. 2006. Isolation of antibiotic-resistant bacteria from the air plume downwind of a swine confined or concentrated animal feeding operation. Environmental Health Perspective. 114: 1032–1037.

González R.A. 1999. Is *Streptococcus pneumoniae* the leading cause of pneumonia of unknown etiology? A microbiologic study of lung aspirates in consecutive patients with community-acquired pneumonia. The American Journal of Medicine. 106(4): 385-390

Greko C. 1999. Antibiotics as growth promoters. Acta Veterinaria Scandinavica. Suppl. 92: 87-100

Hayes J.R., English L.L., Carter P.J., Proescholdt T., Lee K.Y., and D.D. Wagner. 2003. Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. Applied and Environmental Microbiology. 69: 7153–7160.

Hermansson M., Jones G.W., and S. Kjelleberg. 1987. Frequency of antibiotic and heavy metal resistance, pigmentation and plasmids in bacteria of the marine air-water interface. Applied and Environmental Microbiology. 53: 2338-2342.

Herwig R.P., Gray J.P., and D.P. Weston. 1997. Antibacterial resistant bacteria in superficial sediments near salmon net-cage farms in Puget Sound, Washington. Aquaculture. 149: 263-283.

Helms M., Simonsen J., and K. Molbak. 2005. Adverse health effects associated with antimicrobial drug resistance in *Campylobacter* species. The Journal of Infectious Disease. 191: 1050-1055.

Jain R., Rivera M.C., and J.A. Lake. 1999. Horizontal gene transfer among genomes: the complexity hypothesis. Proceeding of the National Academy of Sciences. 96: 3801-3806.

Jensen L.B, Agerso Y., and G. Sengelov. 2002. Presence of erm genes among macrolide-resistant gram-positive bacteria isolated from Danish farm soil. Environmental International. 28 (6): 487–491.

Klein T.M., and M. Alexander. 1986. Bacterial inhibitors in lake water. Applied and Environmental Microbiology. 52(1): 114-118.

Koch A.L. 1981. Evolution of antibiotic resistance gene function. Microbiological Review. 45: 355-378.

Kruse H., and H. Sørum. 2004. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Applied and Environmental Microbiology. 60(11): 4015-4021.

Lemos M. L., Toranzo A.E., and J.L Barja. 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microbiological Ecology. 11:149-163.

Lemos M.L., Dopazo C.P., Toranzo A.E., and J.L. Barja. 1991. Competitive dominance of antibiotic-producing marine bacteria in mixed cultures. Journal Applied of Bacteriology. 71: 228-232.

Lester C.H., Møller N.F., Sørense T.L. Monnet D.L., and A.M. Hammerum. 2006. In vivo transfer of the vanA resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. Antimicrobial Agents and Chemotherapy. 50(2): 596–599.

Levy S. B. 2005. Antibiotics resistances-the problem intensifies. Advanced Drug Delivery Reviews. 57(10): 1446-1450.

Martínez M.L., Pascual A., and G. Jacoby. 1998. Quinolones resistance from a transferable plasmid. Lancet. 351(9105): 797-799.

Marvick, C. 1999. Animal feed antibiotic use raises drug resistance fear. The Journal of American Medical Association. 282: 120-122.

McArthur, J.V., and R.C. Tuckfield. 2000. Spatial patterns in antibiotic resistance stream bacteria: Effects of industrial pollution. Applied and Environmental Microbiology. 66: 3722-3726.

Mudryk Z., and P. Skorczewski. 1998. Antibiotic resistance in marine neustonic and planktonic bacteria isolated from the Gdansk Deep. Oceanology. 40 (2): 123-134.

Mudryk Z.J. 2002. Antibiotic resistance among bacteria inhabiting surface and subsurface water layers in estuarine lake Gardno. Polish Journal of Environmental Studies. 11 (4): 401-406.

Mudryk Z.J. 2005. Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. Marine Pollution Bulletin. 50(1): 80-86.

Norris S., and G.L. Mandell. 1988. The quinolones: history and overview, in Andriole VT (ed): The Quinolones, San Diego, Academic Press Inc: 1- 22.

Olayemi A.B., and F.I. Opaleye. 2005. Antibiotic resistance among coliform bacteria isolated from hospital and urban wastewaters. Journal of Microbiology and Biotechnology. 6 (3): 285-288.

Pegler S., and B. Healy. 2007. In patients allergic to penicillin, consider second and third generation cephalosporins for life threatening infections. Biomedical Journal. 335 (7627): 991.

Peterson A., Andersen J.S., Kaewmak T., and T. Dalsgaard. 2002. Impact of integrated fish farming on antimicrobial resistance in a pond environment. Applied and Environmental Microbiology. 68: 6036-6042.

Phillips I.,Casewell M., Cox T., De Groot B., Friis C., Jones R., Nightingale C., Preston R and J. Waddell. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy. 53: 28-52.

Pichichero M.E. 2006. "Cephalosporins can be prescribed safely for penicillin-allergic patients". The Journal of Family Practice. 55(2): 106–112.

Piddock L.J. 1996. Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy? Journal of Antimicrobial Chemotherapy. 38(1): 1-3.

Rosen P., and R.M. Barkin, eds. Emergency Medicine: Concepts and Clinical Practice. Mosby-Year Book; 1992.

Schwalbe R.S., McIntosh A.C,, Qaiyumi S., Johnson J.A., and J.G Morris Jr. 1999. Isolation of vancomycin-resistant enterococci from animal feed in USA. Lancet. 353: 722.

Schultz M.J. 2004. "Macrolide activities beyond their antimicrobial effects: macrolides in diffuse panbronchiolitis and cystic fibrosis". Journal of Antimicrobial Chemotherapy. 54(1): 21–28.

Seppala H., Klaukka T., and J. Vuopiio-Varkila. 1997. The effect of changes in the consumption of macrolides antibiotics on erythromycin resistance in group A streptococci in Finland. New England Journal of Medicine. 337: 441- 446.

Séveno N.A., Kallifidas D., Smalla K., Van Elsas J.D., Collard, J-M., Karagouni A.D., and E.M. Wellington. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Review in Medical Microbiology. 13(1):15-27.

Silbergeld E.K., Graham J., and L.B. Price. 2008. Industrial Food, Animal Production, Antimicrobial Resistance, and Human Health. Annual Review of Public Health. 151-169

Smith D.L., Harris A.D., Johnson J.A., Silbergeld E.K., and J.G Morris Jr. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Proceedings of the National Academy of Sciences. 99: 6434–6439.

Smith D.L., Dushoff J., and J.G Morris Jr. 2005. Agricultural antibiotics and human health. PLoS Medicine. 2(8): 0731-0735.

Stephenson J. 1996. Icelandic researchers are showing the way to bring down rates of antibiotic-resistant bacteria. Journal of American Medical Association. 275:175.

Soge O.O., Tivoli L.D., Meschke J.S., and M.C. Roberts. 2008. A conjugative macrolide resistance gene, mef(A), in environmental *Clostridium perfringens* carrying multiple macrolide and tetracycline resistance genes. Journal of Applied Microbiology. 106: 34–40.

Teube M. 2001. Veterinary use and antibiotic resistance. Current Opinion in Microbiology. 4:493-499.

Wegener H.C., Aarestrup F.M.,Gerner-Smidt P. and F. Bager. 1999. Transfer of antibiotic-resistant bacteria from animals to man. Acta Veterinaria Scandinavica. 92:51-57.

Van Bambeke F. 1999. Mechanisms of Action. In:Armstrong D, Cohen Journal of Infectious diseases. 7: 1.1-1.14

Wegener H.C. 2003. Antibiotics in animal feed and their role in resistance development. Current Opinion in Microbiology. 6(5): 439–445.

White D. Zhao S., Sudler R., Ayers S, Friedman S., and S. Chen. 2001. The isolation of antibiotic-resistant salmonella from retail ground meats. New England Journal of Medicine. 345: 1147–1154.

Witte W. 1998. Medical consequences of antibiotic use in agriculture. Sciences. 279:996

Young H.K. 1993. Antimicrobial resistance spread in aquatic environments.Journal of Antimicrobial Chemotherapy.31:627–635

Zahid H.M., Mahal Z., and M.R Chowdhury. 2009. Intergenic and intragenic conjugal transfer of multiple antibiotic resistance determinants among bacteria in the aquatic environment of Bangladesh. African Journal of Biotechnology. 8(2): 155-160.

Chapter 1 Patterns of antibiotic resistance of *Enterococcus* spp. in different coastal environments of Puerto Rico

Introduction

Enterococci have been isolated from different aquatic environments and are used as an indicator of the occurrence and transfer of antimicrobial resistance (Peterson *et al.*, 2002). These microorganisms have the ability to tolerate high salt concentrations, thereby increasing their survival in marine environment. Enterococci are also known to acquire antibiotic resistances with relative ease and are able to spread these resistant genes to other species (Rice *et al.,* 1995). Antibiotic resistant enterococci also survive longer in estuarine water than their antibiotic-sensitive counterpart, thus increasing the period of time to spread resistance genes in the estuarine environment (Pettibone *et al.,* 1987). A number of plasmid-carrying strains with multiple resistances have been isolated from marine air-water interfaces (Fergusson *et al*., 2005; Mudryk, 2005). Antibiotic resistances in bacteria mediated by plasmids were found in marine coastal environments of Puerto Rico (Baya *et al.*, 1996). Enterococci are not considered as primary pathogens, but due to their ability to acquire high levels of resistance to antimicrobial agents, they have emerged as nosocomial pathogens worldwide (Kuhn *et al.*, 2003). Several studies have shown that water and sands from beaches with high fecal contamination of human origin may be the potential sources of contamination by pathogens and contribute to the dissemination of bacterial resistance to antibiotics (Cardoso de Oliveira and Watanabe, 2008).

The necessity to establish the incidence of antibiotic resistant *Enterococcus* species (ES*)* in coastal waters in Puerto Rico is important to assess the possible contamination of natural waters with untreated fecal material. Waters contaminated with fecal material may increase the risk of transmission of disease to humans, as well as dissemination of bacterial resistance to antibiotics. The objective of this study was to compare the presence of antibiotic resistant *ES* in the coastal environment of Puerto Rico. To accomplish this objective samples were taken from Barceloneta, Guánica, Guayanilla, and Mayagüez coastal waters.

Material and Methods

Description of Study Sites

Four coastal sites in different areas of Puerto Rico were selected (Figure 1.1). A detailed description of each site given in Table 1.1. Barceloneta has one of the highest concentrations of pharmaceutical industries in the world and is also the site of several dairy industries. Guayanilla was the site of a petrochemical complex in Puerto Rico during the 1980's. Mayagüez is an urban zone and also had tuna factories. Guánica coastal area is relatively free of any industry and is used as a control site. Site coordinates were taken at each sampling point with a global positioning system receiver (Model GPSMAP 175, Garmin International Inc., Olathe, Kansas).

Sampling Strategy

The samples were taken during the morning hours to minimize the effects of the wind, waves, temperature and were stored on ice and transported to the laboratory. A 3.78 L bottle was used to collect each water sample. The sample bottles were washed in soapy water and rinsed thoroughly in tap water and then with distilled water. The bottles were also rinsed twice with water from the sampling site prior to each sampling. Each bottle was submerged in water column near the surface where the water sample was taken.

Water quality analysis

Sea water quality parameters of temperature, salinity, pH, dissolved oxygen, and inorganic nutrients were obtained from each sampling site. Water temperature measurements were obtained by using a field thermometer; salinity with a Sper Scientific refractometer with automatic temperature control (Model 300011). The pH was determined using a pH meter with automatic temperature control (Oaklon Instruments). Dissolved oxygen (DO) was measured with a LaMotte system. The ammonia, nitrate and phosphate nutrients were measured using CHEMets Kit (CHEMetrics, Inc). The Ammonia CHEMets® test employs direct nesslerization. The Nitrate CHEMets® test employs the cadmium reduction method and the Phosphate CHEMets® test employs the stannous chloride chemistry.

Isolation of *Enterococcus* species (ES)

One liter of each sea water sample (in duplicate) was filtered through a 0.45 μm pore size filter. The filters were transferred to Enterococcosel broth (EB) (BBL ™). EB is a specific media containing pancreatic digested casein, peptic digest of animal tissue, yeast extract, oxgall, sodium chloride, sodium citrate, esculin, ferric ammonium citrate, and sodium azide. Filters were incubated for 48 hours at 25 °C. The bacteria were isolated and purified by spread plate technique using *Enterococcus* agar (EA) (BBL ™). From the plates, 90 *Enterococcus* colonies were picked at random and transferred to micro-wells containing 0.2 ml of EB and incubated for another 48 hours.

Resistance to Antibiotics

After 48 hours of growth in EB medium, colonies from the bacterial samples were selected and transferred to the 96 well microplates containing EB and different concentrations of antibiotics. The antibiotics used were streptomycin sulfate, chlortetracycline, and oxytetracycline each with concentrations of 10, 20, 40 μg/ml and with salinomycin (at concentrations of 1, 5, and 15 μg/ml). The values of Minimum Inhibitory Concentration (MIC) for selected strains using various antibiotics were determined by Kirby-Bauer method using the National Committee for Clinical Laboratory Standards (Bauer *et al.*, 1966). MIC values used were obtained from the experimental trials and from review of the literature.

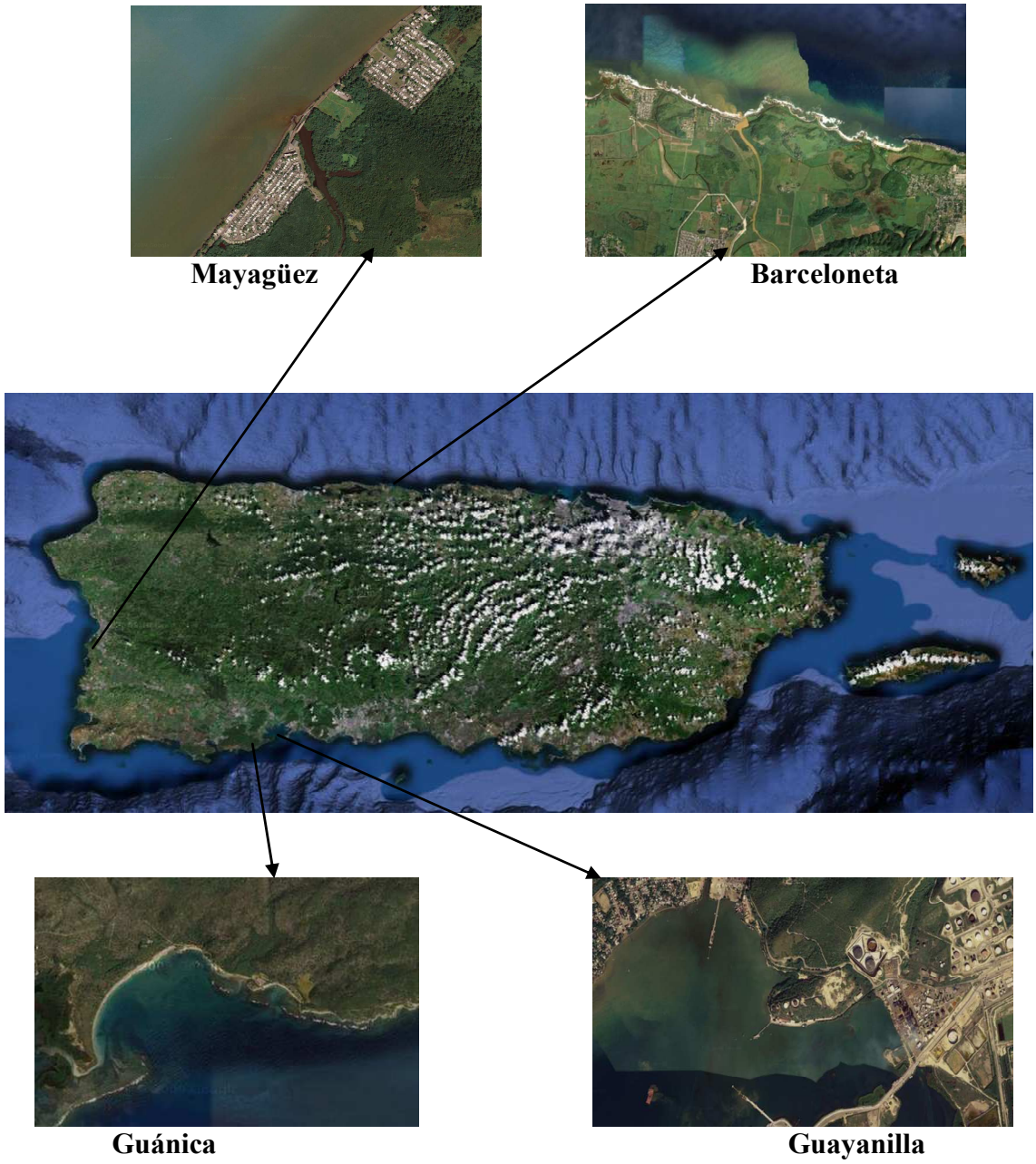


Figure 1.1. Location of the sampling sites from four coastal areas in Puerto Rico.

GPS location in Table 1.1

Table 1.1 Descriptions of Sampling Sites

	Guayanilla	Guánica	Mayagüez	Barceloneta
GPS Location	South Coast of P.R. N 18° 00' 21.0" W 66° 46' 05.9"	Southwestern coast of PR N 17° 57’ 20.0” W 66° 51’ 20.9”	Western Coast of P.R. N 18° 10' 35.9" W 66° 51’ 24.5”	North Coast of P.R. N 18° 28' 51.6" W 66° 32’ 14.0”
Description of Area	Was the site of one of the biggest petrochemical complexes until 1982.	Small town. These sites include natural resources, including the Bosque Seco. The sampling site is near a public beach and resort	The port is the third busiest port on. The sampling area is surrounded by a community. Site of tuna factories.	The area has high concentration of pharmaceutical and dairy industries.
Current Activities	Commercial	Public swimming	Commercial and Urban	Commercial, Urban, Pharmaceutical
Images				

Results

Physical Parameters and nutrient contents of the sampling sites

The values of salinity, temperature, pH, dissolved oxygen, and nutrients are given in Table 1.2. Each value is a mean of three tests. The salinity values ranged from 33-35 PSU. The salinity was highest (35 PSU) in Mayagüez and lowest in Barceloneta (33 PSU). The water temperature ranged from high of 26^{o}C in Mayagüez to low of 24^{o}C in Barceloneta. However, the dissolved oxygen (DO) was lowest in Mayagüez (5.8 mg/L) and highest in Guánica (7 mg/L). The values of nutrients are given in parts per million (ppm). The values of nitrates ranged from 0.2 ppm in Mayagüez and Barceloneta to 0.1 ppm in Guayanilla and for Guánica the values were below measurable limits. The phosphate and ammonia values were 0.15 ppm and 0.10 ppm respectively in Mayagüez and Guayanilla. In Guánica and Barceloneta they were below detectable limits.

Table 1.2 Physical parameters and nutrient concentrations from different coastal environments of Puerto Rico

Physical Parameters	Mayagüez	Guayanilla	Guánica	Barceloneta
Salinity (PSU)	35	34	34	33
Temperature (°C)	26	25	25	24
pH	7.8	8.0	8.1	7.9
Disolved oxygen(mg/L)	5.8	6.2	7	6.4
Nitrate (ppm)**	0.2	0.1	0	0.2
Phosphate (ppm)**	0.15	0.15	0	0
Ammonia (ppm)**	0.10	0.10	0	0

The results are based on the average of three tests; PSU-practical salinity unit, °C-degree Celsius, (mg/L)-milligram per liter, (ppm)-parts per million=1 mg/L.

**Values of zero are indicating that are below detection limit.

Isolation of *Enterococcus* spp. (ES) after enrichment in Enterococcosel broth from coastal environments of Puerto Rico

When the number of ES were isolated and compared from different coastal environments of Puerto Rico, the highest number of *Enterococcus* were found in the Barceloneta coastal environment (1.68 x10^8 CFU/L) followed by Mayagüez (1.04 x 10^7 CFU/L). Lowest numbers of *Enterococcus* (1.24 x 10^6 CFU/L) were isolated from the Guánica coastal environment (Table 1.3).

Table 1.3 Number of *Enterococcus* spp. isolated after enrichments from different coastal environment of Puerto Rico

Sampling Site	Number of *Enterococcus* CFU/L*	Standard Deviation
Mayagüez	**$1.04x10^7$**	**0.03**
Guayanilla	**$9.10x10^6$**	**0.05**
Guánica	**$1.24x10^6$**	**0.02**
Barceloneta	**$1.68x10^8$**	**0.02**

***Average from two dilutions**

To compare the presence of antibiotic resistance of *Enterococcus* bacteria in coastal waters of Barceloneta, Guánica, Guayanilla, and Mayagüez, samples were grown in selective media and antibiotics were added at low to high concentrations as described earlier in methodology in resistance to anibiotics. Oxytetracycline was used at 10, 20, and 40 µg/mL. At low concentration of 10 µg/mL 72% of *Enterococcus* species (ES) were resistance to oxytetracycline in Barceloneta, 11% in Guayanilla, less than 10% in Guánica and Mayagüez. When the concentration of oxytetracycline was increased to 20 µg/mL, over 60% ES were resistant in Barceloneta samples, followed by over 20% in Guayanilla and less than 5% in Guánica and Mayagüez. The percentage of *Enterococcus* resistant to 40 µg/mL was 50% in Barceloneta, less than 20% in Guayanilla, less than 4% in Mayagüez,and none in Guánica (Figure 1.2). In contrast, all sampling sites had ES resistant to both low (10 and 20 µg/mL) and high concentrations (40 µg/mL) of chlorotetracycline. Barceloneta again was the site with the highest percentage of resistant *Enterococcus* bacteria (Figure 1.3). ES resistance to low concentration (10 µg/mL) of streptomycin was found in all sampling sites, but highest percentage of resistant *Enterococcus* (>78%) was found in Barceloneta (Figure 1.4). When *Enterococcus* resistance to high concentration of streptomycin (50 µg/ml) was compared, Barceloneta again had highest percentage (4%) of resistant ES, compared to none in other sampling sites. Resistance to salinomycin at l, 5 and 15 µg/mL was also studied (Figure 1.5). When 1 µg/mL of salinomycin was used, the percentage of resistant *Enterococcus* was 32% in Barceloneta, 12% in Mayagüez, 10% in Guánica and none in Guayanilla. At high concentration of salinomycin (15 µg/mL), percentage of resistant *Enterococcus* was 20% in Barceloneta, 6% in Guánica, 4% in Mayagüez, and none in Guayanilla.

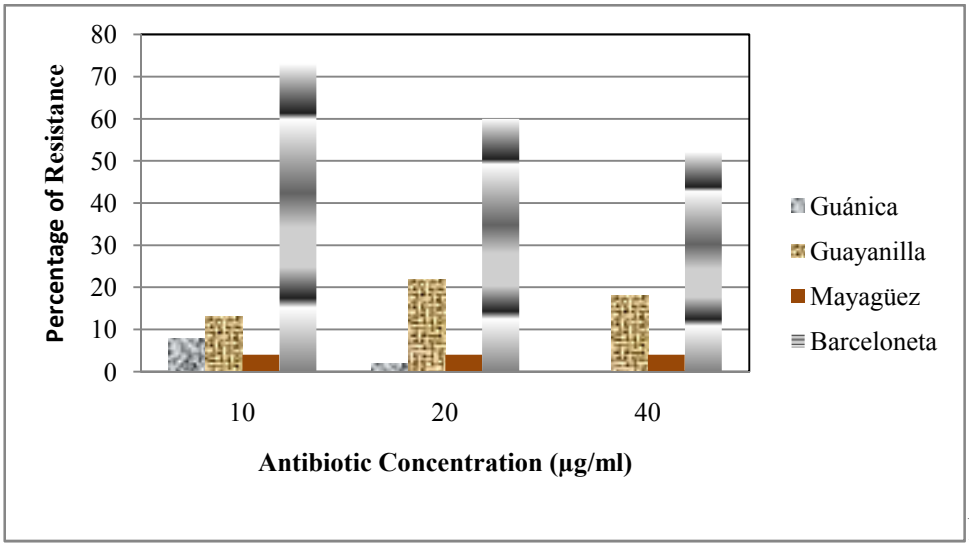


Figure 1.2. Percentages of ES resistant to 10, 20, and 40 µg/mL of oxytetracycline antibiotic in coastal waters of Puerto Rico

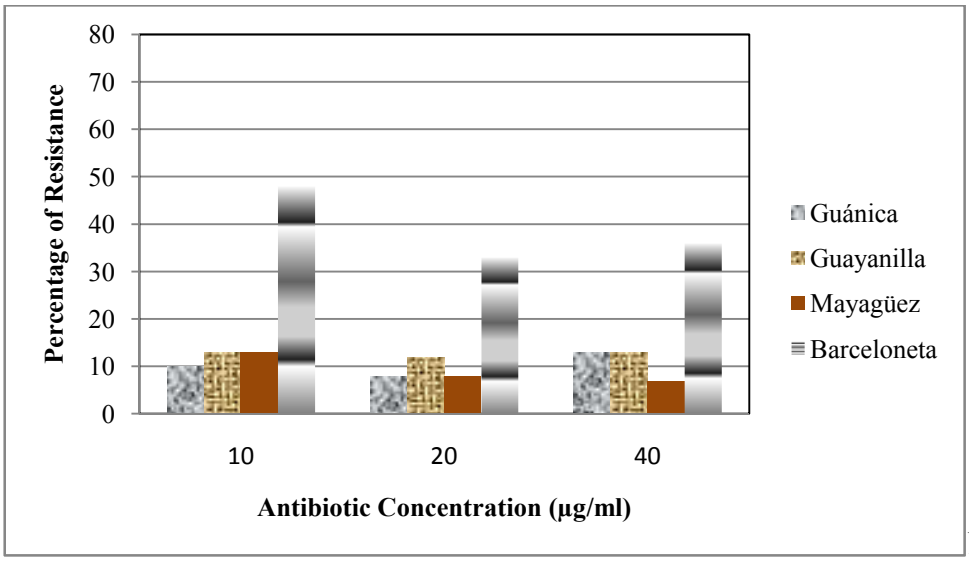
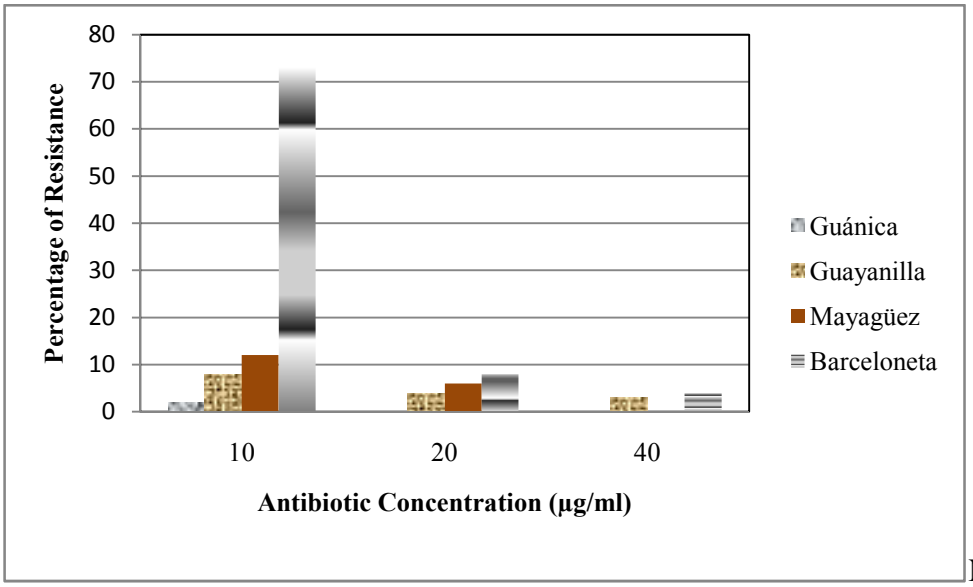
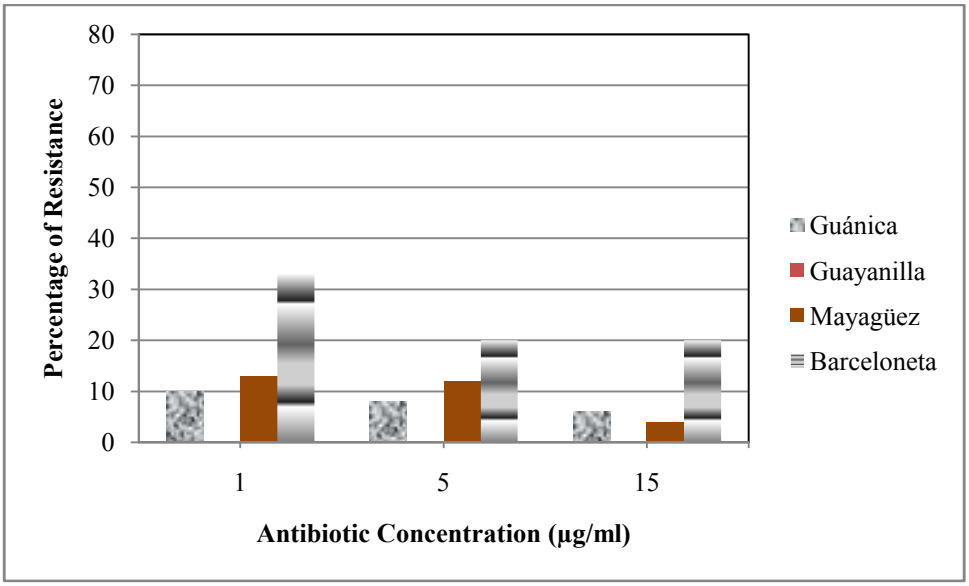


Figure 1.3. Percentages of ES resistant to 10, 20, and 40 µg/mL of chlorotetracycline antibiotic in coastal waters of Puerto Rico



N=90

Figure 1.4. Percentages of ES resistant to 10, 20, and 40 µg/mL of streptomycin antibiotic in coastal waters of Puerto Rico



N=90

Figure 1.5. Percentages of ES resistant to 1, 5, and 15 µg/mL of salinomycin antibiotic in coastal waters of Puerto Rico

Discussion

Most large industrial cities in Puerto Rico are located near the coast. Therefore, the antibiotic resistance of ES in coastal waters of industrial cities of Barceloneta, Guayanilla, and Mayagüez were compared with Guánica coastal water, an area relatively free of industrial pollution. Lowest antibiotic resistant ES as expected were found in the Guánica sampling site selected as control. Some resistant determinant can persist in a population even without selective pressure (Langlois *et al.,* 1988). This might explain the presence of antibiotic resistant ES in Guánica coastal water. Comparing antibiotic resistance to chlortetracycline 40 µg/ml, oxytetracycline 40 µg/ml, and salinomycin 15 ug/mL, the highest percentage of antibiotic resistance ES were found in Barceloneta (Figures 1.2 to 1.5). Comparison of total number of ES after enrichment process in coastal environments of Barceloneta, Guánica, Guayanilla, and Mayagüez indicated the highest number again in Barceloneta followed by Mayagüez. Presence of high numbers of ES in Mayagüez may be explained by input of large quantities of organic waste from tuna factories that were located there during the sampling period. Even though ES numbers in Mayagüez were relatively high, lower percentages of ES were antibiotic resistant. This could be due to lack of antibiotic input sources, e.g. pharmaceutical and dairy industries in Mayagüez coastal waters. The only sources of antibiotic resistance are hospitals and runoff from agricultural land, as well as higher human populations living in the coastal area compared to the Guánica and Guayanilla. Higher number of antibiotic-resistant bacteria in Barceloneta may also be due to heavy concentration of pharmaceutical and dairy industries. After finding higher numbers of antibiotic resistant ES in Barceloneta coastal waters, it was decided to investigate in detail the coastal environment of Barceloneta. To compare antibiotic resistances, samples were taken from the river close to the dairy industry as well as estuary and coastal waters of Barceloneta. The results are discussed in detail in the following chapters.

Literature Cited

Bauer A.W., Kirby W.M., Sherris J.C., and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 45(4): 493–496.

Baya A.M., Brayton P.R., Brown V.L. Grimes D.J., Russek- Cohen E., and R.R. Colwell.1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Applied and Environmental Microbiology. 51: 1285-1292.

Cardoso A. J., and J.M. Watanabe. 2008. Antimicrobial resistance and species composition of ES isolated from waters and sands of marine recreational beach in Southeaster Brazil. Water Research. 42(8): 2242-2250.

Kühn I., Iversen, A., Burman L., Liljequist B.O., Franklic A., Finn M., Aarestrup F., Seyfarth A.M., Blanch A.,Vilano X.,Taylor H., Caplin J.Moreno M., Dominguez L., Herrero I.A.,and R. Möllby. 2003. Comparison of enterococcal populations in animals, humans, and the environment an European study. International Journal of Food Microbiology. 88: 133-145.

Ferguson D.F., Moore Getrich, M.A., and M.H. Zhowandai. 2005. Enumeration and speciation of *Enterococcus* found in marine and intertidal sediments and coastal waters in southern California Journal of Applied Microbiology. 99: 598-608.

Langlois B.E., K.A. Dawson, I. Leak, and D.K. Aaron. 1988. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. Veterinary Microbiology. 18: 147-153.

Peterson A., Andersen J.S., Kaewmak T, Somsiri T., and Dalsgaard. 2002. Impact of integrated fish farming on antimicrobial resistance in a pond environment. Applied and Environmental Microbiology. 68: 6036-6042.

Pettibone G.W., Sullivan S.A., and M.P Shiaris. 1987. Comparative survival of antibiotic-resistant and -sensitive fecal indicator bacteria in estuarine water. Applied and Environmental Microbiology. 53(6): 1241-1245.

Mudryk Z.J. 2005. Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. Marine Pollution Bulletin. 50(1): 80-86.

Rice E.W., J.W. Messer, C.H. Johnson, and D.J. Reasoner. 1995. Occurrence of high-level aminoglycoside resistance in environmental isolates of enterococci. Applied and Environmental Microbiology. 61:374-376.

Chapter 2 Characterization of *Enterococcus* spp. in water and sediments of the Barceloneta-Manatí, Puerto Rico area

Introduction

The enterococci are a complex, diverse, and important group in terms of their interaction with humans. Initially categorized within the *Streptococcus* group, enterococci are now separated as a new genus. Enterococci are gram-positive cocci that may occur singly, in pairs or as short chains. They are facultative anaerobes, capable of growing at temperatures ranging from 10 to 45°C, with an optimum of 35°C. They hydrolyze esculine in the presence of 40% bile salts and can grow in media containing 6.5% NaCl (Facklam *et al*., 2002). They are tolerant to extremes in temperatures, salinity, and pH and are among the most thermotolerant of nonsporulating bacteria (Franz *et al.*, 1999). There are various tests that can be used to identify most enterococci from other catalase negative, and gram-positive cocci (Facklam *et al*., 1999), and biochemical and molecular tests that help to differentiate among species (Carvalho *et al.,* 1998, Manero and Blanch, 1999).

The distribution of *Enterococcus* species in different animal hosts is widely reported. For example, *E. faecalis*, *E. faecium*, *E. hirae,* and *E. durans* are commonly associated with farm animals *(*Devriese *et al.*, 1987). In chickens, the succession of enterococci colonization is age dependent. Dominant species present in chickens are *E. casseliflavus*, *E. gallinarum,* and *E. mundtii*. *Enterococcus avium* was originally described from human feces, but later it was found also in chicken feces (Nowlan and Deibel, 1967). In cattle, the enterocccocal flora consist of *E. faecalis* together with *faecium* and *avium*, but this flora is gradually replaced by *E. cecorum*. In the feces of adult dairy cows, any enterococci species may dominate (Devriese *et al.*, 1996; Devriese *et al.*, 1999). In domestic animals, *E. faecalis* was the most frequent, but other species are *E. avium, E. raffinosus, E .durans, and E. cecorum* (Devriese *et al.*, 1992). The presence of E. *gallinarum* and *mundtii* has been reported in horse and *E. asini* in donkeys (Devriese *et al.,* 1987; De Vaux *et al*., 1998). *Enterococcus seriolicida* is also reported in cattle and in fish as a worldwide fish pathogen (Zlotkin *et al.,* 1998).

Other *Enterococcus* occurs in the colon of nearly all humans, where their numbers can be as high as 10^8 colony-forming units per gram of feces (Huycke *et al*., 1998). *Enterococcus* can cause nosocomial infection and endocarditic, urinary tract infection, and neonatal sepsis (Guzman *et al.,* 1989, Huycke *et al.,* 1998)

The distribution of the *Enterococcus* spp. has been used to determine contamination in beach areas. This was the case in the south Florida recreational beach where *Enterococcus* had been used as an indicator of fecal pollution (Bonilla *et al*., 2007). Enumeration and speciation of enterococci in southern California beaches that were frequently in violation of water quality standards was reported by Fergusson *et al*. in 2005. They found high level of *Enterococcus* in intertidal sediments compared with marine sediments and the predominant species found in both water and sediments were *Enterococcus faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundti*.

In the present study, isolation of *Enterococcus* from water and sediment samples was achieved by using selective media. The samples from river, estuarine, and coastal area were taken during the dry and rainy seasons. Specific aims of this study were: (1) characterize the culturable fraction of *Enterococcus* spp. using microbiological and biochemical techniques; (2) determine if there is an association between the sampling sites and the species recovered; (3) establish if seasonal variation influences species composition of *Enterococcus* in the sampling sites, and (4) characterize *Enterococcus* by DNA sequence analysis.

Materials and Methods

Study Sites

The study area is located on the north coast of Puerto Rico (Figure 2.1). The Manatí River is one of the major rivers in Puerto Rico with a total length of 73 km (Figure 2.2). It originates in the Cordillera Central just north of Barranquitas, and enters the Atlantic Ocean near Barceloneta. The Manatí River estuary (Figure 2.3) is located in the La Boca sector in Barceloneta and the coastal area (Figure 2.4) is west of the estuarine area. Figures 2.2-2.4 indicates the geographical coordinates of sampling sites.

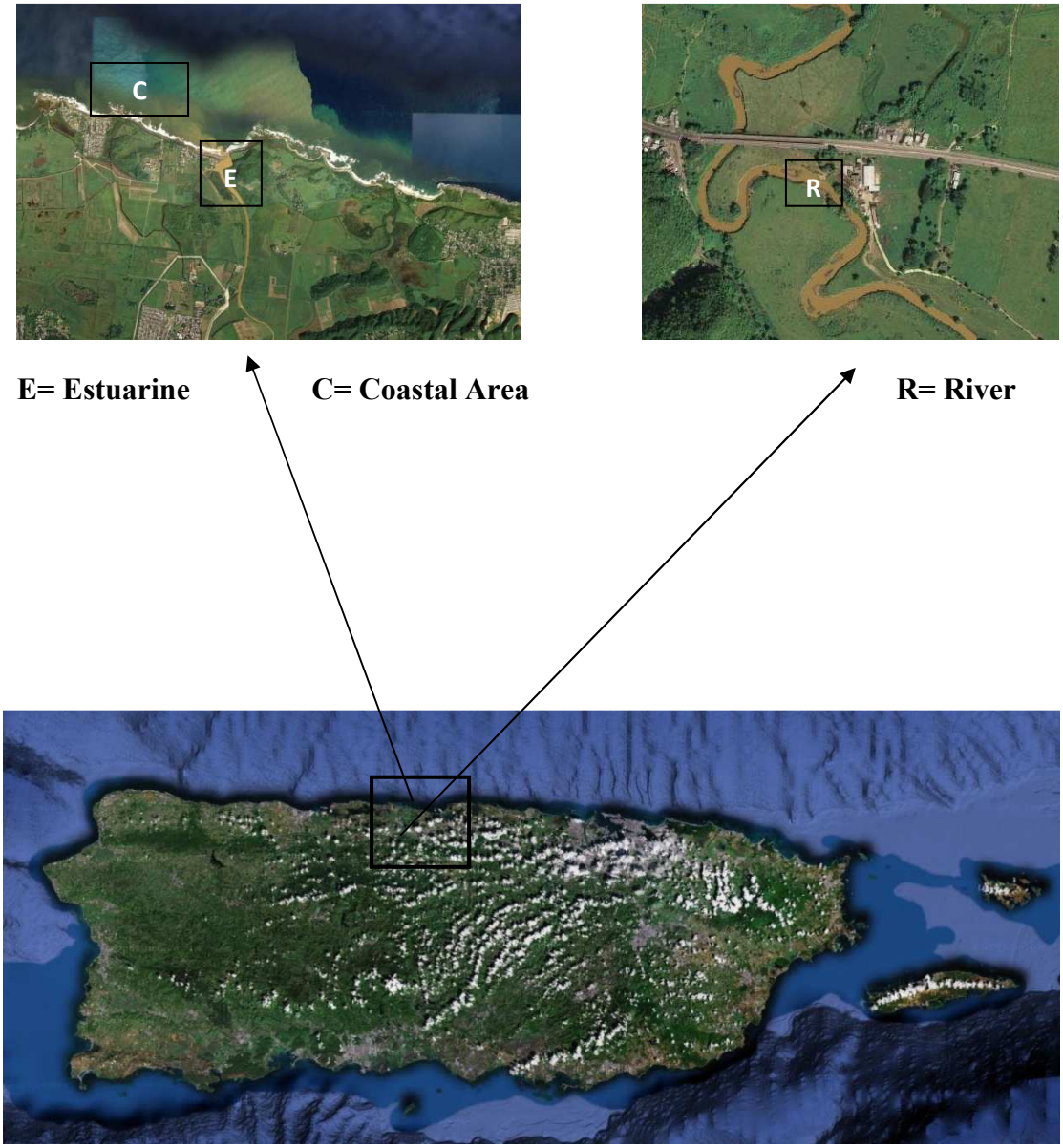


Figure 2.1. Location of sampling sites in Barceloneta, Puerto Rico

Figure 2.2. Location of sampling site in Manatí River

(N 18°25'41.8'', W066°31'30.9'')

Figure 2.3. Location of sampling site in estuarine area

(N18°28'46.5'', W066°32'07.4")

Figure 2.4. Location of sampling site in Barceloneta coastal area

(N18°28'51.6", W066°32'14.0")

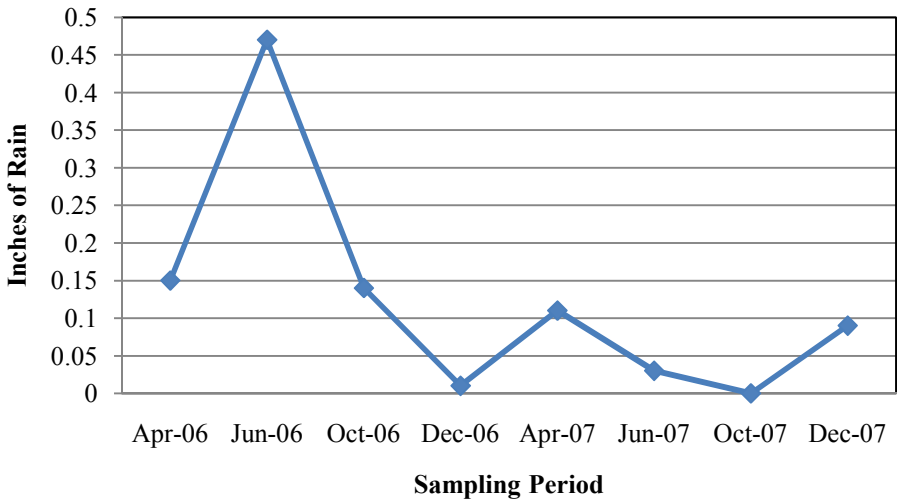


Figure 2.5. Precipitation in the Barceloneta-Manatí area during the sampling period

(Atmos Carib, Research Center at the University of Puerto Rico at Mayagüez)

Sample Collection

The samples were taken during 2006-07 in the months of April, June, October, and December. Precipitation data is shown in the Figure2.5. Plastic bottles of 3.78 L were used to collect water samples. The sample bottles were washed in soapy water and rinsed thoroughly in tap and then in distilled water. The bottles were also rinsed twice with water from each sampling site prior to sampling. Each bottle was submerged in the water column near the surface where the water sample was taken. The samples were taken in duplicates. Sediment samples were taken from river, estuarine, and the coastal area with a grab sampler (Ponar grab sampler). The soil samples near dairy industry were also collected. The samples were transported on ice and processed within six hours after collection.

Water quality analysis

Water quality parameters for river, estuarine, and coastal areas were obtained in duplicate. The instruments and procedures are described in the methodology of water quality analysis in Chapter 1.

Isolation and Identification of *Enterococcus* bacteria

One liter of water sample was filtered through a 0.22 µm GVfilter (Millipore, Durapore®). The filters were transferred to 100 ml of Enterococcosel broth (EB) (BBL ^{TM}) and incubated for 48 hours at 25°C (enrichment medium). The samples later were plated using spread plate technique in Enterococcosel agar (EA) (BBL ^{TM}). From dilutions containing 100-150 colonies, 95 colonies were picked at random and transferred to micro-wells plates containing 0.2 ml of EB and incubated for another 48 hours before running confirmation tests for *Enterococcu*s spp.

Confirmation of *Enterococcus* species

Brain Heart Infusion Agar (BHIA) (Difco) with 6.5% NaCl (Manero and Blanch, 1999) was inoculated with 10 µL of sample from every well showing growth in EB. The plates were incubated for 24-48 hours at 25ºC. The latter temperature was a modification attempting to emulate environmental conditions. After regrowth on BHIA + NaCl catalase test was performed as the final confirmation test for enterococci (Cai, 1999; Holt *et al.*, 2000)

Species composition

The species composition of the enterocccocal isolates from the river, estuarine, and coastal area were tested by their capability to use arginine and ferment mannitol, methyl–α–D glucopyranoside, sorbose, ribose, arabinose, and sucrose (Manero and Blanch 1999; Carvalho *et al*., 1998; Facklam *et al*., 2002). The controls used were *Enterococcus faecalis* (ATCC 29212), E. *faecium* (ATCC35667), E. *durans* (ATCC 6056), E. *avium* (ATCC14025), and E. *gallinarum* (ATCC 49608).

The parameters for richness, diversity and equitability were evaluated to understand the community structure. The total enterocccocal species present at each sampling site was defined as species richness (r). The Simpson's Diversity Index (1 – D) was applied for biodiversity assessment of *Enterococcus* populations and was calculated for each station and time as $D=\Sigma ((n (n-1))/N (N-1))$, where N was the total number of organisms of a particular species and "n" was the total number of enterococci from the particular station for each time (Anderson *et al*., 2006; Cox and Gilmore, 2007). The index represents the probability that two individuals randomly selected from a sample belonged to different species. The value of this index ranges from 0 to 1; the greater the value, the greater the sample diversity. The evenness (equitability) Index was calculated as ED=D/r and was

used to observe if there was a dominant species in the sample. This Index expresses the degree of equal distribution of species based on population density, thus the higher the index, the more uniform the distribution of species. The Paleontological statistics package version. 1.79 (PAST) was used to obtain these parameters (Hammer *et al.,* 2001).

Molecular Analysis

Isolation of genomic DNA

Eighteen colonies from Barceloneta coastal area were isolated and grown in Trypticase Soy Broth (TSB) (Difco) and incubated at 25°C for 24 hrs. The DNA was extracted from cells using lysis buffer (40mM Tris-acetate pH 7.8-8.0, 20 mM sodium-acetate pH 8.0, 1.0 mM EDTA pH 8.0, and 1% SDS) with lysozyme treatment followed by chloroform extraction and ethanol precipitation. The isolated genomic DNA was resuspended in 50 µL of nuclease free water and treated with RNAse (at a final concentration of 20 µg/µL) for 30 minutes at 37ºC (Saano *et al.,*1995). The DNA quality was checked on 0.8% agarose gels after staining with ethidium bromide. All genomic DNA were used as templates for subsequent PCR amplification.

Polymerase chain reaction (PCR), gel electrophoresis and DNA sequencing

The gene encoding the 16S rRNA was amplified by PCR using the combination of forward primer Univ-519-F (5'-CAGCMGCCGCGGTAATWC-3') and the reverse primer Univ-1392-R (5'-ACGGGCGGTGTGTRC-3') (Rodriguez et al., 2006). The reaction mixture consisted of ddH_2O, buffer 1X, 2.5 mM $MgCl_2$, 250 mM dNTP's, primer forward 1 pmol, primer reverse 1pmol, DNA (10 ng), and *Taq* polymerase 0.026 U/µl. PCR reaction consisted of 30 cycles with a denaturation period of 1 min at 94°C, 1 min at 50°C and polymerization for 3 min at 72 °C (Hezayen *et al.,* 2002). PCR amplicons were purified using the MinElute PCR purification kit (USA QIAGEN Inc.), and the product concentration was determined using a 1% agarose gel with markers of *Hind* III. Selected PCR products were sent to Nevada Genomic in the United States. The samples were prepared according to the facility instructions (http://www.ag.unr.edu/genomics/).

Phylogenetic analysis

The sequence of 16S rRNA gene of type organisms for comparison was determined using the seqmatch from Ribosomal Data Project II (http://rdp.cme.msu.edu/). The Note Tab Light was used as an editor program for the sequences. The alignment of the sequences and the construction of a phylogenetic tree were conducted using Mega 4.1 software (Tamara *et al.,* 2007). P distance values were calculated for all pairwise combinations in the multiple alignments and then the distances were assembled into a tree using neighbor-joining method.

Results

Water quality parameters

Physical parameters for the sampling sites are presented in Table 2.1. Each value represents an average of two tests. The salinity ranged from 0 to 35 PSU. The average salinity in the river was zero during all sampling periods. In estuarine conditions, the salinity was zero only during the June 2006 sample and 5 PSU in April 2007. The highest average salinity of 35 PSU was found in the coastal area. The temperature ranged from 25 to 30°C. The average temperature was higher during June and December 2006 with 30°C in estuarine and coastal area stations. Although summer temperatures in all sampling were slightly higher than in winter months, the differences between stations were not statistically significant.

The pH ranged from 7.3 to 8.6 values. These values are within acceptable ranges for good quality standards for the waters bodies monitored. The values of dissolved oxygen ranged between 7.2 mg/L to 10.2 mg/L. The lower average DO value was found in the estuarine area during October 2006 with a value of 6.2 mg/L. In general the DO values are higher in river samples compared to estuarine and coastal area.

The values for nitrate, phosphate, and ammonia are listed in Table 2.1. The average value for phosphate in river samples was 0.2 ppm, and for nitrate 0.5 ppm. The highest values for nitrate were 0.8 ppm in the estuarine station during June and October 2007. The phosphate and ammonia values for coastal water were not detectable in all periods; ammonia was present in river and estuarine samples with a maximum of 0.3 ppm in river samples and 0.1 ppm in the estuarine samples.

Table 2.1 Physical Parameters of water samples from Barceloneta-Manatí

Physical Parameters 2006-2007	River 2006	River 2007	Estuarine 2006	Estuarine 2007	Coastal Area 2006	Coastal Area 2007
Salinity (PSU)						
April	**0**	**0**	**27**	**5**	**33**	**30**
June	**0**	**0**	**0**	**33**	**25**	**35**
October	**0**	**0**	**25**	**33**	**30**	**35**
December	**0**	**0**	**32**	**25**	**35**	**33**
Temperature (°C)						
April	**28**	**25**	**27**	**26**	**27**	**26**
June	**29**	**28**	**30**	**29**	**30**	**29**
October	**29**	**29**	**29**	**30**	**29**	**30**
December	**29**	**28**	**30**	**29**	**30**	**29**
pH						
April	**7.4**	**8.6**	**7.9**	**8.4**	**7.9**	**8.0**
June	**7.9**	**7.4**	**8.1**	**7.8**	**8.0**	**8.4**
October	**8.1**	**7.3**	**7.9**	**7.8**	**8.0**	**8.4**
December	**7.9**	**7.9**	**8.0**	**7.9**	**8.2**	**8.0**
Disolved Oxygen(mg/L)						
April	**9.2**	**9.4**	**8.6**	**8.2**	**6.4**	**8.6**
June	**10.2**	**10**	**9.5**	**8.6**	**9.8**	**8.0**
October	**8.2**	**7.4**	**6.2**	**6.8**	**7.5**	**8.4**
December	**9.5**	**10.2**	**8.8**	**8.6**	**9.2**	**8.4**
Nitrate (ppm)						
April	**0**	**0**	**0.3**	**0.4**	**0.1**	**0.1**
June	**N.D**	**1.0**	**N.D**	**0.8**	**N.D**	**0.1**
October	**0.2**	**1.0**	**0.2**	**0.8**	**0.1**	**0**
December	**1**	**1.0**	**0.4**	**0.2**	**0.1**	**0**
Phosphate (ppm)						
April	**0.15**	**0 .15**	**0**	**0**	**0**	**0**
June	**0.15**	**0.2**	**0.2**	**0.1**	**0**	**0**
October	**0.4**	**0.1**	**0.1**	**0.1**	**0**	**0**
December	**0.1**	**0.2**	**0**	**0.2**	**0**	**0**
Ammonia (ppm)						
April	**0.1**	**0**	**0**	**0**	**0**	**0**
June	**0.1**	**0.2**	**0.1**	**0**	**0**	**0**
October	**0**	**0.3**	**0**	**0**	**0**	**0**
December	**0.2**	**0**	**0**	**0.1**	**0**	**0**

The results are based on the average of two tests. **N.D. = Not determined**

PSU-practical salinity unit, °C-degree Celsius, (mg/L)-milligram per liter, (ppm)-parts per million=1 mg/L

Number of *Enterococcus* spp. after 24 hours in enrichment medium in 2006

Water: When the numbers of *Enterococcus* spp. present in water samples are compared, the highest values after 24 hours in enrichment medium were obtained from river samples collected during April 2006 (1.3×10^{11} CFU/L) and in December 2006 (1.0×10^{11} CFU/L) and the lowest from coastal water samples (Figure 2.6). In all sampling periods the highest number of *Enterococcus* species were always found in river samples, followed by samples from the estuary and then in coastal water.

Sediments: The pattern of *Enterococcus* spp. in sediments during 2006 was similar to water samples where highest number of *Enterococcus* species were always found in the river, followed by the estuary, and then in coastal water sediments (Figure 2.7). The highest number of *Enterococcus* spp. in sediments was obtained during December 2006 (6.0×10^{12} CFU/g for river and 2.9×10^{12} CFU/g in estuarine sediments). However, in the October sample, the number of *Enterococcus* spp. was only slightly lower in river sediments (5.5×10^{10} CFU/g) compared to all other samples during this year.

Number of *Enterococcus* spp. after 24 hours in enrichment medium in 2007

Water: The number of *Enterococcus* spp. after enrichment procedure in water during April 2007 was 8.5×10^{10} CFU/L in the river, 6.9×10^{9} CFU/L in the estuary and 1.3×10^{7} CFU/L in coastal water. During June, sampling the numbers were 6.5×10^{9} CFU/L in the river, 6.3×10^{7} CFU/L in the estuary and 1.2×10^{5} CFU/L in coastal water. During October, sampling the number of *Enterococcus* spp. was 1.6×10^{9} CFU/L in river, 8.8×10^{9} CFU/L in estuary and 1.3×10^{7} in coastal water. In December, the numbers were 1.2×10^{8} CFU/L in river, 7.8×10^{8} CFU/L in estuary and 3.4×10^{7} CFU/L in coastal water (Figure 2.8). Thus in the 2007 sampling periods the highest number of *Enterococcus* species were always found in river, followed by samples from estuary and then in coastal water (Figure 2.8).

Sediments: The number of *Enterococcus* spp. after enrichment procedure in April 2007 were 4.6×10^{12} CFU/g in the river, 3.9×10^{12} CFU/g in the estuary, and 6.5×10^{8} CFU/g in coastal sediments. In June, the numbers were 4.65×10^{11} CFU/g in the river, 4.95×10^{11} CFU/g in the estuary and 4.9×10^{8} CFU/g in coastal sediments. In December, the number of *Enterococcus* spp. was 4.9×10^{11} CFU/g in river, 5.5×10^{11} CFU/g in the estuarine samples and 7.5×10^{10} CFU/g of coastal sediments. However, the numbers were higher in October 2007 in estuary (5.5×10^{13}

CFU/g) and coastal environments ($1.3x10^{13}$ CFU/g) compared with the others sampling during 2006 and 2007 (Figure 2.9).

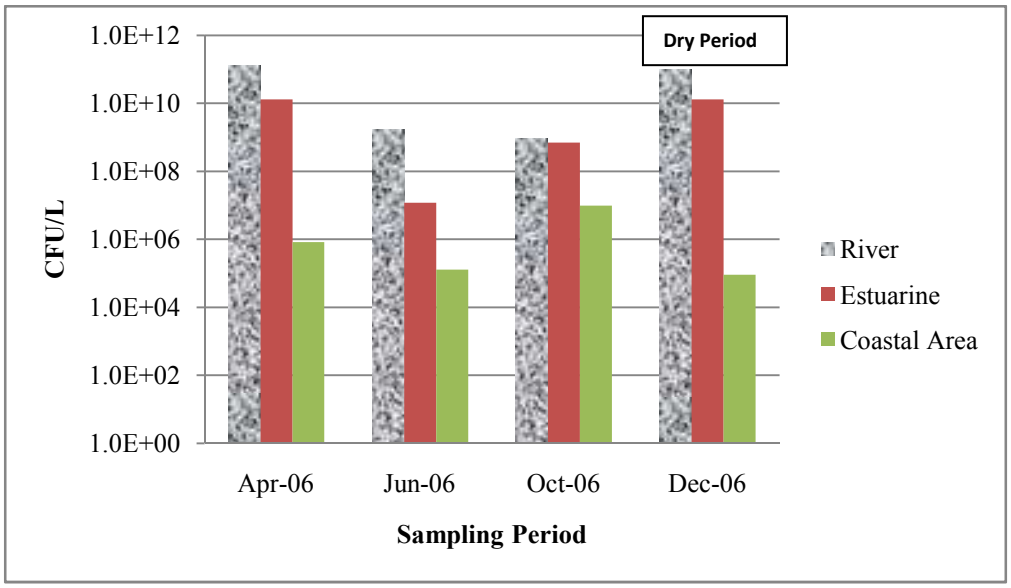


Figure 2.6. Number of *Enterococcus* spp. from Barceloneta water samples after enrichment during 2006

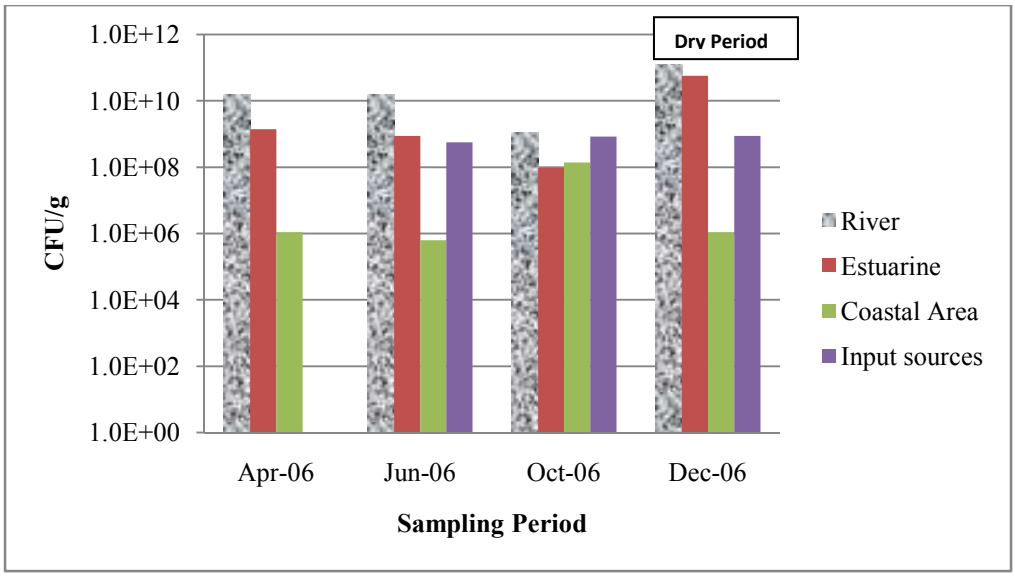


Figure 2.7. Number of *Enterococcus* spp. from Barceloneta sediment samples after enrichment during 2006

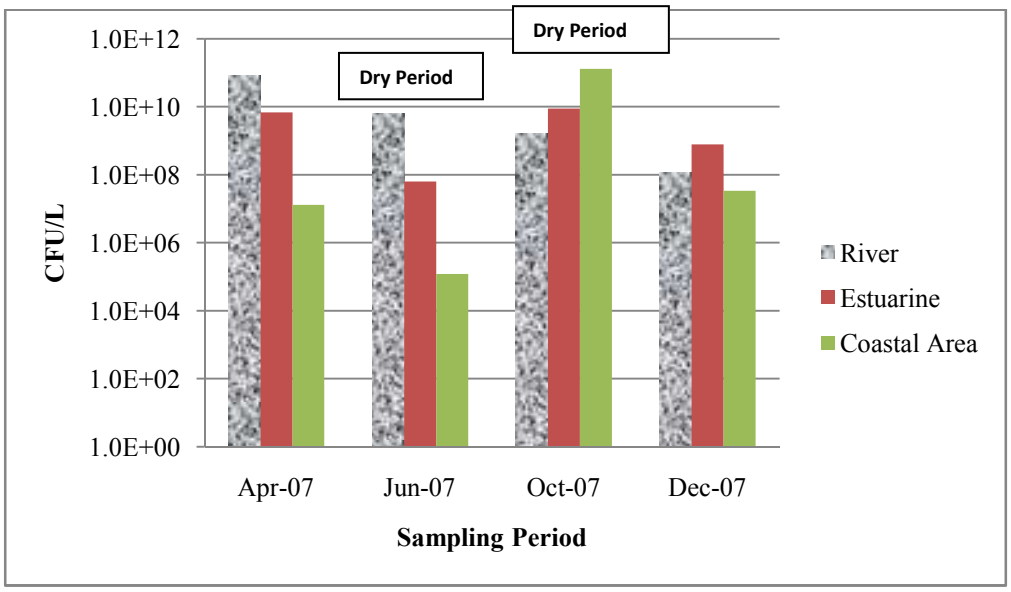


Figure 2.8. Number of *Enterococcus* spp. from Barceloneta water samples after enrichment during 2007

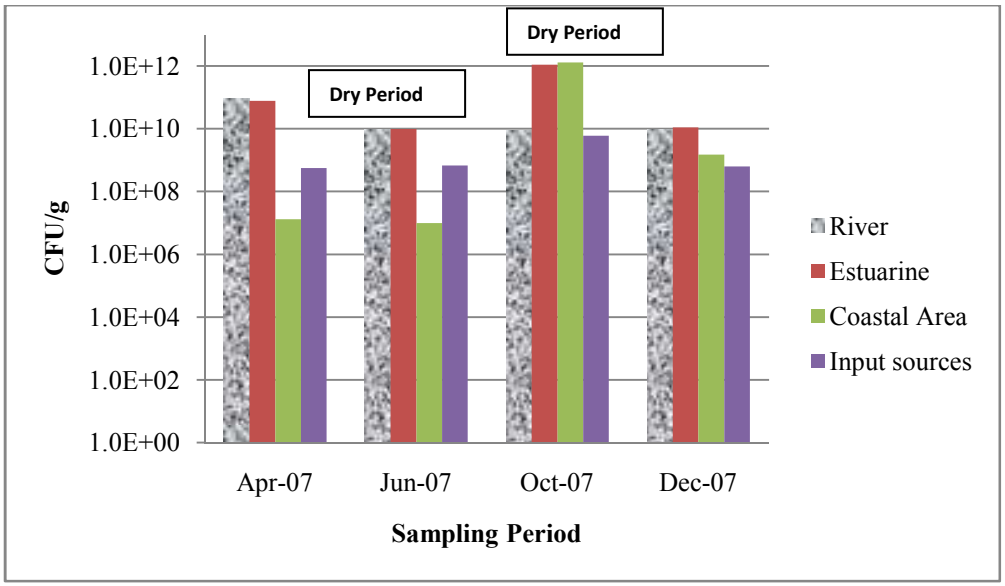


Figure 2.9. Number of *Enterococcus* spp. from Barceloneta sediment samples after enrichment during 2007

Characterization of *Enterococcus* species

A total of 4560 isolates of *Enterococcus* spp. were characterized during the 2-yr study. During each sampling period, 570 isolates were characterized. A total of fifteen different species of *Enterococcus* were identified. The relative abundance and the species richness of *Enterococcus* from different sampling sites are shown in Tables 2.2 and 2.3. The tables 2.6-2.11 shows *Enterococcus* species isolated at the different stations during 2006 and 2007. *E. faecalis* was the only one present in all samples.

In river waters ten different *Enterococcus* species were obtained. The predominant species were *E. faecalis*, followed by *E. durans* and *E. hirae*. The species of *E. porcinus*, *E. canis*, *E. sulfureus*, *E. dispar*, and *E. avium* were not isolated. During April 2006 was the period where were the highest number of species were obtained. *E. gallinarum* was only found in river waters. On the case of river sediments, species of *E. canis, E. haemoperoxidus, E. dispar, E. villorum, and E. cecorum* were isolated in only one sample. In river sediments the numbers of *E. faecium* were less compared with river waters and estuarine samples. Species of *E. sulfureus, E. gallinarum*, and *E. avium* were not isolated from river sediments (Table 2.7).

In estuarine waters, twelve different *Enterococcus* species were isolated. *E. faecalis* was the dominant species followed by *E. hirae* and *E. faecium*. Species of *E. asini, E. canis, E. dispar*, and *E. villorum* were isolated only in one sample in estuarine waters. In October 2007, a dry period, only two different species were isolated in water and sediments. In estuarine sediments, eleven different species were obtained. *E. faecalis* and *E. hirae* were the dominant species. *E. asini, E. cecorum, E. porcinus E. canis, E. dispar, and E. faecium* were isolated in only one sample. The numbers of species in estuarine sediments were lower than the species in estuarine waters, except in the sample of June 2006, where five species were isolated from sediments and four from the water column (Table 2.9).

In coastal samples, the highest number of species (13) was obtained. *E. avium* and *E. sulfurous* were only isolated from coastal waters, with *E. faecalis*, *E. seriolicida*, and *E. hirae* being the dominant species. The species *E. canis*, *E. sulfureus* and *E. faecium* were isolated only once. December 2007 was a rainy period and had the fewest isolated species (11) from the coastal area. The species of *E. faecium*, *E. dispar* and *E. porcinus* were isolated once on sediments (Table 2.11). The numbers of species in sediments were lower than coastal waters in all sampling. The diversity

values are presented in Table 2.4. High values of Simpson's Index represent high sample diversity (Appendix A). Thus samples of seawater, followed by the estuarine water, are the sites with higher diversity of species. There are differences in terms of occurrence of predominant species in the different periods. *E. faecalis* is the dominant species in all the periods followed by *E. hirae, E. durans, E. seriolicida* and *E. canis.* See Table 2.5 for the results of the predominant species in the different times.

Some of the presumptive *Enterococcus* could not be identified to species level using biochemical characterization. The samples containing the highest percentage of unidentified isolates were from the estuarine water samples during June 2007 with 13.33%, followed by 13% in the river and estuarine sediments during October 2006 and June 2007 respectively.

Table 2.2 Number of times in (%) *Enterococcus* spp. was found during each sampling

Species	River Water	River Sediment	Estuarine Water	Estuarine Sediment	Coastal Water	Coastal Sediment
E. asini	25	25	12.5	12.5	37.5	37.5
E. cecorum	25	12.5	37.5	12.5	25	25
E. faecalis	100	100	100	100	100	100
E. durans	87.5	25	25	25	75	50
E. seriolicida	25	50	50	37.5	87.5	37.5
E. hirae	87.5	75	62.5	62.5	87.5	75
E. porcinus		25	25	12.5	25	12.5
E. canis	-	12.5	12.5	12.5	12.5	25
E. sulfureus	-	-	-	-	12.5	-
E. gallinarum	12.5	-	-	-	-	-
E. faecium	50	25	50	12.5	12.5	12.5
E. haemoperoxidus	12.5	12.5	25	25	50	37.5
E. dispar	-	12.5	12.5	12.5	37.5	12.5
E. avium	-	-	-	-	12.5	-
E. villorum	25	12.5	12.5	-	-	-

- indicates no sampling Number of different smples (n) = 8

Table 2.3 Species Richness (r) during rainy and dry periods

Sampling site	April 2006 (R)	June 2006 (R)	October 2006 (R)	December 2006 (D)	April 2007 (R)	June 2007 (D)	October 2007 (D)	December 2007 (R)
River Water	9	4	4	4	4	3	3	5
River Sediment	3	5	6	2	4	4	3	3
Estuarine Water	8	4	5	3	3	4	2	4
Estuarine Sediment	5	5	5	3	1	3	2	2
Coastal Water	8	5	5	6	5	6	6	5
Coastal Sediment	6	5	4	3	3	6	4	3

(R) = Rainy periods or the rain prior to sampling. (D) = Dry periods or no rain prior to sampling.

Table 2.4 Simpson Diversity Index of *Enterococcus* spp. in different stations and seasons

Station	April 2006	June 2006	October 2006	December 2006	April 2007	June 2007	October 2007	December 2007
River Water	0.59	0.61	0.25	0.40	0.19	0.23	0.13	0.69
Estuarine Water	0.77	0.67	0.72	0.52	0.27	0.63	0.06	0.66
Coastal Water	0.78	0.73	0.43	0.80	0.34	0.60	0.59	0.76
River Sediment	0.55	0.64	0.68	0.48	0.44	0.41	0.3	0.29
Estuarine Sediment	0.40	0.64	0.69	0.52	0.02	0.10	0.10	0.10
Coastal Sediment	0.65	0.75	0.59	0.40	0.32	0.6	0.7	0.22

Values higher than 0.70 underlined

Table 2.5 Predominance of species during different sampling periods

Period of Sampling	Predominance of Species
April 2006	*E. faecalis, E. hirae,* and *E. durans*
June 2006	*E. faecalis, E. canis*. and *E. durans*
October 2006	*E. faecalis,* and *E. hirae*
December 2006	*E. faecalis, E. hirae,* and *E. seriolicida*
April 2007	*E. faecalis, E. hirae,* and *E. durans*
June 2007	*E. faecalis* and *E. hirae*
October 2007	*E. faecalis*
December 2007	*E. durans, and E. hirae*

Table 2.6 Number of species (%) in Barceloneta-Manatí river waters

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	2.23	0	0	0	0	0	0	6.67
E. cecorum	2.23	0	0	0	2.23	0	0	0
E. faecalis	57.78	51.11	4.44	73.33	86.67	84.44	91.11	13.33
E. durans	2.23	6.67	2.23	2.23	2.23	2.23	0	20
E. seriolicida	2.23	10.99	0	0	0	0	0	0
E. hirae	13.33	0	84.44	3.34	5.55	4.44	2.23	46.67
E. porcinus	0	0	0	0	0	0	0	0
E. canis	0	0	0	0	0	0	0	0
E. sulfureus	0	0	0	0	0	0	0	0
E. gallinarum	2.23	0	0	0	0	0	0	0
E. faecium	6.67	31.11	5.55	21.11	0	0	0	0
E.haemoperoxidus	2.23	0	0	0	0	0	0	0
E. dispar	0	0	0	0	0	0	0	0
E. avium	0	0	0	0	0	0	0	0
E. villorum	0	0	0	0	0	0	2.23	11.12
N.I.	7.78	0	3.33	0	2.22	7.78	3.33	2.22

N.I.-not identified

Table 2.7 Number of species (%) in Barceloneta-Manatí river sediments

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	0	0	0	0	2.20	4.39	0	0
E. cecorum	0	2.34	0	0	0	0	0	0
E. faecalis	55.56	20	33.33	64.44	71.11	73.33	82.22	11.23
E. durans	0	53.33	0	0	0	0	0	6.73
E. seriolicida	0	0	2.29	26.39	8.79	0	2.20	0
E. hirae	32.19	0	22.22	0	6.60	2.20	13.33	82.22
E. porcinus	0	0	2.29	0	0	4.39	0	0
E. canis	0	11.11	0	0	0	0	0	0
E. sulfureus	0	0	0	0	0	0	0	0
E. gallinarum	0	0	0	0	0	0	0	0
E. faecium	11.73	14.07	0	0	0	0	0	0
E.haemoperoxidus	0	0	36.71	0	0	0	0	0
E. dispar	0	0	2.29	0	0	0	0	0
E. avium	0	0	0	0	0	0	0	0
E. villorum	0	0	0	0	0	2.22	0	0
N.I.	0	0	0	8.89	11.11	13.33	3.33	0

N.I.-not identified

Table 2.8 Number of species (%) in Barceloneta-Manatí estuarine waters

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	6.60	0	0	0	0	0	0	0
E. cecorum	0	0	2.20	0	0	0	0	40
E. faecalis	24.44	22.22	17.50	62.22	82.22	51.11	95.56	22.22
E. durans	0	46.67	0	0	0	0	0	32.99
E. seriolicida	2.20	0	24.44	0	0	26.67	0	4.40
E. hirae	30.61	0	40	12.67	14.66	6.60	0	0
E. porcinus	4.40	0	0	0	0	2.20	0	0
E. canis	0	10.99	0	0	0	0	0	0
E. sulfureus	0	0	0	0	0	0	0	0
E. gallinarum	0	0	0	0	0	0	0	0
E. faecium	6.59	17.52	0	23.22	0	0	4.40	0
E.haemoperoxidus	17.78	0	8.79	0	0	0	0	0
E. dispar	6.59	0	0	0	0	0	0	0
E. avium	0	0	0	0	0	0	0	0
E. villorum	0	0	0	0	2.20	0	0	0
N.I.	0	2.22	6.67	1.11	0	13.33	0	0

N.I.-not identified

Table 2.9 Number of species (%) in Barceloneta-Manatí estuarine sediments

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	0	0	2.20	0	0	0	0	0
E. cecorum	2.25	0	0	0	0	0	0	0
E. faecalis	73.33	16.07	4.40	62.22	97.78	93.33	93.33	93.33
E. durans	0	53.33	0	0	0	0	0	2.25
E. seriolicida	2.25	0	35.56	21.11	0	0	0	0
E. hirae	6.73	0	37.78	15.83	0	2.20	2.20	0
E. porcinus	0	0	0	0	0	2.20	0	0
E. canis	0	9.18	0	0	0	0	0	0
E. sulfureus	0	0	0	0	0	0	0	0
E. gallinarum	0	0	0	0	0	0	0	0
E. faecium	0	16.07	0	0	0	0	0	0
E.haemoperoxidus	8.98	0	6.60	0	0	0	0	0
E. dispar	0	4.59	0	0	0	0	0	0
E. avium	0	0	0	0	0	0	0	0
E. villorum	0	0	0	0	0	0	0	0
N.I.	6.67	0	13.33	0	2.22	2.22	4.44	4.44

N.I.-not identified

Table 2.10 Number of species (%) in Barceloneta-Manatí coastal waters

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	24.44	0	0	0	0	2.20	2.20	0
E. cecorum	5.28	0	0	0	4.40	0	0	0
E. faecalis	17.60	22.46	19.79	21.11	77.78	40	10.99	10.56
E. durans	3.52	15.72	0	4.43	2.23	0	13.19	20.24
E. seriolicida	7.04	37.78	4.40	24.44	4.44	46.7	0	31.11
E. hirae	31.11	0	2.20	15.83	6.67	2.20	57.78	21.99
E. porcinus	3.52	0	0	11.61	0	0	0	0
E. canis	0	6.73	0	0	0	0	0	0
E. sulfureus	1.76	0	0	0	0	0	0	0
E. gallinarum	0	0	0	0	0	0	0	0
E. faecium	0	17.78	0	0	0	0	0	0
E.haemoperoxidus	0	0	71.11	17.78	0	2.20	2.20	0
E. dispar	0	0	2.20	0	0	4.40	2.20	0
E. avium	0	0	0	0	0	0	0	15.39
E. villorum	0	0	0	0	0	0	0	0
N.I.	6.67	0	0	4.44	4.44	2.22	11.11	0

N.I.-not identified

Table 2.11 Number of species (%) in Barceloneta-Manatí coastal sediments

Species	Apr-06	Jun-06	Oct-06	Dec-06	Apr-07	Jun-07	Oct-07	Dec-07
E. asini	17.78	0	0	0	0	2.20	30.78	0
E. cecorum	6.03	0	0	0	4.40	0	0	0
E. faecalis	6.03	37.78	8.792	73.33	80	40	15.40	11.11
E. durans	12.07	15.56	0	0	2.20	0	0	2.25
E. seriolicida	0	17.60	0	15.83	0	46.67	0	0
E. hirae	53.33	0	28.58	10.56	0	2.20	10.99	86.67
E. porcinus	0	0	4.44	0	0	0	0	0
E. canis	3.02	11.73	0	0	0	0	0	0
E. sulfureus	0	0	0	0	0	0	0	0
E. gallinarum	0	0	0	0	0	0	0	0
E. faecium	0	11.61	0	0	0	0	0	0
E.haemoperoxidus	0	0	59.38	0	0	2.20	37.78	0
E. dispar	0	0	0	0	0	4.40	0	0
E. avium	0	0	0	0	0	0	0	0
E. villorum	0	0	0	0	0	0	0	0
N.I.	2.22	5.55	6.66	0	13.33	2.22	4.44	0

N.I.-not identified

Molecular Results

Figure 2.10 shows the product of DNA extraction from seven of eighteen strains. Only thirteen colonies had a product that was adequate to perform the Polymerase Chain Reaction (PCR). The PCR product (Figure 2.11) was used in cleaning procedure using PCR Cleanup System and sent to Nevada Genomic Facility. An alignment and phylogenetic tree was constructed with the sequences generated by Nevada Genomic facilities, using the Neighbor Joining method and p-distance mode (Figure 2.12). With the phylogenetic tree, we can observe the relationships of our strain with that of the *Enterococcus* spp. Strains 101, 111 and 104 have homology with *E. hirae.* Strain 109 and 110 can be correlated with *E. casseliflavus*. Strains 112, 103, 105, 106 and 108 have 99% of homology with *E. faecalis*. The out-group used were *Clostridium uliginosum* (AJ276992.1) and *Shigella flexneri* (X969631). *Shigella* and *Clostridium* are both gram-negative and were selected because both are phylogenetically outside the group of species specifically being studied.

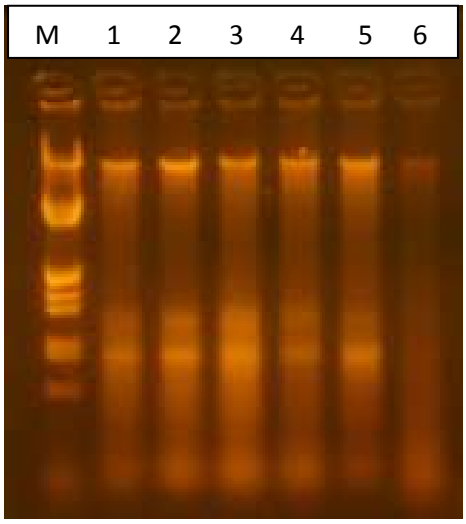


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Figure 2.10. Genomic DNA extraction from six strains isolated from Barceloneta-Manatí coastal water samples.

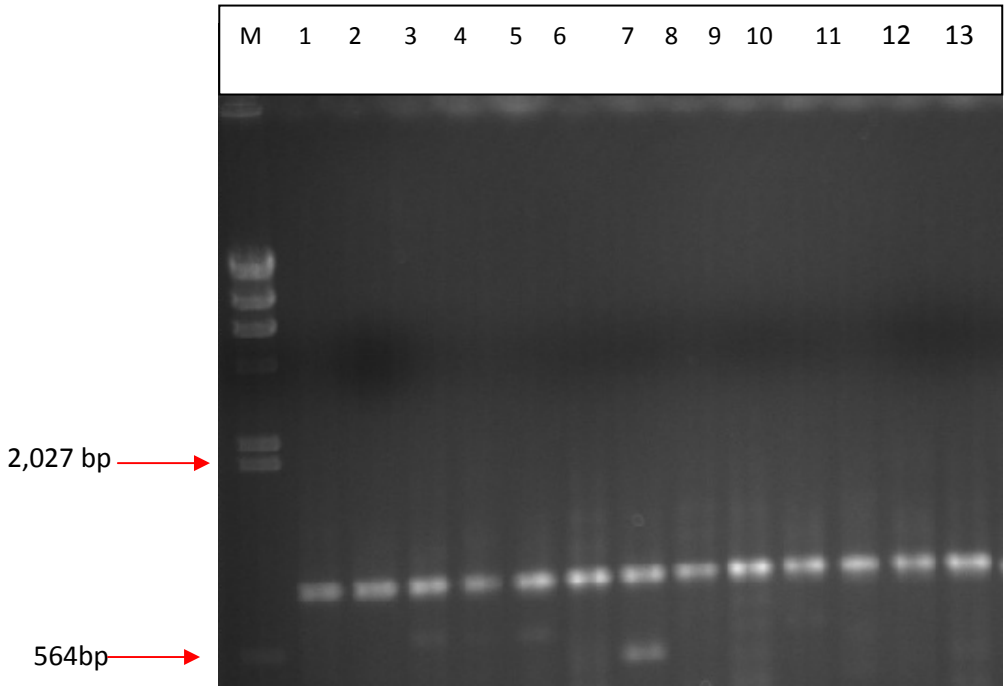


Figure 2.11. PCR products from Barceloneta-Manatí coastal water samples. PCR amplicons were purified using the MinElute PCR purification kit (USA QIAGEN Inc.), and the product concentration was determined using a 1% agarose gel with markers of *Hind* III.

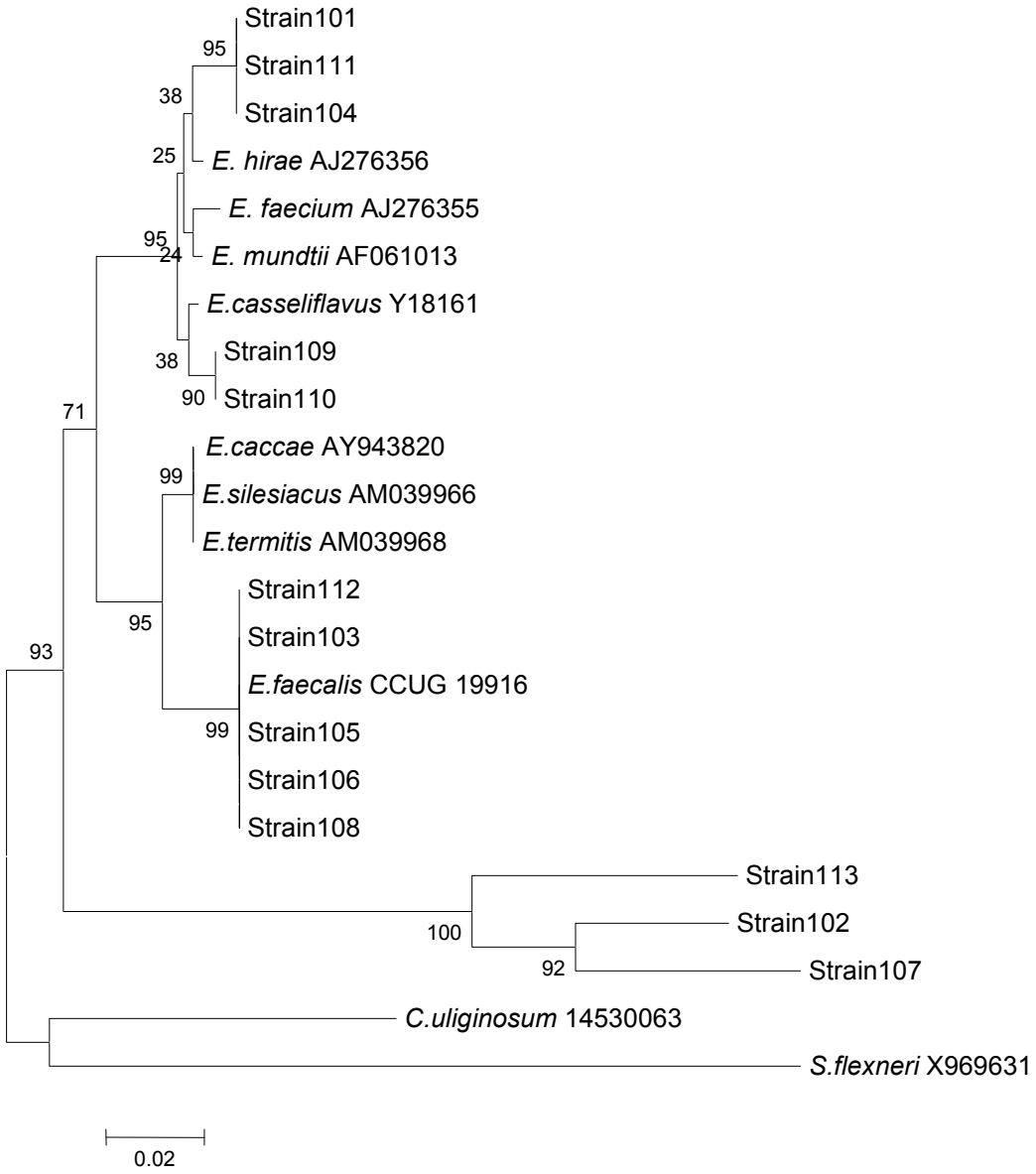


Figure 2.12. Phylogenetic tree using Neighbor Joining Method and p-distance model of strains isolated from Barceloneta-Manatí coastal waters.

Discussion

Most industrial cities and towns in Puerto Rico are located on the coast. The coastal town of Barceloneta has one of the largest concentrations of pharmaceutical industries in Puerto Rico and is also the site of several of large dairy farms. *Enterococcus* spp. isolated from river, estuarine, and coastal environments in Barceloneta-Manatí area were analyzed to evaluate the diversity of *Enterococcus* present in the three environmental samples. Knowledge of predominant species present at a specific site could be useful in developing methods using enterococci as microbial sources tracking.

Althoung minor differences were found in water quality parameters, there are indications that in the tropics, even slight variations in water and sediment temperature can affect the survival, persistence, and regrowth of indicator bacteria (Noble *et al., 2004*; Boehm, 2007). Certain species of enterococci are favored by certain temperatures (Domig *et al.,* 2003). Because constant room temperature of 25^{o}C was used to culture the bacteria, this may have adversely affected the growth of some *Enterococcus* species, which grow best at other temperatures. Bonilla *et al.* (2006) noted that poor isolation from sewage samples of *E. faecium* and *E. gallinarum* may be due to laboratory temperature and culture medium. Similarly, research by Lleo *et al.* in 2005 indicated that different strains of *Enterococcus* have differences in the resuscitation capacity from viable but non culturable state (VBNC) to culturable state. For example *E. faecalis* and *E. hirae* that can be resuscitated within 60 days while *E. faecium* cannot.

In Barceloneta, the salinity is controlled by the quantity of rain in this area. This is observed during samplings of June 2006 and April 2007 collected after heavy rains where the lowest salinities in estuarine and coastal areas were observed. The presence of *E. canis* during June 2006 is important because low salinity may have contributed to its prevalence. In general, higher species diversity was obtained after heavy rainfall. For example, during October 2006 when wet weather conditions prevailed, six species of *Enterococcus* were isolated in river sediment samples compared to December sample during the dry season with only two species at the same site. Similar results were observed by Rivera (2008) and Harwood (2007) in which abundance and diversity of enterocccocal species was affected by environmental conditions, with more diverse populations during increased precipitation.

Knowledge of the variation of *Enterococcus* spp. distribution and factors that contribute to these is important in clinical and environmental science. Phylogenetic analysis of PCR amplification from colonies from coastal samples with an approximate size of 873 bp revealed that evaluated strains belong to Bacteria domain and Firmicutes phylum. The predominant genus within this group is *Enterococcus*. These results support the isolation of *Enterococcus* in selective media. Approximately 80% of the strains that were sequenced support the initial isolation of *Enterococcus* in selective media. This confirms that with *Enterococcus* agar, additional tests are necessary to confirm the isolation of *Enterococcus.* This can be further confirmed by catalase test and regrowth on Brain Heart Infusion Agar.

A comparison of enterococci distribution in river, estuarine, and coastal areas showed that *E. faecalis* dominates in all three environments. The species dominance varies among samples and sampling periods suggesting increments in the abundance of a variety of species. The reason for the dominance of *E. faecalis* could be the capacity of this strain to survive for longer periods. The variation between the species obtained from water and sediment samples can be correlated to factors such as precipitation, human intervention, and the presence of specific vectors like domestic farm animals or birds (Nichols *et al*., 2008). Ahmed *et al.* (2005) found that non-point sources representing domestic and wild animals can contain *Enterococcus.* During December 2007, *E. avium* was isolated from the coastal environment and during this period increased populations of wild birds were observed during sample collection.

The role of aquatic sediments as a sink and possible source of pollutants in marine systems is recognized. Sediments serve as a substrate for enterococci spp. and offer a suitable environment in which these indicator bacteria can survive and proliferate. Increased populations of *Enterococcus* spp. in the sediment of all three environments tended to support this hypothesis. The numerical variation of *Enterococcus* in the sediments could be due to the type of soil where the *Enterococcus* was isolated. Fergusson *et al.* (2005) obtained higher number of *Enterococcus* isolates in sediments from intertidal compared to marine zones and this was correlated to the type of soil. Cools *et al.* (2001) evaluated the survival of *E. coli* and *Enterococcus* spp. in soils of various textures, suggesting that sandy soil does not favor the survival of *Enterococcus* compared with loamy soil.

Relative abundance of *Enterococcus* species in the three environments were compared after growing the bacteria in enrichment medium for 24 h. The highest values in river samples may have

been due to the proximity of this site to the dairy industry. Feces in water runoff containing higher number of *Enterococcus* may enter into the river after the rain. Predominant species in tropical Barceloneta coastal water, in order of occurrence were *E. faecalis, E. hirae* and *E. seriolicida. E. hirae* is a member of animal microflora and can cause humans infections; *E. seriolicida* is a freshwater and saltwater fish pathogen. The abundance distribution of *Enterococcus* spp. in tropical Barceloneta water is different than that found in California coastal waters, where the most abundant *Enterococcus*, in diminishing order, were *E. faecalis, E. faecium and E. hirae.*

Compared to river and estuarine environments, the coastal area has the lowest number of *Enterococcus* spp., but has high variability of species according to the Simpson Index. Previous studies in different European countries suggested higher diversity of enterococci in environmental samples influenced by warm-blooded animals (Kühn *et al.*, 2005). In the study thirteen different species in coastal waters were found versus nine species in sediments a possible explanation for this can be the resuspension of *Enterococcus* spp. in the water column that were trapped in sediments. This resuspension may also be due to tides and wave action in the coastal zone of Barceloneta. Ferguson *et al*. (2005) studied enterococci distribution in intertidal and marine sediments and coastal waters at California bathing beaches and, determined that the distribution of species present in water samples was comparable to those found in sediments. This study recognized that resuspension of enterococci that are persistent in sediments may contribute to the levels of indicator bacteria and may be the cause for failure to meet beach water quality standards. Pathogenic microorganisms associated with sediment particles have the possibility of being resuspended back into the water column due to natural turbulence or human recreational activity (Irvine and Pettibone, 1993; Obiri-Danso and Jones, 2000; Bonilla, 2007).

Petersen and Dalsgaard (2003) established that species composition of *Enterococcus* in tropical aquatic environments is influenced by fecal and antimicrobial pollution. Our studies confirm this, and in addition, suggest that species composition in the Barceloneta-Manatí area may be determined by physical factors such as precipitation, salinity, human intervention, and due to the presence of specific types of vectors such as domestic and farm animals. In conclusion, the results obtained in this study show a large temporal and spatial variation in enterocccocal abundance and community composition in Barceloneta-Manatí.

Literature Cited

Ahmed W., Neller R., and M. Katouli. 2005. Host species–specific metabolic fingerprint database for enterococci and *E.coli* and its application to identify sources of fecal contamination in surface waters. Applied and Environmental Microbiology. 71(8): 4461-4468.

Anderson M.A., Whitlock J.E., and V.J. Harwood. 2006. Diversity and distribution of *Escherichia coli* genotypes and antibiotic resistance phenotypes in feces of humans, cattle, and horses. Applied and Environmental Microbiology. 72: 6914-6922.

Boehm A.B. 2007. *Enterococcus* concentration in diverse coastal environment exhibit extreme variability. Environmental Science and Technology. 41(25): 8227-8232.

Bonilla T.D., Nowosielski K., Cuvelier M., Hartz A., Green M., Esiobu N., McCorquodale D.S., Fleisher J.M., and A. Rogerson. 2007. Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. Marine Pollution Bulletin. 54(9): 1472-82.

Bonilla, T.D., Nowosielski K., Esiobu N., McCorquodale D.S., and A. Rogerson. 2006. Species assemblages of *Enterococcus* indicate potential of fecal bacteria at a south Florida recreational beach. Marine Pollution Bulletin. 52: 800–815.

Cai, Y. 1999. Identification and characterization of *Enterococcus* species isolated from forage crops and their influence on silage fermentation. Journal of Dairy Sciences. 82: 2466-2471.

Cardoso de Olivera A.F., and J.M Watanabe Pinhata. 2008. Antimicrobial resistance and species composition of *Enterococcus spp*. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. Water Research. 42(8-9): 2242-2250.

Carvalho M.G.S., Texeira L.M., and R. R Facklam. 1998. Use of test for acidification of methyl-α-D-glucopyransoside and susceptibility to efrotomycin for differentiation of strains of *Enterococccus* and related genera. Journal of Clinical Microbiology. 36: 1584-1587.

Cox C.R., and M.S. Gilmore. 2007. Native microbial colonization of *Drosophila melanogaster and* its use as a model of *Enterococcus faecalis* pathogenesis. Infection and Immunity.75: 1565–1576.

Cools D., Merckx R., Vlaassak K., and J. Verhaegen. 2001. Survival of *E. coli* and *Enterococcus* spp., derived from pig slurry in soils of different textures. Applied Soil Ecology. 17(1): 53-62.

De Vaux A., G. Laguerre, C. Civies, and H. Prevost. 1998. *Enterococcus asini* sp nov. isolated sp from the caecum of donkeys. International Journal of Systematic Bacteriology. 48: 383-387.

Devriese L.A., Van De Kerckhove A., Kilpper-Balz R., and K.H Schleiffer. 1987. Characterization and identification of *Enterococcus* species isolated from the intestine of animals. International Journal of Systematic Bacteriology. 37: 257-259.

Devriese L.A., Cruz Colque J.L., De Herdt P., and F. Haesebrouck. 1992. Identification and composition of the tonsillar and anal enterococcal flora of dogs and cats. Journal of Applied and Bacteriology.7: 421-425.

Devriese L.A., Ieven M., Goossens H., Vandamme P., Pot B., Hommez J., and F. Haesebrouck 1996. Presence of vancomycin –resistant enterococci in farm and pet animals. Antimicrobial Agents and Chemotherapy. 40: 2285-2287.

Devriese L.A., Vandamme P., Pot B., Vanrobaeys M., Kersters K., and F. Haesebrouck. 1999. Differentiation between *Streptococcus gallolyticus* strains of human clinical and veterinary origins and *Streptococcus bovis* strain from the intestinal tract of ruminants. Journal of Clinical Microbiology. 36: 3520-3523.

Doming K. J., Mayer H.K., and W. Kneifer. 2003. Methods used for the isolation, enumeration, characterization and identification of *Enterococcus* spp. International Journal of Food Microbiology. 88(2)147-164.

Facklam R.R., D.F. Sahm, and L.M. Teixeira. 1999. *Enterococcus. In* P.R. Murray,E.J.Baron, Pfaller M.A., Tenover F.C., and R.h.Yolken(ed), Manual of Clinical Microbiology,7th ed. ASM Press, Washington D.C. p. 297-305.

Facklam R.R., Carvalho M.S., and L.M. Texeira. 2002. The enterococci: pathogenesis, molecular biology, and antibiotic resistance. In M.S. Gilmore (ed). History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci. p. 1-54.

Ferguson D.M., Moore, D.F., Getrich, M.A., and M.H. Zhowandai. 2005. Enumeration and speciation of *enterococci* found in marine and intertidal sediments and coastal water in southern California. Journal of Applied Microbiology. 99: 598-608.

Franz C.M., Holzapfel W.H., and M.E. Stiles. 1999. Enterococci at the crossroads of food safety? International Journal of Food Microbiology. 47: 1-24

Guzman C.A., Pruzzo C., LiPira G., and L. Calegari. 1989**.** Role of adherence in pathogenesis of *Enterococcus faecalis* urinary tract infection and endocarditis. Infection and Immunity. 57(6): 1834-1838.

Hammer Ø., Haper D.A.T., and P. D. Ryan. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4:9.

Harwood V.J. 2007. Assumptions and limitations associated with microbial source tracking. *In* Emerging issues in food safety: Microbial Source Tracking. Ed J.W. Santo Domingo and M.J. Sadowsky. ASM Press. Washington, D.C. p. 33-64.

Holt J.G.,Krieg N.R., Sneath P.H.A., Staley J.T., and S.T. Williams. 2000. Bergey's Manual of Determinative Bacteriology. 9th ed. Lippincott Williams and Wilkins. Wolters Kluwer Co.528.

Huycke M.M., Sahm D.F., and M.S. Gilmore.1998. Multiple-drug resistant enterococci; the nature of the problem and an agenda for the future. Emerging Infection Diseases. 4: 239-249.

Irvine K.N., and G.W. Pettibone. 1993. Dynamics on indicator bacteria populations in sediment and river water near a combined sewer outfall. Environ Sciences and Technology. 14(6): 531-542.

Kuntz R.L., Hartel P.G., Rodgers K., and W. I. Segars. 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Research 38: 3551–3557.

Kühn I., Iversen A., Finn M., Greko C.,Burman L.G., Blanch A.R., Vilanova X., Manero A., Taylor H., Caplin J., Domínguez L., Herrero I.A., Moreno M.A., and R. Möllby. 2005. Occurrence and relatedness of vancomycin-resistant enterococciin animals, humans, and the environment in different European regions. Applied and Environmental Microbiology. 71: 5383-5390.

Manero A., and A. Blanch 1999. Identification of *Enterococcus* species with biochemical key. Applied and Environmental Microbiology. 65(10): 4425-4430.

Nichols C., Rukovets B., Scott T., Mitchell B., and E. Pizzuto. 2008. Identifyng the origin, and impact of temperature on *Enterococcus* concentrations, persistence, and regrowth through microbial source tracking and monitoring at a long island sound bathing beach. Monitoring Conference 2008 Water Environment Federation. 57-75.

Niemi R.M., Niemelä S.I., Bamford D.H., Hantula J., Hyvärinen T., Forsten T., and A.Raateland. 1993. Presumptive fecal streptococci in environmental samples characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Applied and Environmental Microbiology. 7: 2190–2196.

Noble R.T., Lee, I.M., and K.C. Schiff. 2004. Inactivation of indicator microorganisms from various sources of fecal contamination in seawater and freshwater. Journal of Applied Microbiology. 96:464-472.

Nowlan S.S., and R.H. Deibel. 1967. Group Q streptococci. Ecology, serology, physiology, and relationship to established enterococci. Journal of Bacteriology. 94:291-292.

Obiri-Danso K., and K. Jones. 2000. Intertidal sediments as reservoirs for negative *Campylobacter, Salmonellae* and fecal indicators in three EU recognized bathing waters in northwest England. Water Research. 34(2): 519-27.

Petersen A., and A. Dalsgaard. 2003. Species composition and antimicrobial resistance genes of Enterococcus spp., isolated from integrated and traditional fish farms in Thailand. Environmental Microbiology. 3(5): 395-402.

Rivera T. Y. 2008. Isolation and Identification of Fecal Pollution Indicators Enterococcus spp from sea grass Thalassia testudinum at La Parguera Puerto Rico and the use of enterococci for microbial sources. Master Thesis. University of Puerto Rico. Mayagüez Campus. 94 pp.

Rodriguez E.M., Pérez E., Schadt C., Zhou J., and A.Massol-Deya. 2006. Microbial diversity and bioremediation of hydrocarbon-contaminated aquifer (Vega Baja, Puerto Rico). International Journal of Environmental Research on Public Health. 3(3):292-300.

Saano A., Tas E., Piippola S., Lindstrom K., and J. Van Elsas. 1995. Nucleic acids in the environment: methods and applications. Trevors, J.T. and J. Van Elsas, (eds) Berlin: Springer. p. 49-67.

Wheeler A. L, Hartel P.G., Godfrey D.G., Hill J.L., and W. I. Segars. 2002. Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. Journal of Environmental Quality 31:1286-1293.

Zlotkin A., Eldar A., Ghittino C., and H.Bercovier. 1998. Identification of *Lactococcus garvieae* by PCR. Journal of Clinical Microbiology. 36 (4): 983-985.

Chapter 3 Multiple antimicrobial resistance patterns of *Enterococcus* spp. in waters and sediments of Barceloneta-Manatí, Puerto Rico

Introduction

Enterococcus has evolved resistance to antibiotics by the acquisition of antibiotic resistance genes on plasmids or transposons from other organisms or by spontaneous mutation that give the enterococci increased level of resistance (Arias and Murray, 2002). There are reports that enterococci isolated from the poultry environment are frequently resistant to antimicrobials used to treat human infections (Van den Bogaard *et al.,* 2002). The pattern of antibiotic resistance within *Enterococcus* species (ES) is different when they are isolated from the poultry environment. *E. faecalis* was highly resistant to lincosamide, macrolides, and tetracycline antibiotics, while *E. faecium* was resistant to fluoroquinolones and penicillin (Hayes *et al.,* 2004).

Increased numbers of multiple antibiotic resistances of enterococci in fresh and marine waters increases health risks for humans because the treatment of infection caused by these bacteria will be difficult if they are already resistant to multiple antibiotics. It is important to understand the multiple antibiotic resistant patterns and the survivability of these bacteria to help in the assessment of potential risks associated with the consumption of raw or partially cooked food. This is based on the results from other investigations that found that animals may serve a reservoir of vancomycin resistant enterococci (Bates *et al.,* 1994). Thus, in order to control multiple-drug resistant enterococci, it is necessary to have a better understanding of the interaction between enterococci, the natural environment, hospital environments, and human beings (Huckey *et al.*, 1998; Silbergeld *et al.,* 2008).

Mechanism of resistance of penicillin, tetracycline, and vancomycin

There are multiple mechanisms by which enterococci acquired resistance (Vivek and Chow, 2002). The intrinsic resistance to ß lactam antibiotic is due to the low affinity of their penicillin binding proteins (PBPs) for the ß-lactam agent. The resistance differs between ß-lactam, with penicillin generally having the most activity, followed by carpenems and cephalosporin having the least activity. Aminoglycoside acts primarily by interfering with protein synthesis by binding to the

16S rRNA of the 30S ribosomal subunit. *Enterococcus* species intrinsically possesses low level of resistance to aminoglycoside by limiting transport of the drugs across the cell membrane. Aminoglycoside is not effective as immunotherapy against enterococci. When cell wall active agents such as penicillin or a glycopeptides is combined with the aminoglycoside, the uptake of the aminoglycoside into the cell is dramatically increased, resulting in synergetic killing of the *Enterococcus* spp.

The glycopeptides antibiotics like vancomycin are used to treat infections due to resistant gram-positive organisms. The type of antibiotics acts by inhibiting cell wall biosynthesis on the substrates of the peptidoglycan pentapeptide precursors. The appearance and dissemination of vancomycin resistance in humans can be attributed in part to the widespread use of vancomycin in clinical practice and glycopeptides use in animal husbandry. Although strains of vancomycin resistant enterococci in animals have been extensively studied, less is known of multiple antibiotic resistant of this organism (Oquri *et al.,* 2001).

The tetracycline resistance is present in at least 60 to 65% of enterococci clinical isolates even though these antibiotics are not routinely used to treat enterococci infections. Tetracycline inhibits protein synthesis by interfering with the binding of aminoacyl-tRNA to the ribosome. There are two major mechanisms of tetracycline resistance in enterococci, (i) active efflux of the drug across the cell membrane, and (ii) ribosomal protection. Resistance to tetracycline was first detected in the 1950's and became more apparent by the 1970s when it was widely reported among Enterobacteriaceae, staphylococci, streptococci, and *Bacteroides* spp.

The purpose of the present study is to determine if the *Enterococcus* species isolated from waters and sediments of Barceloneta-Manatí area are resistance to multiple antibiotics (combination of tetracycline, penicillin, and vancomycin). Additionally, it was important to determine if the site where multiple antibiotics are routinely used, e.g. dairy industries, also has higher number of *Enterococcus* spp. resistant to multiple antibiotics. Abundance of multiple antibiotic resistant enterococci in areas near dairy industries (point source) may reflect the risk of finding relatively higher number of antibiotic resistant strains in water and sediments of river, estuary, and coastal waters of Barceloneta, Puerto Rico.

Material and Methods

Selection of Antibiotics

Penicillin, tetracycline, and vancomycin (Sigma, Aldrich) were selected because their widespread use in the dairy industry.

Isolation of Bacteria Resistant to Antibiotics

Detailed description of isolation and identification of *ES* is given in the previous chapter. Briefly, one liter of water sample was filtered through 0.22 µm filters. Filters were incubated for 48 h at 25^{o}C and plated on agar with appropriate dilutions. Colonies were picked up at random and transferred to microplate containing 0.2 mL of Enterococcosel medium. After 48 h, the bacteria showing positive growth were transferred to a brain heart medium and catalase tests were performed. Forty five colonies of characterized ES from water and sediment samples were transferred to microplate containing Enterococcosel broth with antibiotics. A total of 270 colonies were evaluated in each sampling period.

Minimum Inhibition Concentration (MIC)

MIC of each antibiotic was determined using parameters developed by the National Council of Clinical Laboratory Standards (NCCLS). MIC for each antibiotic was determined by using the Kirby Bauer Method (Figure 3.1). The antibiotics and concentrations used were tetracycline (0.1, 4, 10, 30, and 40 µg/mL), penicillin (0.01, 0.05, 1.0, and 15 µg/mL) and vancomycin (0.05, 1.0, 10, 20, and 30 µg/mL).

Multiple Antibiotic Resistances (MAR)

Individual colonies of *Enterococcus* spp. were transferred into each well of 96 well microplates containing Enterococcosel broth, and incubated for 48 hours at 25°C. After 24 hours the colonies were transferred to microplates with Enterococcosel broth containing different antibiotics. The following was the composition of the microplates: #1 Enterococcosel broth + penicillin (5µg/mL) + tetracycline (10µg/mL), #2 Enterococcosel + penicillin (5µg/mL) + vancomycin (10 µg/mL), #3 Enterococcosel + vancomycin (10µg/mL) + tetracycline (10µg/mL), #4 Enterococcosel + Penicillin (5µg/mL) + tetracycline (10µg/mL) + vancomycin (10ug/mL), and #5

Enterococcosel + Penicillin (15µg/mL) + tetracycline (40µg/mL) + vancomycin (20ug/mL). After 24 hr, the colonies with positive growth in the selective medium containing combinations of antibiotics indicated their resistance to multiple antibiotics (See Figure 3.2).

Statistical Analysis for Evaluation of Antibiotic Resistances

Descriptive statistical methods using frequencies and percentages, and Z-test were used to evaluate the equality of proportions to the abilities of *Enterococcus* spp. resistance to different antibiotics.

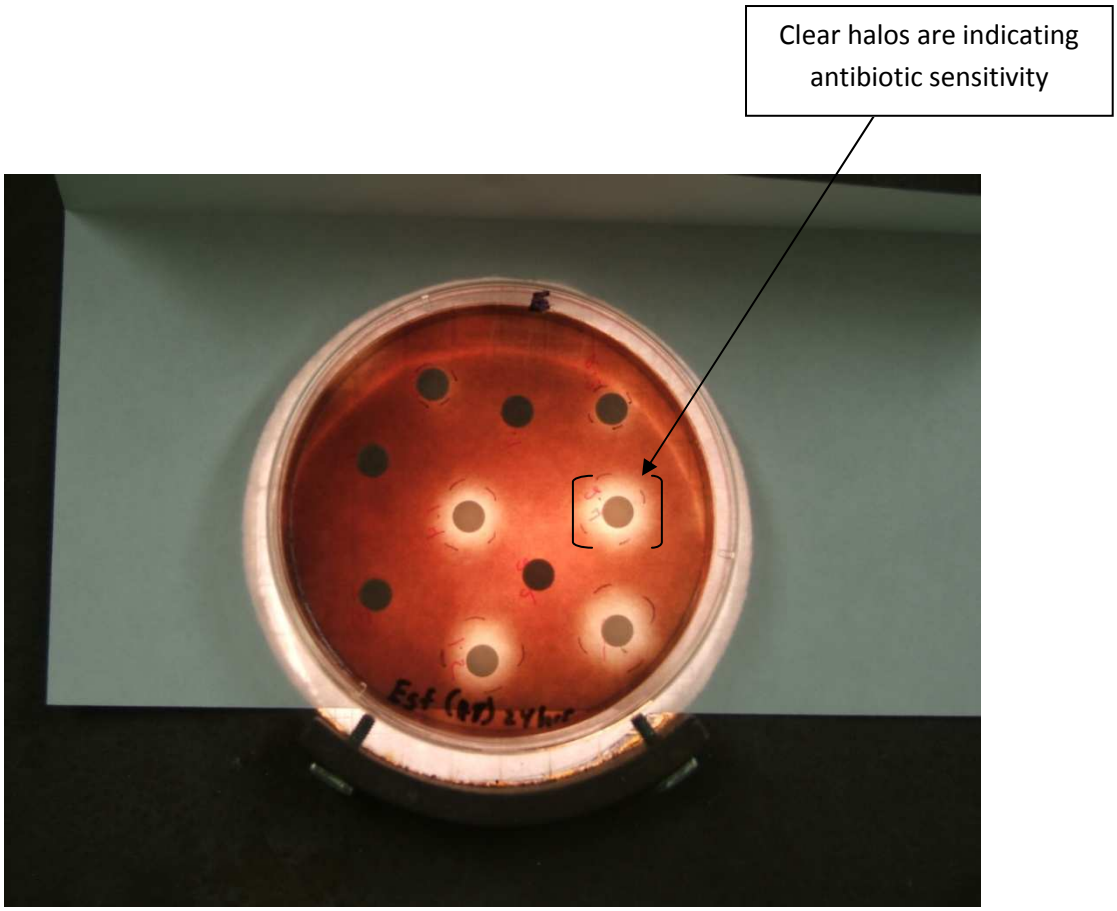


Figure 3.1. Resistant *Enterococcus* spp. isolated from the estuarine sample using the Kirby Bauer technique.

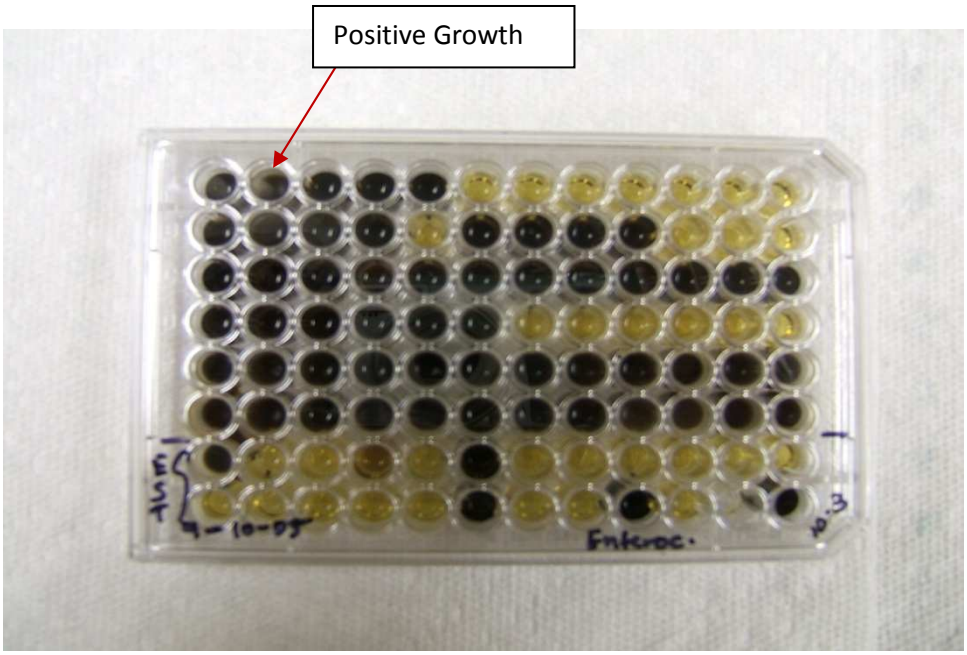


Figure 3.2. Microplate containing Enterococcosel broth inoculated with sample from estuarine water. The dark wells indicate *Enterococcus* spp. growth

Results

Determination of MIC of antibiotics

MIC of penicillin, tetracycline, and vancomycin antibiotics was determined. Various concentrations of each antibiotic were tested against isolated *Enterococcus* species (ES). The exact concentrations tested for each antibiotic were determined during preliminary work (data no shown). MIC for both tetracycline and vancomycin was 10 µg/mL and for penicillin 5 µg/mL.

Percentage of multiple antibiotic resistant (MAR) *Enterococcus* spp. (ES) in water and sediment samples

The MAR *Enterococcus* species (ES) isolated from Barceloneta-Manatí river, estuarine, and coastal water samples during 2006-07 were analyzed. The antibiotics penicillin, tetracycline and vancomycin were used in combination. Data presented in Table 3.1 show that ES isolated from waters of the three environments characterized differences in levels of resistance to combination of the two antibiotics. For example, in water samples collected during April 2006, when penicillin was used with tetracycline (P+T), 93% of *ES* were resistant to these two antibiotics in river water compared to 91% in estuarine water and 31% in coastal water. However, when penicillin +

vancomycin (P + V) were used, the percentage of *ES* resistant to these two antibiotics was 53% in river water, 27% in estuarine water, and 9% in coastal water. Percentage of *ES* resistant to tetracycline + vancomycin (T + V) was 62%, 24, and 73% in river, estuary, and coastal waters respectively (Table 3.1). The percentage of resistance of *ES* varied in different samples in river, estuarine, and coastal water. In the June 2006 sample, the resistance to the combination of same antibiotics was generally less in coastal water than in river and estuarine water. The sample in June 2006 was collected after heavy rain; total number of *ES* resistant to antibiotics was low in river water during this sampling period. A possible explanation may be dilution of the bacterial numbers due to heavy rain. The results show (Table 3.3) that there were no significant differences in resistance to combination of the three antibiotics between *ES* isolated from river and estuarine water. Average levels of resistance of ES to combinations of the three antibiotics were also lower in coastal sediments compared to ES isolated from estuarine sediments. In April 2006, 93% of ES were resistant to combinations of two antibiotics (P+T, P+V, and T+V) in river sediments compared to 80% in estuarine sediments and about 50% in coastal sediments. In June 2006 samples the percentage of ES resistant to combination of the three antibiotics was also much lower in river sediments (Table 3.2).

Percentages of ES resistant to three antibiotics (P+T+V) at low and high concentrations were also compared in water and sediments samples from the three environments (Table 3.3). The result show there was no significant difference in ES resistance to P+T+V in the three environments at low concentrations. However, at high concentrations of P+T+V the percentage of ES resistant to these antibiotics was lower in coastal water and sediments.

Based on 5-day average rainfall data prior to sampling indicated that samples can be divided into the dry or rainy period (Table 3.4). Thus, the samples collected in December 06, June 07, and October 07 with average rainfall of less than 0.1 inch were considered to be during the dry period and samples collected during April 06, June 06, October 06, April 07, and December 07, with average rainfalls of 0.15, 0.47, 0.14, 0.11 and 0.12 inch respectively during the rainy period. Comparison of resistance to combinations of two antibiotics P+T, P+V, and T+V during the rainy period indicated that resistance to P+T and P+V was higher in river as well as estuarine water and sediments (Table 3.5). Over 50% of ES were resistant to P+T, about 45% resistant to P+V, and about 32% resistant to T+V in the two environments. However, in coastal water and sediments,

there was not much difference in resistance to P+T, P+V, and T+V. Thus, in these environments the percentage of ES resistance to the antibiotics was generally lower than in river and estuarine water and sediments. About 42% of ES were resistant to P+V, about 39% resistant to P+T, and 29% resistant to T+V in coastal water and sediments. In river and estuarine water ($Z = 2.37$) (See statistical analysis in Appendix B) and sediments ($Z = 4.09$) during dry period (Table 3.6) comparatively higher percentages of ES were resistant to P+T than during the rainy period. Thus, in the two environments about 70% of ES was resistant to P+T compared to about 54% to P+V ($Z = 3.13$) and about 42% to T+V ($Z = 3.18$). In coastal water and sediments the percentage of ES resistant to P+T and P+V was significantly higher than to T+V. In these two environments about 40% of ES were resistant to P+T, about 42% resistant to P+V, and only about 29% ($Z = 2.21$) resistant to T+V.

Table 3.1 Resistance (%) to combination of antibiotics in waters

Sampling Period	Antimicrobial Agents	River	Estuarine	Coastal water
April 2006	Tet +Vanc	62	24	73.3
	Pen +Tet	93	91	31.11
	Pen+ Vanc	53	27	8.89
June 2006	Tet +Vanc	0	9	17
	Pen +Tet	8	53	27
	Pen+ Vanc	0	27	6.7
October 2006	Tet +Vanc	20	16	20
	Pen +Tet	31	18	6.6
	Pen+ Vanc	22	31	18
December 2006	Tet +Vanc	34	36	9
	Pen +Tet	49	53	31
	Pen+ Vanc	18	11	13.3
April 2007	Tet +Vanc	53	62	31
	Pen +Tet	93	84	53
	Pen+ Vanc	42	36	27
June 2007	Tet +Vanc	80	42	38
	Pen +Tet	82	91	64
	Pen+ Vanc	64	56	49
October 2007	Tet +Vanc	30	38	26
	Pen +Tet	51	58	47
	Pen+ Vanc	69	100	96
December 2007	Tet +Vanc	4	13	29
	Pen +Tet	49	53	31
	Pen+ Vanc	87	100	97

Pen=penicillin (5µg/ml), Tet= tetracycline (10 µg/ml), Van= vancomycin (10 µg/ml)
n=45

Table 3.2 Resistance (%) to combination of antibiotics in sediments

Sampling Period	Antimicrobial Agents	River	Estuarine	Coastal Area
April 2006	Tet +Vanc	82	82	78
	Pen +Tet	100	97	53.3
	Pen+ Vanc	97	62	18
June 2006	Tet +Vanc	0	16	8.9
	Pen +Tet	5	40	17
	Pen+ Vanc	0	18	4.44
October 2006	Tet +Vanc	13	10	16
	Pen +Tet	24	16	10
	Pen+ Vanc	18	26	13
December 2006	Tet +Vanc	42	27	26.6
	Pen +Tet	76	87	24.4
	Pen+ Vanc	27	17	16
April 2007	Tet +Vanc	60	71	40
	Pen +Tet	82	97	71
	Pen+ Vanc	40	27	18
June 2007	Tet +Vanc	69	53	31
	Pen +Tet	87	84	71
	Pen+ Vanc	47	69	53
October 2007	Tet +Vanc	31	29	16
	Pen +Tet	64	69	42
	Pen+ Vanc	76	98	93
December 2007	Tet +Vanc	8	20	17
	Pen +Tet	27	26	20
	Pen+ Vanc	96	100	91

Pen=penicillin (5µg/ml); Tet= tetracycline (10 µg/ml); Van= vancomycin (10 µg/ml)

n=45

Table 3.3 Resistance (%) to combination of antibiotics in Barceloneta–Manatí Puerto Rico

Date of Sample	Antimicrobial Agents	Percentage of Resistant Isolates from estuarine		Percentage of Resistant Isolates from estuarine		Percentage of Resistant Isolates from coastal area	
		Water	Sediment	Water	Sediment	Water	Sediment
Apr-06	Pen +Tet +Van (LC)	31	69	80	100	56	71
	Pen + Tet +Van (HC)	7	16	7	16	0	4
Jun-06	Pen +Tet +Van (LC)	0	0	14	13.3	11	4.4
	Pen + Tet +Van (HC)	0	0	0	0	0	0
Oct-06	Pen +Tet +Van (LC)	27	22	18	16	13.3	11
	Pen + Tet +Van (HC)	9	6	11	8	4.4	3
Dec-06	Pen +Tet +Van (LC)	16	23	11	18	4.4	6.7
	Pen + Tet +Van (HC)	6.67	16	0	4.4	0	4.4
Apr-07	Pen +Tet +Van (LC)	38	44	47	58	16	4
	Pen + Tet +Van (HC)	9	13	13	18	4	4
Jun-07	Pen +Tet +Van (LC)	27	17	64	49	16	27
	Pen + Tet +Van (HC)	24	16	18	16	7	4.4
Oct-07	Pen +Tet +Van (LC)	76	49	69	71	67	58
	Pen + Tet +Van (HC)	38	27	29	22	13	18
Dec-07	Pen +Tet +Van (LC)	53	60	53	47	37	29
	Pen + Tet +Van (HC)	13	9	16	9	8	7

LC= low concentration; Pen= penicillin (5 µg/ml), Tet=tetracycline (10µg/m), Van= vancomycin (10µg/ml) HC = high concentration; penicillin (15 µg/ml), tetracycline (40µg/ml) and vancomycin (20µg/ml);(n=45*Enterococcus*)

Table 3.4 Average rainfall 5 days prior to sampling

April 2006 (rainy)	June 2006 (rainy)	October 2006 (rainy)	December 2006 (dry)	April 2007 (rainy)	June 2007 (dry)	October 2007 (dry)	December 2007 (rainy)
March 29= 0.00	May 29= 0.00	October 25= 0.45	December 6= 0.00	March 30= 0.33	June 1= 0.18	October 26= 0.00	November 29= 0.24
March30= 0.00	May 30= 1.30	October 26= 0.00	December 7= 0.00	March31= 0.16	June2= 0.00	October 27= 0.00	November 30= 0.10
March31= 0.06	May 31= 1.00	October 27= 0.00	December 8= 0.00	April 1= 0.03	June 3= 0.00	October28= 0.00	December 1= 0.12
Apri l1= 0.34	June 1= 0.10	October 28= 0.19	December 9= 0.00	April 2= 0.00	June 4= 0.00	October 29= 0.00	December 2= 0.06
April 2= 0.39	June 2= 0.44	October 29= 0.18	December 10= 0.00	April 3= 0.05	June 5= 0.00	October 30= 0.00	December 3= 0.03
April 3= 0.10	June 3= 0.00	October 30= 0.00	December 11= 0.10	April4= 0.04	June 6= 0.00	October 31= 0.00	December 4= 0.2
AVE= 0.15	**AVE= 0.47**	**AVE= 0.14**	**AVE= 0.01**	**AVE= 0.11**	**AVE= 0.03**	**AVE= 0.0**	**AVE= .12**

Table 3.5 Comparison of resistance (%) to two antibiotics of *Enterococcus* spp. isolated from water and sediments of Barceloneta-Manatí during rainy periods

River Water (Wet Period)

	P+T	T+V	P+V
April 2006	93	62	53
June 2006	8	0	0
October 2006	31	20	22
April 2007	93	53	42
December 2007	49	4	87
Average	**55**	**28**	**41**

River Sediment (Wet Period)

	P+T	T+V	P+V
April 2006	100	82	97
June 2006	5	0	0
October 2006	24	13	18
April 2007	82	60	40
December 2007	27	8	96
Average	**48**	**33**	**50**

Estuarine Water (Wet period)

	P+T	T+V	P+V
April 2006	91	24	27
June 2006	53	9	27
October 2006	18	16	16
April 2007	84	62	36
December 2007	53	13	100
Average	**60**	**25**	**41**

Estuarine Sediment (Wet Period)

	P+T	T+V	P+V
April 2006	97	82	62
June 2006	40	16	18
October 2006	16	10	26
April 2007	97	71	27
December 2007	26	20	100
Average	**55**	**40**	**47**

Coastal Water (Wet period)

	P+T	T+V	P+V
April 2006	31.11	73.3	8.89
June 2006	27	17	6.7
October 2006	6.6	20	18
April 2007	53	31	27
December 2007	31	29	97
Average	**30**	**34**	**32**

Coastal Sediment (Wet period)

	P+T	T+V	P+V
April 2006	53.3	78	18
June 2006	53.3	8.9	4.4
October 2006	10	16	13
April 2007	71	40	18
October 2007	20	17	91
Average	**34**	**32**	**29**

P=penicillin (5µg/ml), T= tetracycline (10 µg/ml), V= vancomycin (10 µg/ml)

Table 3.6 Comparison of resistance (%) to two antibiotics of *Enterococcus* spp isolated from water and sediments of Barceloneta-Manatí during dry periods

River Water (Dry Period)

	P+T	T+V	P+V
December 2006	49	34	18
June 2007	82	80	64
October 2007	51	30	69
Average	**61**	**48**	**50**

River Sediment (Dry Period)

	P+T	T+V	P+V
December 2006	76	42	27
June 2007	87	69	47
October 2007	64	31	76
Average	**76**	**47**	**50**

Estuarine Water (Dry Period)

	P+T	T+V	P+V
December 2006	53	36	11
June 2007	91	42	56
October 2007	58	38	100
Average	**67**	**37**	**56**

Estuarine Sediment (Dry Period)

	P+T	T+V	P+V
December 2006	87	27	17
June 2007	84	53	69
October 2007	69	29	98
Average	**80**	**36**	**61**

Coastal Water (Dry Period)

	P+T	T+V	P+V
December 2006	31	9	13
June 2007	64	38	49
October 2007	47	26	96
Average	**47**	**24**	**52**

Coastal Sediment (Dry Period)

	P+T	T+V	P+V
December 2006	24	27	16
June 2007	71	31	53
October 2007	42	16	93
Average	**46**	**25**	**54**

P=penicillin (5µg/ml), T= tetracycline (10 µg/ml), V= vancomycin (10 µg/ml)

Statistical Analysis

Percentage frequency of ES resistant to combinations of antibiotics for the three environment samples were calculated (See appendix B). Similarly, percentage frequency for rainy and dry periods was also calculated. In water during dry periods, 41.03% of *Enterococcus* spp. was resistant to combination of two and three antibiotics compared to 30.96% during the rainy period. Thus, in water samples during dry periods higher numbers of ES were resistant to combinations of antibiotics than during the rainy period. This is confirmed by a Z value of 3.89. The frequency of ES resistant to antibiotics in sediments during the dry period was 41.14% and during the rainy period 32.5%. Z value of 3.3 indicates there is significant difference in resistance to antibiotics in dry and rainy period sediments also.

Detailed Z values for ES resistance to combinations of antibiotics P+T, P+V, and T+V in river, estuary and coastal waters, and sediments are given in Appendix B. These values confirm the significant differences in antibiotics resistance discussed in previous section.

Relative abundance of MAR *Enterococcus* species (ES) in river, estuary, and coastal waters, and sediments

Relative abundance of multiple antibiotic resistance ES isolated from the three environments was analyzed. The antibiotics penicillin, tetracycline, and vancomycin were used and MIC for each of these antibiotics was determined. MIC for both tetracycline and vancomycin was 10 μg/mL and for penicillin 5 μg/mL. A comparison of MAR enterococci distribution in river, estuarine, and coastal waters, and sediments showed that *E. faecalis* dominates in river, estuarine, and coastal waters and sediments (Table 3.7). Predominant MAR species in the three environments in order of occurrence were *E. faecalis*, *E. hirae*, *E. durans,* and *E. faecium.*

Table 3.7 MAR *Enterococcus* spp. isolated from different sampling sites and sampling periods

	RIVER														* ESTUARINE													
	E. faeca		***E. hira***		***E. faeci***		***E. seriol***		***E. dura***		***E. haem***		***E. disp***		***E. faeca***		***E. hira***		***E. faeci***		***E. seriol***		***E. dura***		***E. haem***		***E. disp***	
Sampling Period	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**
April 2006	X	X	X	X		X			X						X	X	X	X	X			X			X	X	X	
June 2006															X	X			X	X				X				
October 2006	X	X	X		X				X								X	X			X							
Decem. 2006	X	X			X			X							X		X	X		X								
April 2007	X	X	X	X				X							X	X		X										
June 2007	X	X	X												X	X	X	X			X							
October 2007	X	X	X												X	X												
Decem. 2007	X	X	X						X	X					X	X							X					

W= Water sample S=Sediment Sample

E. faeca=E. faecalis, E.hira=E. hirae, E. faeci=E. faecium, *E. seriol= E. seriolida*, *E. dura= E. durans*, *E. haem= E. haemoproxidus* and, *E. disp= E. dispar*

Continuation...Table 3.8 MAR Enterococcus spp. isolated from different sampling sites and sampling periods

	COASTAL AREA ***													
	E. faeca		***E. hira***		***E. faeci***		***E. seriol***		***E. dura***		***E. haem***		***E. disp***	
Sampling Period	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**	**W**	**S**
April 2006	X	X	X	X		X			X					
June 2006														
October 2006	X	X	X		X				X					
Decem. 2006	X	X			X			X						
April 2007	X	X	X	X				X						
June 2007	X	X	X											
October 2007	X	X	X											
Decem. 2007	X	X	X						X	X				

E.faeca=E. faecalis, E.hira=E. hirae, E. faeci=E. faecium, *E. seriol= E. seriolida*, *E. dura= E. durans*, *E. haem= E. haemoproxidus* and, *E. disp= E. dispar*

**** E. asini was found on October 2007 in coastal sediments.*

Discussion

The frequency with which resistant enterococci are isolated from the environments is useful in monitoring the development of resistance resulting from the usage of antimicrobial agents in animal production. The intrinsic resistance of many marine bacteria is known, but a high level of antibiotic resistance in marine organisms might result from terrestrial bacteria with antibiotic resistant plasmids entering the seawater. This may explain the observed prevalence of resistance in the marine coastal water in Barceloneta, Puerto Rico. Investigations have established that terrestrial bacteria entering seawaters with antibiotic resistant plasmids may be responsible for the incidence of resistance genes in the marine environments. Coincident plasmids for antimicrobial resistance in marine bacteria have been isolated from polluted and unpolluted Atlantic Ocean samples (Baya *et al,* 1986). It is also well established that samples from different sites had different resistance to different antibiotics. Bacteria resistant to a combination of antibiotics including kanamycin, chloramphenicol, gentamicin, and tetracycline were isolated from sewage effluent samples.

The penicillin and tetracycline antibiotics have been widely used in the Barceloneta dairy industry as prophylaxes and treatment. Research has indicated that antibiotic resistance in isolates of *Listeria monocytogenes* may depend on factors such as previous exposure, the type of antibiotic used in the locality, and incidence of plasmids in the isolates (Hadom *et al.,* 1993). After years of antibiotic use it is possible that the normal flora in the human or animal intestinal track can develop antibiotic resistance and antibiotic resistance genes can be shed in feces. Raw sewage flowing into bodies of water may introduce antibiotic-resistant bacteria into the environment. To test this hypothesis a total of 2160 isolates of *Enterococcus* were characterized to species level and were tested for resistance to multiple antibiotics. Seven species of multiple antibiotic resistant *Enterococcus* were identified. Predominant species resistant to multiple antibiotics in the river, estuary, and coastal waters, and sediments in order of occurrence was *E. faecalis* followed by *E. hirae*. Different *Enterococcus* species may have developed resistant to different antibiotics. Evidence of this came from a study by Hayes *et al.* (2004) where they observed that *E. faecalis* developed more resistance to licosamide, macrolides, and tetracycline antibiotics, while isolates of *E. faecium* were observed to be more frequently resistant to fluoroquinolones and penicillin.

Higher percentages of enterococci were resistant to penicillin combined with tetracycline in river water and sediments than in estuary and coastal water. This is to be expected because the river is near to dairy industries where penicillin and tetracycline are heavily used. After October 2007, *Enterococcus* was also equally resistant to the combination of vancomycin and penicillin antibiotics. Investigations revealed that after the summer 2007, there was an outbreak of infection of *Bacillus* spp. and mycoplasma on the dairy farm. Penicillin and ramoplanin, a glycopeptides like vancomycin, was used to treat the animals.

Studies by Bitttencourt *et al.* (2007) found that in the freshwater environment, percentages of bacteria resistant to antibiotics differed between rainy and dry periods. This was confirmed by the present study in which higher percentages of ES were resistant to multiple antibiotics during the dry period than during the rainy period, even though total numbers of ES isolated during rainy periods were higher than during the dry period. A possible explanation may be that during rainy periods, there is input of nutrients from water runoff, combined with resuspension of sediments in the water. During rain energy of tides and waves can bring more organic material and resuspend sediments in the estuary and coastal waters. This may also amplify the resuspension of isolated ES attached to bio-films in the sediments. During rainy periods, total numbers of ES may increase, but survivability of resistant ES may decrease, leading to lower numbers of antibiotic-resistant bacteria in all three environments. Anderson and Levin (1999) found that bacteria sensitive to antibiotics have higher survival rates than antimicrobial resistant bacteria in surface waters during the dry season, concluding that acquisition of antimicrobial resistant phenotypes reduce bacteria fitness. Cummings *et al*. 2008 found that during rainy periods, antibiotic resistant genes were introduced into coastal waters during heavy rains and that genetic determinants were present during dry periods, but at levels below the PCR detection limit. Only after the enrichment of the media with tetracycline they were able to isolate the resistance determinants.

The predominance of antibiotic resistant *E. faecalis* may be due to the introduction of fecal material into the estuarine environment. During rainy period sewage overflow was evident. The presence of multiple antibiotic resistant species e.g. *cecorum, villorum* and *avium* on river, estuarine and coastal waters in December 2007 may be due to presence of horse, goats, and great quantities of birds during this specific sampling period. We found higher number of organisms in

sediment from river, estuarine, and coastal area that in waters from the same places. The sediments may provide a steady environment in which the proliferation can be facilitated. The sediments may also provide protection and the nutrients resulting in higher numbers of organisms that are multiple antibiotics resistant. Another possible reason for higher number of multiple antibiotic-resistant bacteria in sediments may be that resistant determinants are acquired differently within sediment and seawater environments. Support for this argument come from studies by Neela *et al.* 2007 that showed that resistance determinants in *Vibrios* spp are acquired differently within the sediment and sea water environments.

Large quantities of antibiotics are used in dairy industry in Barceloneta, Puerto Rico as growth promoters, prevention and control of infections. Mostly penicillin and tetracycline are used in large quantities. This is reflected in higher resistance of ES to these two antibiotics at minimum inhibitory concentrations (MIC), while resistance to vancomycin is low in all three environments. Even though penicillin and tetracycline were mostly used in Barceloneta, there are reported uses of vancomycin during outbreak of infection in dairy animals (Goméz, 2007). That might explain the presence of vancomycin resistant ES in all three environments. The distribution of antibiotic resistance in order of occurrence is river, estuarine and coastal water. The resistance is slightly higher near the dairy industry water and sediments than in estuarine or in coastal water and sediments. It is interesting to note the presence of ES resistance to all three antibiotics in coastal water and sediments, thus increasing the chances of transferring the resistance to marine bacteria.

Literature Cited

Anderson D.I., and B. Levin. 1999. The biological cost of antibiotic resistance. Current Opinion in Microbiology. 2: 489–493.

Arias C.A., and B.J Murray. 2002. Mechanism of antibiotic resistance in enterococci. Journal The Jornal of Biological Chemistry. 277: 358.

Bates J., Jordens J., and D.T Griffiths. 1994. Farm animals as a putative reservoir for vancomycin –resistant enterocccocal infection in man. Journal of Antimicrobial Chemotherapy. 34: 507-516.

Baya A.M., Barayton P.R., Brown V.L., Grimes D.J., Russek-Cohen E., and R.R. Colwell. 1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Applied and Environmental Microbiology. 51: 1285-1292.

Bittercourt-Lima C.I., Cursino L., Gonçalves-Dornelas H., Pontes D.S, . Nardi .M.D., Callisto M., Chartone-Souza E., and A.M.A. Nascimento. 2007. Genetic and molecular research of multiple antimicrobial resistances in enterobacteria isolate from pristine freshwater. Genetic Molecular Research (6)3: 510-521.

Cumming D. E. 2008. Resitance in coastal wetland sediments of urban and agricultural watersheds. Health Marine Ecosystem. p. 1-2.

Goméz, E. 2007. Personal communication.Situation about dairy industries in Barceloneta.

Hadom K., Hachlor H., Schrffner A., and F.A. Kayser.1993. Genetic characterizations of plasmid- encode multiple antibiotic resistances in a strain of *L.monocytogenes* causing endocarditic. European Journal of Clinical Microbiology and Infection Disease. 12: 928-937.

Hayes J. R., English L.L, Carr L.E., Wagner D.D., and S.W. Joseph. 2004. Multiple antibiotic resistance of *Enterococcus* spp. isolated commercial poultry products environment. Applied and Environmental Microbiology. 70(10): 6005-6011.

Huycke M.M., Sahm D.F., and M.S Gilmore. 1998. Multiple drug resistant enterococci: The nature of the problem and an agenda for the future. Emerging Infection Diseases. 4(2): 239-249.

Neela F.A., Nonaka L., and S. Suzuki. 2007. The diversity of multi-drug resistance profiles in tetracycline resistant *Vibrio* species isolated from coastal sediments and seawaters. The Journal of Microbiology. 45(1): 64-68.

Oquri T, Misawi S., Nakamura A., Igari J., Ishii Y., and K.Yomaguchi. 2001. Epidemiological study on vancomycin–resistant enterococci from fecal sample in the east area of Japan. Journal of the Japanese association for Infection diseases.75(7): 541-50.

Silbergeld E.K., Graham J., and L.B. Price. 2008. Industrial food animal production, antimicrobial resistance, and human health. Annual Review of Public Health. 29: 151-169.

Van den Bogaard, A.Willems E.R., London N., Top J., and E. E.Stobberingh. 2002. Antibiotic resistance of fecal enterococci in poultry, poultry farmers and poultry slaughterers. Journal of Antimicrobial Chemotherapy**.** 49: 497-505.

Vivek K. and J.W. Chow. 2002. Chapter 2. The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance. Edited by Michael S. Gilmore, 2002. ASM. Washington Press.

Chapter 4 Analysis of multiple antibiotic resistant strains in coastal sediments of Barceloneta using culture independent techniques

Introduction

To study the composition of the microorganism in the community, both culture independent and culture dependent techniques are used. Since only 0.1-10% of the total community can be cultivated, the uses of culture independent techniques are useful to evaluate the structure of a community. Analysis of the small subunit of DNA is used because of low rate of evolution and highly conserved regions (Engebretson and Moyer, 2003). The 16S rRNA sequences are ubiquitous and highly conserved, and have become the standards for many phylogenetic studies. In contrast to physiological properties, gene sequences provide an objective metric for evolutionary diversity (Pace, 1997).

Two approaches are used to evaluate the sediment microbial community in the presence of antibiotics, (1) the terminal restriction fragment length polymorphism technique (TRFLP) and (2) construction of a clone library. The profiles generated with these two techniques aided in better understanding the grouping of resistant population, which can't be detected by cultivation.

The TRFLP technique developed in 1997 by Lui *et al.* has been a useful tool to better understand the composition and phylogenetic diversity in different environments. This technique has been used for the characterization of communities exposed to different environmental conditions, e.g. changes in the microbial communities that fix nitrogen in forest soil (Yeager *et al.*, 2005). Others have used this technique to characterize communities that were exposed to hydrocarbon, metal, and chemical contaminants (Ayala *et al.,* 2004). The TRFLP technique produces distinctive patterns of the community that can be used to evaluate the similarities and diference in different communities as well as temporal and spatial changes in the community structure in response to environmental changes. In this technique, the DNA is extracted from the environmental samples and the 16S rRNA gene is amplified. Usually the forward primer is fluorescent label. The amplified products are digested with restriction enzymes and the proximal products are separated by weight on a polyacrilamide gel. Each T-RF's generated is defined like

one Operational Taxonomic Unit (OTU's) and can be inferred like one population inside the community (Engebreston and Moyer, 2003 and Osborne *et al.,* 2006).

The second technique for the identification of the community structure is to construct 16S rRNA gene metagenomic clone libraries. Through this approach it is possible to amplify the 16S rRNA genes obtained directly from environmental DNA through Polymerase Chain Reaction (PCR) based techniques, cloning and sequencing of such environmental genes. With the sequences obtained phylogenic analyses are generated to help in the characterization of the community.

The following were the objectives of the present study:

(1) Evaluate the survivability of multiple antibiotic resistance strains in Barceloneta coastal sediments.

(2) Characterize multiple antibiotic resistant (MAR) strains.

(3) Determine dominant group and demonstrate the variation of organisms that could contribute to the spreading of resistance.

(4) Study the changes associated within the community due to the presence of antibiotics.

In the sediment samples, antibiotics were introduced and variation within the community was observed with the help of TRFLP. The samples without antibiotics were the controls. Predominance of the taxonomical groups before and after use of antibiotics was determined.

Material and Methods

Collection of sediment sample and microcosm preparation

Barceloneta sediment samples were collected in whilpark packs during the sampling period of June 2006. After collection the samples were transported to the lab. Some samples were frozen until DNA extraction; others were used for the construction of the systems to make the evaluation of the communities using T-RFLP. The controls and treatment systems were prepared as followed:

Table 4.1 Preparation of the systems for T-RFLP

Systems	Description	Contents
Systems #1 and #2	Controls	Sediments (20g) + 20mL sterile water + nutrient
Systems #3 and #4	Treatment 1	Sediments (20g) + 20mL sterile water + nutrient+ Penicillin (10µg)
Systems #5 and #6	Treatment 2	Sediments (20g) + 20mL sterile water + nutrient + Penicillin (10µg) + Tetracycline (20µg)
Systems #7 and #8	Treatment 3	Sediments(20g) + 20mL sterile water + nutrient + Penicillin (10µg) + Tetracycline (20µg) + Vancomycin (20µg)

The controls and experimental systems were place on shaker at 40 rpm for one week. After one week DNA was extracted from systems 1, 3, 5 and 7, the systems 2, 4, 6 and 8 were left on the shaker for one additional week. After this period the DNA was extracted.

DNA extraction and PCR amplification of 16S rRNA genes

DNA was extracted from 0.5 g of soil using Q-biogene FastDNA® SPIN soil kit (MP Biomedicals USA). PCR reactions for 16S rDNA bacterial genes were amplified using the primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1392R (5'-

ACGGGCGGTGTGTRC-3') (Sullivan *et al*., 2002). For PCR reaction 1 µl of DNA template, 12.5 µl of GoTaq® Green Master Mix, 2X (Promega, Madison, WI), 2.5 µl of primer 27F (10 ρM/µl) 0.5 µl of primer 1392R (50 pM/µl), .25 µl of BSA, and 8.25 µl nuclease free water were used in a 25 µl reaction volume. The following steps were employed: 1 cycle at 95°C for 5min; 28 cycles, each consisting of 94°C for 1 min, annealing at 55°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 7 min. PCR reactions was performed in PTC-100 Petier Thermal Cycler (MJ Research). The amplification products were visualized in 1% agarose gel electrophoresis with ethidium bromide, which were run at 90 V for 30 min and observed using an ultraviolet transilluminator.

Terminal Restriction Fragment Length Polymorphism of 16S rRNA amplicons

The T-RFLP technique primer 27F was labeled with Infra Red Dyes IR700 (LI-COR, Lincoln, NE). The PCR reaction using the labeled primer was: 1 cycle at 95°C for 3 min; 25 cycles, each consisting of 94°C for 1 min, annealing at 50°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 7 min. PCR products were separately digested with the following enzymes: *Hae* III, *Rsa*, and *Msp* I. Each digestion reaction consisted of 1.5 µl of 10X reaction buffer, 1 unit of each restriction enzyme, 5 µl of PCR product, and 3.5 µl of double distilled water (ddH_2O) for a total reaction volume of 10 µl. The digestions were incubated at 37°C for 4h followed by 10 min at 65°C to inactivate the enzymes and a 4°C final step. An aliquot of 2 µl of restriction product was mixed 1:1 with IR2 stop solution (LI-COR, Lincoln, NE) and 1 µl was electrophoresed in a LI-COR Biosciences NEN®DNA Analyzer Model 4300 (LI-COR, Lincoln, Inc.) The acrylamide gel was prepared to a final concentration of 5.5% by mixing acrylamide gel matrix (KB_{Plus}-LICOR) with BT buffer, adding 150 µl of 10 % ammonium per-sulfate and 20 ml of 15% of TEMED. The samples were denatured at 95ºC for 5 minutes and kept at 4ºC until loading the gel. A gel pre-run consisted in a run of 20 min with 1X buffer (KB_{Plus}-LICOR) with the following parameters: 1,500 V voltage, 40 mA current, and 40W of power. After pre-run, 1µl of each denatured sample was loaded, and the gel was run for 3 hours using the same pre-run parameters. A KB_{Plus}-LICOR molecular sizing standard of 50-700 bp was used. Band analysis is performed to obtain T-RFLP profiles using Gel-Pro Analyzer 4.5 (Media Cybernetics, Silver Spring, Maryland, USA). Fragments of T-RFLP were analyzed to determine the size and

abundance of the fragments. The fluorescent terminals were compared with a standard of 50-700 bp molecular weight.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

The first part of the analysis of the communities was generated by the evaluation of the electropherogram after the selection of adequate bands and peaks. The samples treated with different enzymes were evaluated according to the peaks generated by optical density between the 50 and 700 bp. The T-RFLP profiles were evaluated using one scripts written on CLisp (Haible *et al.,* 2005) and *PEAKS* for determining real peak bands (Caro, 2008). The final binned matrix was used to compare microbial communities from different samples. The matrix was exported to Paleontological statistics package ver. 1.79 (PAST) (Hammer *et al.,* 2001). The similarity of the community was determined using correspondence analysis (CA). This analysis provides a graphic representation of the sample. Scatter plots generated by CA were used to identify grouping of t-RFLP microbial community profiles.

Cloning of 16S rRNA gene and sequencing

The DNA product from the system 1 (control system) and system 7 (system with three antibiotics) (Table 4.1) were used for PCR and to construct clone libraries. The primers used to construct two clone libraries were 1392R (5'-ACGGGCGGTGTGTRC-3') and 27F (5'-AGA GTTTGATCMTGGCTCA-3') (Sullivan *et al.,* 2002). PCR were performed as described before. A molar ratio of 3:1 PCR product to vector was cloned using pGEM®-T Vector System (Promega) as described by the manufacturer. Positive clones were identified by white/blue selection in Luria Bertani agar (LB) with 100 µg/ml ampicillin, 0.5 mM IPTG, and 80 µg/ml X-Gal (LB/Amp/IPTG/Xgal). Positive clones were grown in 5ml of LB liquid media and plasmid extraction was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega). The DNA concentration of plasmid isolation was measured spectrophotometrically at 260 nm and inserts were sequenced using SP6 and T7 vector primer. The sequences of the promoter were: T7 (5'-ATTTAGGTCACACTATAGAA-3') and SP6 (5'-TAATACGAC TCACTATAGGG-3'). A quantity of 12 µg/ml with a concentration of 50 ng/ml of purified PCR products were sent to the Nevada Genomic facility for sequencing.

Sequence and phylogenetic analysis of clone library

The sequences obtained from the Nevada Genomics facilities were submitted to pipeline analysis. Vectors sequences were deleted and the sequences were submitted to quality analysis to remove quimeric sequences. Clone sequences were analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Only sequences that were inserted in forward orientation during pGEM cloning were used to phylogenic analysis. Reference sequences were obtained by submitting clone sequences in Sequence Match tool (rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) at the Ribosomal Data Project (RDP). The alignment of the clone sequences and the construction of phylogenetic tree were made using Mega 4.1 software (Tamara *et al.,* 2007). The phylogenic trees were made using the Neighbor-joining (NJ) method and *p*-distance model with a bootstrap of 1000. For each clone library a libcompared was made using algorithms of the RDP to make a comparison in term of taxonomy present in both libraries.

Results

DNA extraction and PCR amplification of 16S rRNA genes

The DNA from Barceloneta sediments was extracted using a kit supplied by Qbiogene Company (www.qbiogene.com). The results are shown in the Figure 4.1. Other extraction kits (MO BIO, Carlsbad, CA) were also used for the extraction but the product was not enough for PCR reaction. The PCR using universal primers were 27 F and 1392 R. The amplification of PCR product was successfully obtained. Because of the presence of additional low intensity bands obtained in PCR, it was necessary to purify the PCR product from agarose gel (Figure 4.2). The PCR products were purified using Wizard SV Gel and PCR Cleanup System Purification Kit (Promega Inc.).

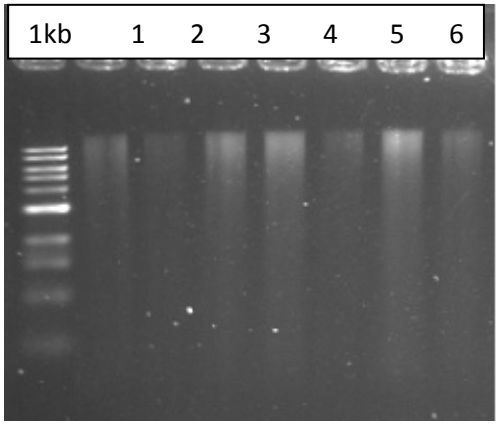


Figure 4.1 Genomic DNA Extraction from Barceloneta sediments. M=Marker 1Kb (1) control sample; (2) one antibiotic after one week; (3) one antibiotic after two weeks; (4) two antibiotics after one week; (5) two antibiotics after two week; and (6) three antibiotics after one week

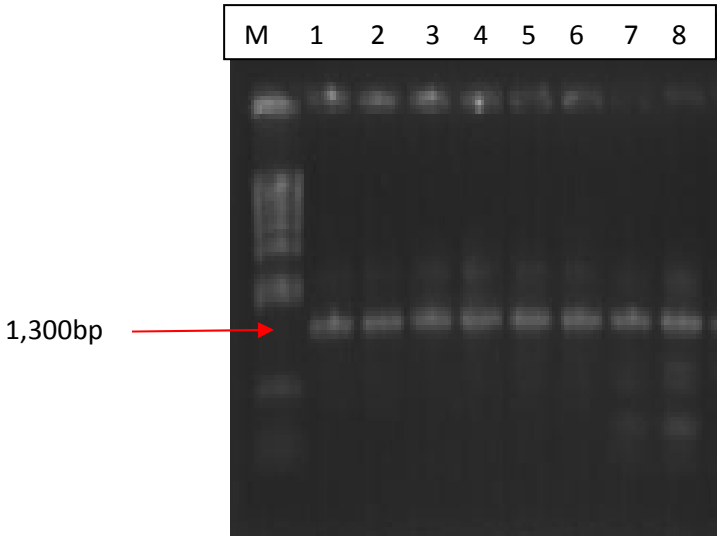


Figure 4.2 PCR products from Barceloneta sediments with the different systems. M=marker 1kb (1) control sample after one week; (2) control sample after two weeks; (3) one antibiotic after one week; (4) one antibiotic after two weeks; (5) two antibiotics after one week; (6) two antibiotics after two week; (7) three antibiotics after one week; and (8) three antibiotics after two weeks.

Terminal Restriction Fragment Length Polymorphism of 16S rRNA amplicons

The PCR with the labeled primer 27F was modified because the amplification was not obtained in sediment samples with the same PCR cycle using the 27F unlabelled primer. The modifications consisted of decreasing the number of cycles from 28 to 25 and decreasing the annealing temperature from 55 to 50°C. After the amplification the samples were digested using the enzymes *Hae* III, *Rsa*, and *Msp* I. After the digestion the T-RFLP profiles were obtained in a LI-COR Biosciences NEN®DNA Analyzer Model 4300 (LI-COR, Lincoln, Inc). The Figure 4.3 present the gel generated in the LI-COR DNA Analyzer.

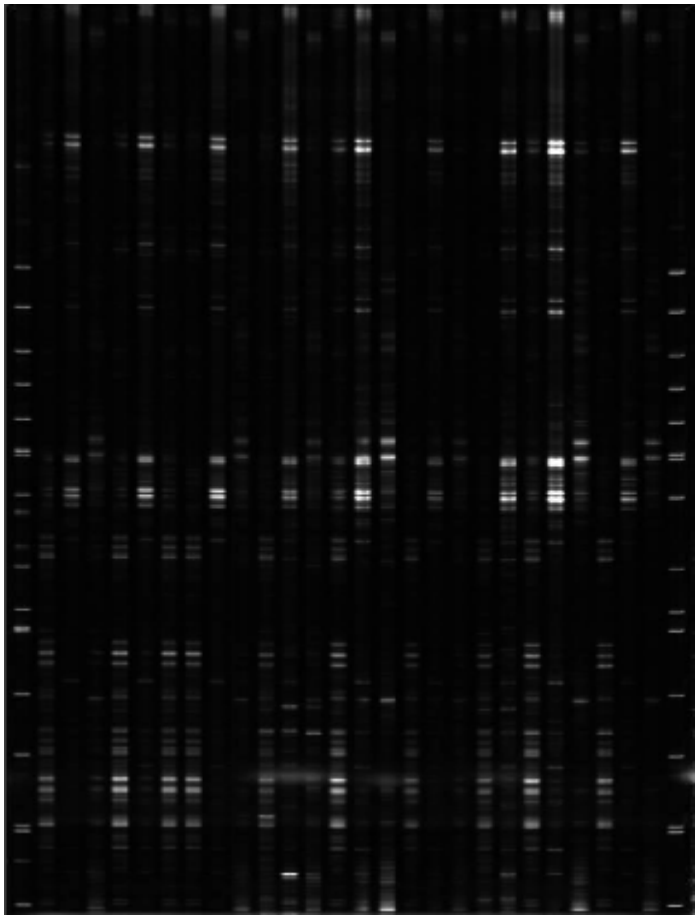


Figure 4.3 Image of gel generated by Lycor DNA Analyzer®

Marker =M (KB_{Plus}-LICOR molecular sizing standard of 50-700 bp)

Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis

Tables 4.2 to 4.4 show the electropherograms peaks obtained for each sample with each enzyme used. The enzymes used were *Rsa*, *Hae* III and *Msp* I. The peaks were evaluated after the exclusion of bands that did not correspond to real peaks or correspond to a molecular weight between 50 and 700 bp. Among the restriction endonucleases used for the tRFLP, *Rsa* gave us better resolution of fragments, although with *Hae* III the variation between the same samples in terms of the intensity of optical density are more evident. The majority of the TRF's that are seen in the control are also evident in the treated sample, but treated samples have some additional fragments. (For generation electropherograms peaks see Appendix 3)

When *MSP* I endonuclease is used in control sample after one week, OTU's between 50-100 bp, 100-204 bp, and 300-460 bp are noted (Table 4.2). Comparatively, this enzyme shows lowest generation of OTU's. After two weeks, the majority of the OTU'S are lost and the remaining OTU are of low intensity. In treated sample with one and two antibiotic new peaks are seen after one week between 530-600 bp. Two peaks (r^{22} 460-530 bp and r^{41}100-204 bp) are present in all samples at every stage, although after two weeks the intensity is lower. With this enzyme, the presence of a new OTU in 460 bp (r^{23}) is generated only in the treated samples and is conserved even after two weeks.

With the *Hae* III enzyme in the control group, OTU's between 100-204 bp, 300-460 bp, 460-530 bp are found, and an additional peak at 650 bp (r^{13}) is observed (Table 4.3). In all treated samples in the initial stage, the same OTUs between 364-460 bp are observed, but intensity of the peaks at the final stage decreased. Some peaks disappeared in the treated samples compared to the control sample. The samples treated with two antibiotics show additional peaks at <100 bp (r^{50}) and between 300-364 bp (r^{32}). There is also disappearance of peak at <100 bp (r^{50}) in samples treated with three antibiotics. In each of the samples at each stage, a peak at 364 bp (r^{29}) was present, although after two weeks, the size of the peak decreased.

With *Rsa* endonuclease, OTU's between 50-110 bp, 100-204 bp, and 300-460 bp were obtained in the control sample (Table 4.4). In the sample treated with one antibiotic for one week, the number of OTU's decreased and the optical density of the peaks also decreased. After two weeks incubation with one antibiotic (final stage), the majority of OTU's present between

300 bp and 460 bp disappeared; only r^{33} and r^{35} peaks remained. Initially there was no difference in OTU's in samples treated with three antibiotics. The intensity of peaks with three antibiotics after one and two weeks was similar to samples treated with one or two antibiotics. However, the intensity of the peaks decreased after two weeks in samples treated with one or two antibiotics, but not in samples treated with three antibiotics. A new OTU at 300 bp (r^{34}) was also evident in all treated samples. The same peak was appeared in the control sample after two weeks. In all the samples, a peak <100 bp (r52) was present and remained in all stages in all the samples, although after two weeks, the intensity was lower.

In general, the diversity in microbial populations in samples treated with one or two antibiotics was higher than the sample treated with three antibiotics. Also the distribution of peaks appeared to be similar to the control samples, even though some new OTU's are apparent in the treated samples. The most diverse community of the treated sample corresponds to the samples treated with one antibiotic (penicillin). In these samples, new OTU's, highest peaks, and higher variations were found compared to two and three antibiotics. Shared peaks in control and treated samples showed the presence of consistent community before and after treatment. The abundance of peaks generated by the electropherograms indicated the presence of different taxonomic groups in the sediment samples of Barceloneta, Puerto Rico.

Table 4.2 Peaks obtained in the electropherograms with *Msp* I

Treatment	530 bp-700 bp	300 bp-530 bp	100 bp-300 bp	< 100 bp
Control (T1)		24,27,29,32,33,35	37,38,39,40,41,43,45,46,47	49,50,52,53,55
Control (T2)		22,24	41,43	49,50
1antibiotic (T1)	11,12,14,15	17,18,19,22,23,24,27, 29	41	49,50,52,55
1 antibiotic (T2)		22,23	41	
2 antibiotics (T1)	14,15	17,18,19,22,23,27	41	55
2 antibiotics (T2)		22,23	41	
3 antibiotics (T1)		22,23	38,39,41	49,50,52
3 antibiotics (T2)		22,23	41	

T1=Initial stage T2=Final Stage

Table 4.3 Peaks obtained in the electropherograms with *Hae* III

Treatment	530 bp-700 bp	300 bp-530 bp	100 bp-300 bp	<100 bp
Control (T1)	10,12,13,16	18,22,23,24,25,26,27,28,29,30,33	40,41	
Control (T2)	13	24,25,26,27,28,29,34	41,43	48,49
1antibiotic (T1)	12,13	20,22,24,25,26,27,28,29,33	40,43	48
1 antibiotic (T2)	13	22,24,26,27,28.29		
2 antibiotics (T1)	12,13	23,24,25,26,27,28,29,32	40	48,49,50
2 antibiotics (T2)	12,13	24,26,27,28,29		
3 antibiotics (T1)	13	23,24,25,26,27,28,29,32	40	
3 antibiotics (T2)		23,24,25,26,27,28,29		

T1=Initial stage T2=Final Stage

Table 4.4 Peaks obtained in the electropherograms with *Rsa*

Treatment	300 bp-530 bp	100 bp-300 bp	<100 bp
Control (T1)	23,24,26,27,28,32,33,35	37,39,40,41,43,45,46,47	49,50,51,52,53,55
Control (T2)	27,28,32,33,34	38,39,41,42,43,47	49,50,51,52,55
1antibiotic (T1)	32,33,34	37,38,39,40,41,43,44,45,46	49,50,51,52,53,55
1 antibiotic (T2)	33,35	38,40,43,46	49,50,52,55,
2 antibiotics (T1)	32,33,34	37,38,39,40,41,42,43,46	49,50,51,52,55
2 antibiotics (T2)	35	39,40,41,42,43,45,47,48	49,50,51,52,53,55
3antibiotics (T1)	32,33,34	37,38,39,40,41,43,45,46	49,50,52,55
3 antibiotics (T2)	32,35	37,39,40,41,43,46	49,50,52,55

T1=Initial stage T2=Final Stage

The correspondence analysis in the Figure 4.4 represents the difference in aggregation of OTU's of Barceloneta from the different systems. Disappearance of OTU's in treated samples indicates that there was a difference between community structures in treated versus control systems. The dispersion of data represents community variations. Concentration of OTU data points can be correlated with the number of OTU's shared by the different systems. The samples treated with one and two antibiotics are more related to the control community, as shown by the closer distribution. The community represented by the system with three antibiotic, may be distinct compared to other systems and is demonstrated by the separation from other systems.

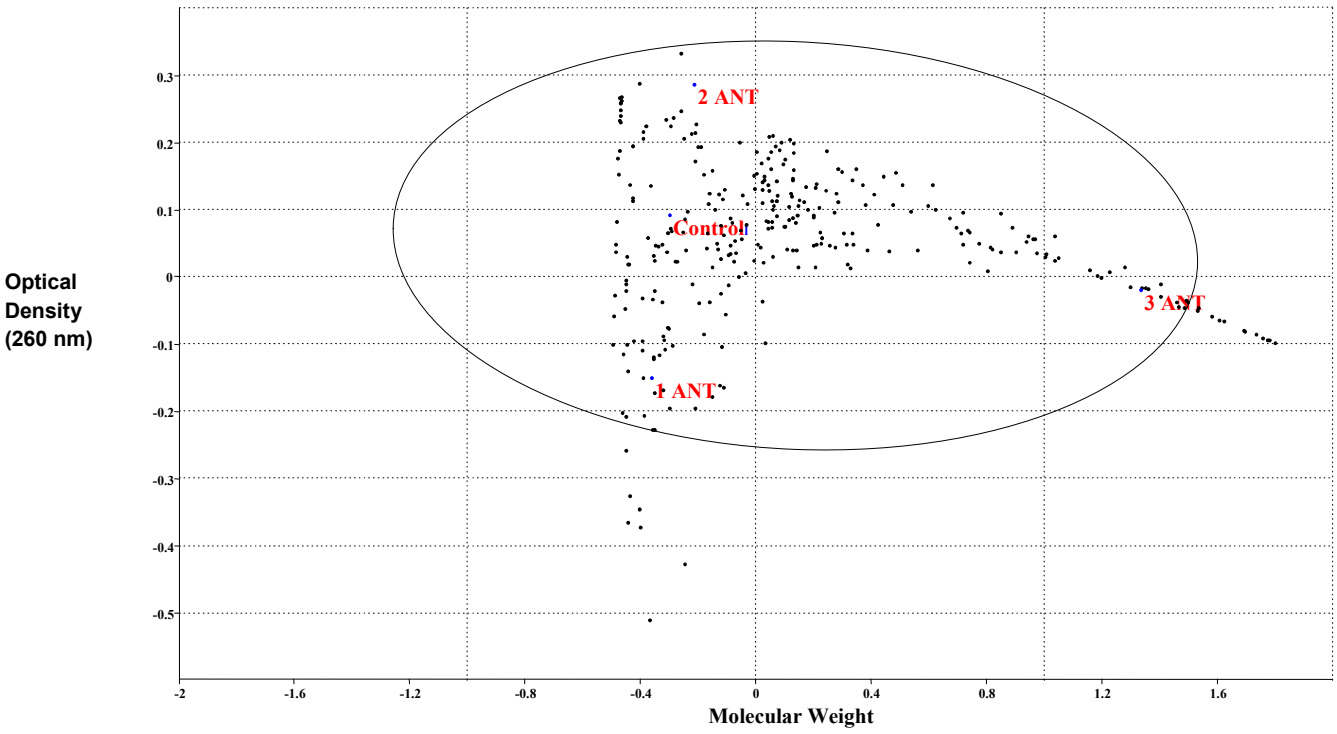


Figure 4.4. Correspondence analysis of 16S rRNA T-RFLP profiles from the sediment community of Barceloneta.

Cloning of 16S rRNA gene and sequencing

Two clone libraries were obtained from the control systems and the systems with the three antibiotics to evaluate the variability of the communities in the Barceloneta sediments. Figure 4.5 shows the genomic DNA extracted from the control and antibiotic treated system. The DNA was used to generate the PCR (Figure 4.6) and cloned using pGEM®-T Vector System from Promega Company. Positive clones were selected with the cloning system and plasmid extraction was performed using Wizard® Plus SV Minipreps DNA Purification System (Promega). Figures 4.7 and 4.8 show some of the purified plasmids. The purified PCR plasmid was sent to Nevada Genomic and the results of the sequences were used for construction of clone libraries and phylogenetic analysis.

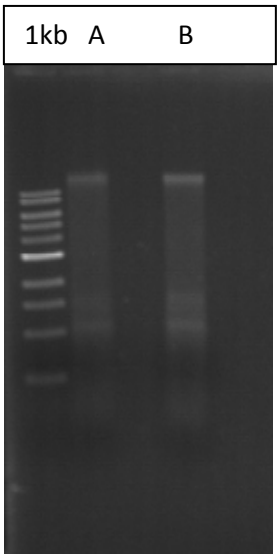


Figure 4.5. Genomic DNA extraction from Barceloneta sediments.
M=Marker (1 Kb) A= control sample B= treatment sample (antibiotics)

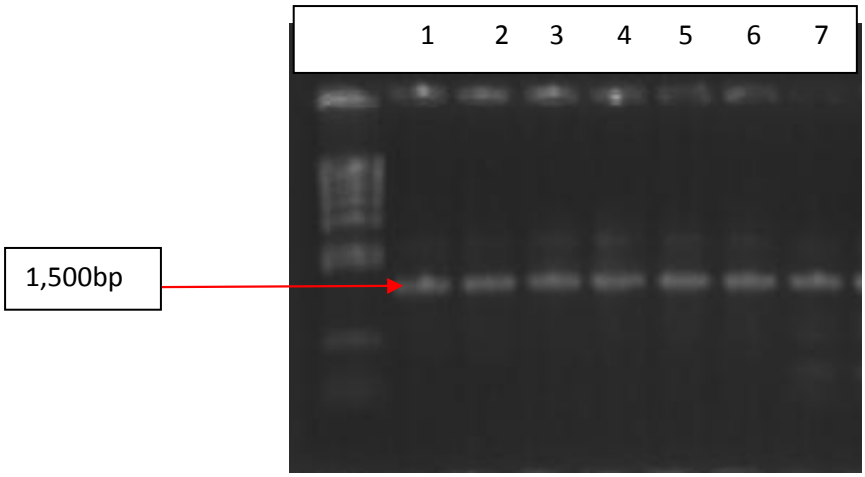


Figure 4.6. PCR product that was cloned with pGEM®-T Easy Vector System. M=Marker: DNA/EcoRI+Hind III lane 1= positive control; lane 2-4 (Master Mix Library 1); and lane 5-7 (Master Mix Library 2)

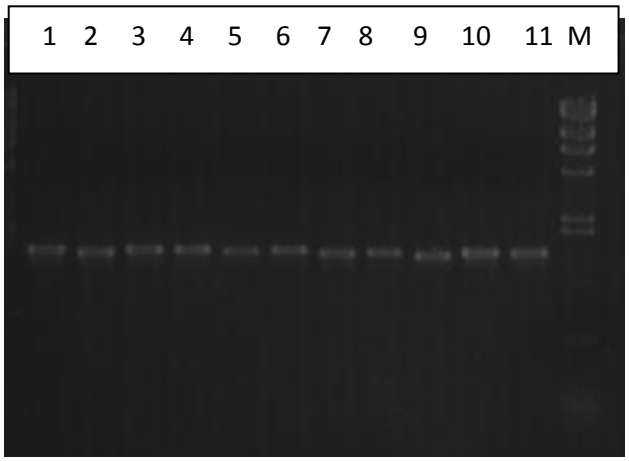


Figure 4.7 Plasmid extraction from eleven samples from library 1 (control)

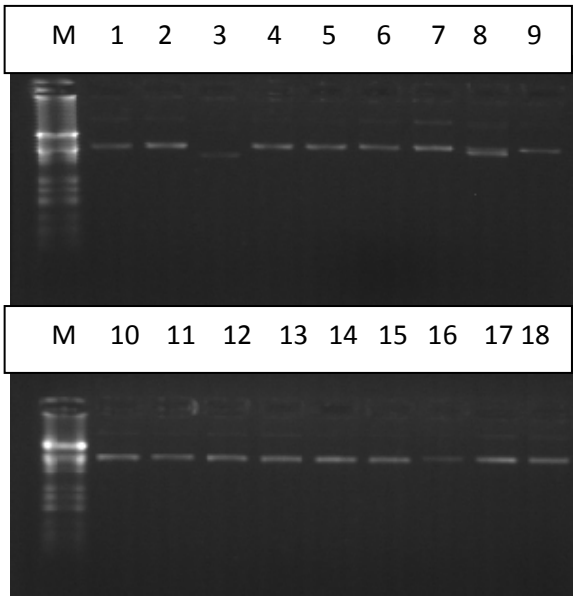


Figure 4.8 Plasmid extraction from eighteen samples from library 2 (with antibiotics)

Sequence and phylogenetic analysis of clone library

Using clone libraries, community composition in Barceloneta-Manatí sediments was determined in the control samples as well as samples treated with antibiotics. With the phylogenetic analysis conducted by Mega 4 software, two trees were obtained representing two clone libraries (Figures 4.9-4.10). The first clone library corresponded to 50 clones and the second consisted of 48 clones that were antibiotic resistant. The phylogenetic analysis of the first tree revealed that the majority of the strains recovered from the Barceloneta sediments belonged to the Phylum Actinobacteria, Proteobacteria, and Plantomycetes. Other phylogenetic groups represented in library 1 are Acidobacteria and Nitrospirae. The second tree represents microorganisms that are resistant to antibiotics (Figure 4.10). This group is dominated by Proteobacteria, with delta and gamma Proteobacteria the most dominant, but the Phyla Nitrospirae was not present in the second tree.

The clones analyzed from the control and treated samples were clustered in different OTU's. The clones analyzed from the control sediment sample from Barceloneta-Manatí were

clustered in eight different OTU's. Clones AO1, HO5, and HO2 corresponded to OTU one**.** This OTU presented two clusters closely related to *Pseudomonas* spp. and *Schlegelella thermodepolymer*a from the Phylum Proteobacteria. The second OTU was represented by GO4 and formed a cluster with Sphingobacteriales bacterium from the Phylum Bacteroidetes. The third OTU was represented by B04, E04, G01, C01, C03, and D02, and formed two clusters with independent branches that were similar to organisms within the Phylum Acidobacteria. D05 represented the fourth OTU clustered with *Acidimicrobium ferooxidans* from the Phylum Actinobacteria. B05 corresponded to OTU five related to *Candidatus Magnetobacterium* from the Phylum Nitrospira. The clone E03 and A03 corresponded to the Phylum Chloroflexi related to *Dehalococcoides* spp. and represent the OTU six. OTU seven corresponded to A06, CO5, F02, and F03 that are clustered with *Plantomyces* spp. The OTU eight is represented by an independent branch of uncultured clone.

The clones analyzed from treated sediment samples were clustered in six different OTU's. 2A06, 2GO4, 2C01, 2B04, represented the first OTU with representatives of the Proteobacteria Phylum. 2A06, and G04 form a branch with sulfur oxidizing bacterium, 2C01 with *Hialiangium tepidum*, and B04 with *Desulfarculus baarsii*. The second OTU was represented by 2FO6 from the Bacteroidetes phylum and formed a cluster with *Cytophaga* spp. The clone 2G01 and 2H01 represented the third OTU and formed an independent branch near *Rubrobacter xylanophilus* which belonged to the Actinobacteria phylum. The four OTU was represented by 2GO5 from the Phylum Firmicute, forming a branch with *Streptococcus salivarus.* 2FO4 was the fifth OTU and was near the cluster of *Dehalococcoides* spp. from the Chloroflexi phylum. Twenty-three clones formed the sixth OTU. These clones were clustered in an independent distinctive branch. These clones were associated with uncultured clones according to the similarity analysis.

. Phylogenetic trees constructed from both clone libraries indicate an aggregation of uncultured clones, indicating that the sequences of the clones cannot be compared with reference strains. Additional strains were evaluated with this clone but classification was not possible. The alignment of the sequences with the reference strains can be the reason for not obtaining separation between the clones in the libraries. Evaluation of individual strains with gene sequences banks indicated that they are more closely related to uncultured strains. According to

an algorithm develop by the RDP, the clones were categorized by the S_{ab} score value that show how close are other organisms in the sequence bank (Tables 4.5 and 4.6).

We compared the two libraries using the *libcompared* algorithm developed by RDP II. Proteobacteria was dominant in both libraries. The percentages of Proteobacteria obtained were 46.8% in library 1 and 19.4% in library 2. Environmental sequences belonging to the □-Proteobacteria were most abundant in both libraries (11.1% Lib 1 and 28.1% Lib 2) (Figures 4.11 and 4.12). The Phylum proteobacteria was favored when antibiotics were present in the system in comparison with other phylogenetic groups (Figure 4.12). The class of δ-proteobacteria (3.1%) only could be identified within the library where the DNA was exposed to the antibiotic treatments and β-proteobacteria (5.6%) only was identified in the control library. Representatives of the Phylum Chloroflexi where characterized within the original community but not in the treated sample. Clones that belong to the Phylum Firmicutes were obtained within the clone library that was treated with antibiotics.

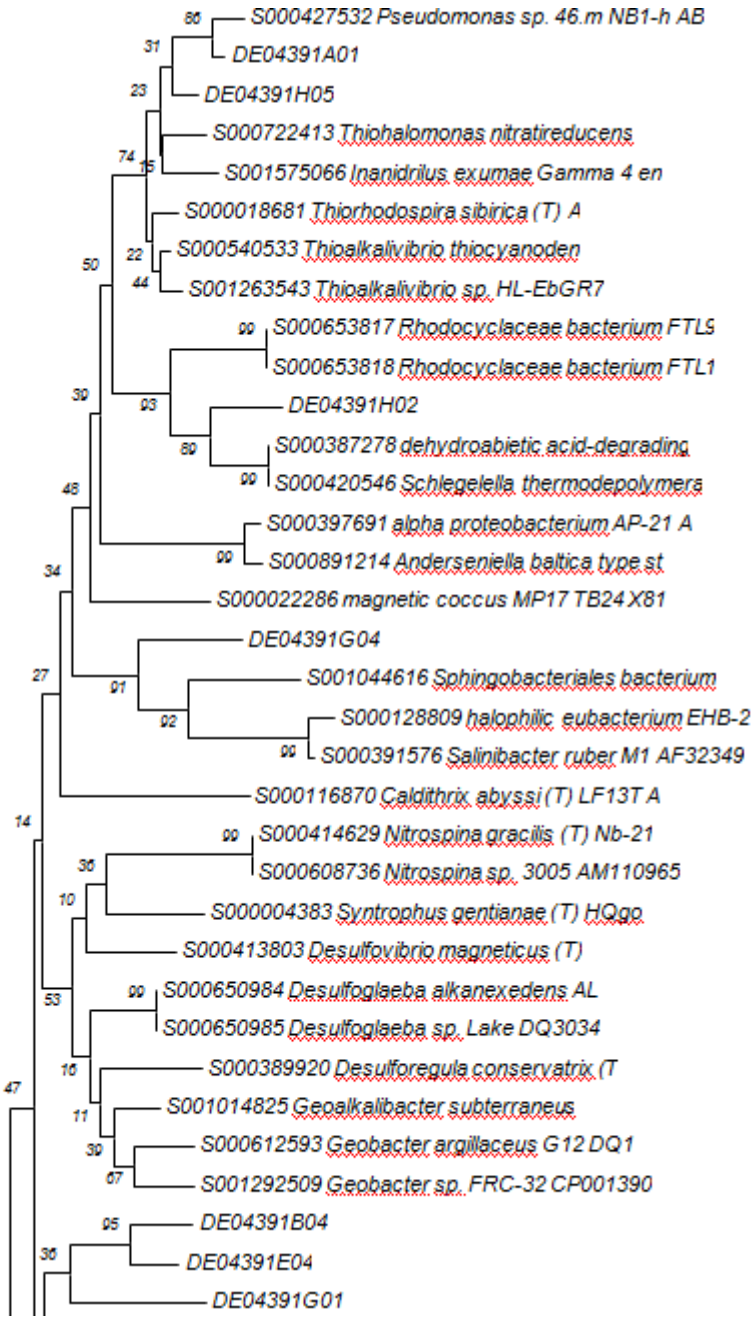
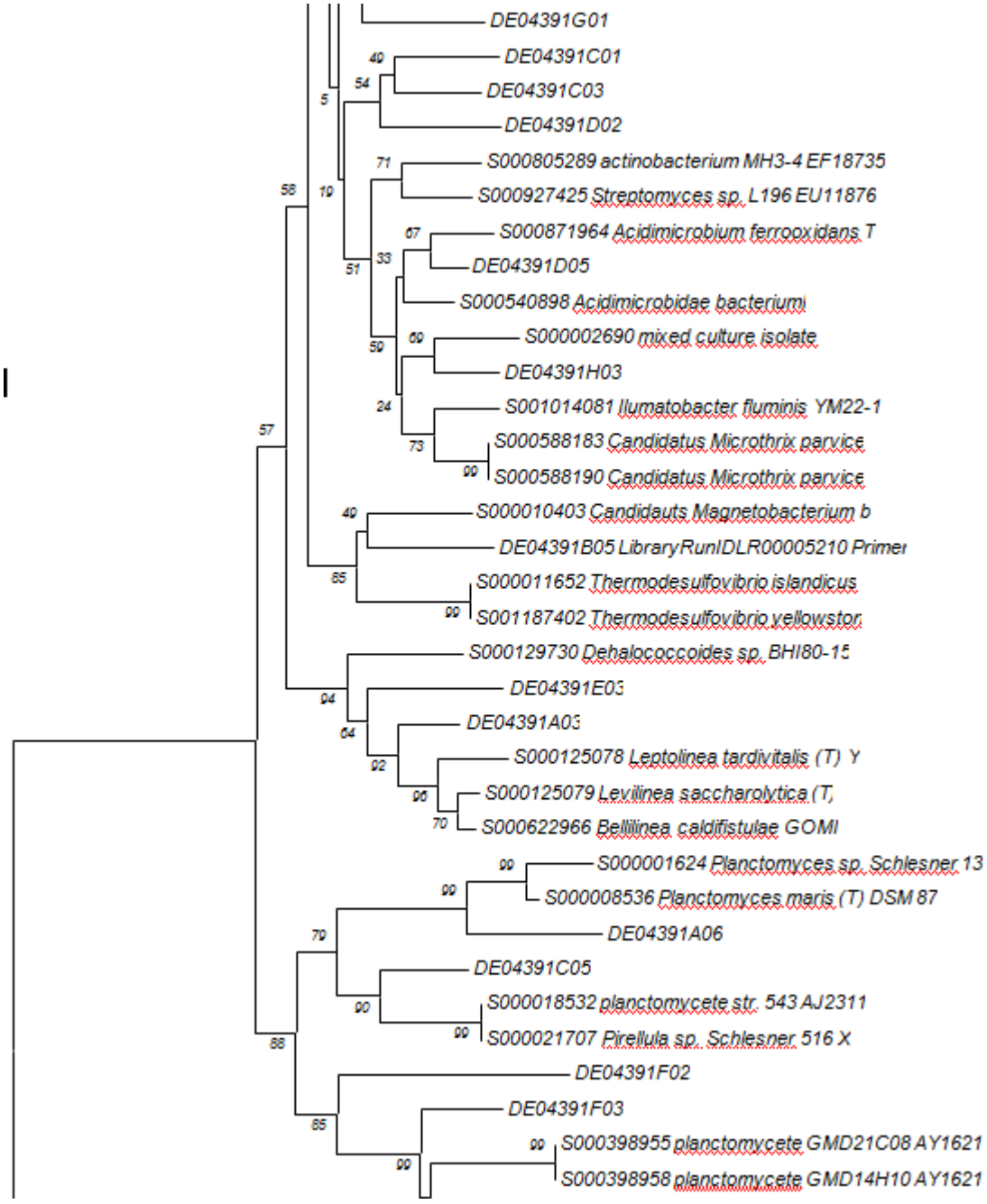


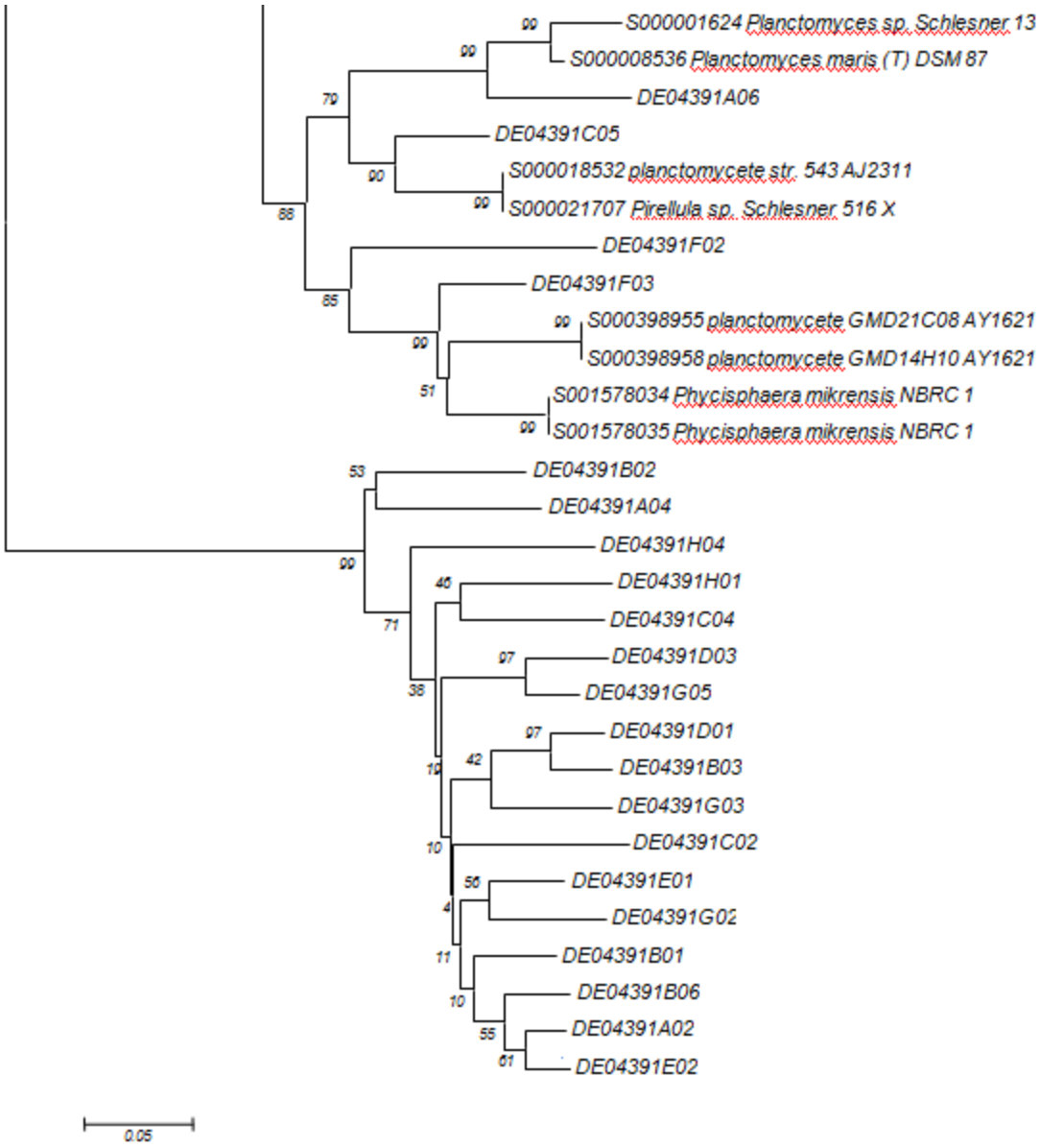
Figure 4.9. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library I isolates from the Barceloneta coastal sediment.

Bar represents 1 substitution per 100 nucleotides.



Continuation of Figure 4.9. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library I isolates from the Barceloneta coastal sediment.

Bar represents 1 substitution per 100 nucleotides.



Continuation of Figure 4.9. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library I isolates from the Barceloneta coastal sediment.

Bar represents 1 substitution per 100 nucleotides.

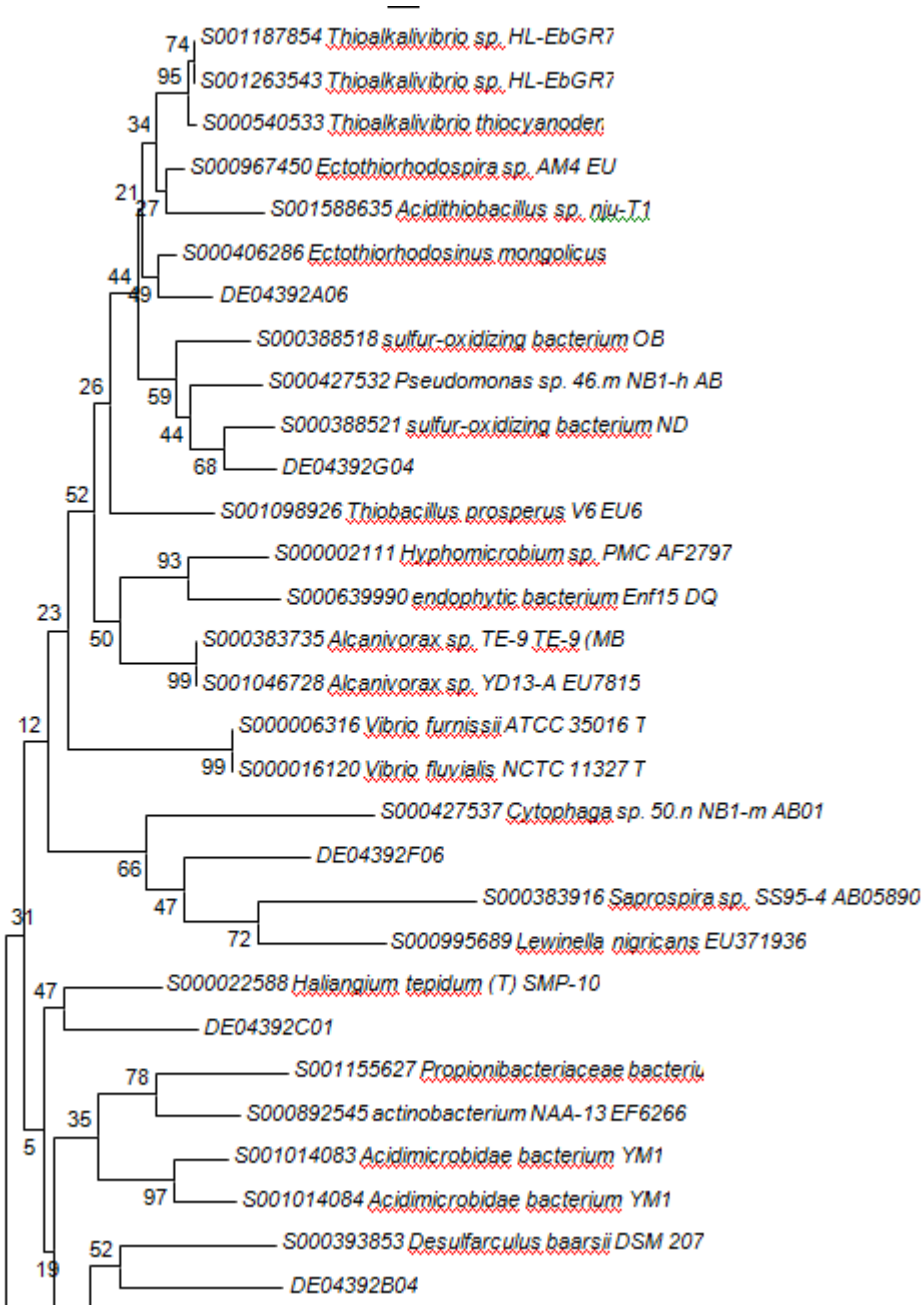
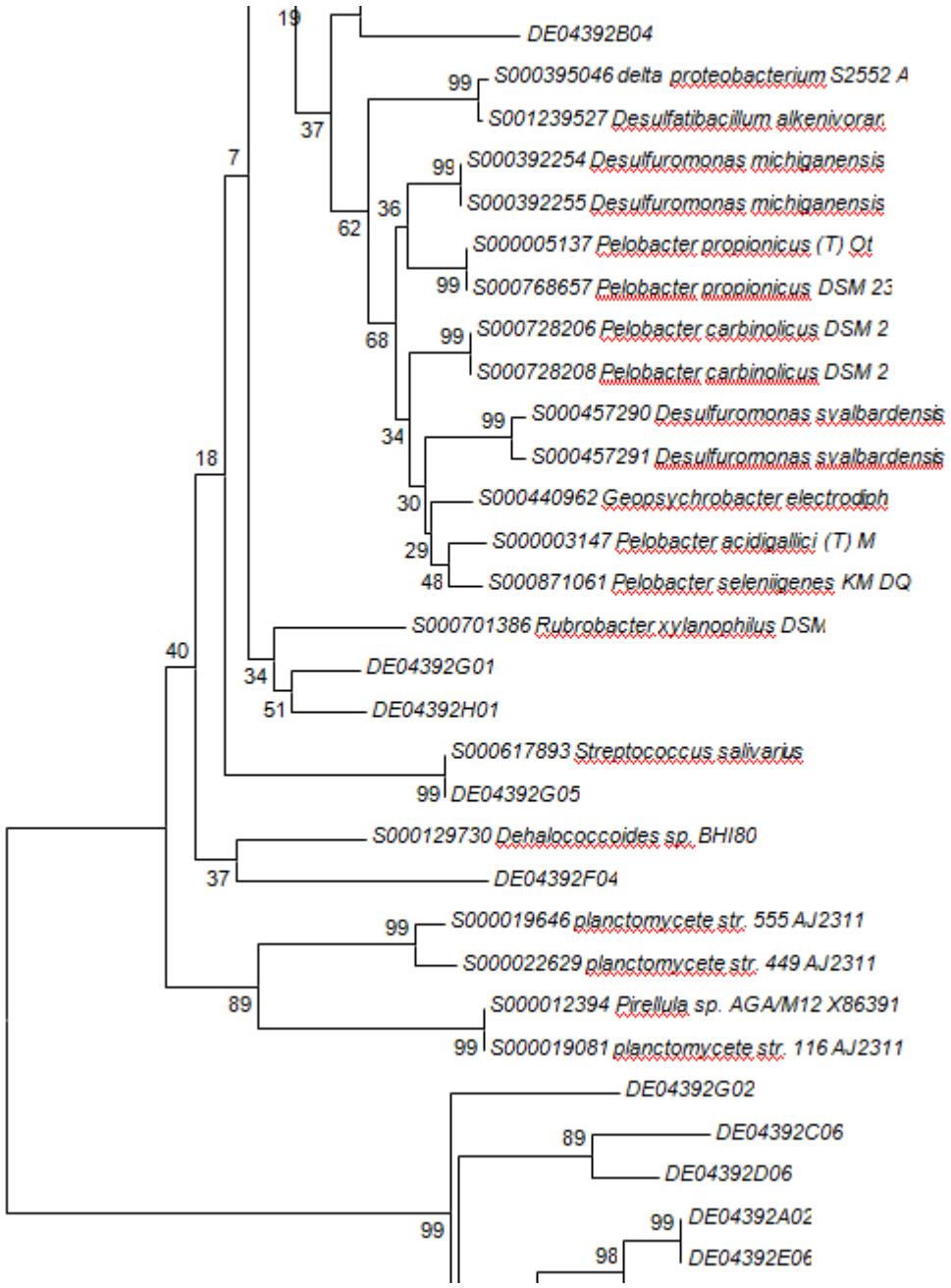


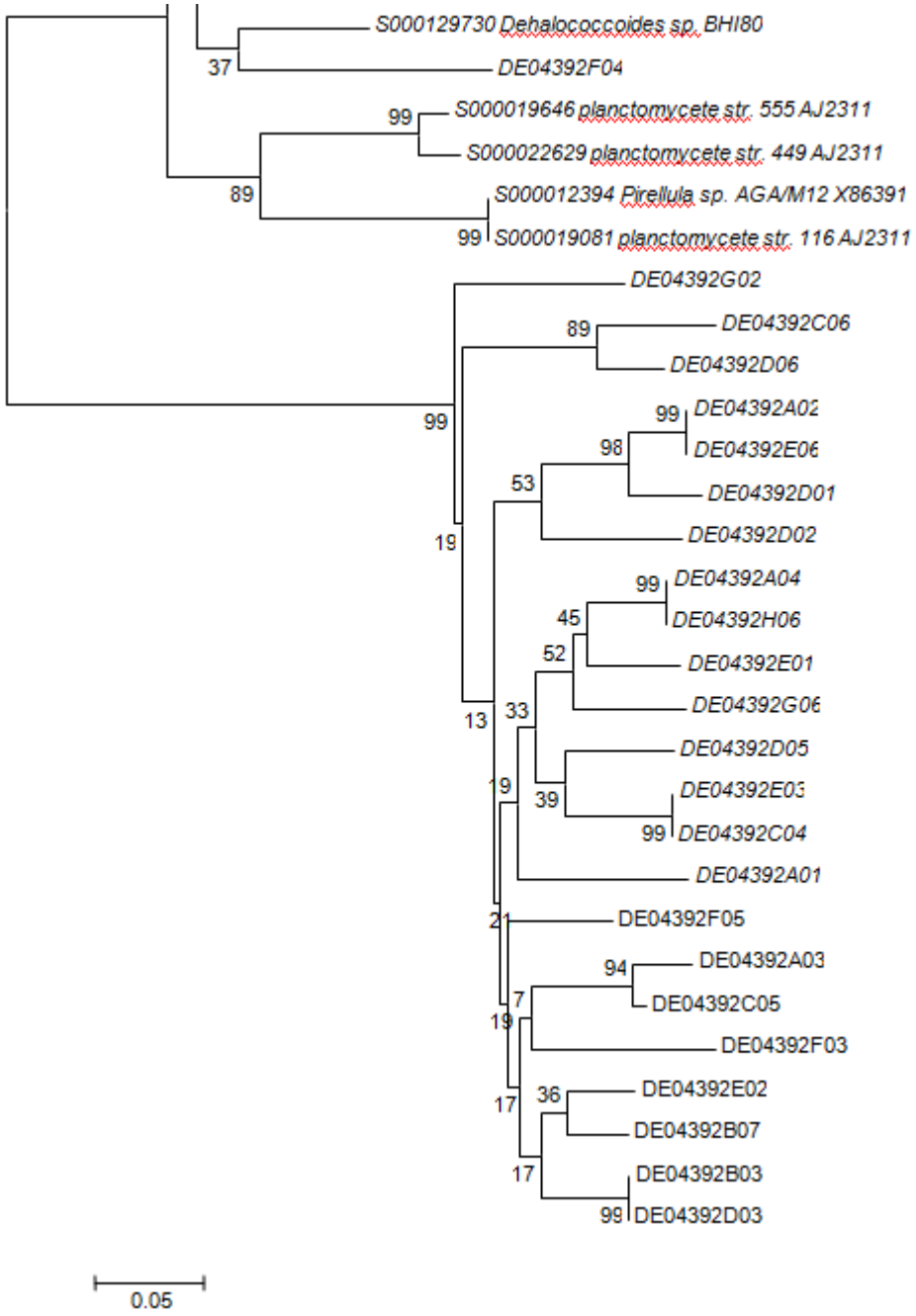
Figure 4.10. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library II isolates from the Barceloneta coastal sediment.

Bar represents I substitution per 100 nucleotides**.**



Continuation of Figure 4.10. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library II isolates from the Barceloneta coastal sediment.

Bar represents I substitution per 100 nucleotides.



Continuation of Figure 4.10. Neighbor-joining distance tree using 16S rRNA gene showing the phylogenic relationship of the clone library II isolates from the Barceloneta coastal sediment.

Bar represents I substitution per 100 nucleotides

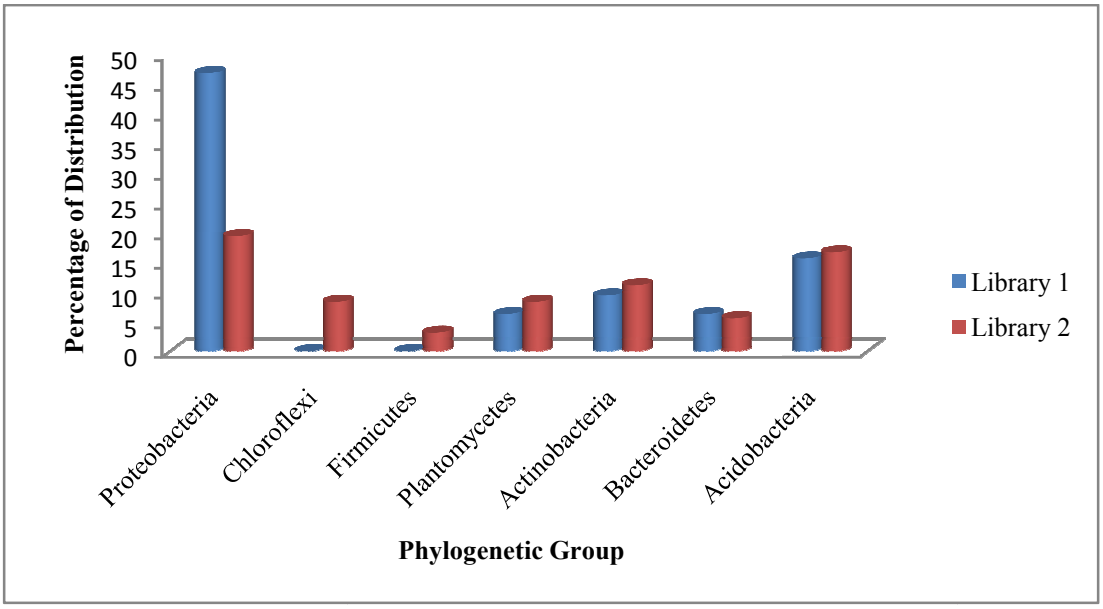


Figure 4.11. Distribution of Phylogenetic groups in Barceloneta-Manatí sediments.
Library 1 (Control); Library 2 (antibiotic treatments)

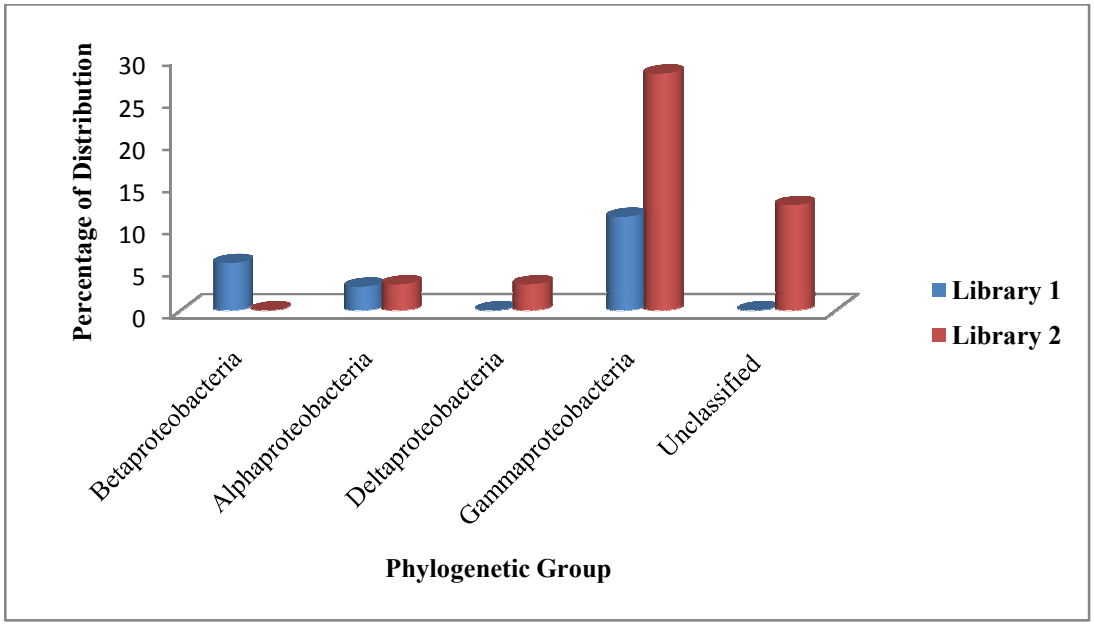


Figure 4.12. Distribution of the Phylum Proteobacteria in Barceloneta-Manatí sediments.
Library 1 (Control); Library 2 (antibiotic treatments)

Table 4.5 Characterization of bacterial phylotypes detected in clone library 1 (no treatment) according to the RDP-II Seqmatch tool

Phylotype of Clone	Organism	S_{ab} Score	Accesion Gene Bank
	Phylum Proteobacteria		
	Betaproteobacteria		
DE04391H02	uncultured bacterium	0.835	EF999367
DE04391G03	uncultured bacterium	0.887	EU799924
	Alphaproteobacteria		
DE04391G02	Uncultured alpha proteobacterium	0.771	DQ431900
	Gammaproteobacteria		
DE04391D01	uncultured bacterium	0.841	EU491745
DE04391A01	uncultured bacterium	0.859	EU491366
DE04391B03	uncultured bacterium	0.885	DQ513032
DE04391H05	uncultured bacterium	0.955	EU652572
	Phylum Actinobacteria		
DE04391D03	uncultured actinobacterium	0.924	AM259898
DE04391H03	uncultured bacterium	0.847	GQ246284
DE04391D05	uncultured bacterium	0.728	EU491214
	Phylum Acidobacteria		
DE04391A02	uncultured bacterium	0.888	EU491394
DE04391E02	uncultured Acidobacteria	0.934	DQ289910
DE04391G01	uncultured bacterium	0.692	GQ246392
DE04391B04	uncultured Acidobacteria	0.874	FJ20523
DE04391E04	uncultured bacterium	0.854	EU491394
DE04391B06	uncultured Acidobacteriaceae	0.899	AM040134
	Phylum Plantomycetes		
DE04391F02	uncultured bacterium	0.775	GQ246412
DE04391F03	unidentified bacterium	0.773	AY34441
DE04391C04	uncultured planctomycete	0.806	EU246803
DE04391A06	uncultured bacterium	0.658	FJ203277
DE04391C05	uncultured bacterium	0.809	EU488059
	Phylum Chloroflexi		
DE04391B02	uncultured Chloroflexi bacteriu	0.728	EU246818
	Phylum Bacteroidetes		
DE04391C02	uncultured Cytophaga sp	0.866	DQ889917
DE04391G04	uncultured Cytophaga sp	0.789	DQ889917
	Phylum WS_3		
DE04391D02	uncultured bacterium	0.764	EU286999
DE04391B01	uncultured bacterium	0.806	FJ712436

DE04391H04	***Phylum Nitrospira***		
DE04391B05	uncultured bacterium	0.902	EU385706
	uncultured bacterium	0.734	EU734975
	Phylum Unclassified		
DE04391C01	uncultured bacterium	0.727	EU617821
DE04391H01	uncultured bacterium	0.808	AJ966584
DE04391E01	uncultured bacterium	0.842	EU617821
DE04391C03	uncultured Gemmatimonadetes	0.874	DQ431883

Table 4.6 Characterization of bacterial phylotypes detected in clone library 2 (antibiotic treatment) according to the RDP-II Seqmatch tool

Phylotype of Clone	Organism	S_{ab} Score	Accesion Gene Bank
	Phylum Proteobacteria		
	Deltaproteobacteria		
DE04392H01	uncultured organism	0.560	DQ396062
DE04392G02	uncultured bacterium	0.719	GQ246399
DE04392C01	uncultured Haliangiaceae bacterium	0.654	FJ516990
DE04392E01	uncultured bacterium	0.868	EU287158
DE04392H01	uncultured organism	0.560	DQ396062
DE04392D03	unidentified bacterium	0.801	AF317744
DE04392B03	unidentified bacterium	0.800	AF317744
	Alphaproteobacteria		
DE04392A01	uncultured alpha proteobacterium	0.967	DQ431901
	Gammaproteobacteria		
DE04392H06	uncultured gamma proteobacterium	0.921	AB294936
DE04392G06	Vibrio furnissii	0.990	X76336
DE04392E03	uncultured bacterium	0.941	EU491375
DE04392A04	uncultured gamma proteobacterium	0.924	AB294936
DE04392C04	uncultured bacterium	0.934	EU491375
DE04392G04	uncultured bacterium	0.913	EU652530
DE04392D05	uncultured bacterium	0.972	EU491392
DE04392A06	uncultured bacterium	0.768	DQ823216

	Phylum Actinobacteria		
DE04392A02	Acidimicrobidae bacterium	0.922	AB360345
DE04392D01	uncultured actinobacterium	0.798	AM935387
DE04392E06	uncultured bacterium	0.918	EF659441
	Phylum Acidobacteria		
DE04392G01	Acidobacterium sp	0.814	EU373917
DE04392E02	uncultured bacterium	0.751	EU542539
DE04392G01	uncultured Acidobacterium sp	0.814	EU373917
DE04392A03	uncultured bacterium	0.808	EU617737
DE04392C05	uncultured bacterium	0.816	EU287125
	Phylum Bacteroidetes		
DE04392F03	uncultured Bacteroidetes bacterium	0.683	FJ205289
DE04392F05	uncultured Acidobacteria bacterium	0.874	FJ205234
DE04392F06	uncultured Bacteroidetes bacterium	0.551	FJ205345
	Phylum Plantomycetes		
DE04392C06	uncultured planctomycetes	0.861	DQ289931
DE04392D06	uncultured bacterium	0.845	EF157256
	Phylum WS_3		
DE04392B07	uncultured bacterium	0.736	EU135567
DE04392B04	uncultured bacterium	0.645	DQ351773
	Phylum Firmicutes		
DE04392G05	Streptococcus salivarius	1.000	AM157419
	Phylum Unclassified		
DE04392D02	uncultured bacterium	0.663	DQ394955
DE04392F04	uncultured bacterium	0.745	DQ394955

Discussion

Only a fraction (<1%) of naturally occurring microorganisms in the marine environment is cultivable. Therefore, it was necessary to develop independent culture techniques to detect the microbial community in the environment (Hugenholtz *et al*, 1999). One of the promising culture independent techniques for community analysis is by terminal restriction fragment length polymorphism (T-RFLP). Interpretations of individual peaks obtained by T-RFLP using several enzymes for digestion presents a problem. Several researchers have suggested each peak may represent multiple groups. In the present study, the T-RFLP technique was only used to evaluate changes in the community composition in the presence of single or multiple antibiotics. For this purpose, it is not important to know if one peak represents more than one group; at least it can be established that one peak corresponds to an OTU. Correspondence analysis (CA) established that the introduction of antibiotics into a coastal marine sediment sample promoted variability compared to a control that is not treated with antibiotics. The CA analysis demonstrated that when one or two antibiotics were used, enrichment of members of the community occurred, as well as increased taxonomic groups. Overall, there was a decline in microbial community in the sediments exposed to three antibiotics. Although the community diversity seemed similar to the control group, the presence of some new taxonomic groups was evident.

T-RFLP patterns revealed shared similarities in the community under different treatments, but with a unique pattern for each treatment. This can be due to several factors: (1) only communities having biological and genetic mechanisms are able to survive and establish in a disturbed community; (2) the number of resistant organisms can increase to higher levels that may mask the recovery of other groups; (3) the community can adapt to the exposure to antibiotics, but only strains that are resistant would develop mechanisms to survive long periods of exposure. Another importance for the T-RFLP generated profile system is the generation of community profiles that can be compared for further analysis in the sampling site.

Work done by Ayala *et al.* (2004) and Caro, (2008) found that the variability in microbial communities in the system is lowered with the introduction of contaminants like pesticides and explosives. Cotto (2007) also found that variability in microbial communities decreases in sea grass environments exposed to anthropogenic activities. The present study indicated that community structure also differed depending on the antibiotics used. For example, when

penicillin is used, more OTU's and increased concentrations of peaks are observed, compared to the combination with two and three antibiotics, but lower compared to the control. In samples with multiple antibiotics, community structure was similar to the control samples; but there was a decrease in the taxonomic unit and the intensity of peaks that was correlated with the less abundance. However, new fragments are generated, indicating change in community structure. These results are similar to those obtained by Guardabassi *et al*. (2000) where he showed that the diversity of the microbial community in an aquatic environment was reduced when the community was exposed to water from fish farms with a recent history of antimicrobial treatment. Increase in species of *Acinetobacter* and decrease of previously abundant species was observed after the treatment with oxolic acid.

Other promising culture independent technique used to determine composition of marine communities in Barceloneta-Manatí coastal sediments is by clone libraries of 16S rRNA. With this technique, it is possible to amplify the 16S rRNA gene obtained directly from environmental DNA through PCR, cloning, and sequencing environmental genes. Using this technique Fuhrman *et al.* (1993) reported that marine microbial communities contain novel, uncultured species that are widespread in the major oceans of the world. Although the clones of both libraries could be limited by the number and size of the samples collected, cell lysis, nucleic acid extraction, PCR amplification, and cloning, the methodology is useful to characterize some OTU's that could be present in the T-RFLP (Wintzigerode *et al.,* 1997). The sequences generated sometimes showed limited variation for members of closely related taxa. A possible reason for not obtaining the separation of some groups of clones was the conserved nature of these genes. Another reason for some gene sequences not showing high similarity (>97%) with database sequences could be that the prokaryotic community samples were composed of novel organisms that haven't been characterized. The absence of a group in the library does not necessarily indicate that the organism is not present; the results only suggest that an organism may not be present in the samples. To establish the absence of a group in a sample, it is necessary to evaluate higher coverage of organisms in the Barceloneta sediments requiring evaluation of higher number of clones. It is important to note the presence of Firmicutes phylum where *Enterococcus* belongs. The presence of this group demonstrates that *Enterococcus* and other organism associated with this group have the ability of antibiotic resistance.

The phylogenetic analysis revealed that most of the OTU's are related to bacteria associated with marine sediments. The related organisms in the library have physiological and metabolic capacity for growth and survival in the sediment of Barceloneta. Analysis of 16S rRNA gene revealed a dominance of members of the Phylum Proteobacteria. In previous research, α-proteobacteria has been isolated from marine sediments that represented 55% of the evaluated community (Cottrell and Kirchman, 2000). Organisms from these phyla, like marine aerobic *Pseudoalteromonas* and *Marinomonas*, have antibiotic resistance (Gorshkova and Ivanova, 2001).

In the clone library of the treated sample, *Enterococcus* spp. of the Phylum Firmicutes is present in lower numbers compared to other phylogenetic groups, indicating that other microorganisms may be responsible for antibiotic resistance. It is important to mention the presence of organisms related to *Streptococcus* and *Bacillus* in the libraries that may have the ability to conjugate and acquire the ability of antibiotic resistance. Also, the dominance of gamma proteobacteria resistant to antibiotic in samples of sediments is important because inside this group are important groups of disease-causing bacteria such as, *Vibrios* spp, *Salmonella* spp, *Psedudomonas* spp, and members of enterobacteriaceae group.

Knowing the composition of the community in sediments of coastal environments may provide a base to establish which other organisms, in addition to cultivable strains, are responsible for the introduction of resistance to antibiotic genes in the environment and eventually provide a source for resistance within humans. Knowing the structure of a community may also help us develop new ways to control the factors that promote the development of resistance. This may be useful information for a community faced with decision making regarding the uses of our resources and how our behavior may impact those resources.

Literature Cited

Ayala R., Castiller H.S., Criddle C., and Y.J. Tiedje. 2004. Correspondence between community structure and function. Applied and Environmental Microbiology. 70: 4950-4960.

Caro Q.A. 2008. Diversity and microbial community structure at a former military ranges in vieques (Puerto Rico). Master Thesis. University of Puerto Rico. Mayaguez. 149 pp.

Couto R. M. 2000. Endophytic prokaryotic diversity associated with sea grass beds of *Thalassia testudinum* from Cabo Rojo, Lajas, and Vieques, Puerto Rico. Master Thesis. University of Puerto Rico. Mayaguez. 311 pp.

Engebretson J.J., and C.L., Moyer. 2003. Fidelity of select restriction endonucleases in determine microbial diversity by terminal-restriction fragment length polymorphism. Applied and Environmental Microbiology 69: 4823-4829.

Gorshkova N.M., and E. P. Ivanova. 2001. Antibiotic Susceptibility as a taxonomic characteristic of proteobacteria of the genera *Alteromonas, Pseudoalteromonas, Marinomonas*, and *Marinobacter*. Russian Journal of Marine Biology. 27(2): 116–120.

Guardabassi L., Dalsgaard A., Rafatelly M., and J.E. Olsen. 2000. Increase in the prevalecence of oxolinic acid resistant *Acinetobacter* spp. observed in a stream receiving the effluent from a freshwater trout farm following the treatment with oxolinic acid-medicated feed. Aquaculture. 188: 205-218.

Haible B., Stoll M., and S. Steingold. 1992-2008. CLISP: an ANSI Common Lisp Implementation. University and Munich Univesity. [Online.]

Hammer Ø., Haper D.A.T., and P. D. Ryan. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontology Electronica. 4:9.

Lui W.T., Marsh T.L., Cheng H., and L.J. Forney. 1997. Characterization of microbial diversity by determines terminal restriction fragment length polymorphisms of gene encoding 16S rRNA. Applied and Environmental Microbiology 63: 4516-4522.

Osborne C., Rees G.N., Bernstein Y., and P.H. Janssen. 2006. New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. Applied and Environmental Microbiology. 72: 1270-1278.

Pace N., 1997. A molecular view of microbial diversity and the biosphere. Science. 276(5313): 734-740.

Tamura K, Dudley J. Nei M., and S. Kumar. 2007. Mega 4: molecular evolutionary genetics analysis (MEGA) software version 4.0 Molecular Biology and Evolution 24:1596-1599.

Yeager, C.M., Northup D.E., Grow C.C., Bams S.M., and C.R. Kuske. 2005. Changes in nitrogen –fixing and ammonia oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. Applied and Environmental Microbiology. 71: 2713-2722.

CONCLUSIONS

The main objective of this research was to investigate as to why coastal waters of Barceloneta have higher number of multiple antibiotic-resistant bacteria compared with other coastal waters in Puerto Rico. This objective was achieved by finding a probable source (dairy industry) where higher number of *Enterococcus* spp. were found and where antibiotics are routinely used and then follow the presence of multiple antibiotic resistance *Enterococcus* spp. from the river near the dairy industry (point source) to estuarine and then in coastal water and sediments. Seasonal variations in occurrence of multiple antibiotic resistant *Enterococcus* spp. were also evaluated. Change in community composition with and without antibiotics was evaluated by molecular techniques. The presence of multiple antibiotics resistant other than *Enterococcus* was also evaluated in Barceloneta coastal water sediments by using molecular techniques. Following are general conclusions from this research

Chapter1

- Compared to Guánica, Guayanilla, and Mayagüez, the coastal waters of Barceloneta have higher number of *Enterococcus* and also higher numbers of organisms resistant to multiple antibiotics. This may be due to selective pressure exerted by the dairy industry where antimicrobial agents are routinely used. This hypothesis was further investigated.

Chapter 2

- The river water near the dairy industry has higher numbers of *Enterococcus* bacteria compared to estuarine and coastal areas. Higher numbers of *Enterococcus* were found during the rainy season. This could be due to feces from cattle entering the river during rainfall.
- *E. faecalis* was dominant in all environments evaluated. Compared to other research, we found lower numbers of *E .faecium* in some samples that may be due to lower temperatures.
- Abundance and diversity of *Enterococcus* species was affected by environmental factors with more diverse populations during increased precipitation.
- Presence of some species of *Enterococcus* in the Barceloneta area can be associated to the presence of specific vectors types.

- The recovery of *Enterococcus* in sediments was higher in river and estuarine samples, but lower in coastal samples this could be due to differences in soil type.
- Distribution of *Enterococcus* spp. in order of occurrence was *E. faecalis, E. faecium* and *E. hirae.* Thus in tropical waters the distribution of *Enterococcus* spp. is different than reported in other coastal environments.
- In general, the composition of *Enterococcus* spp in the tropical environment of Barceloneta may be influenced by: (1) precipitation (2) salinity (3) temperature (4) presence of specific type of vectors and (4) influence of antimicrobial pollution.

Chapter 3

- Analysis of multiple antibiotic resistance (MAR) indicated that the river has higher number of MAR *Enterococcus* spp., followed by the estuarine and coastal areas.
- The recoveries of MAR organisms are affected by the period of sampling. During the rainy period, the number of MAR microorganisms decreased, probably due to the dilution of bacteria after rainwater.
- The predominance of MAR species in the three environments in order of occurrence was *E. faecalis, E. hirae*, *E. durans an*d *E. faecium.*
- Higher percentages of *Enterococcus* were resistant to the combination of penicillin and tetracycline in river water and sediments than in estuarine and coastal environments. The high levels of antibiotic resistance in the river may be due to penicillin and tetracycline usage by the dairy industries for prophylaxis and infections treatment of cattle.
- *Enterococcus* was equally resistant to the combination of penicillin and vancomycin only after the October 2007 sampling. Investigation revealed that after the summer 2007, cattle were treated with high concentrations of penicillin and ramoplanin (a glycopeptides like vancomycin) due to an outbreak of Mycoplasma.
- The presence of lower numbers of MAR *Enterococcus* spp. in coastal environment is important because it increases the risk of transfer of resistance to marine microorganisms. This in turn can adversely affect human health.

Chapter 4

- The majority of the strains recovered from Barceloneta sediments belonged to the Phylum Actinobacteria, Proteobacteria, and Plantomyces. In the samples treated with antibiotics, the phyla of proteobacteria was most dominant.
- The samples treated with antibiotics had the least OTU's compared to the sample without antibiotics.
- The introduction of antibiotics in the coastal marine sediments decreased the variability in microbial community compared to the control samples.
- In the clone library of the antibiotic treated sample Phylum Firmicutes which includes *Enterococcus* spp., this genera is present in lower numbers compared to other groups, indicating that other organisms can account for the ability and transfer of resistance.

Appendix

Appendix A
Statistical Analysis of Diversity from *Enterococcus* spp from Barceloneta-Manatí.

April 2006

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	10	8	9	3	6	7
Individuals	95	95	95	95	95	95
Dominance_D	0.4045	0.2222	0.2152	0.4436	0.5908	0.3462
Shannon_H	1.497	1.813	1.868	0.9415	0.9755	1.431
Simpson_1-D	0.5955	0.7778	0.7848	0.5564	0.4092	0.6538
Evenness_e^H/S	0.447	0.766	0.7193	0.8546	0.4421	0.5973
Menhinick	1.026	0.8208	0.9138	0.303	0.6092	0.7035
Margalef	1.976	1.537	1.749	0.4362	1.093	1.306
Equitability_J	0.6503	0.8718	0.8501	0.857	0.5445	0.7352
Fisher_alpha	2.82	2.082	2.423	0.5851	1.414	1.72
Berger-Parker	0.6	0.3158	0.3196	0.5612	0.7526	0.5354

June 2006

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	4	5	5	5	5	6
Individuals	95	95	95	95	95	95
Dominance_D	0.39	0.33	0.27	0.3571	0.3609	0.2476
Shannon_H	1.132	1.332	1.497	1.265	1.289	1.65
Simpson_1-D	0.61	0.67	0.73	0.6429	0.6391	0.7524
Evenness_e^H/S	0.7755	0.7578	0.8937	0.7087	0.726	0.8679
Menhinick	0.4041	0.5077	0.5077	0.5	0.5051	0.6124
Margalef	0.6543	0.8744	0.8744	0.8686	0.8724	1.095
Equitability_J	0.8166	0.8277	0.9302	0.7861	0.8011	0.9209
Fisher_alpha	0.8386	1.117	1.117	1.108	1.114	1.419
Berger-Parker	0.5204	0.4742	0.3814	0.53	0.5408	0.3854

October 2006

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	5	6	5	6	6	5
Individuals	95	95	95	95	95	95
Dominance_D	0.7494	0.2799	0.5703	0.3155	0.3121	0.4067
Shannon_H	0.6322	1.51	0.8654	1.338	1.417	1.193
Simpson_1-D	0.2506	0.7201	0.4297	0.6845	0.6879	0.5933
Evenness_e^H/S	0.3764	0.7542	0.4752	0.6351	0.6873	0.6595
Menhinick	0.5051	0.6092	0.5051	0.6092	0.6092	0.488
Margalef	0.8724	1.093	0.8724	1.093	1.093	0.8595
Equitability_J	0.3928	0.8426	0.5377	0.7466	0.7907	0.7413
Fisher_alpha	1.114	1.414	1.114	1.414	1.414	1.093
Berger-Parker	0.8571	0.4124	0.7245	0.3711	0.3814	0.5619

December 2006

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	4	4	7	3	3	3
Individuals	99	98	96	98	98	98
Dominance_D	0.5958	0.4761	0.1936	0.5132	0.4756	0.5977
Shannon_H	0.7514	0.9448	1.831	0.8466	0.9136	0.7515
Simpson_1-D	0.4042	0.5239	0.8064	0.4868	0.5244	0.4023
Evenness_e^H/S	0.53	0.6431	0.8912	0.7773	0.8311	0.7067
Menhinick	0.402	0.4041	0.7144	0.303	0.303	0.303
Margalef	0.6529	0.6543	1.315	0.4362	0.4362	0.4362
Equitability_J	0.542	0.6816	0.9408	0.7706	0.8316	0.684
Fisher_alpha	0.8364	0.8386	1.737	0.5851	0.5851	0.5851
Berger-Parker	0.7374	0.6327	0.25	0.6531	0.6327	0.7449

April 2007

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	5	3	6	5	2	4
Individuals	97	98	97	98	99	99
Dominance_D	0.8031	0.7268	0.6544	0.5525	0.976	0.6736
Shannon_H	0.5242	0.5166	0.8706	0.9626	0.09749	0.6651
Simpson_1-D	0.1969	0.2732	0.3456	0.4475	0.02404	0.3264
Evenness_e^H/S	0.3378	0.5588	0.3981	0.5237	0.5512	0.4862
Menhinick	0.5077	0.303	0.6092	0.5051	0.201	0.402
Margalef	0.8744	0.4362	1.093	0.8724	0.2176	0.6529
Equitability_J	0.3257	0.4702	0.4859	0.5981	0.1406	0.4798
Fisher_alpha	1.117	0.5851	1.414	1.114	0.355	0.8364
Berger-Parker	0.8866	0.8367	0.7938	0.7245	0.9798	0.8081

June 2007

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	4	5	7	6	4	7
Individuals	97	98	98	98	99	98
Dominance_D	0.7669	0.3696	0.3974	0.5835	0.8903	0.3974
Shannon_H	0.5509	1.232	1.2	0.9376	0.3097	1.2
Simpson_1-D	0.2331	0.6304	0.6026	0.4165	0.1097	0.6026
Evenness_e^H/S	0.4337	0.6855	0.4741	0.4256	0.3408	0.4741
Menhinick	0.4061	0.5051	0.7071	0.6061	0.402	0.7071
Margalef	0.6558	0.8724	1.309	1.091	0.6529	1.309
Equitability_J	0.3974	0.7654	0.6165	0.5233	0.2234	0.6165
Fisher_alpha	0.8409	1.114	1.725	1.41	0.8364	1.725
Berger-Parker	0.866	0.5204	0.4694	0.7449	0.9394	0.4694

October 2007

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	4	2	7	4	3	5
Individuals	98	99	97	100	99	96
Dominance_D	0.8665	0.9336	0.4008	0.6954	0.8913	0.2987
Shannon_H	0.3548	0.1726	1.332	0.6268	0.2794	1.416
Simpson_1-D	0.1335	0.0664	0.5992	0.3046	0.1087	0.7013
Evenness_e^H/S	0.3565	0.5942	0.5414	0.4679	0.4408	0.8239
Menhinick	0.4041	0.201	0.7107	0.4	0.3015	0.5103
Margalef	0.6543	0.2176	1.312	0.6514	0.4352	0.8764
Equitability_J	0.2559	0.249	0.6846	0.4522	0.2543	0.8797
Fisher_alpha	0.8386	0.355	1.731	0.8342	0.5837	1.121
Berger-Parker	0.9286	0.9596	0.5876	0.82	0.9394	0.3854

December 2007

	Riv. Wat	Est.Wat	Sea Wat	Riv. Sed	Est. Sed	Sea Sed
Taxa_S	6	4	5	3	3	3
Individuals	98	98	97	99	99	99
Dominance_D	0.3049	0.3333	0.2348	0.7073	0.8913	0.7795
Shannon_H	1.465	1.208	1.562	0.584	0.2809	0.4479
Simpson_1-D	0.6951	0.6667	0.7652	0.2927	0.1087	0.2205
Evenness_e^H/S	0.721	0.8368	0.9533	0.5977	0.4414	0.5217
Menhinick	0.6061	0.4041	0.5077	0.3015	0.3015	0.3015
Margalef	1.091	0.6543	0.8744	0.4352	0.4352	0.4352
Equitability_J	0.8174	0.8715	0.9703	0.5316	0.2556	0.4077
Fisher_alpha	1.41	0.8386	1.117	0.5837	0.5837	0.5837
Berger-Parker	0.4694	0.4082	0.3196	0.8283	0.9394	0.8687

Apendix B

Frequencies of *Enterococcus* that are MAR and Z- test for analysis of proportion.

Water samples	River	Estuarine	Coastal waters
V+P (dry season)	22.7	25	23.7
V+P (rainy season)	18.4	19.8	14.2
P+T (dry season)	27.3	30	21.3
P+T (rainy season)	22.6	24.6	14
V+T (dry season)	22.3	17.3	11
V+T (rainy season)	12.6	12.2	15.4
V+P+T (low concentration) (dry season)	17.7	21.7	13.3
V+P+T (low concentration) (rainy season)	13.4	20.6	12
V+P+T (high concentration) (dry season)	10.3	10.5	3
V+P+T (high concentration) (rainy season)	3.4	4.2	1.6

Frequencies of Enterococcus that are MAR on water samples n=45

Sediment samples	River	Estuarine	Coastal waters
V+P (dry season)	22.3	27.7	24.3
V+P (rainy season)	22.2	20.4	12.6
P+T (dry season)	34	36	20.7
P+T (rainy season)	20.8	24.6	15.8
V+T (dry season)	21.3	16.3	11
V+T (rainy season)	14.2	17.4	14
V+P+T (low concentration) (dry season)	10.7	20.7	13.7
V+P+T (low concentration) (rainy season)	17.2	20.8	10.6
V+P+T (high concentration) (dry season)	8.7	6.3	4
V+P+T (high concentration) (rainy season)	3.6	4.2	1.6

Frequencies of Enterococcus that are MAR on sediment samples n=45

Z test for analysis of proportions. The test values are considered using the frequencies results from water and sediments.

River and Estuarine waters and sediments during dry period comparatively higher percentage of ES was resistant to P+T than in rainy period. Z=2.37 in water and 4.09 in sediments

	River water	Estuarine Water	River Sediment	Estuarine Sediment
Dry Period	$27.3/45$	$30/45$	$34/45$	$36/45$
Rainy Period	$22.6/45$	$24.6/45$	$20.8/45$	$24.6/45$

Dry versus rainy on water sample

$$\frac{27.3+30}{90}=\frac{57.3}{90}=0.64=64\%$$

$$\frac{22.6+24.6}{90}=\frac{47.2}{90}=0.52=52\%$$

Dry versus rainy on sediment sample

$$\frac{34+36}{90}=\frac{70}{90}=0.78=78\%$$

$$\frac{20.8+24.6}{90}=\frac{45.4}{90}=0.50=50\%$$

$$Z=\frac{0.64-0.52}{\sqrt{\frac{(0.64)(0.36)}{90}+\frac{(0.50)(0.50)}{90}}}=2.37$$

$$Z=\frac{0.78-0.50}{\sqrt{\frac{(0.78)(0.22)}{90}+\frac{(0.50)(0.50)}{90}}}=4.09$$

In the two environments about 70% of ES was resistant to P+T compared to about 54 % to P+V and about 42% to T+V

River and Estuarine

P+T versus P+ V Z= 3.13
P+T versus T+V Z= 5.48

	River	Estuarine
P+T (water dry)	27.3	30
P+T (sediment dry)	34	36
P+T (water rainy)	22.6	24.6
P+T (sediment rainy)	20.8	24.6
	104.7	115.2

	River	Estuarine
P+V (water dry)	22.3	27.7
P+V(sediment dry)	22.7	25
P+V(water rainy)	22.2	20.4
P+V (sediment rainy)	18.4	19.8
	85.6	92.9

	River	Estuarine
T+ V (water dry)	22.3	17.3
T +V (sediment dry)	21.3	16.3
T + V (water rainy)	12.6	12.2
T +V (sediment rainy)	14.2	17.4
	70.4	63.2

River and Estuarine Water and Sediments

P+T $\frac{104.7+115.2}{360} = 0.61$ P+T $\frac{104.7+115.2}{360} = 0.61$

P+V $\frac{85.6+92.9}{360} = 0.495$ T+V $\frac{70.4+63.2}{360} = 0.37$

Z=3.13 Z= 3.18

Comparison P +T versus P+V on estuarine and River during dry period

$$Z = \frac{0.61 - 0.495}{\sqrt{\frac{(0.61)(0.39)}{360} + \frac{(0.495)(0.505)}{360}}} = 3.13$$

$$Z = \frac{0.61 - 0.41}{\sqrt{\frac{(0.61)(0.39)}{360} + \frac{(0.37)(0.63)}{360}}} = 3.18$$

There is significative difference between P+T and P+V on both sites.
In coastal water and sediment ES resistant to P+T was slightly lower than P+V

Coastal Water
P+T versus P+V

P+T (dry)=21.3	P+V(dry)=23.7	T+V (dry) =11
P+T(rainy)=14	P+V (rainy)=14.2	T+V (rainy)=15.4
35.3/90=0.39	37.9/90=0.42	26.4/90=0.28

Coastal Sediment
P+T versus P+V

P+T (dry)=20.7	P+V(dry) 24.3	T+V(dry)=11
P+T(rainy)=15.8	P+V (rainy)12.6	T+V (rainy)=14
36.5/90=0.41	36.9/90=0.42	25/90=0.28

P+ Tw+ P+Ts

$$\frac{35.3 + 36.5}{180} = \frac{71.8}{180} = 0.40$$

P+ Vw+ P+Vs

$$\frac{37.9 + 36.9}{180} = \frac{74.8}{180} = 0.42$$

$$Z = \frac{0.42 - 0.40}{\sqrt{\frac{(0.42)(0.58)}{180} + \frac{(0.40)(0.60)}{180}}} = 0.41$$

There is not significative difference between the antibiotic combination of P+T and P+V in coastal water and sediments.

P+ Tw+ P+Ts

$$\frac{35.3 + 36.5}{180} = \frac{71.8}{180} = .40$$

T+ Vw+ T+Vs

$$\frac{26.4 + 25}{180} = \frac{51.4}{180} = 0.29$$

$$Z = \frac{0.40 - 0.29}{\sqrt{\frac{(0.40)(0.68)}{180} + \frac{(0.29)(0.71)}{180}}} = 2.21$$

There is significative difference between the antibiotic combination of P+T and T+V in coastal water and sediments.

In water during dry period 41.03% of *Enterococcus* spp. was resistant to combination of two and three antibiotics compared to 30.96% during rainy period. Thus, in water samples during dry period higher numbers of ES were resistant to combination of antibiotics than in rainy period. This is confirmed by Z value of 3.89.

All antibiotics in water
Dry Period
P+V
River 22.7 Estuarine 25 Coastal Waters 23.7 =71.4/135= 52.9%
P+T
River 27.3 Estuarine 30 Coastal Waters 21.3 =78.6/135=58.2%
T+V
River 22.3 Estuarine 17.3 Coastal Waters 11 =50.6 /135=37.48%
P+T+V (low)
River 17.7 Estuarine 21.7 Coastal Waters 13.3 =52.7/135=39.04%
P+T+V (high)
River 10.3 Estuarine 10.5 Coastal Waters 3 =23.8/135= 17.63%

277/675=41.03%

Rainy Period
P+V
River 18.4 Estuarine 19.8 Coastal Waters 14.2 =52.4/135=38.81%
P+T
River 22. Estuarine 24.6 Coastal Waters 14 =61.2/135=0.453%
T+V
River 12.6 Estuarine 12.2 Coastal Waters 15.4 =40.2/135=29.77%
P+T+V (low)
River 13.4 Estuarine 20.6 Coastal Waters 12 =46/135=34.07%
P+T+V (high)
River 3.4 Estuarine 4.2 Coastal Waters 1.6 =9.2 /135=6.81%

209/675=30.96%

$$Z = \frac{0.410 - 0.309}{\sqrt{\frac{(0.410)(0.59)}{675} + \frac{(0.309)(0.691)}{675}}} = 3.89$$

There are significative differences between periods. In water samples the dry periods have more resistance to antimicrobial agents compared to rainy period.

All antibiotics in sediments
Dry Period
P+V
River 22.3 Estuarine 27.7 Coastal Sediments 24.3 =74.3/135= 55%
P+T
River 34 Estuarine 36 Coastal Sediments 20.7 =90.7/135=67.19
T+V
River 21.3 Estuarine 16.3 Coastal Sediments 11 =48.6 /135=36%
P+T+V (low)
River 10.7 Estuarine 20.7 Coastal Sediments 13.7 =45.1/135=33.41%
P+T+V (high)
River 8.7 Estuarine 6.3 Coastal Sediments 4 =19/135= 14.07%

277.7/675=41.14%

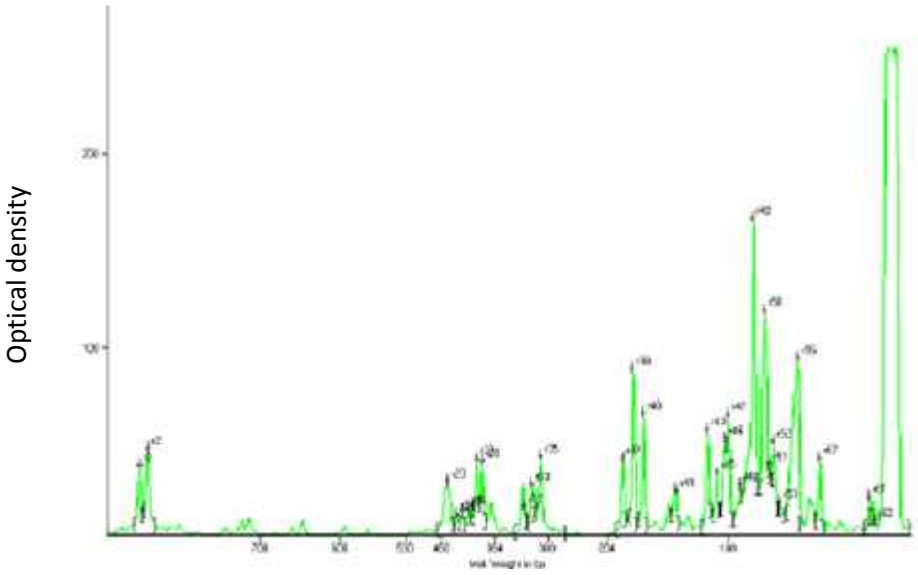
Rainy Period
P+V
River 22.2 Estuarine 20.4 Coastal Sediments 12.6 =55.2/135=40.88%
P+T
River 20.8 Estuarine 24.6 Coastal Sediments 15.8 = 61.2/135=45.33%
T+V
River 14.2 Estuarine 17.4 Coastal Sediments 14 = 45.6/135=33.78%
P+T+V (low)
River 17.2 Estuarine 20.8 Coastal Sediments 10.6 =48.6/135=36%
P+T+V (high)
River 3.6 Estuarine 4.2 Coastal Sediments 1.6 = 9.4/135=6.96%

220/675=32.5%

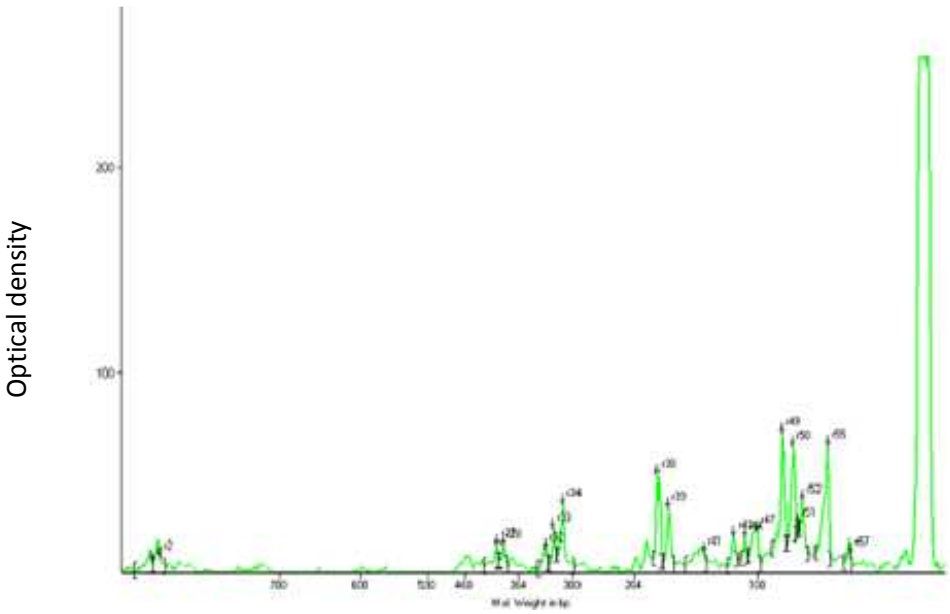
$$Z = \frac{0.411 - 0.335}{\sqrt{\frac{(0.411)(0.588)}{675} + \frac{(0.335)(0.665)}{675}}} = 3.3$$

There are significative differences between periods. In sediment samples the dry periods have more resistance to antimicrobial agents compared to rainy period.

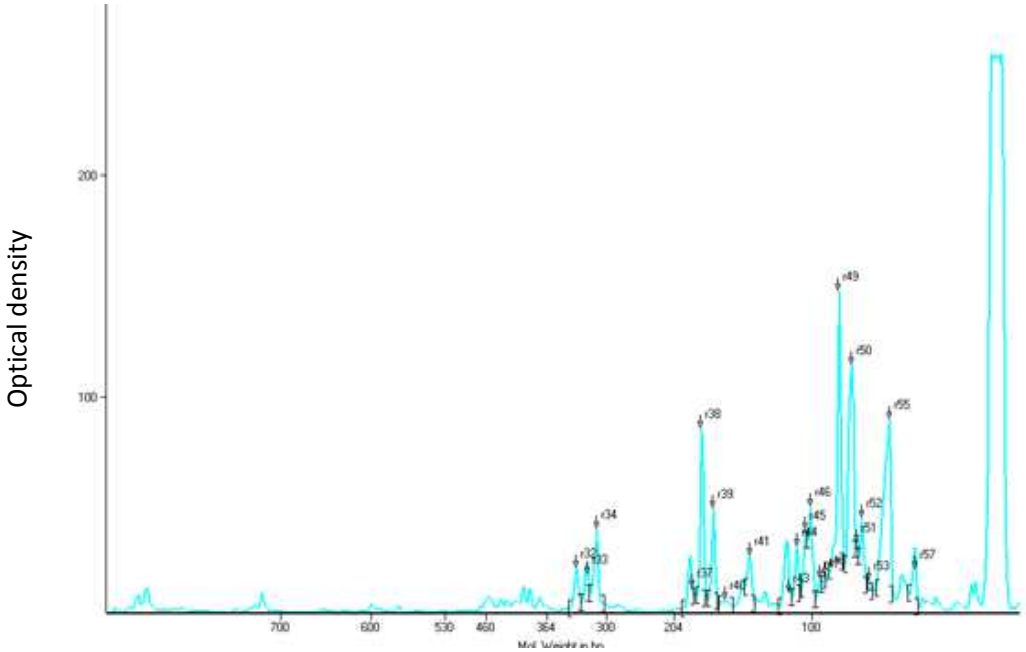
Appendix C
Electropherograms generated with the tRFLP



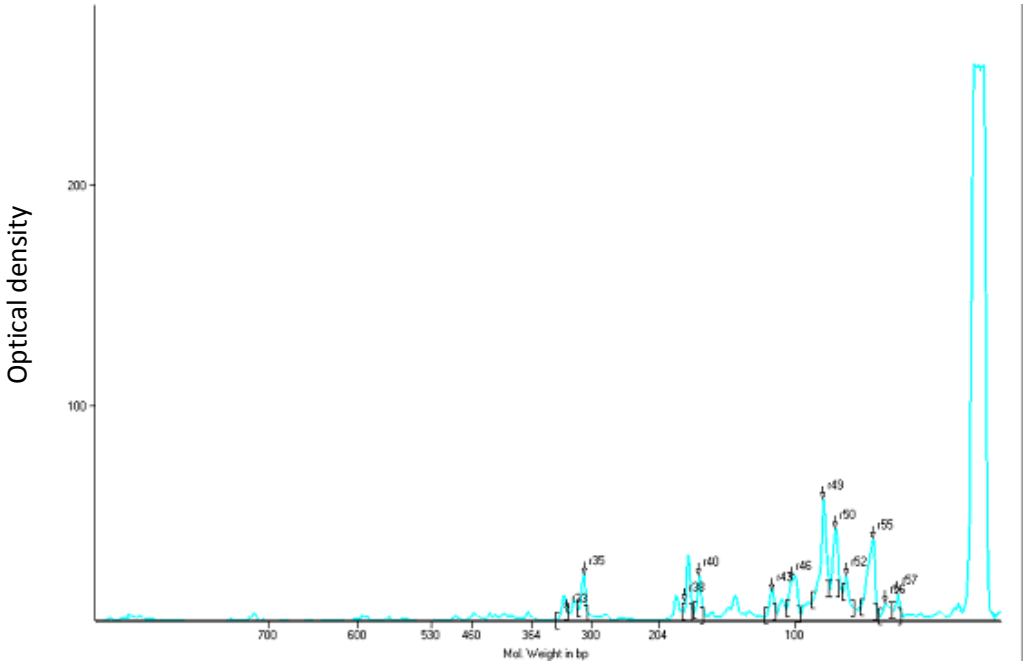
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments without antibiotics (Control after 1 week) generated by digestion with *Rsa.* (Lane 14)



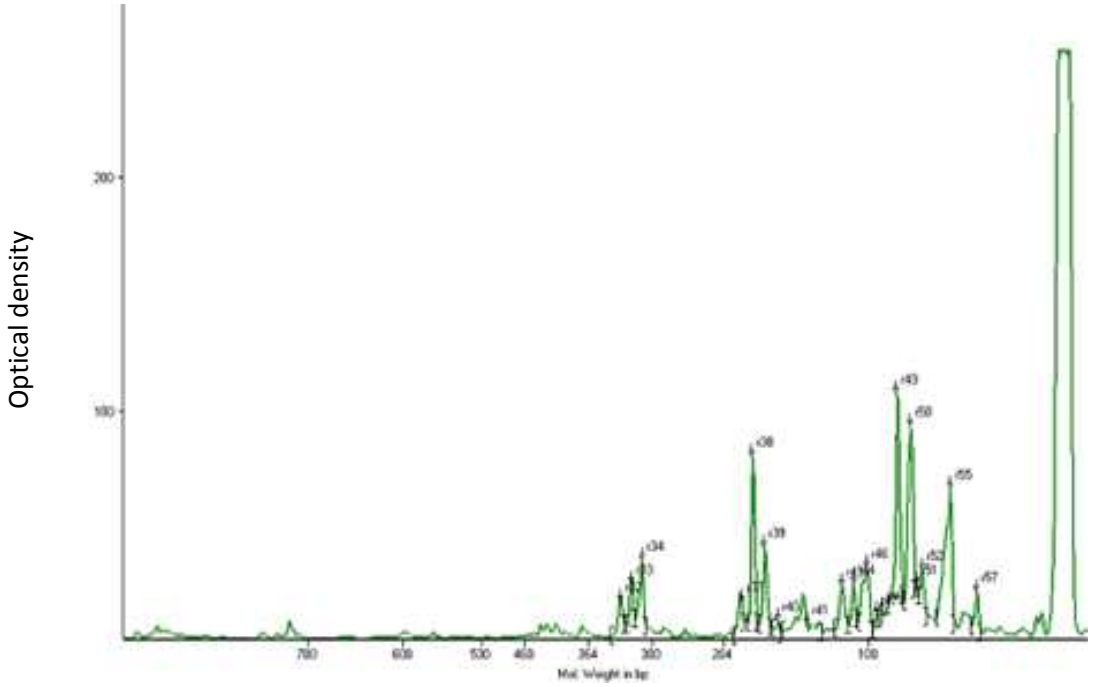
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments without antibiotics (Control final stage**)** generated by digestion with *Rsa.* (Lane 2)



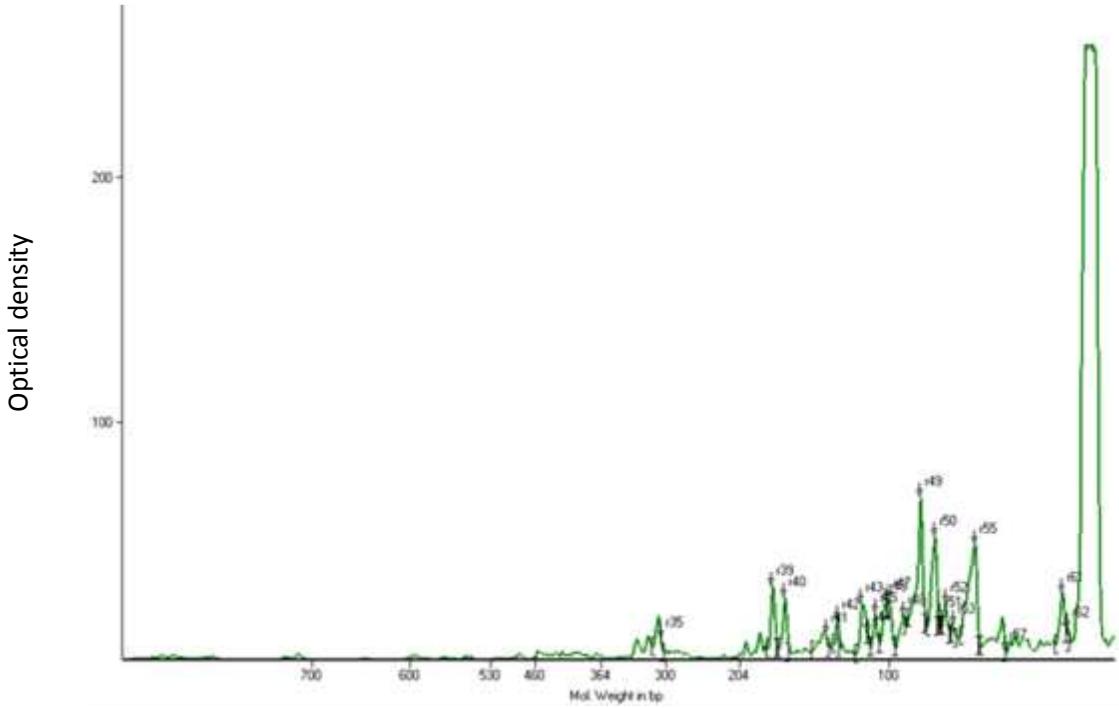
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **one antibiotic (initial stage)** generated by digestion with *Rsa.* (Lane 5)



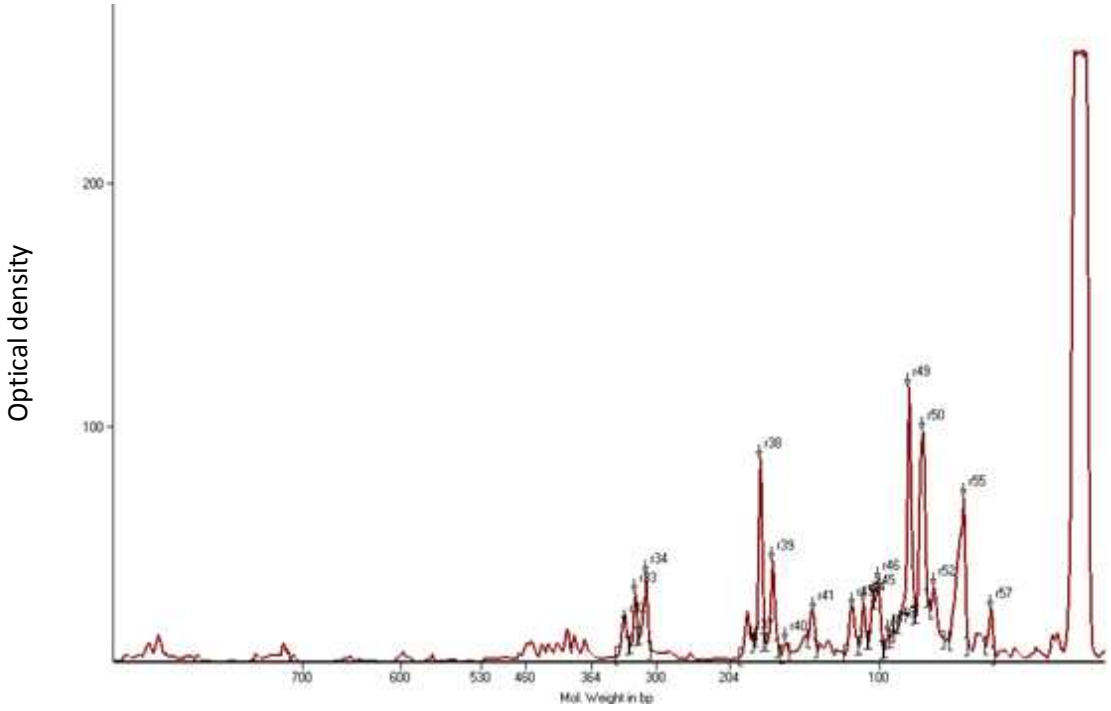
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **one antibiotic (final stage)** generated by digestion with *Rsa.* (Lane 17)



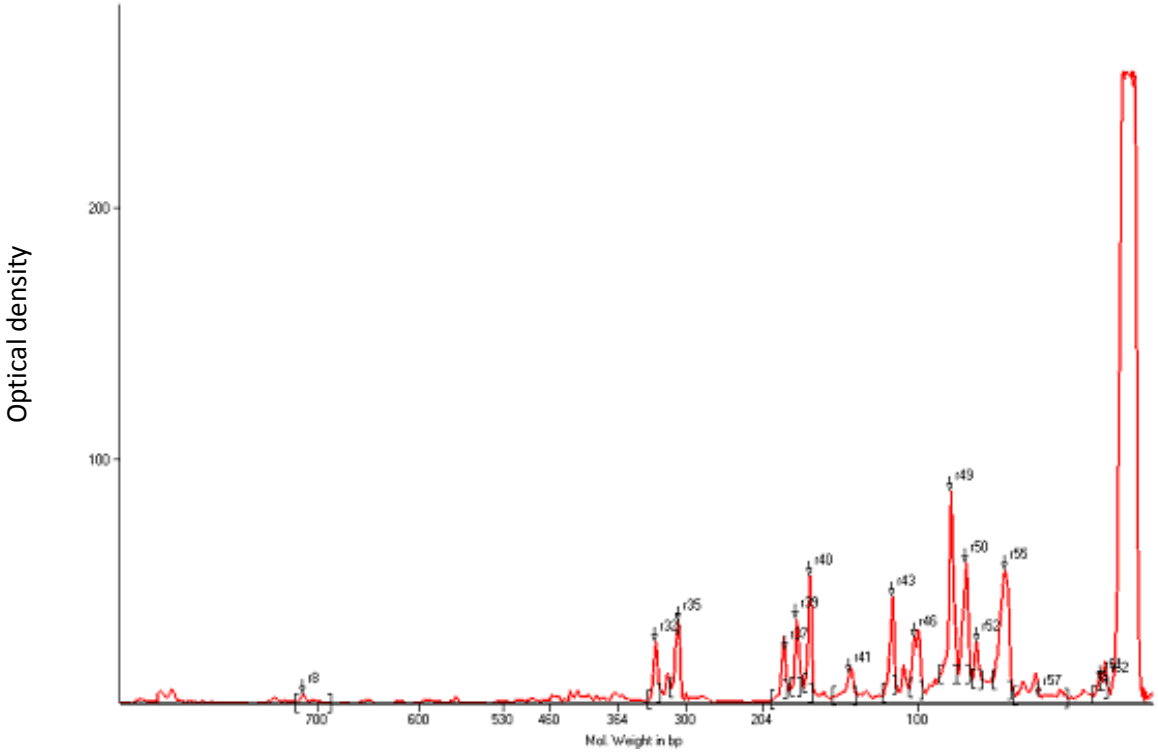
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotics (after one week)** generated by digestion with *Rsa.* (Lane 8)



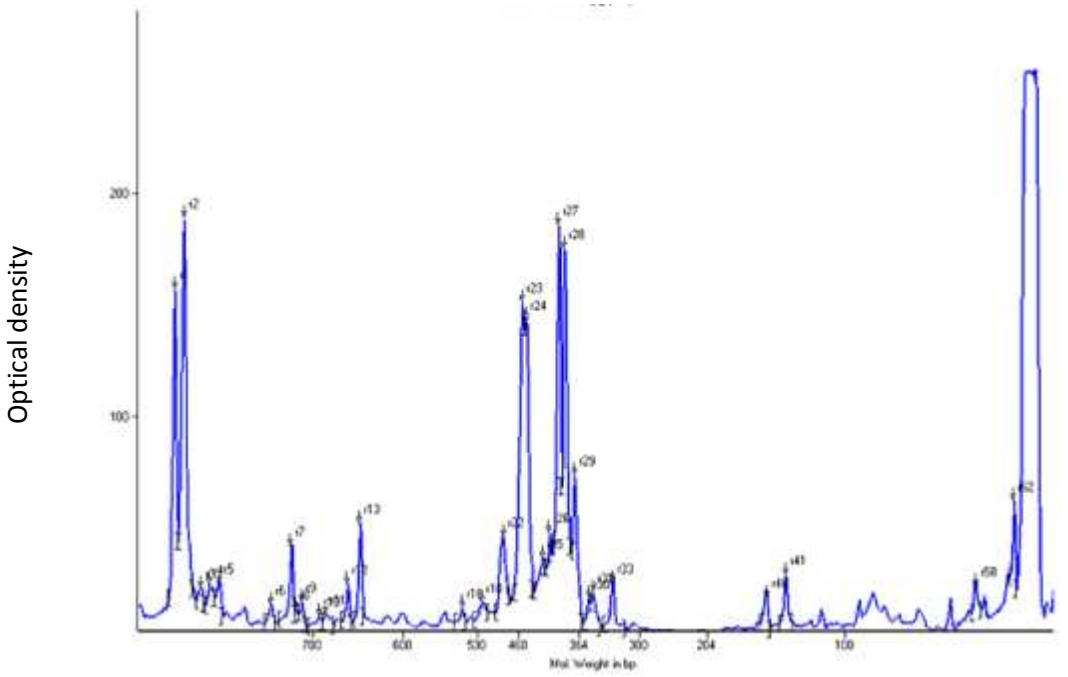
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotics (final stage)** generated by digestion with *Rsa.* (Lane 20)



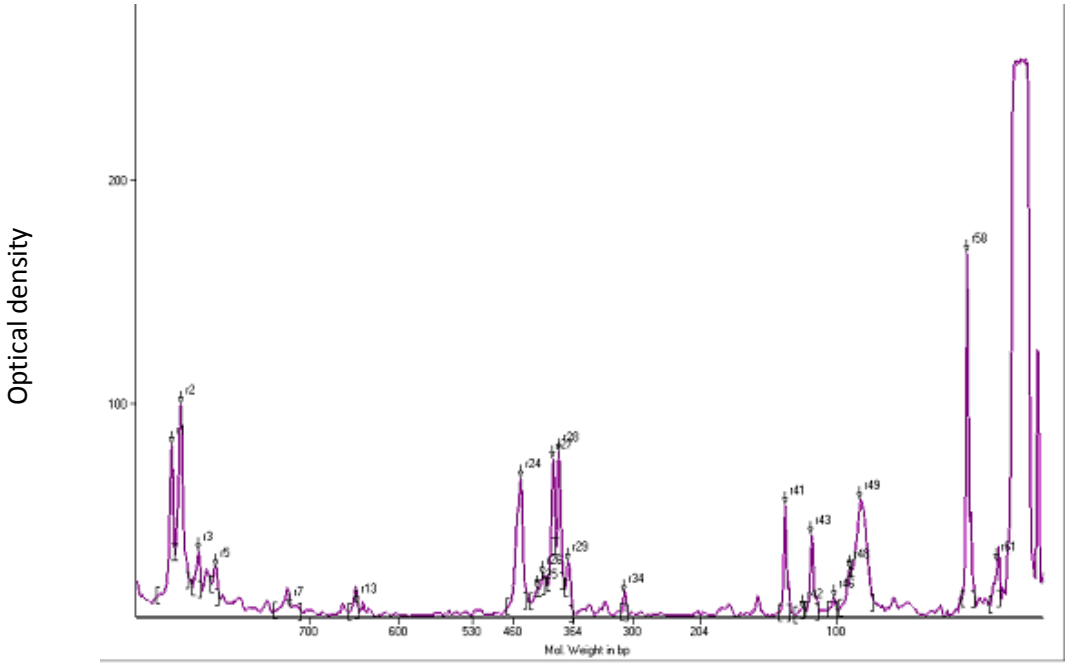
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments **with three antibiotics (after one week)** generated by digestion with *Rsa.* (Lane 7)



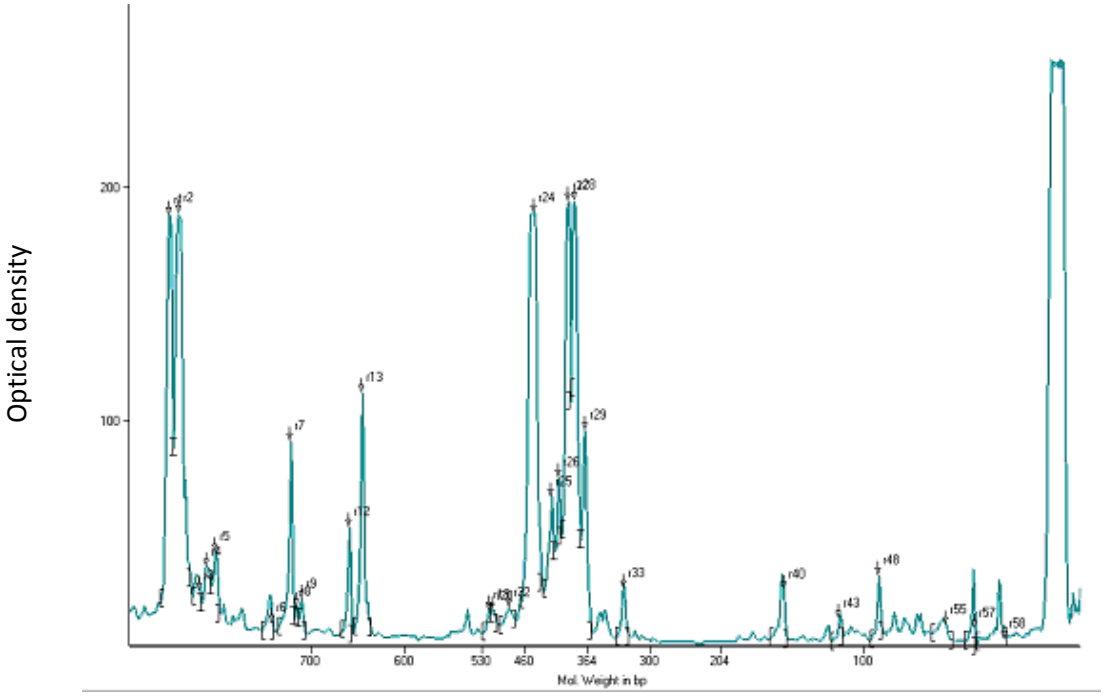
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **three antibiotics (final stage)** generated by digestion with *Rsa.* (Lane 25)



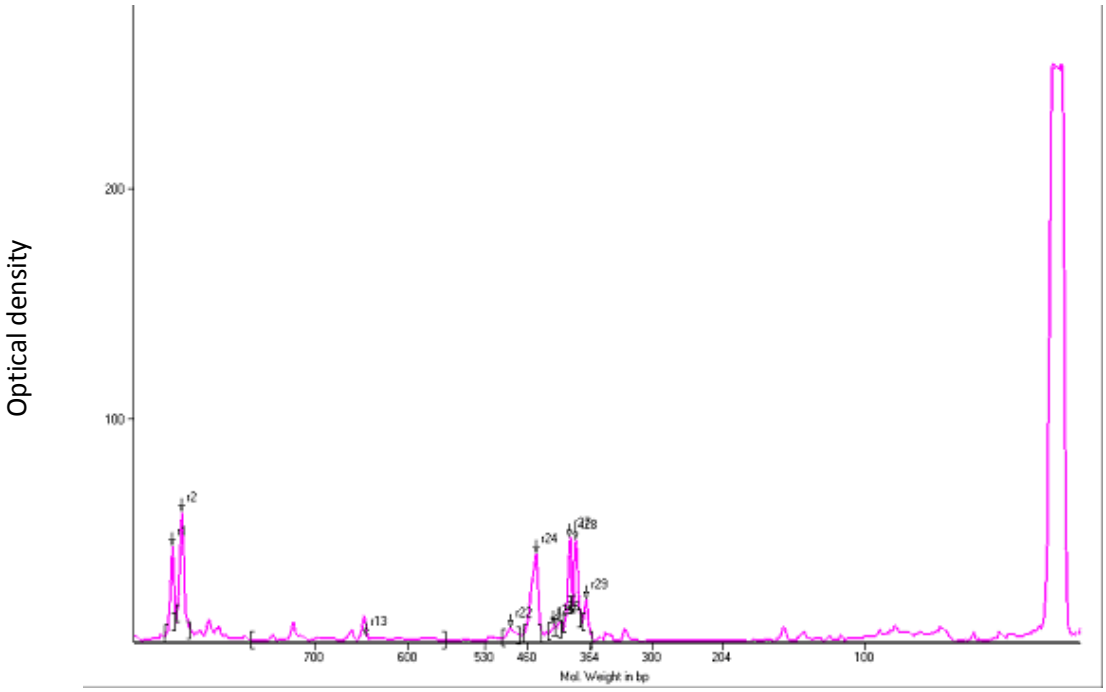
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments **without antibiotic (control after one week)** generated by digestion with *Hae.* (Lane 15)



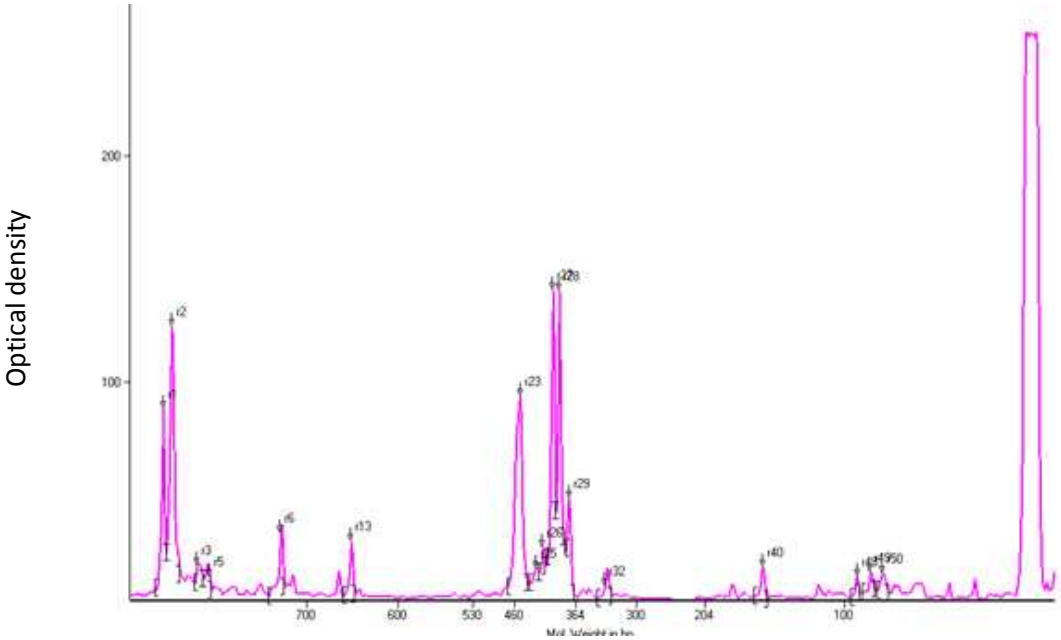
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments **without antibiotic (control final stage)** generated by digestion with *Hae.* (Lane 12)



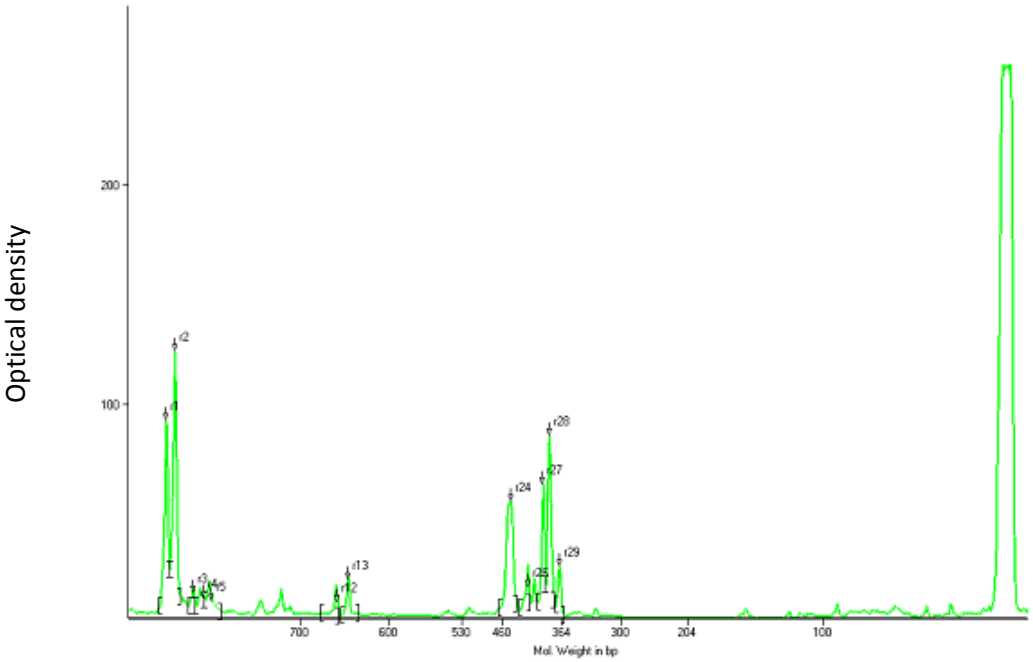
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **one antibiotics (after one week)** generated by digestion with *Hae* (Lane 23)



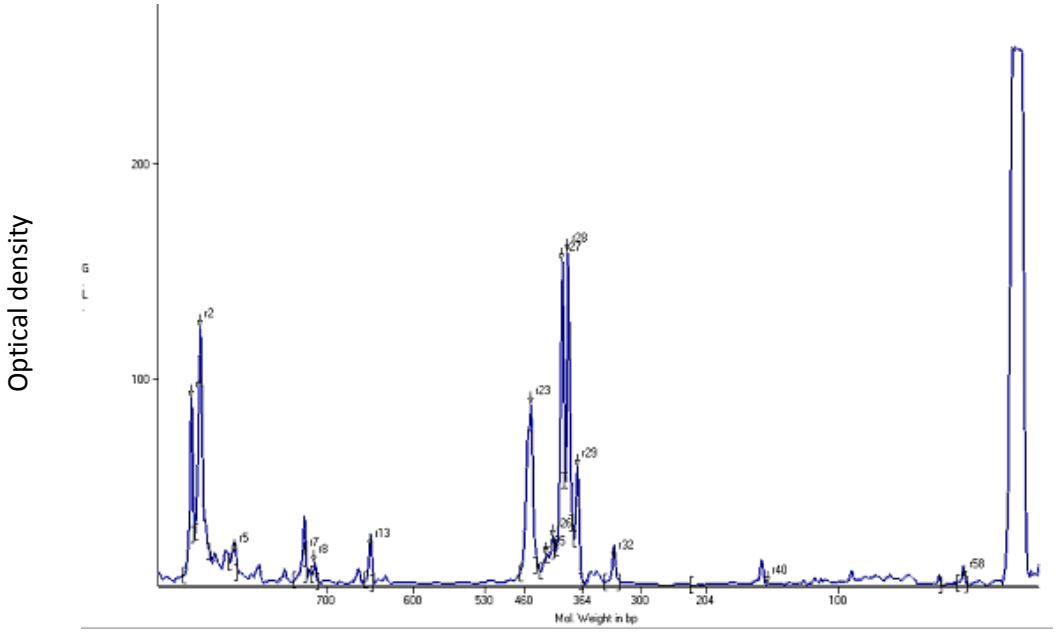
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **one antibiotic (final stage)** generated by digestion with *Hae.* (Lane 18)



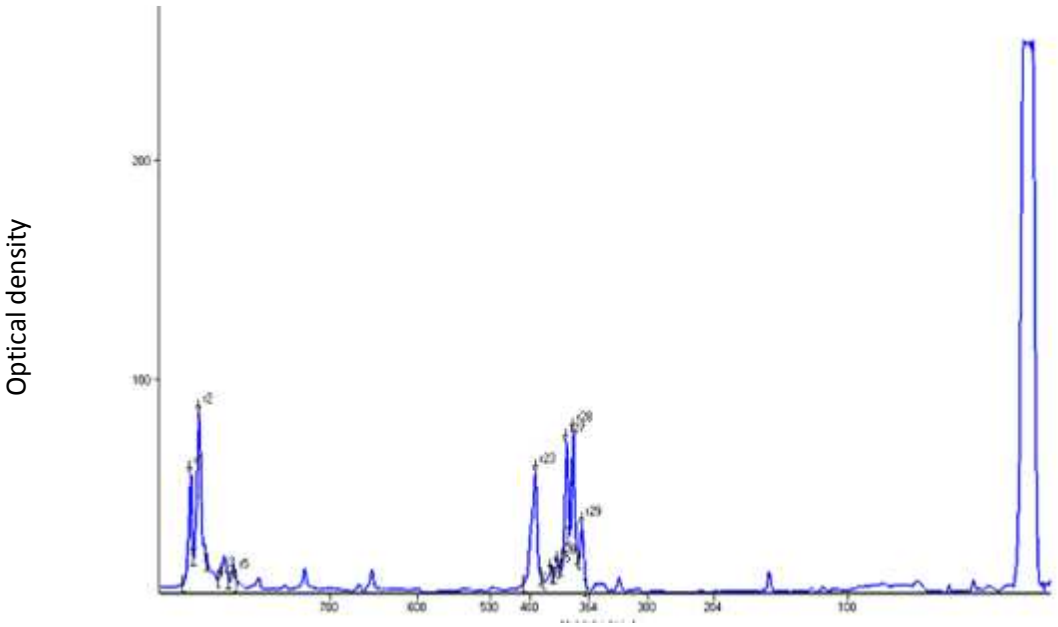
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotics (after one week)** generated by digestion with *Hae.* (Lane 6)



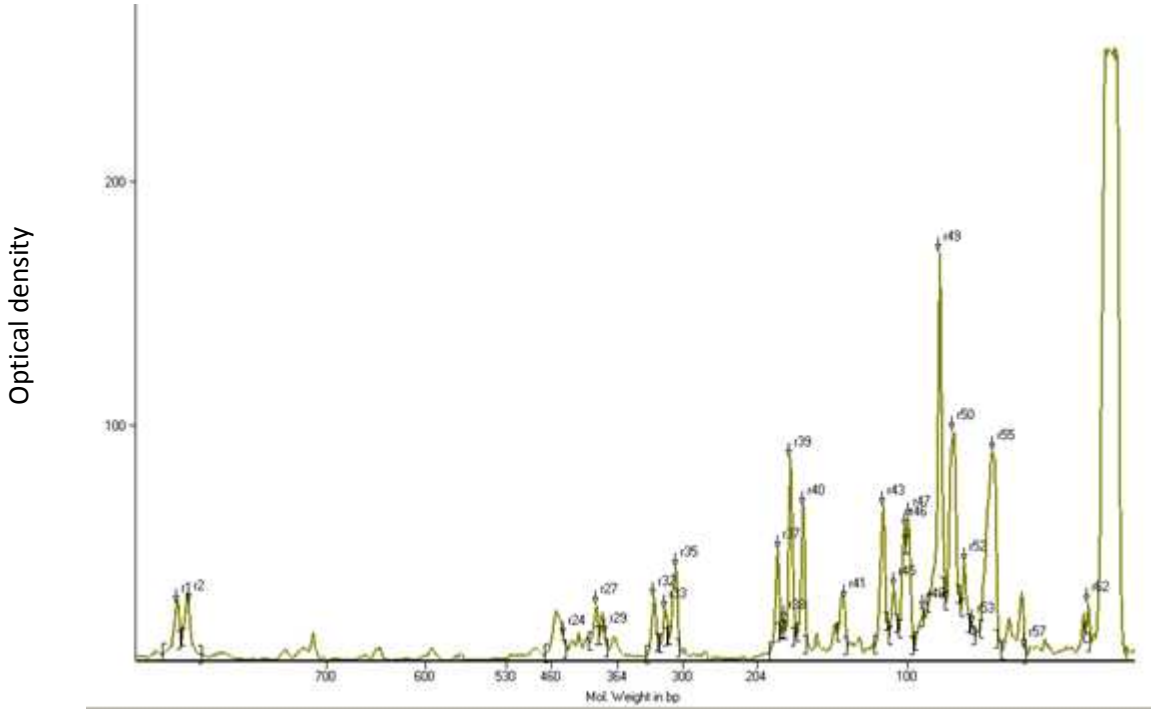
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotics (final stage)** generated by digestion with *Hae.* (Lane 26)



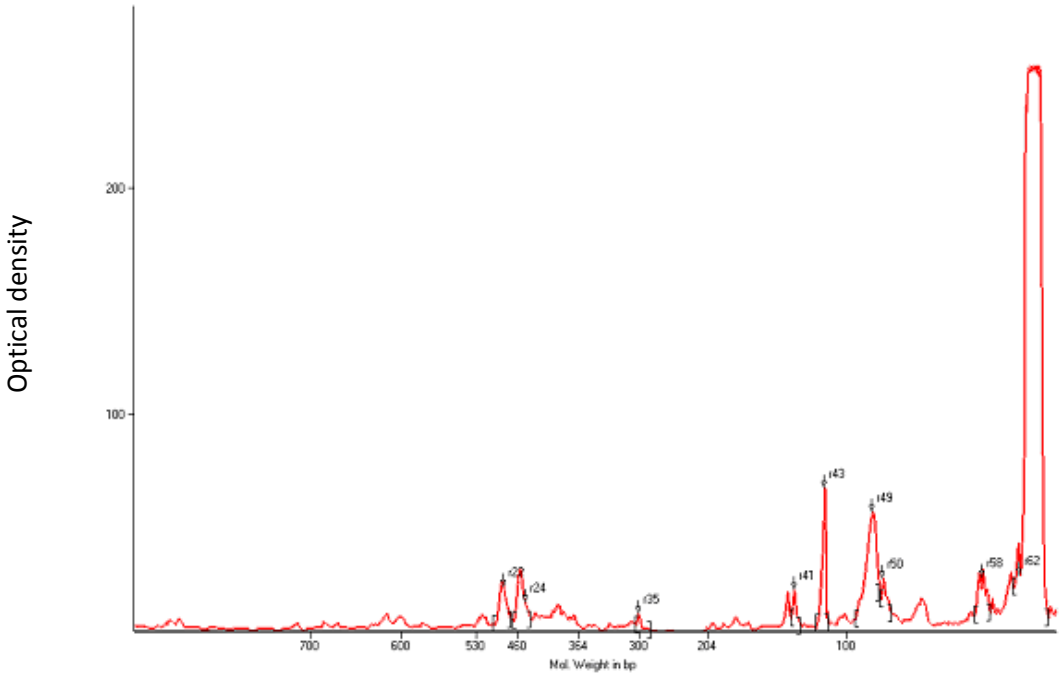
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **three antibiotics (after one week)** generated by digestion with *Hae.* (Lane 9)



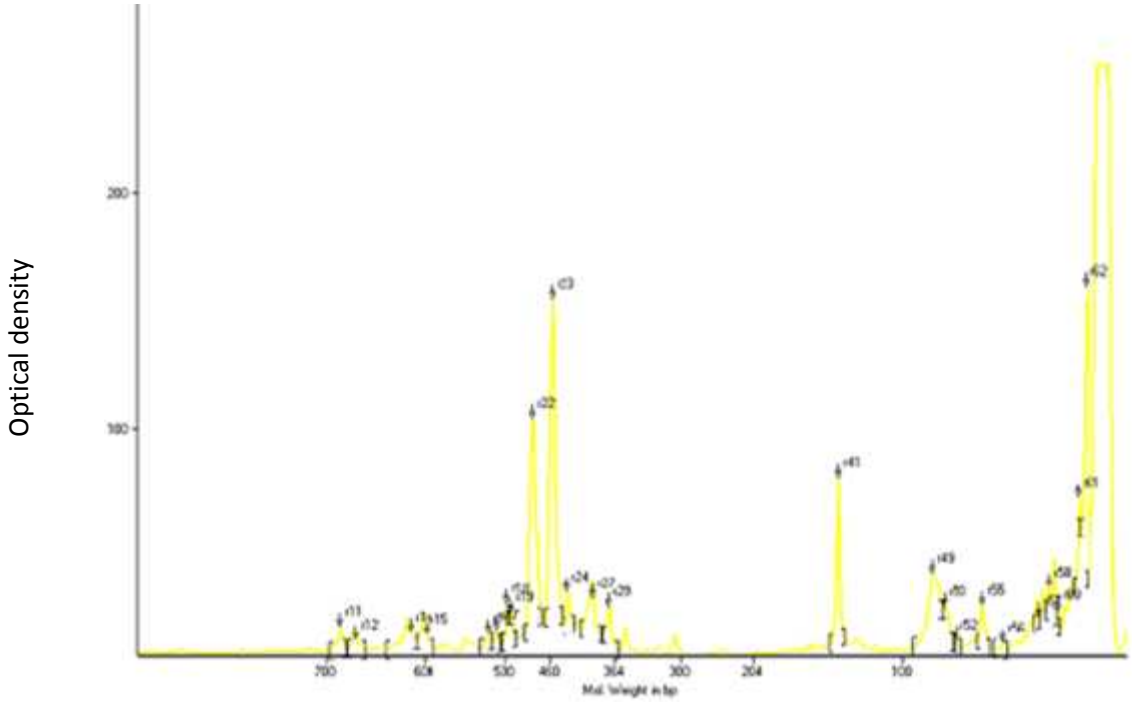
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **three antibiotics** (**final stage)** generated by digestion with *Hae.* (Lane 3)



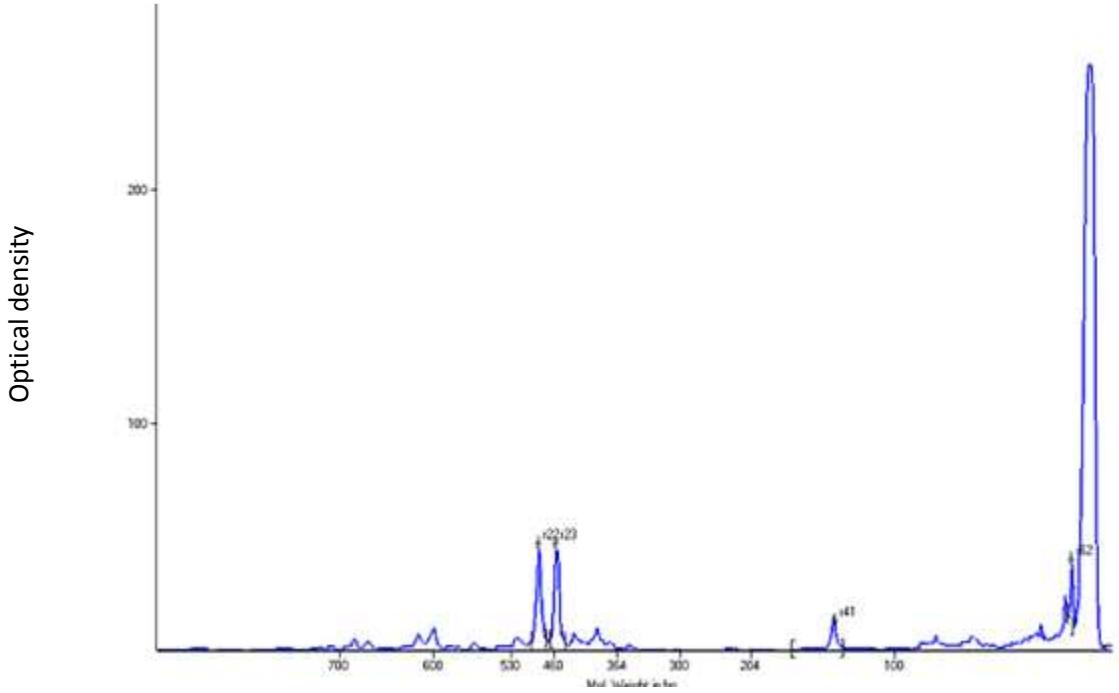
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments **without antibiotic (control after one week)** generated by digestion with *Msp I.* (Lane 22)



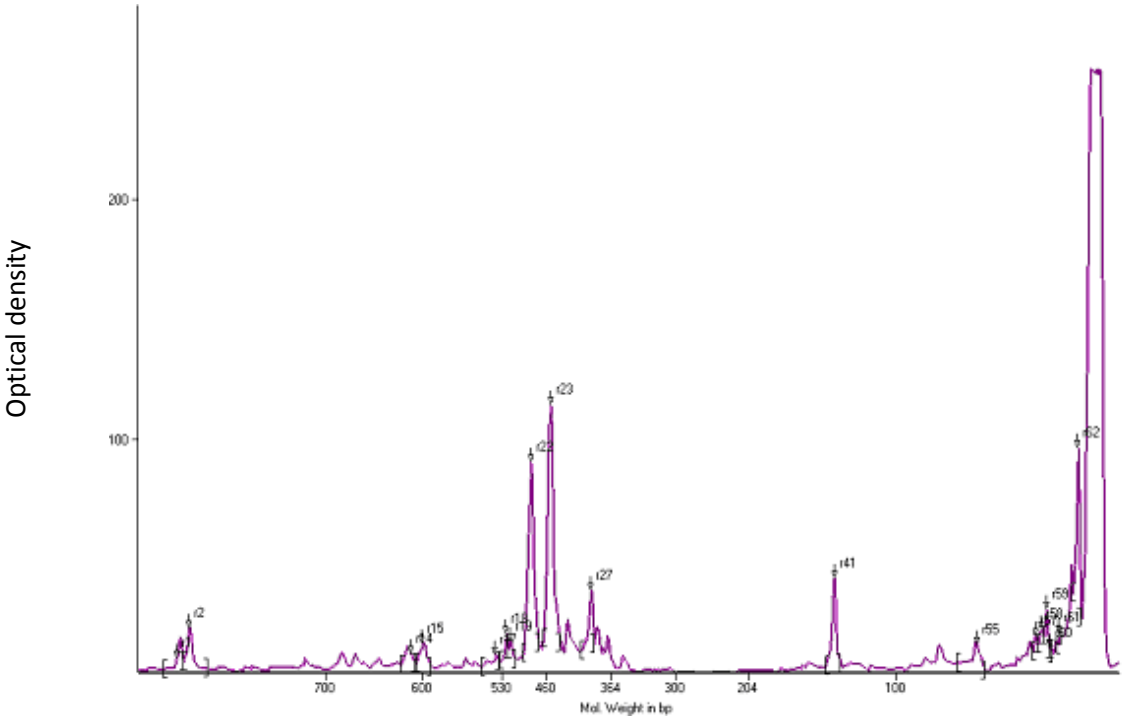
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments **without antibiotic (control final stage)** generated by digestion with *Msp I.* (Lane 13)



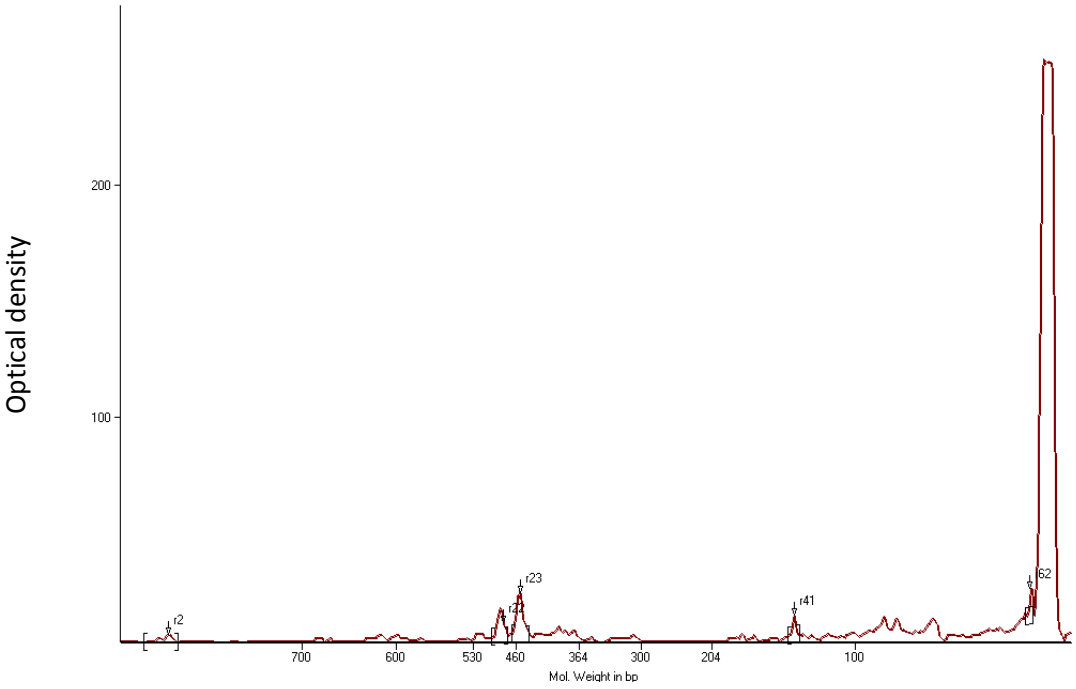
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **one antibiotic (after one week)** generated by digestion with *Msp I.* (Lane 16)



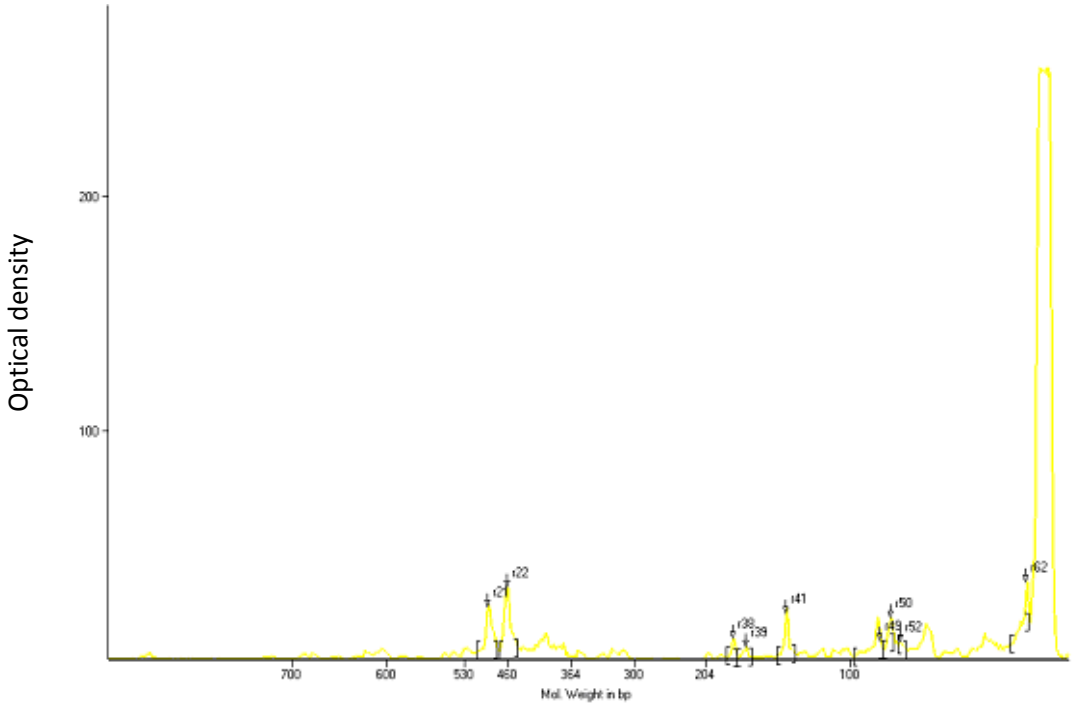
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with one **antibiotic (final stage)** generated by digestion with *Msp I.* (Lane 27)



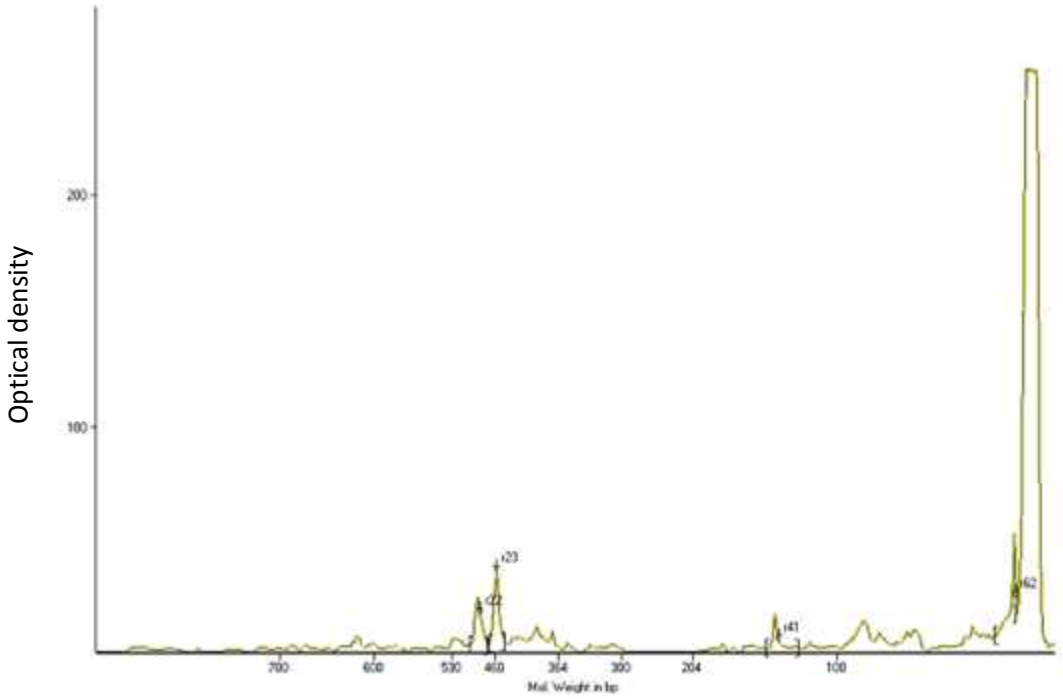
Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotic (after one week)** generated by digestion with *Msp I.* (Lane 24)



Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **two antibiotics (final stage)** generated by digestion with *Msp I.* (Lane19)



Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **three antibiotics (after one week)** generated by digestion with *MspI.* (Lane 4)



Electropherograms of T-RFLP patterns from communities of Barceloneta sediments with **three antibiotics (final stage)** generated by digestion with *Msp I* (Lane 10)