INSTITUTO TECNOLÓGICO DE COSTA RICA

SCHOOL OF CHEMISTRY

DEPARMENT OF ENVIRONMENTAL ENGINEERING

UNIVERSITY OF CENTRAL FLORIDA

COLLEGE OF SCIENCES

DEPARTMENT OF CHEMISTRY

Final Project of Graduation to complete the requirements for the bachelor's degree in Environmental Engineering

"Study of the capacity of bacterial species isolated from oil contaminated marine sediments cores to colonize and produce biofilm on virgin PE and PET microplastics under controlled marine environment."

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TEC | Tecnológico de Costa Rica

Liberia, Guanacaste, April 2021

“Study of the capacity of bacterial species isolated from oil contaminated marine sediments cores to produce biofilm and colonize virgin PE and PET microplastics under controlled marine environment”

Report presented to the School of Chemistry at Tecnológico de Costa Rica as a partial requirement to complete the protocols in order to obtain the degree in Environmental Engineering

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DEDICATION

"Agradezco a Dios por permitirme culminar esta etapa de mi vida y por todas las experiencias vividas. Dedico este trabajo a mi familia, por ser mis pilares, motivarme a siempre continuar y nunca rendirme en mis sueños. Mi papá, Róger Rodríguez Araya, quien ha sido un ejemplo de perseverancia, de paciencia y fe. Mi mamá, Azálea Cordero Castro, por enseñarme a ser fuerte, independiente y disciplinada. Mi hermano, Róger Antonio Rodríguez Cordero, por sus buenos consejos, apoyo incondicional y motivación. Además, agradezco a mi tía Leda Cordero, y mi familia en Cartago, por ser mi hogar por muchos años. Y finalmente, dedico mi tesis muy especialmente a mi abuelita Anita Castro, por su amor tan dulce que siempre me dio, por sus oraciones y por ser mi ángel desde el cielo. Gracias a ella por todas sus enseñanzas, que me han permitido convertirme en la persona que hoy soy".

"I thank God for allowing me to complete this stage of my life and for all the experiences I have had. I dedicate this work to my family, for being my pillars, motivating me to always continue and never give up on my dreams. My father, Róger Rodríguez Araya, who has been an example of perseverance, patience, and faith. My mom, Azálea Cordero Castro, for teaching me to be strong, independent, and disciplined. My brother, Róger Antonio Rodríguez Cordero, for his good advice, unconditional support, and motivation. In addition, I thank my aunt Leda Cordero, and my family in Cartago, for being my home for many yearsAnd finally, I dedicate my thesis especially to my grandmother Anita Castro, for her sweet love that she always gave me, for her prayers and for being my angel from heaven. Thanks to her for all her teachings that have allowed me to become the person I am today".

ACKNOWLEDGMENTS

“Estoy profundamente agradecida con la Dr. Beazley por recibirme en su laboratorio, por creer en mí y siempre ayudarme de cualquier manera posible. También agradezco sinceramente a mi tutor, el Dr. Céspedes Camacho, por su apoyo desde el inicio hasta el final de mi proyecto y por siempre motivarme a dar lo mejor de mí. Y a todos mis amigos que hicieron de mi etapa universitaria una de las mejores experiencias de mi vida. Finalmente, estoy muy especialmente agradecida con Nicholas Young, por su apoyo y amor incondicional en cada momento y por ayudarme a alcanzar mis metas cuando las circunstancias no fueron las mejores (COVID-19).”

“I am deeply grateful to Dr. Beazley for welcoming me into her laboratory, for believing in me and always helping me in any way she could. I also sincerely thank my tutor Dr. Céspedes Camacho for his support from the beginning to the end of my project and for encouraging me to always do my best. And to all my friends who made my university life one of the best experiences of my life. Finally, I am especially grateful to Nicholas Young, for his unconditional support and love in every moment and for helping me to achieve my goals when circumstances were not the best (COVID-19)”

LIST OF ACRONYMS AND ABBREVIATIONS

ASW	Artificial seawater
BLAST	Basic logical alignment search tool
CRA	Congo red agar
CV	Crystal violet
DDTS	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DWTP	Drinking water treatment plants
EG	Ethylene glycol
GESAMP	Group of Experts on the Scientific Aspects of Marine Environmental Protection
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
MMT	Million metric tons
MPs	Microplastics
M10b	Brackish marine medium
NB	Nutrient Broth
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PE	Polyethylene
PET	Polyethylene terephthalate
P_F	Forward primer
POPs	Persistent organic pollutants
PP	Polypropylene
P-P&A fibers	Polyester, polyamide, and acrylic fibers
P_R	Reverse primer
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
SI	System of Units
TCBS	Thiosulfate-citrate-bile salts-sucrose
tdh	Thermostable direct hemolysin
tlh	thermolabile hemolysin
TPA	Terephthalic acid
trh	Thermostable direct hemolysin-related hemolysin
UCF	University of Central Florida
UV	Ultraviolet
vide infra	See below
vide supra	See above
WWTP	Wastewater treatment plants
1M	Marsh 1
2M	Marsh 2
3M	Marsh 3
1S	Subtidal 1
2S	Subtidal 2
3S	Subtidal 3

LIST OF SYMBOLS

%	percentage
µm	micrometer
mm	millimeter
µg	microgram
g	gram
ppm	parts per million
°	degrees
cm	centimeter
NaCl	sodium chloride
mL	milliliter
°C	degree Celsius
ng	nanogram
1:1	dilution
µL	microliter
OD_{595}	optical density at 595 nanometers
nm	nanometer
≥	greater than or equal to
≤	less than or equal to
<	less than
x	concentration
rpm	revolutions per minute
S	Svedberg unit
+	positive
-	negative

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ABSTRACT

From the estimated 5.25 trillion floating plastic particles in the ocean, 92% are microplastics (MPs). Their surfaces can be colonized by bacteria, which make these polymers potential vectors for the dispersion of pathogens through marine environment and high food chains. This study shows results of the ability of bacterial isolates from marine sediments to colonize PE and PET MPs under controlled marine environment. The presence of presumptive *Vibrio parahaemolyticus* was examined by TCBS and the biofilm production capacity was determined by Congo red agar and Crystal violet tests. MPs were analyzed under SEM and the species identification was carried out by qPCR, PCR and DNA sequencing. No *V. parahaemolyticus* were present in the analyzed samples and only 22% of the total samples were positive in both CV and CRA tests. However, both PE and PET MPs exhibited bacterial adhesion in all sequenced samples, and PE presented the highest biofilm production. It was determined for the first time the biofilm production capacity of *M. yunnanensis, V. dokdonensis, B. flexus* and the presumptive *F. arsenicus* and *F. nanhaiensis* on both polymers. In addition to *B. safensis* ability to produce biofilm on PE, *B. pumilus* attachment to PET and the biofilm production capacity of the presumptive *B. aquamaris* and *B. vietnamensis* on PE. These new findings may serve as an insight for future studies regarding the ability of the identified species to colonize MPs under marine environment, which might represent a possible risk due to their high persistence and potential pathogenicity.

Keywords: PLASTIC POLLUTION, MICROPLASTICS, POLYETHYLENE, POLYETHYLENE TEREPHTHALATE, MARINE SEDIMENTS, BACTERIA, BIOFILM

1 INTRODUCTION

The production of plastics has increased significantly responding to a globally growing demand [1]. In 2015, around 407 million metric tons (MMT) of plastic were produced worldwide [2], and about 21 MMT of plastic waste were estimated to enter the ocean in 2016 [3]. Nevertheless, if current production and use trends continue, it is estimated that plastics generation could rise to approximately 2 billion metric tons by 2050 [1], [4], and it could reach a total of 90 MMT of plastic waste entering the marine environment by 2030 [3]. Moreover, due to COVID-19 pandemic, existing global efforts aimed to reduce plastic waste have been affected [5], since there has been a high demand for plastic products such as face masks, face shields, gloves, sanitizer bottles and non-reusable plastic for packaging of delivered products [5].

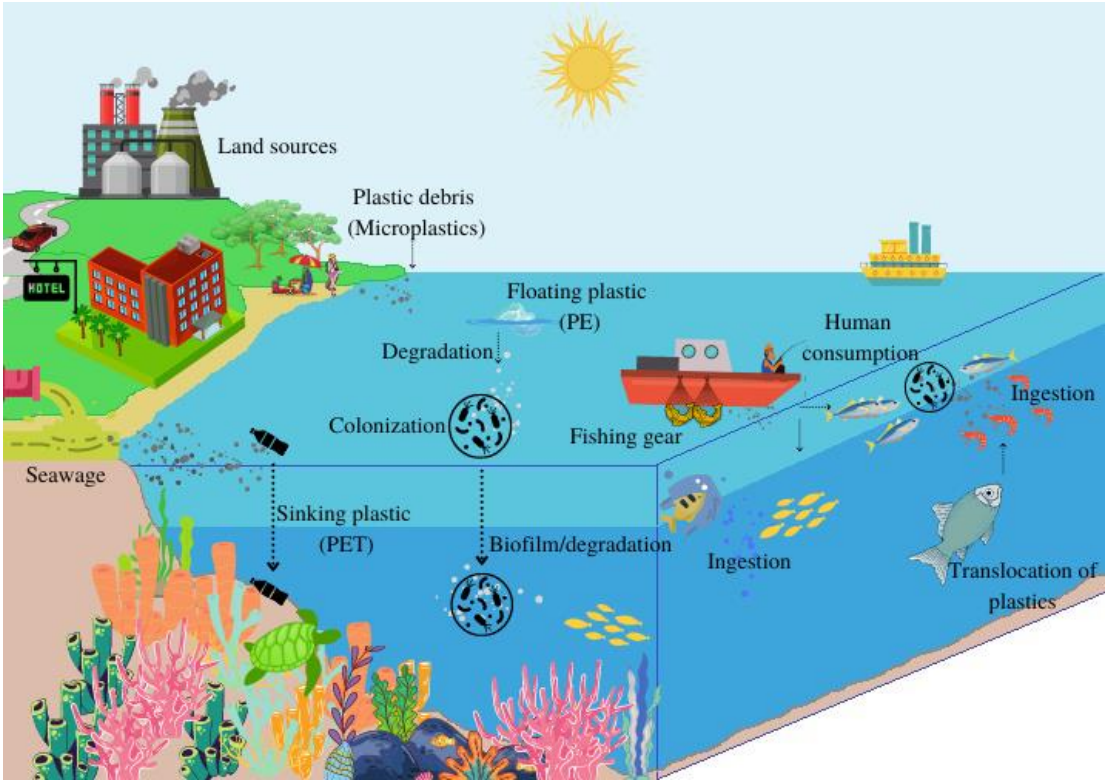

Figure 1. Lifecycle of plastics (land-marine environment). Adapted from [6].

Plastic debris enter the ocean through inland waterways, wastewater outflows or transport by wind and tides [7] (Figure 1). Polyethylene (PE) and polyethylene terephthalate (PET) are two of the most common plastics founded in the ocean [8], [9]. These polymers are usually

synthesized from fossil fuels [10], and it takes many years to break down the everyday items such plastic bottles (450 years) or plastic bags (20 years) [11]. Also, some MPs possess additive chemicals (e.g. plasticizers and flame-retardants) [9] that might contain hazardous substances, representing potential health problems [10], [12], [13]. Once plastics are in the environment, may considerably affect wildlife thorough ingestion or entanglement [14].

At least 5.25 trillion plastic particles were estimated to be floating on the sea [15], of which 92% are microplastics (MPs) [15], [16]. These small particles (i.e. < 5 mm) are either man made MPs such resin pellets, micro-beads, plastic powders [17], or can be generated as a result of weathering and fragmentation of larger plastic debris [9], [17]. Their hydrophobic surfaces can be rapidly colonized by microorganisms such as bacteria (i.e. by biofilm production), which make these polymers potential vectors for the dispersion of pathogens through marine environment and high food chains [6], [18], [19]. Since MPs are easily ingested by many commercial species [20] (e.g. fishes, oysters [21] and shrimps [22]), this could represent a possible pathway for seafood contaminated with MPs [23], [24] and pathogenic bacteria [25] to be ingested by humans. Furthermore, this situation could also imply repercussions for the shellfish and tourism industry [21], [26].

Biofilm production on MPs may contribute to its biodegradation [19] or to increase the density, promoting these to sink through the water column [27], [28]. The persistent occurrence of MPs in different environments such as sea, marine species [20], soils [29], air [30], sewage [31], drinking water [32] and even human placentas [33] is a major concern that requires great attention.

The aim of this work is to explore the relationships between non-weathered MPs and different bacteria species isolated from oil contaminated marine sediments from the Gulf of Mexico. For this purpose, the presence of presumptive *Vibrio parahaemolyticus* and *Vibrio cholerae* was examined by Thiosulfate-citrate-bile salts-sucrose (TCBS) test. Biofilm production capacity of the isolated colonies was determined by means of Congo red agar (CRA) and Crystal violet (CV) assays. Moreover, the ability of the bacterial isolates to colonize PE and PET MPs under controlled marine environment conditions was studied using scanning electron microscopy (SEM). Species identification was carried out by polymerase chain

reaction (PCR), quantitative polymerase chain reaction (qPCR) and deoxyribonucleic acid (DNA) sequencing.

Understanding the interactions between MPs and biofilms would provide crucial insights into plastic degradation and potential MPs impacts in food webs [34], needed for future research.

2 OBJECTIVES

2.1.1 General objective

Study the ability of different bacterial species, isolated from oil contaminated marine sediments, to colonize virgin (non-weathered) microplastics under controlled marine conditions.

2.1.2 Specific objectives

- Determine the capacity of bacterial species isolated from marine sediments to produce biofilm using Congo red and Crystal violet assays.

- Analyze the ability of bacteria species isolated from marine sediments to colonize polyethylene and polyethylene terephthalate microplastics under controlled marine conditions.

3 LITERATURE REVIEW

3.1 PLASTIC POLLUTION

The global demand for plastics has exponentially increased over the past 70 years. China, Asia (excluding China), Europe and North America are the most dominant regions in plastic manufacturing worldwide [1]. Due to their versatility, resistant and low economic cost, plastics have become attractive and suitable for many applications such as packaging [35], building and construction, transportation, electrical, consumer products, industrial machinery, textiles and others [2], [10].

Plastics are a group of synthetic polymers formed by long chains of monomers, usually synthesized from fossil fuels or biomass (biopolymers) [10]. They are primarily classified as thermoplastics (i.e. capable of being deformed by heating) and thermoset (i.e. cannot be re-moulded) [36]. Also, some plastics have additive chemicals such as plasticizers, flame-retardants, antioxidants, lubricants, pigments, ultraviolet (UV) stabilizers, and others, that could be including during plastics manufacture [12], [9]. However, these additives might contain hazardous substances (e.g. bisphenol A, boric acid and phthalate) and can be found in significant concentrations in some polymers, which represents a potential health risks as endocrine disrupting [10], [36] and cancer-causing agents [13].

There are different types of plastics, such as PE, PET, polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC); in addition to polyester, polyamide, and acrylic (P-P&A) fibers [2], to include the most common ones, which due to their widespread and very common use are highly likely to be disposed of in both terrestrial and aquatic environments [35], [37].

In 2015 it was estimated that 407 MMT of primary plastic (i.e. from virgin materials) where produced, of which most corresponded to PE (29%), PP (17%), P-P&A fibers (14%), PVC (9%) and PET (8%) [2], [9] (Figure 2). Moreover, industrial sectors with the highest production were packaging (36%), building and construction (16%), and textiles (14%) [2]. However, plastics generation might increase to approximately 2000 MMT by 2050 if current tendencies remains the same [1], [4].

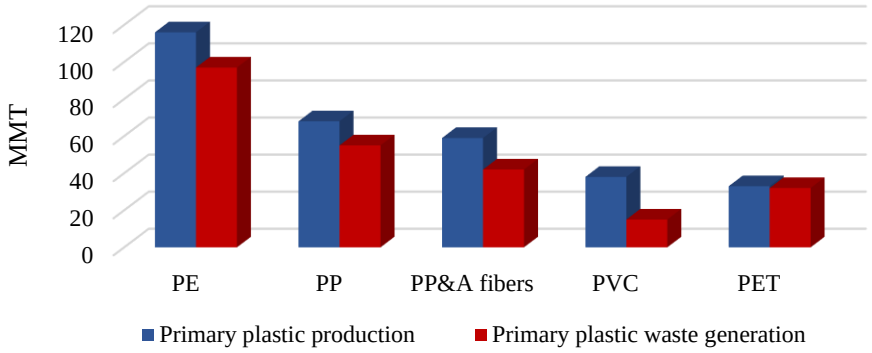

Figure 2. Global primary plastics production and primary waste generation of the most common plastics in 2015. Adapted from: [2]

In addition, 302 MMT of plastic waste were recorded in 2015, and it was mainly constituted by PE (32%), PP (18%), P-P&A fibers (14%) and PET (11%) [2] (Figure 2). Currently, 40% of all plastic is placed in landfills, 25% is incinerated, 19% is considered unmanaged dumps or leaks, 13% is recycled and about 3% is rejected or lost [38]. Nevertheless, it was estimated that by 2050 around 12000 MMT of plastic waste (i.e. cumulative since 1950) might be accumulated in landfills or environment if worldwide waste management does not improve [2]. On top of this, in 2017 the Chinese government imposed a ban on the import of plastic waste and other materials into China, which over the las three decades was the biggest importer (i.e. also trader) of global plastic waste [39]. This fact has had a considerable change on plastic waste management [40] and plastic waste trade flows over the world [39].

Unfortunately, mismanaged plastic waste is entering the ocean through inland waterways, wastewater outflows or transport by wind and tides [7], which can generate negative impacts on wildlife and humans [20]. A recent study, based on solid waste, economic status, and population density from 192 coastal countries, indicated that approximately 4.8 to 12.7 MMT of plastics waste entered the ocean in 2010 [7]. Moreover, in 2016 another investigation, founded on 173 countries waste generation, estimated that 19 to 23 MMT of plastic waste ended up in aquatic ecosystems, and it was predicted that 90 MMT of plastic waste may enter the sea by 2030 [3]. As a consequence, it is expected that there could be more plastic in the sea than fish biomass by 2050 [38].

3.2 MICROPLASTICS IN MARINE ENVIRONMENT

3.2.1 Microplastics description

Plastic debris that has been fragment into smaller particles are called MPs [41], originally designated as those pieces smaller than 5 mm [4], [5]. However, this term has been defined in different ways by various researchers [35], according to their size range proposals [37]. In addition, some scientists have disputed that MPs should be determined based on the International System of Units (SI), as particles smaller than 1 mm (1000 μm) [9]. However, due to the lack of an international agreement about plastic debris sizes and sampling [17], [43], the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) decided to reach a consensus for plastic marine litter definition based on existing research (Table 1) [9]. Therefore, a diameter between 5 mm and 1 μm was recommended as a size range for microplastics. This would prevent excluding any relevant study previously published [9].

Table 1. Classification of plastic marine litter. Adapted from [9].

Class	Common size ranges
Macroplastics	25 – 1000 mm
Mesoplastics	5 – 25 mm
Microplastics	< 5 mm
Nanoplastics	< 1 μm

There are two categories of MPs which are based on how they were originally manufactured [17]. “Primary” MPs are those intentionally fabricated for a specific purpose or as a predecessor to a diversity of products [43]. These include micro-beads used in cosmetics, personal care products such, toothpaste [44], hand and facial cleaners [45], in addition to plastic powders for moulding [46], resin pellets used during plastics production and others [29], [47]. “Secondary” MPs are generated as a consequence of the fragmentation and weathering of larger plastic materials [43], which might happen during product use (e.g. paint flakes and textile fibers [9]) or when they have been deposited into the environment [48].

MPs in marine environment are primarily composed of PE, PP, and PS, which tend to float, in addition to PET, PVC and polyamide (nylon) MPs which, conversely, are more likely to sink [17]. Moreover, the majority of MPs entering the marine environment are mostly from secondary category [49].

3.2.2 Behaviors and distribution of MPs in marine environment

The behavior and distribution of MPs through the global marine environment can be discussed under three main behavioral mechanisms: physical (migration, sedimentation and accumulation), chemical (degradation, adsorption and absorption) and biological (biofilm, biodegradation, ingestion and translocation) [19]. In this way, it is possible to understand and effectively address the problems generated by the presence of MPs in the environment.

3.2.2.1 Physical

Migration relates to the transport of MPs across the oceans due to wind, tide, or even natural phenomena such as tsunamis [50]. Moreover, sedimentation depends on the density of the plastics, however, MPs density may increase during their stay in the aquatic environment due to the colonization by microorganisms, metals or organic litter adherence [27], [28], allowing the vertical transport of the plastic particles through the water column [19], [8]. Also, a higher density can cause the MPs to sink to the bottom of the marine sediments [44]. Nevertheless, the MPs accumulation could be considered as temporal (i.e. plastic waste available to enter the ocean from land) and spatial (i.e. extension of MPs in the global marine environment) [19]. The temporal accumulation depends on the disposal of the plastic waste from the land to the ocean, while the spatial accumulation is controlled by abiotic and biotic factors according to the marine environment (*vide supra*). Still, high concentrations of floating plastics in the centers of the five ocean gyres (i.e. North Pacific, North Atlantic, South Pacific, South Atlantic and Indian Ocean) has been evidenced [15], [47]. These accumulations of plastic marine litter are commonly referred to as "garbage patch", which is comparable to a large island of garbage [51].

3.2.2.2 Chemical

Moreover, degradation refers mainly to a modification in the properties of the polymers, induced by environmental factors [19], and is ordinally classified as photo-oxidative degradation, thermal degradation, ozone-induced degradation, mechanochemical degradation and catalytic degradation [52]. In addition, it has been shown that MPs can absorb persistent organic pollutants (POPs) [53] such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDTs) [19]. PAHs concentrations from 0.001 to 9.3 ppm (µg/g-plastic fragment) were detected in PE and PP fragments recollected from open ocean and beaches from different locations such as Vietnam, Japan, United States of America, and Costa Rica [54]. Also, PCBs (0.001 – 0.436 ppm) and DDTs (0.0002 – 0.124 ppm) concentrations were detected [54]. Additionally, the accumulation of heavy (toxic) metals has been demonstrated, showing a high affinity with MPs [13]. Heavy metals such lead, chromium and zinc have showed significant adsorption to PE and PVC [55]. A recent study, carried out in Canada, investigated the interaction of MPs with crude oil and how these, due to the formation of MPs-oil-dispersant agglomerates, may reduce the effectiveness of oil dispersants applied during oil spill response operations[56].

3.2.2.3 Biological

Although there is a very interesting interaction between metals, chemical additives, and MPs, it has also been demonstrated that the surfaces of these microparticles can be rapidly colonized by bacteria [57]. Moreover, they can become excellent vectors for the dispersion of pathogenic microorganisms through the aquatic environment [18].

Due to synthetic MPs are chemically and physically dissimilar from naturally substrates, they represent a new sort of substrate to microorganisms [58]. Their surface could promote multicellular aggregates, usually referred to as biofilm [59], and may even become an “artificial microbial reef” formed from a wide microbial community (e.g. heterotrophs, autotrophs, predators, and symbionts) currently known as “Plastisphere” [60].

Biofilms are determined as “matrix-enclosed bacterial population adherent to each other and/or to surfaces or interfaces” [61]. Within these microcolonies, bacteria can build up in organized communities with functional heterogeneity [62]. Bacterial cells generate

extracellular polymers (i.e. exopolysaccharides) to create complex matrixes [61] that will allow survival in hostile environments [62] mainly caused by biotic and abiotic stress (e.g. pH, salinity and temperature) [63]. This exopolysaccharides biosynthesis consists of assimilation of a carbon substrate, intracellular synthesis and finally its exudation out of the bacterial cell [63], [64]. In the first stages of biofilm formation, the bacteria are in a stable union with cells of the same or different species, thus forming microcolonies of single and mixed species [61]. Once a considerable number of cells have joined together, the biofilm begins to mature by producing an extracellular matrix, which also contributes to the final architecture of the microbial community [59].

In addition, the regulation of bacterial genes is carried out through quorum-sensing, which responds to the cell population density, and occurs by the creation, secretion, and detection of autoinducers (i.e. extracellular signal molecules). Bacteria use quorum-sensing circuits to control physiological functions such as motility, conjugation, competence, sporulation, virulence and biofilm formation [65]. However, the biofilm structure is related to chronic infections, antibiotic and bactericide resistance [62].

However, the high molecular weight and hydrophobicity have made plastics a resistant material to biodegradation (excluding biopolymers) . As mentioned before, petroleum-based MPs degradation is limited by specific abiotic factors (e.g. UV radiation and temperature) [66]. However, it has been demonstrated that there is a variety of microorganisms (mainly bacteria and fungi) capable of partially degrading polymers such as PE [67], PS [68], PP, [69], PVC [70] and PET [71]. These microorganisms (*vide infra*) have been isolated from marine water, soil contaminated by crude oil, guts of plastic-eating worms and others [72]. Nevertheless, they are unable to completely decompose plastics by metabolic or enzymatic action [66].

Due to their small sizes, MPs can be easily ingested by a wide variety of marine species [20], [73]. Several studies have shown the ingestion of MPs from commercially marine species such as mollusks [73], [74], shrimps [22], [75], [21], fishes and oysters [21]. Therefore, there is concern that MPs contaminated by pathogenic bacteria could be present in high food chains as the mentioned seafood species or others, which could become a vector to expose the consumer to these pathogens [18]. In addition, recent studios in Portugal have estimated a

human intake of about 842 MPs particles per year, from fish consumption (i.e. 15600 g fish muscle/year) [23]. Furthermore, many studies have stated the presence of marine debris into the digestive tract of sea turtles [14], [76], [77], [78], as well as in different seabirds species [14], [79]. Some of them are displayed in Table 2, as well as the MPs detected.

Table 2. Some of the species of marine life that have evidenced ingestion of plastic marine debris.

Animal	Marine species	Location	Plastic	References
Mollusk	*Crassostrea gigas*	United Kingdom	PS	[74]
	Sinonovacula constricta	East China	PE	[21]
Shrimp	*Fenneropenaeus indicus*	India	Polyester, polyamide, PE	[22]
	Paratya australiensis	Australia	Rayon, polyester	[75]
	Parapenaeopsis hardwickii	East China	PE	[21]
Fish	*Larimichthys crocea*	East China	PET	[21]
	Scomber colias	Portugal	PE, polyester	[23]
Sea turtle	*Chelonia mydas*	Brazil, Atlantic Ocean, American Samoa and Hawaii	Bags, fragments	[14], [76], [77]
	Lepidochelys olivacea	American Samoa and Hawaii	Bags, fragments, netting, rope	[77]
Sea bird	*Macronectes giganteus*	Brazil	Fragments, pellets	[14]
	Puffinus griseus	Brazil	Fragments, pellets	[14]
	Larus dominicanus	Uruguay	PE, PP	[79]

There are different studies about the translocation of MPs to other tissues once ingested [19]. Investigations with mussels (*Mytilus edulis)* has shown the presence of PE microparticles in their gills, stomach and digestive glands after 3 hours of exposure to MPs [80]. Also, translocation of PS microparticles from their gut cavity to the circulatory system has been evidenced after 3 days of exposure and persisted over 48 days [74].

Tropic transfer of MPs has also been investigated. Crabs *(Carcinus maenas)* fed with mussels *(Mytilus edulis),* previously exposed to fluorescent PS microspheres, presented MPs in their stomach, hepatopancreas, ovaries and gills [81]. In addition, zooplankton labelled with ingested microspheres (10 μm fluorescent PS) was offered to mysid shrimp (*Neomysis integer),* demonstrating the transfer of MPs from one trophic level to a higher one after incubating these organisms together for 3 hours [82].

3.2.3 Other impacts of microplastics

Although the presence of MPs in seafood represents a potential risk to food safety, the risks to human health cannot yet be accurately estimated due to the complexity of MPs and their toxicity [73]. However, their presence in food and water intended for human consumption deserves attention [32]. There are studies related to the occurrence of MPs in drinking water sources and potable water [32]. For example, in the Czech Republic, it was observed an average range of 1473 to 3605 plastic particles per liter in raw water from three drinking water treatment plants (DWTP), where the MPs size were approximately 1 to 10 μm and mainly composed of PET, PP and PE (fibers and fragments) [83]. However, this composition may vary from one plant to another according to the characteristics of the raw water and its origin.

In addition to the contamination generated, MPs could compromise standard methods of water disinfection. Chlorination efficiency may decrease due to the presence of MPs suspended in water, acting as protective substrates for pathogenic microorganisms [84], [85], also, plastic debris could be oxidized by ozone and reduce the amount of available ozone applied to react with bacteria in water [85], [86]. Nevertheless, the wastewater treatment plants (WWTP) should have the capability to trap MPs coming from households (e.g. laundry and cosmetic use), industries, and others, to prevent MPs from entering natural aquatic environments [32]. Microplastics such as low-density polyethylene (LDPE), high-density polyethylene (HDPE), PP, PS are commonly found in a WWTP [31], which has made them one of the main sources of MPs due to the large number of fibers from clothing and plastic beads from personal care products [31]. However, nowadays there is no legislative limit for the content of MPs in drinking water and wastewater, still, different techniques and methods

have been tried to remove plastics debris from water such as coagulation/flocculation, sedimentation, filtration, magnetic extraction [83] and electrocoagulation [87]. Even so, there is a lacking of further research, regulation [84], efficient technology and treatment method aimed at removing MPs from water [32].

Besides that, different studies suggested an ubiquitous existence of MPs in soil environments [29], which might affect soil biophysical environment if there is a considerable concentration of MPs [49]. However, their presence is possibly conditioned by the sources and hydrogeographic characteristics. Also, there is a lack of standard methods for extraction and identification of MPs in soils [29].

3.3 PROPERTIES AND BACTERIAL COLONIZATION OF PE AND PET MICROPLASTICS

3.3.1 Properties of PE and PET

PE, PP, PS, PET and PVC MPs are the most abundant in marine environment (*vide supra*). Table 3 lists some of the characteristics of the two plastics of interest in this study: PE and PET. Besides being two of the most common plastic marine debris, they possess distinctive properties, which allows a comparison of two different possible "plastisphere".

Table 3. Principal characteristics of PE and PET.

Properties	PE	PET	References
Thermoclassification	Thermoplastics	Thermoplastics	[10]
Specific gravity	0.91-0.95	1.34-1.39	[9]
UV resistance	Low	Good	[88]
Oxidation resistance	Low	Good	[88]
Polarity	Nonpolar	Polar	[89]
Enzymatic degradation potential	Low	High	[6]
Mainly uses	Milk/juice jugs and bags	Bottles and strapping	[35]

Many studies have confirmed that PE is probably the most common plastic type founded in the ocean [8]. HDPE has a specific gravity of 0.94 - 0.95, and it is mainly used for milk and juice jugs [35], [9]. LDPE possesses a specific gravity of 0.91 – 0.93 and its principal application are plastic bags and six-pack rings [35]. On the other hand, PET is also one of the most regularly used plastics and has a specific gravity of 1.34 – 1.39, which allows the MPs to sink in a marine environment, due to its higher density [9]. However, PET applications are focused on bottles and strapping [9]. LDPE and HDPE present a low resistance to UV/oxidation, while the PET has a good UV/oxidation resistance [88], which might influence the degradation process.

Solar radiation can induce different types of degradation in floating MPs principally [90]. The visible spectrum leads to thermal degradation [6], while UV light induces photodegradation of polymers into monomers [91], and infrared radiation causes thermal oxidation of polymer chains [92]. However, the biodegradation of polymers includes enzymatic processes capable of hydrolyzing the plastic into oligomers and later monomers. PE contains very stable main structures that are difficult to degrade, conversely, PET is considered more susceptible to hydrolysis and the catalytic enzymes of such reactions [6].

Moreover, it has been considered that the polarity of these polymeric surfaces can influence bacterial colonization in the sea, either due to the adsorption of a conditioned or different film that favors colonization or by directly attracting more bacteria [89].

3.3.2 PE and PET colonization

Plastisphere communities have indicated a considerable difference from other surfaces (*vide supra*), where MPs can be rapidly colonized by bacteria and considered as a novel ecological habitat in the marine environment [60], [58].

Some of the bacteria commonly related to the colonization of plastic marine debris, mainly PE and PET are listed on Table 4.

Table 4. Bacterial classes and orders commonly found in plastic marine debris. Adapted from:[93].

Class	Order	Genus	Plastic	References
Alphaproteobacteria	Rhodobacterales	-	PE, PP, PS	[60], [94], [95], [96], [97]
Flavobacteria	Flavobacteriales	*Tenacibaculum*, *Algibacter*	PE, PS, PET	[94], [96], [98], [99], [100]
Sphingobacteriia	Sphingobacteriales	*Tunicatimonas*	PE, PP	[94], [60]
Epsilonproteobacteria	Campylobacterales	*Arcobacter*	LDPE	[57]
Gammaproteobacteria	Vibrionales	*Vibrio*	PE, PP, PS	[60], [96], [95], [97], [101]
	Alteromonadales	*Colwellia*, *Shewanella*	LDPE, PET	[57], [98]
	Pseudomonales	*Pseudomonas*	PE, PET	[102], [103], [104]
Bacilli	Bacillales	*Bacillus*	PE, PET	[102], [103], [105]

Phylos such as Proteobacteria (e.g. Alphaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria classes) and Bacteroidetes (e.g. Epsilonproteobacteria and Sphingobacteriia classes), are commonly reported in the literature as MPs colonizers [6]. In addition, Cyanobacteria phyla [106] and orders such as Rhodobacterales, Flavobacteriales, Sphingobacteriales, Campylobacterales, Vibrionales and Alteromonadales have been regularly found on plastic marine debris.

PE and PP MPs from the North Atlantic were found to have eukaryotic (e.g. Diatoms and ciliates) and bacterial microbiota built on these polymers [60]. The presence of photosynthetic microorganisms such cyanobacteria (e.g. *Phormidium* and *Rivularia*) and some diatoms [60] that are frequently known as biofilm formers (e.g. *Navicula*, *Nitzschia* and *Sellaphora*) [107], was evident on the plastics surface. Bacterial cells were visualized in surface pits and, curiously, the genus *Vibrio* showed a 24% dominance over a PP sample [60].

Diatoms have shown to dominate the biofilm present in PE MPs after one week in seawater [6]. Later, after two weeks exposure, the concentration of diatoms decreases (but persists) as other microorganisms such as cyanobacteria and heterotrophic bacteria adhere to the plastic. After four weeks, the microbial community continues to diversify into a complex matrix [6].

Moreover, it is considered that primary MPs colonizers are those who sensing the synthetic polymer surface and might impact the formation and dynamics of the microbial community [58]. In addition, a study in the United Kingdom also revealed that bacteria within coastal marine sediments can quickly colonize LDPE MPs. Rod and spirilla shaped bacterial cells were observed after 15 days, where *Arcobacter sp.* and *Colwellia sp.* were the most abundant [57], which also have previously been associated with hydrocarbon degradation in marine environments [108], [109], [57].

However, different studies have recognized Bacteroidetes and Proteobacteria phylum as a very common group in PET colonizing bacterial community [98]. In addition, PET biodegradation has been related to *Ideonella sakaiensis sp.* (Proteobacteria) which is able to produce enzymes (PETase) to transform PET into terephthalic acid (TPA) and ethylene glycol (EG) monomers [110]. Moreover, PET bottles in the North Sea (UK) were colonized by a mixed assemblage of microorganisms (prokaryotic and eukaryotic), where Thiotrichales and Alteromonadales orders and Flavobacteria class were highly associated with the biofilm, in addition to diatoms and cyanobacteria [94].

Due to taxonomic differences between marine sediments, water column and "plastisphere" microbial communities, MPs can serve as a vector for the introduction of nonnative species into new environments [94]. However, in addition to all mentioned research on plastics debris extracted from marine environments, there are also studies on virgin MPs (non-weathered) to better understand the microbiological behavior on plastic surfaces under controlled conditions.

Some examples of studied bacteria on virgin PE and PET are displays on Table 5, in addition to the relevance of such species with respect to their behavior with the polymer (e.g. biodegradation) or pathogenicity.

Table 5. Bacterial species studied on virgin PE and PET microplastics.

Plastic	Species	Relevance	References
PE	*Vibrio crassostreae*	oyster disease	[111]
	Rhodococcus ruber	biodegradation	[112]
	Kocuria palustris	LDPE biodegradation	[67]
	Bacillus pumilis	LDPE biodegradation	[67]
	Bacillus subtilis	LDPE biodegradation	[67]
	Bacillus gottheilii	biodegradation	[68]
	Bacillus cereus	biodegradation	[68]
PET	*Mycobacterium avium*	pathogen	[113], [114]
	Serratia plymuthica	Biodegradation	[71]
	Bacillus gottheilii	Biodegradation	[68]
	Bacillus cereus	Biodegradation	[68]

3.4 MARINE SEDIMENTS

Marine sediment consists of a mixture of material deposited on the seafloor and its sources are controlled by factors such as wind, ocean circulation, and water depth [115].

In addition, marine sediment is one of the most important ecosystem components for microbial life in the coastal aquatic environment since it provides biotic and abiotic surfaces for bacterial growth. The concentration of organic matter is at least 10,000 times higher than in the water column, making it highly attractive to microorganisms such as bacteria [116]. Variables such as temperature, salinity and organic content in the sediment are some of the determinants of bacterial composition in the sediments [117].

In some marine environments, the sediments have been contaminated by oil spills (e.g. Deepwater Horizon oil spill in 2010 [118]), however, bacteria such as Sphingomonads,

Caulobacters, Oceanospirilla, Nitrosococci, Colwelliae and Cycloclasticus play an important role in oil biodegradation [118]. Moreover, Bacillus species are commonly inhabit marine sediments and related to oil-contaminated sediments *(vide infra)* [119].

3.5 *VIBRIO* AND *BACILLUS* SPECIES

Different studies have suggested the potential transmission of pathogenic organisms through MPs [60]. One of them is the bacteria of the genus *Vibrio*, which are usually very abundant in aquatic environments such as estuaries, marine coastal waters, aquaculture, and marine sediments [120], [121]. These microorganisms are classified as Gram-negative, chemoorganotrophic, and facultative anaerobes [101], [120]. Also, they are commonly rod-shaped cells with a polar flagellum that affords their mobility [120].

An important characteristic of this species is the ability to colonize different marine organisms, while taking advantage of the nutrients released by them (e.g. sugars, amino acids and lignin [122]) [123], [124], so, it can be found in various marine species of high commercial demand (e.g. under- cooked fish, crustaceans, and mollusks [125]). [126].

However, there are some *Vibrio* species that are pathogenic for both marine organisms and humans [122]. Among the most harmful species are *Vibrio cholerae* and *Vibrio parahaemolyticus*, which are generally associated with extra-intestinal infections (wounds), and diarrheal infections [122]. Nevertheless, foodborne infections are highly associated with the ingestion of raw or under-cooked seafood [125].

There is a simple way to identify *V. cholerae* and *V. parahaemolyticus* using the selective TCBS agar. This agar contains a mixture of bile salts and sodium thiosulfate to achieve an alkaline pH for the inhibition of Gram-positive bacteria, sucrose as a fermentable carbohydrate, and thymol blue and bromothymol blue as indicators of pH changes [127], [128].

Another group of potentially pathogenic bacteria related to MPs are from the genus *Bacillus*. They are rod shaped bacteria, classified as Gram-positive, endospore-forming, aerobic or facultatively anaerobic [129]. In addition, these microorganisms are characterized by being

ubiquitous species in the environment and capable of degrading a variety of substrates such as cellulose, starch, pectin, proteins, agar and hydrocarbons [129].

Different species of *Bacillus* form spores as a survival mechanism due to starvation. Inactive spores can remain in the environment for years because of their peculiar structure and components [130]. Moreover, spores awaken rapidly in a suitable environment and the germinated spores have the potential to cause disease or food spoilage [130]. In fact, these microorganisms are commonly found in the dairy industry, causing persistent contamination and deterioration of products [131], since their capacity to produce biofilm and spores makes these bacteria difficult to eliminate [130]. However, *Bacillus* species can also be crucial for effective industrial processes such as wastewater treatment or food fermentation [131].

Some of the different *Bacillus* spp. that have been found as part of microplastic bacterial communities in marine environments are: *B. cereus* (pathogenic), *B. sphericus, B. pumilis*, *B. halodenitrificans* [105], *B. safensis* [132] and *B. thuringiensis*, to mention the most common ones. In the case of *Vibrio* spp. is important to remark the followings: *V. natriegens* [60], *V. aestuarianus, V. splendidus* [96], *V. parahaemolyticus, V. fluviales* and *V. alginolyticus* [101].

Besides that, it has been shown that species such as *B. aquimaris, B. megaterium*, and *B. pumilus*, isolated from oil-contaminated sediment in Indonesia, were related to the degradation of total petroleum hydrocarbons [119]. Furthermore, *B. aquimaris* has also demonstrated a great potential to degrade PAHs [133], which highlights possible opportunities for bioremediation.

Therefore, research on the function of different bacteria on hydrocarbons or oil derivatives, such as plastic, is of great importance. In addition, the increase of plastics marine debris requires greater attention. Their different behaviors and properties, explained above, can play an important role for the study of MPs and their consequences on the environment. However, this study is mainly focused on a biological/chemical behavior, such as bacterial interaction with virgin PE and PET microplastics under controlled marine conditions, to determine the biofilm production capacity of the studied bacteria isolated from oil-contaminated sediments.

Moreover, there are two useful techniques that can be employed to study the potential biofilm production capacity of these bacteria. Firstly, CV solution is commonly used to visualize the adhesion of bacterial cells or biofilm, generally on glass tubes or 96-well microtiter plates with medium, which can be a qualitative or quantitative assay [134], [135]. And secondly, CRA is characterized by its rich content of brain-heart infusion broth, supplemented with sucrose and Congo red dye, which acts as a stain to evidence the presence of exopolysaccharides [136].

4 MATERIALS AND METHODOLOGY

4.1 SAMPLE COLLECTION

Microorganisms were isolated from sediment samples previously collected in May 2018 from the Chandeleur Islands (Figure 3) in the Gulf of Mexico (29.895448°, -88.827780°). This chain of uninhabited islands was impacted by the Deepwater Horizon oil spill in 2010, and oil was visually found buried in sediment eight years after the spill [118].

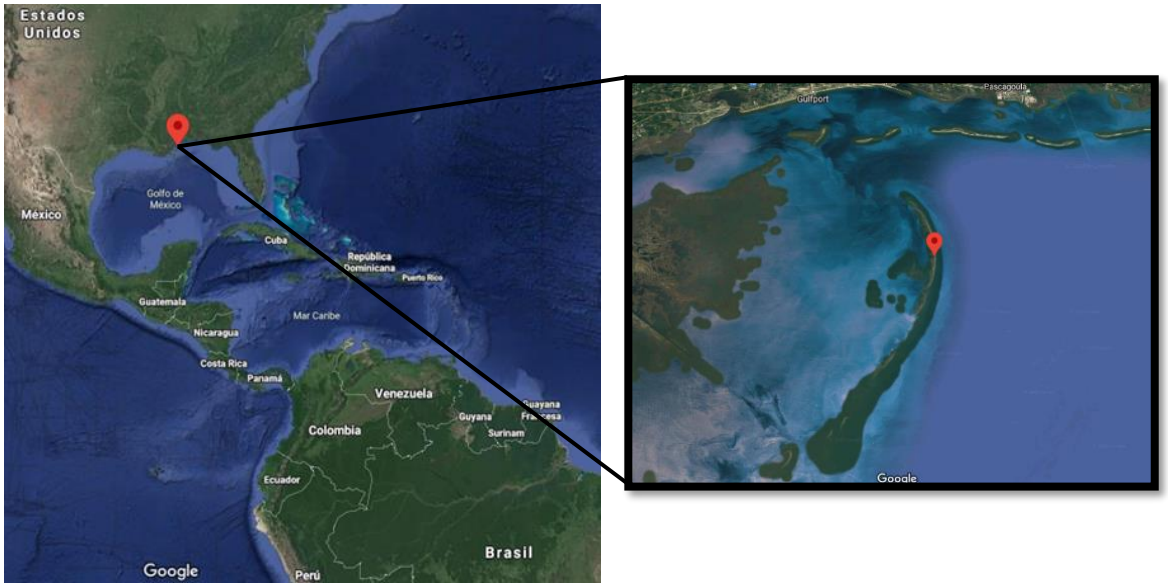

Figure 3. Chandeleur Islands, Louisiana, United States, located in the Gulf of Mexico (29.895448°, -88.827780°). The sediments cores were collected from the indicated area. Images taken from Google maps.

Triplicate sediment cores were collected from two types of sediments (Figure 4): Subtidal (S) and Marsh (M), were classified as Subtidal 1 (1S), Subtidal 2 (2S), Subtidal 3 (3S), Marsh 1 (1M), Marsh 2 (2M), and Marsh 3 (3M).

Figure 4. Subtidal (S) sediment cores were collected within the unvegetated foreground. Marsh (M) sediment cores were collected within the marsh vegetation. The darker spots in the subtidal show the buried tar mat where oil remains from the oil spill. Photo courtesy of Tatarlw and Klelnhulzen

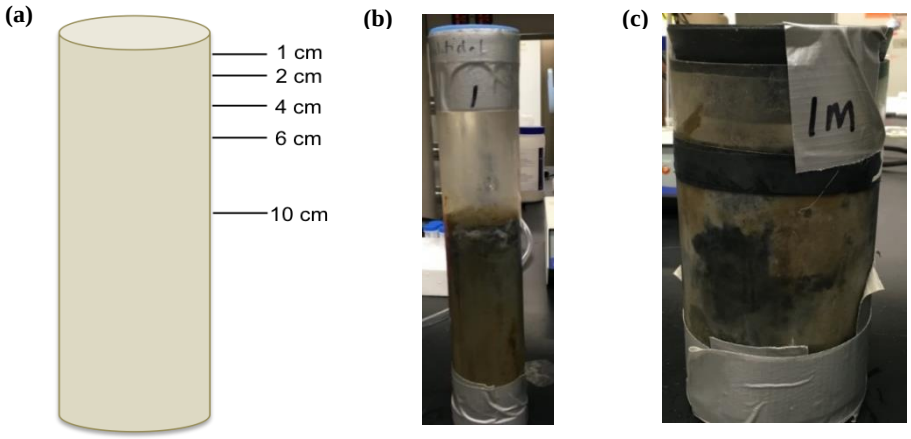

Figure 5. (a) Schematic of a sediment core showing the sections extruded measured in cm down from the sediment-water interface; (b) subtidal core; and (c) marsh core. Photos courtesy of M. Beazley.

The sediment cores were extruded in sections at different depths: 0-1, 1-2, 2-3, 3-4, 4- 6, 6-8, 8-10, 10-12 and 12-14 cm (Figure 5). Therefore, the sediment samples were classified according to their origin and location.

The following activities were carried out in the laboratory of Dr. Melanie Beazley at University of Central Florida (UCF), Department of Chemistry, Physical Sciences Building room 207.

4.2 ISOLATION OF COLONIES FROM SUBTIDAL AND MARSH SEDIMENTS

Approximately 2 grams from each sediment section was suspended in 2 mL of a sterile 0.85% sodium chloride (NaCl) solution and subsequently mixed vigorously with a vortex mixer. Then, 50 µL of this suspension was spread [137] on Nutrient Broth (NB) + 1% NaCl agar plates using a L-shaped Drigalsky, so there was a plate for each sediment location. These were properly labeled and incubated at 30°C for 24 hours.

Difco™ Nutrient Broth (Becton, Dickinson and Company) + 1% NaCl (Fisher Chemicals) agar plates were prepared by adding 15 g of Bacto ™ Agar (Becton, Dickinson and Company), 10 g of NaCl and 8 g of Nutrient Broth in one liter of ultra-pure water and sterilizing by autoclave.

The next step was to select 50 different colonies of each plate and patching onto a new agar, which consisted of taking a small amount of the colony with help of a sterilized stick and passing it to a new NB + 1% NaCl agar plate. The 50 selected colonies of the original plate were placed in order and numbered according to a grid. This process was performed for a total of 54 plates (Subtidal and Marsh) previously incubated. The new patched plates with the 50 selected colonies were incubated overnight at 30°C and subsequently stored at 4 °C.

4.3 TCBS ISOLATION TEST

TCBS agar was used to isolate presumptive *Vibrio* species, such as *Vibrio cholerae* and *Vibrio parahaemolyticus* mainly. The preparation of the TCBS consisted of suspending 44.5 g of Difco™ TCBS Agar (Becton, Dickinson and Company) in 500 mL of ultrapure water and then stirring and bringing to boil carefully [138]. The TCBS agar was then poured in Petri dishes to cool.

Small amounts of the colonies from the previous NB + 1% NaCl patched plates were cultivated on the TCBS and incubated overnight at 30°C. After the incubation period the samples were classified according to the color of the colonies. Green color was an indicator of presumptive *V. parahaemolyticus*, and yellow colonies indicated the presence of presumptive *V. cholerae* [138]. Due to many samples, those that were the most representative

were selected; the largest and greenest. Therefore 25 Subtidal samples and 25 Marsh samples were chosen to continue the research.

4.4 FREEZER STOCKS

All the samples that showed green growth on TCBS (presumptive *V. parahaemolyticus*) were store. To this, 1 ml of NB + 1% NaCl was placed in a sterile 1.5 ml conical microtubes with cap and then, using a sterilized stick, a small amount of the sample from the NB + 1% NaCl plate patch was added and mixed completely.

All the microtubes were incubated overnight at 30°C and 100 rpm. Subsequently, 180 µl of dimethyl sulfoxide (Alfa Aesar) + 80% glycerol (Fisher Chemical) (1:1) were added to each tube to store at -30°C.

4.5 THREE ZONE STREAK ISOLATION

After selecting the most representative samples, according to the TCBS test (*see section 4.3*), the process of three-zone streak [137] was made to achieve isolation of individual colonies.

This method consisted of spreading the sample across a portion of the plate in three distinct zones. Each zone overlaps with a small amount of the previous zone creating a dilution effect. From the last zone a single streak was taken to the empty space of the plate with the goal to achieve isolation of individual colonies.

After obtaining individual colonies, these were transferred and incubated again to NB + 1% NaCl plates by picking and patching. These where the 50 samples used in the subsequent tests.

4.6 qPCR AND PCR

The boil prep preparation consisted of adding 1 mL of NB + 1% NaCl to a sterile 1.5 mL conical microtubes with cap. Then using a sterilized stick, a small amount of the sample was added. These were heated to 100°C for 10 minutes to open the DNA strands. After cooling down they were stored in the freezer as a DNA reservoir for qPCR and PCR samples.

PCR and qPCR amplification were conducted for thermolabile hemolysin (*tlh),* thermostable direct hemolysin (*tdh)* and thermostable direct hemolysin-related hemolysin (*trh)* genes.

4.6.1 qPCR

The final concentrations of the qPCR master mix components were: PowerUp™ SYBER™ Green Master Mix (Thermo Fisher Scientific) 1x, forward primer (P_F) and reverse primer (P_R) 500 nM (Invitrogen, Thermo Fisher Scientific), DNA 10 – 100 ng and 3 µL of Molecular biology grade water (Corning). The final reaction volume was 20 µL and the reactions were run on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). See primer sequence in *Appendix 1*.

The thermal-cycling program for the mentioned genes (*vide supra*) was as follows: denaturation at 94°C for 3 minutes, followed by 40 cycles consisting of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, and a final elongation of 72°C for 5 minutes.

4.6.2 PCR

The final concentrations of the PCR master mix components contained: Buffer 1x (Promega), deoxyribonucleotide triphosphate (dNTP) 200 nM (Promega), P_F and P_R 250 nM (Invitrogen, Thermo Fisher Scientific). In addition to 0,2 µL of GoTaq® DNA Polymerase (Promega), DNA 0.2-2 ng/µL and 15.1-13.3 µL of Molecular biology grade water (Corning) for a final reaction volume of 25 µL. The reactions were run on a Veriti™ 96-well Thermal Cycler (Thermo Fisher Scientific). See primer sequence in *Appendix 1* and Master Mix recipe in *Appendix 2.*

The thermal-cycling program for the mentioned genes (*vide supra*) was as follows: denaturation at 95°C for 2 minutes, followed by 40 cycles consisting of 95°C for 15 seconds, 59°C for 1 minute, and a final elongation of 60°C for 1 minute.

The electrophoresis was carried out with SeaKem® LE Agarose gel (Lonza) 1%. Sodium Borate Buffer 1x was used to separate the DNA bands. EZ-Vision® One Dye-as-Loading Buffer (VWR Life science) and Quick-Load® 100 bp DNA Ladder (New England BioLabs Inc) were used to visualize and analyze the bands.

4.7 BIOFILM TEST WITH CONGO RED AGAR

CRA was used to determine if the bacterial isolates were capable of biofilm production. The preparation consisted of adding 52 g of Bacto™ Brain Heart Infusion (Becton, Dickinson and Company), 36 g of sucrose (VWR Chemicals, LLC) [139], and 10 g of Bacto™ Agar (Becton, Dickinson and Company) in 950 mL of ultrapure water. Then, 0.8 g of Congo red (VWR Chemicals, LLC) was placed in a separate Erlenmeyer glass in the remaining 50 mL of water. After both solutions were autoclaved, they were allowed to cool, and then mixed together and poured into petri dishes.

Each of the 50 samples (25 Subtidal and 25 Marsh) were individually cultivated on the CRA plates and incubated overnight at 30°C. Those samples with a black growth were considered as possible biofilm-producing colonies, and the samples that showed red growth were determined as non-biofilm-producing colonies [140].

4.8 PLASTIC SAMPLES PREPARATION

Two types of plastic were used: PET and PE.

- PET: A green soda bottle was washed with soap and water, then cut into small pieces of 5x5 mm (Figure 6.a), which were dipped in 95% ethanol, washed with ultrapure water and dried. In addition, some PET fibers from a pet toy were sterilized in the same way.
- PE: A clean bottle of milk was flash frozen with liquid nitrogen and then broken into small pieces between 2 mm and 5 mm (Figure 6.b) with a coffee grinder, which were cleaned and sterilized in the same way as PET.

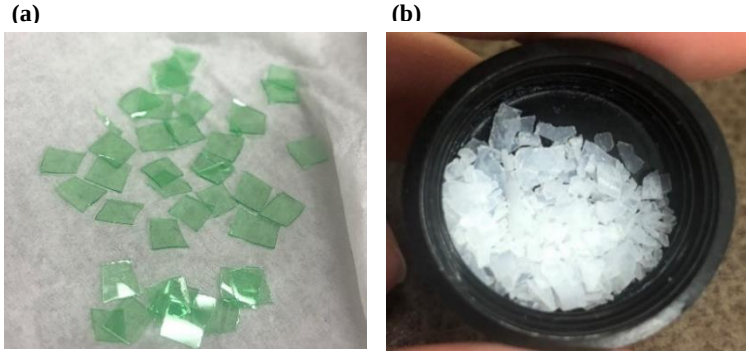

Figure 6. (a) 5x5 mm pieces of PET. (b) 2mm – 5mm pieces of PE.

4.9 BIOFILM ASSAY ON MICROPLASTICS

A brackish marine medium (M10b) was prepared to simulate a saline and nutrient-rich environment where the presumptive *V. parahaemolyticus* could survive and grow in contact with the microplastic. This medium is composed of the following ingredients: 4 g of Tryptone (Fisher BioReagents^{TM}), 2.5 g of yeast extract (Fisher BioReagents^{TM}), 300 mL of artificial seawater (2xASW) at salinity of approximately 15 ppt and 700 mL of ultrapure water. The 2xASW was made of 35. 1 g of NaCl, 1.5 g of potassium chloride (KCl) (Fisher Chemicals), 24.7 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Fisher Chemicals) and 2.9 of calcium chloride dihydrate ($CaCl_2.2H_2O$) (Fisher Chemicals) [118]. Its preparation consisted of adding the ingredients in order and shaking it moderately until complete dissolution.

4.9.1 6-well plates:

The first biofilm production test was carried out on PET (bottle and fiber) microplastics. It was developed in triplicate for both types of PET, to which three different samples (M21, S14 and 3M 12-14 #16) were added individually in each of the wells. Each sample was incubated for 5 hours at 30°C and 150 rpm in a glass tube containing 5 mL of M10b medium.

Three 5x5 mm pieces of bottle PET was added to three separate wells, and likewise a small portion of PET fiber was placed in the remaining wells. Then the 6-well plate was filled with 7 mL of M10b medium.

A second test was also carried out with another 6-well plate. Similarly, three pieces of bottle PET and the fiber were placed in the wells with the medium, but two wells were left as a control (one of each type of PET) and only one sample was seeded in the remaining wells.

Both plates were incubated at 30°C for approximately one month as a first reference test. The fibers were analyzed under the SEM.

4.9.2 Glass tubes

The 50 samples were previously grown at 30°C and 150 rpm for approximately 5 hours in glass test tubes containing 5 mL of M10b. Subsequently, two pieces of bottle PET and 2 mL

of M10b were added. The tubes were incubated with cap at 30°C and 150 rpm for approximately 4 weeks before being analyzed in the SEM.

4.9.3 96-well microtiter plate

All 50 samples were grown overnight at 30°C and 150 rpm in a sterile 1.5 mL conical microtubes with cap containing 1 mL of M10b. Three MPs pieces were introduced in each well of the 96-well microtiter plate, there was a plate for PET and another one for PE. Then 100 µl of M10b were placed in each well with a multichannel pipette and subsequently 100 µl of the prepared isolate samples were added in triplicate into each specific well of the plate.

Between each of triplicate trials, three sterile well (no bacteria) were left, acting as a negative control. The 96-well microtiter plate were closed and incubated at 30°C for 10 days. These microplastic were later analyzed in the SEM.

4.10 BIOFILM ASSAY WITH CRYSTAL VIOLET

The samples were grown following the procedure in section 4.9.3. After two days of incubation the medium was removed with a multichannel pipette, trying not to touch the wells to avoid dislodging the biofilm. Subsequently, all the wells were washed three times with phosphate-buffered saline (Corning®). Following this, 200 µL of 1% Crystal violet (Fisher Chemical) was added to each well, allowed to stand for 15 minutes [135]. Finally, they were washed three times with water and dried at room temperature. All wells were filled with 200 µL of 96% ethanol [140] (Figure 7).

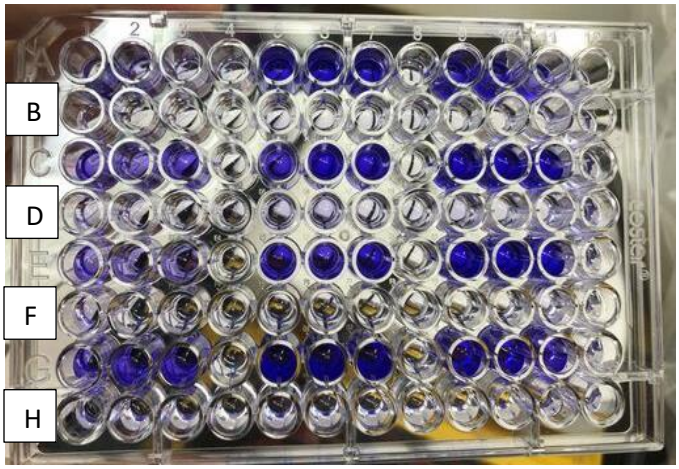

Figure 7. Top view of a 96-well microtiter plate filed with 200 µL of 96% ethanol. Rows B, D, F and H acted as controls, as did the wells in the middle of each triplicate sample.

The absorbance was measured at 595 nm (OD_{595}) with an iMark® Microplate absorbance reader. The biofilm production results were classified according to a modification of the method described by Abdallah [134]. This considered $OD_{570} \geq 1$ as highly positive biofilm production capacity. However, due to the high OD_{595} values obtained, a new selection criterion was considered in this study. The biofilm formation was finally interpreted as positive ($2.9 \leq OD_{595} \leq 4$), inconclusive ($1 \leq OD_{595} < 2.9$) or low/negative ($OD_{595} < 1$). Also, the concentration of bacteria in the samples was not measured, therefore, this essay was determined as a qualitative detection of biofilm.

4.11 SCANNING ELECTRON MICROSCOPE

The microplastics incubated in the 96-well microtiter plate were extracted with tweezers and were prepared for analysis with SEM. These were washed with phosphate-buffered saline (PBS) and left overnight (15 hours) in Glutaraldehyde 4% in 0.1 M sodium cacodylate buffer (Fisher). Then, they were fixed with different alcohol percentages: 25%, 50%, 75%, 95% and 100% (10 minutes each) and subsequently dried [122].

The samples were sputtered coated with gold, using a Quorum® Technologies EMS 150 T ES Iridium Sputter Coater (Figure 8.a), then the microplastic samples were analyzed in the ZEISS Gemini® SEM (Figure 8.b), following the protocols of Material Characterization Facility at UCF.

(a) **(b)**

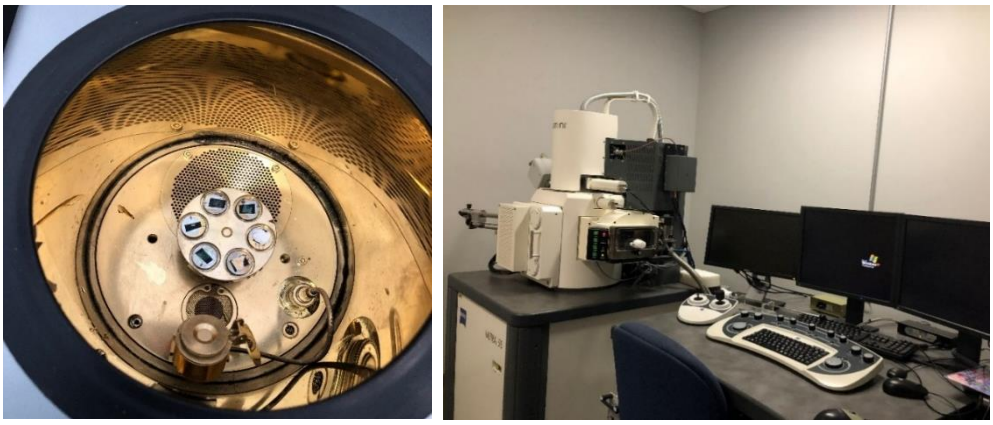

Figure 8. Analysis of microplastic samples in Materials Characterization Facility at UCF. a) Six fixed microplastic samples inside the Sputter Coater, before the process starts. b) ZEISS Gemini® SEM.

4.12 DNA SECUENCING

For this step 500 mL of NB + 1% NaCl medium and NB + 1% agar plates were prepared. Subsequently, freezer stocks on the selected samples (S4, S10, S18, S24, M17, M21) were thawed on ice and spread on the NB + 1% NaCl plates.

The growths from the original stocks of S10, S18 and S24 samples showed multiple species (colonies of different appearance) on the spread plates, therefore the three-zone streak method was used to obtain isolated colonies.

Isolated colonies were transferred to 5 mL of NB + 1% NaCl in glass culture tubes and grown in a shaker incubator for 2 days at 30°C and 100 rpm. New freezer stocks were prepared from 1 mL of the freshly grown samples according to the procedure described in section 4.4. From the remaining sample, 2 mL was used to extract whole genomic DNA using Quiagen DNeasy UltraClean Microbia Kit. The concentration of extracted DNA (ng/µL) was confirmed on an Invitrogen ™ Qubit ™ 3.0 Fluorometer (Thermo Fisher Scientific)

Prescreening of the DNA quality was performed by PCR, using the GoTaq® DNA Polymerase (Promega) with the 27F and 1492R primers (IDT) for the 16S ribosomal ribonucleic acid (rRNA) component. The master mix for the reaction is included below (*see Appendix 3*).

Subsequently, electrophoresis procedure was carried out with 0.7% Agarose gel of SeaKem® LE Agarose (Lonza) and following the procedure in section 4.6.2. The DNA bands were visualized on a Fotodyne Foto/Phoresis UV Transilluminator (*see Appendix 4*).

High quality PCR products were prepared following the previously mentioned procedure, except ExTaq® DNA Polymerase (Promega) was used instead of GoTaq®. The product bands were excised from the gel and the product was recovered with a Zymoclean Gel DNA Recovery Kit. The concentration of purified PCR product was confirmed on an Invitrogen ™ Qubit ™ 3.0 Fluorometer.

Samples were sent to the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for Sanger sequencing. The sequencing data was processed using

MEGA-X and the sequence similarity was determined with the Basic logical alignment search tool (BLAST) on the NIST database to identify the bacterial species.

5 RESULTS AND DISCUSSION

5.1 ISOLATION OF COLONIES FROM SUBTIDAL AND MARSH SEDIMENTS

A large growth of colonies was obtained (10-300 approximately) from the spread plates. Therefore, accurate colony count could not be obtained. However, patched plates (with 50 randomly selected colonies) were stored as an immediate reserve of the samples for future testing (Figure 9).

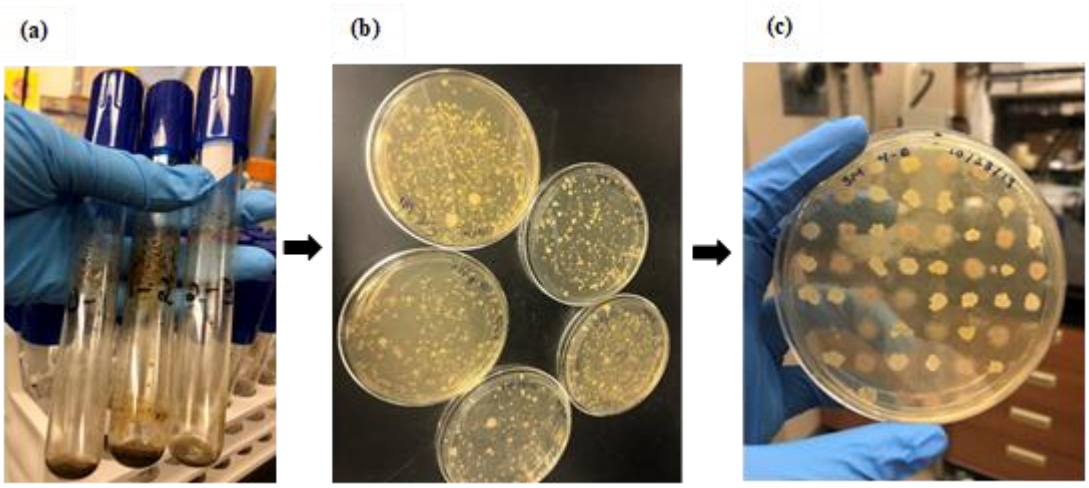

Figure 9. a) Sediment samples suspended in NaCl, b) Spread plates, c) NB + 1% NaCl patched plate of a Marsh core sediment.

5.2 TCBS ISOLATION TEST

Vibrio sp. are considered one of the most common pathogens of seafood. In addition, they can be found in aquaculture systems, which can function as reservoirs of pathogenic species [6]. That is why in this study interest was taken to isolate the potentially pathogenic *V. parahaemolyticus* [122], [125], [141], [142].

Those colonies that grew green on the TCBS plates were counted as presumptive *V. parahaemolyticus* and the yellow ones as presumptive *V. cholera* [138]. However, this classification was complicated to carry out on several occasions since some green colonies were overlapped by a yellow color (Figure 10.a), maybe due by sucrose-fermenting bacteria [143]. Moreover, a few colonies took more than 24 hours to grow, therefore their temperature was increased to 35°C.

(a) (b)

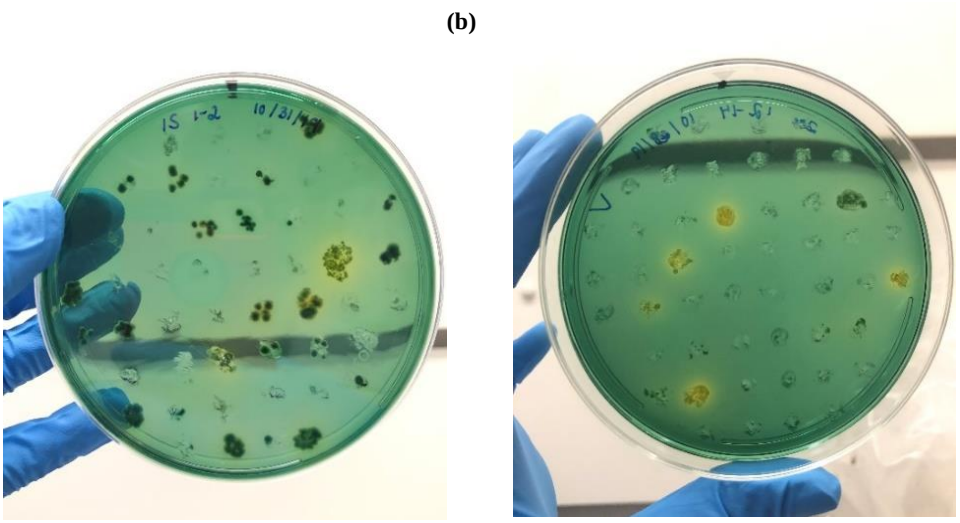

Figure 10. TCBS patched plates with 50 colonies. (a) 1S 1-2 sediment core sample shows an extensive green colony growth. (b) 2S 12-14 sediment core sample shows a poor colony growth but with a predominance of yellow strains.

All the colonies on TCBS patched plates (Figure 10) were counted and classified. *Appendixes 5* and *6* show the colony count results for Marsh and Subtidal sediments correspondingly, where the amount of presumptive *V. cholera* and *V. parahaemolyticus* colonies in each sediment core and depths are indicated.

Marsh sediment cores showed a predominance of presumptive *V. cholera* (64%). In core 1M, the presence of *V. cholera* was found in deeper locations, while in middle depths there were more *V. parahaemolyticus*. In addition, the 2M core contained more colonies of *V. parahaemolyticus* than *V. cholera*, that were more evenly distributed throughout the sediment depths. However, in section 3M almost seven times more colonies of presumptive *V. cholera* were found, distributed at different depths.

In contrast, all sediment cores from the Subtidal cores showed a predominance of *V. parahaemolyticus* colonies (61%). In core 1S the *V. parahaemolyticus* colonies were quantified in all depths, but the *V. cholera* colonies were found in the near-surface locations. However, core 2S showed a general distribution of the colonies across the different depths, although the *V. cholera* colonies were found mainly deep in the sediment core. Lastly, in core 3S there was a mainly growth of *V. parahaemolyticus*.

Moreover, Figure 11 presents a total count by locations (cm) of the colonies from Marsh and Subtidal sediments on TCBS agar, this allows an overview of the presumptive *V. cholera* and *V. parahaemolyticus* in the sediment cores. The figure below shows a predominance of *V. cholera* in Marsh cores sediment, with a higher concentration at depths of 6-8 cm and 8-10 cm, which reported 75 and 102 colonies correspondingly, while only 6 and 2 colonies of *V. parahaemolyticus* were counted respectively. However, shallower locations, between 2-6 cm, showed a larger growth of *V. parahaemolyticus*.

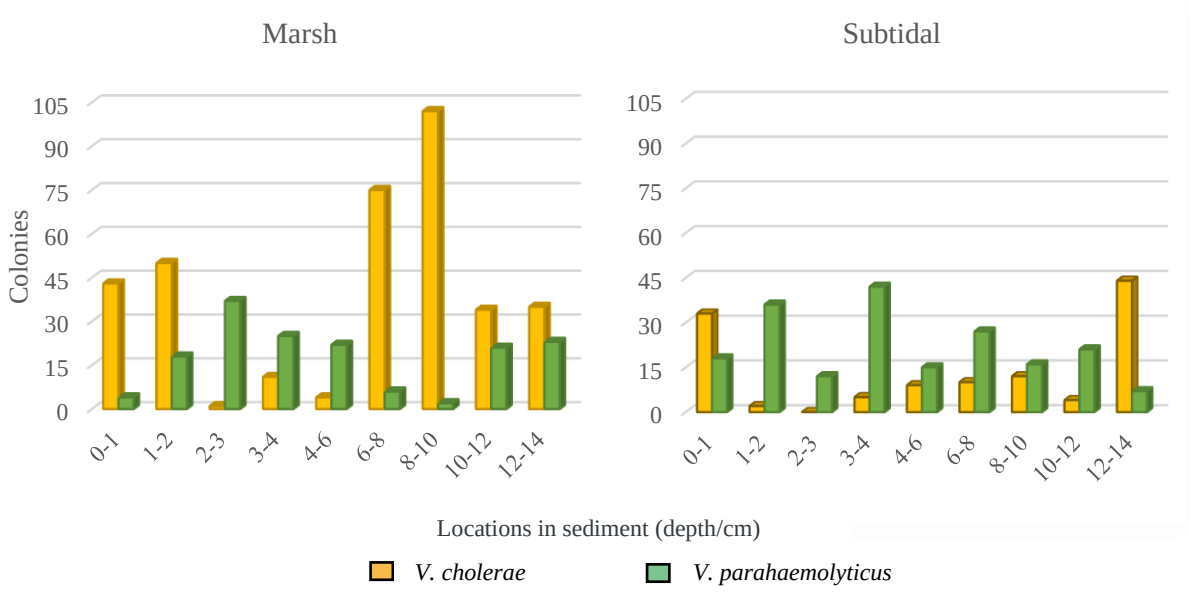

Figure 11. Results of counting of Vibrio sp. in Marsh and Subtidal sediments on TCBS agar.

For Subtidal samples, Figure 11 displays a total count by locations of the colonies from Subtidal sediment on TCBS agar, which shows a predominance of *V. parahaemolyticus* colonies, except for 0-1 cm and 12-14 cm, where these colonies were significantly low. However, the largest number of *V. parahaemolyticus* colonies were found at 1-2 cm and 3-4 with 36 and 42 colonies correspondingly.

Sediment can be considered the most important ecosystem constituent for microbial life in the coastal aquatic environment since it offers biotic and abiotic surfaces and nutrients for bacterial growth [116], which is probably why the concentration of bacteria in marine sediments may exceeds by approximately three orders of magnitude the concentration in the

water-column [144]. Although samples from the water-column were not analyzed in this study, that could be related to the total colonies of presumptive *V. parahaemolyticus* and *V. cholerae* obtained from Subtidal and Marsh sediments, where 69% of all isolated colonies were from Marsh sediment cores and only 31% from Subtidal.

In addition, there are some factors that might affect the distribution of the *Vibrio* sp. through the sediments such as: surface temperature, salinity, nutrients, and others. However, the temperature turned out to be the most incident within all variants [116]. *Vibrio sp.* might be mostly present in the water-column through the warmer months; however, sediments are contemplated to be potential reservoirs when conditions in the water-column are rough [117].

Since the desired species to be isolated was *V. parahaemolyticus*, those colonies that grew greener on the TCBS plates were selected to be used in the following analysis. Each isolation number was linked to the type of sediment core (1S, 2S, 3S, 1M, 2M, 3M), its location or depth (1-14 cm) and its specific number according to its position on the NB + 1% NaCl patched plate (*see Appendix 7*).

Additionally, these selected samples were further isolated by three-zone streak isolation, to achieve isolation of single colonies for the following study methods.

5.3 QPCR AND PCR

V. parahaemolyticus possesses the *tlh* gene as a species marker [145]. In addition, *V. parahaemolyticus* virulence can be related to *tdh* and *trh* genes [146], [147].

After running the first samples in the qPCR, no significant results were obtained. Due to this, it was decided to repeat the test in the PCR. Nevertheless, the samples were running several times, but again no results were obtained, which indicated that the bacterial isolates were detected as *tlh* negative, in the same way for the *tdh* and *trh* genes.

Consequently, no virulence factors were found either. Therefore, it was considered that *V. parahaemolyticus* was not detected in the analyzed samples.

However, it was determined that the three-zone streak method should have been performed before and not after the TCBS test, to guarantee that the isolated singles colonies were used

on the TCBS agar and subsequent methods. Thus, the samples were subsequently analyzed by DNA sequencing (*vide infra*).

5.4 BIOFILM PRODUCTION

CRA and CV methods were carried out to achieve a qualitative detection of biofilm production under controlled environmental conditions at the laboratory. In addition, the results of both tests were compared to identify the possible samples with the highest biofilm production capacity.

5.4.1 Biofilm test with Congo red

The 50 cultivated plates were analyzed according to the colonies grown on it. Figure 12.a shows a positive result for biofilm production, while Figure 12.b illustrates no evidence of biofilm [140].

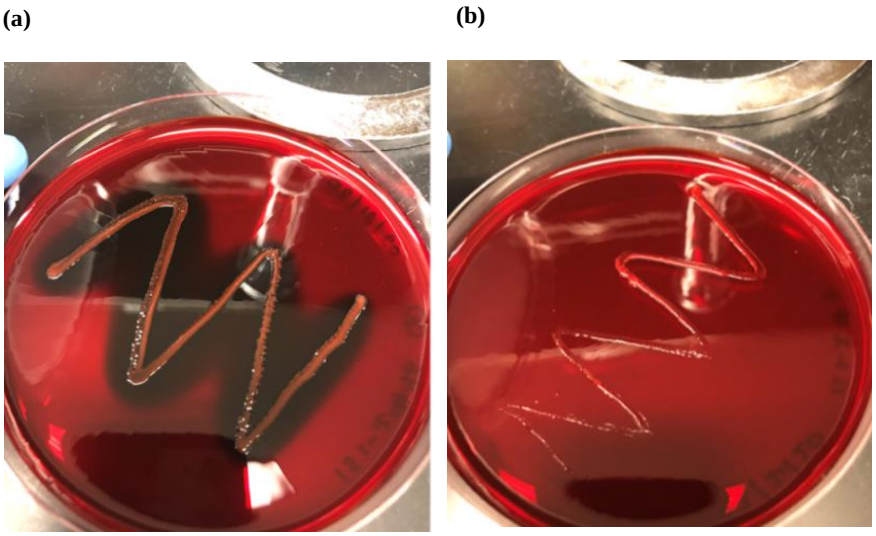

Figure 12. CRA incubated plates. (a) Black growth indicates positive result for biofilm production. (b) Red growth indicates no biofilm production.

According to the assay results (*see Appendix* 8), samples S1, S7, S9, S10, S12, S14, S15, S16, S18, S19, S20, S21, S22 and S23 were positive for biofilm production. While for the Marsh samples those that indicated positive results were M1, M2 M3, M6, M8, M9, M10, M12, M13, M14, M18, M21, M23 and M25. Based on this information, 56% of both, Subtidal and Marsh samples showed a biofilm production.

As described by Freeman et al. [136], CRA method uses Brain-Heart Infusion broth supplemented with sucrose as a highly nutritive medium. In addition, Congo red has been widely used as a stain to show the presence of exopolysaccharides of aquatic Gram-negative bacillus [136]. However, its efficacy has been questioned by different studies, therefore, some researches have made improvements to the method proposed by Freeman [139], [148], [149]. Currently, CRA is one of the most common methods for biofilm detection due to its simplicity and low cost [150], [151].

Through these results, it was possible to create an overview of those colonies that might be more likely to colonize MPs due their ability to produce biofilm.

5.4.2 Biofilm essay with Crystal violet

After washing the CV out of the wells and filling them with 96% ethanol, the absorbance (OD_{595}) of the 50 samples was measured on triplicate with an iMark® Microplate absorbance reader. The results were corrected by subtracting the average of the blanks from each replica obtained (*see Appendix 9 and 10*).

For Marsh results, Figure 13 shows that samples such as M15, M18, M19, M21, M22 and M23 presented OD_{595} values higher than 2.9 nm, and therefore were considered as positive for biofilm production capacity, also it should be noted that very high OD_{595} values were found. In addition, samples M5, M6, M8, M9, M12, M20 and M25, were classified as inconclusive ($1 \leq OD_{595} < 2.9$), which can be interpreted as a possible biofilm production. On the other hand, the lowest values corresponded to M1, M7, M14, M16 and M17. However, M2, M3, M4, M10, M11, M13 and M24 showed inconsistencies in the values (wide errors bars), which generated ambiguity in the results. Therefore a 52% of Marsh samples were considered as positive biofilm producers.

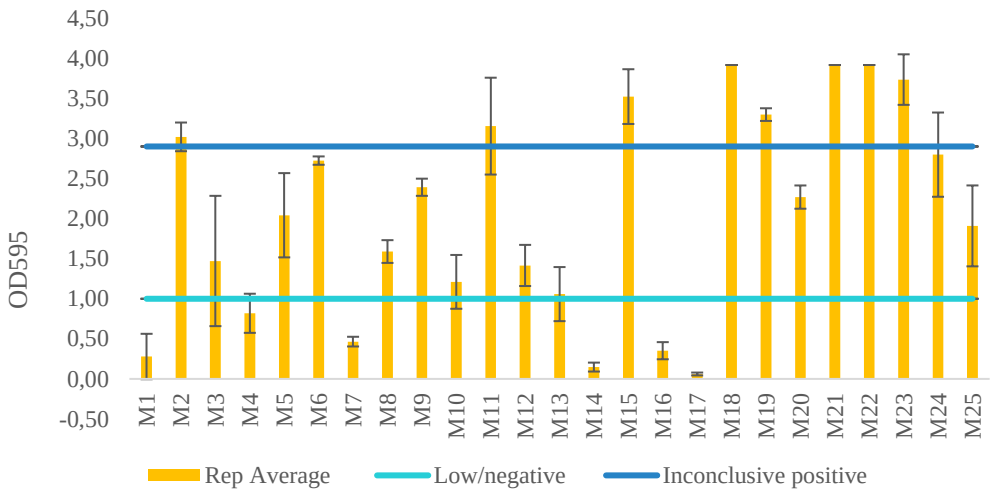

Figure 13. CV results for Marsh samples. OD_{595} > 2.95: highly positive biofilm production capacity. OD_{595} < 1: Low/negative biofilm production capacity, which also refers to the original method described by Abdallah [134]. Inconclusive values: 1 ≤ OD_{595} < 2.9. Error bars corresponds to standard deviation.

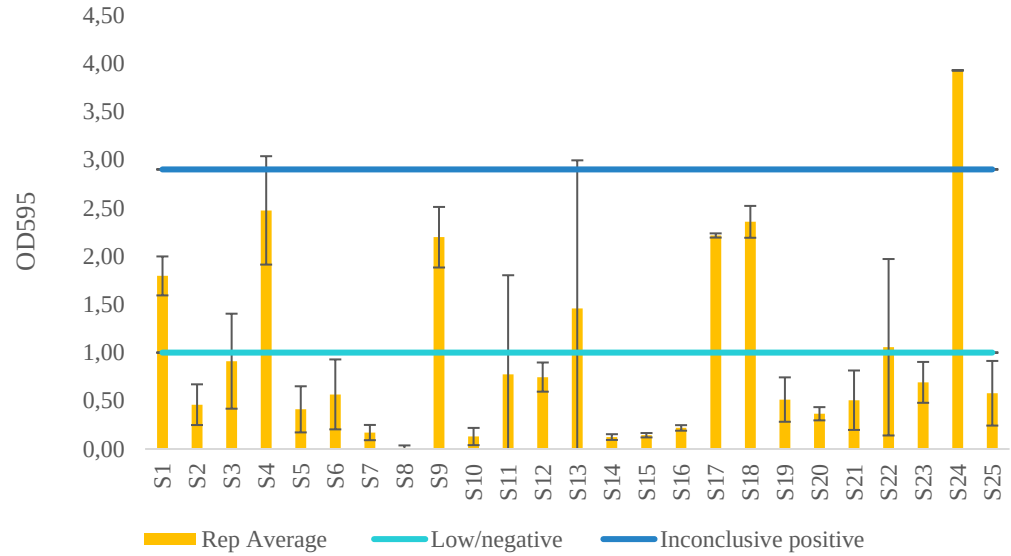

Figure 14. CV results for Subtidal samples. OD_{595} > 2.95: highly positive biofilm production capacity. OD_{595} < 1: Low/negative biofilm production capacity, which also refers to the original method described by Abdallah [134]. Inconclusive values: 1 ≤ O OD_{595} < 2.9. Error bars corresponds to standard deviation.

In relation to Subtidal results (Figure 14), the strain S24 had the highest OD_{595} value (4.0 nm). This is followed by samples S1, S9, S17 and S18 which showed an inconclusive biofilm production. Then, the lowest or negative values corresponded to samples S2, S5, S6, S7, S8, S10, S14, S15, S16, S19, S20, S21, S23 and S25 so this could suggest a low biofilm

production capacity in Subtidal colonies. However, there is ambiguity in S3, S4, S11, S13, and S22. Therefore, only 20% of the samples were classified as positive biofilm producers.

Based on the CV results, a variety of responses were evident from Marsh and Subtidal samples, which could suggest the presence of different species of bacteria in the samples. Moreover, a possible contamination with CV remains and an unequal concentration of the added bacteria are considered as possible contributing factors to the errors. However, it is important to emphasize that the purpose of this test was to perform a qualitative detection of biofilm production capacity.

5.4.3 Congo red vs Crystal violet

The samples that were classified as presumptive positive biofilm producers for both tests were: S1, S9, S18, M6, M8, M9, M12, M18, M21, M23 and M25, which corresponds to a 22% of all samples. This evidence the response of some bacteria to only produce biofilm in a single medium, either CV or CRA. For the results of CV were considered both highly positive biofilm production capacity and inconclusive positives values.

Of the total number of samples, 36% showed a presumed capacity to produce biofilm according to the CV test. In addition, 52% of total samples corresponded to Marsh samples and only 20% to Subtidal. On the other hand, 56% of all samples were positive according to the CRA, where an equal result of 56% was obtained for Subtidal and Marsh. For both tests, the Marsh samples were those with the highest capacity to produce biofilm.

Contrary to the CRA, the CV method was based on the growth of bacteria in the M10b medium, allowing the replication of conditions under which the samples were incubated with the MPs. Moreover, this method has been widely used as a reference in several quantitative adherence assays for biofilm detection (e.g. tissue culture treated plates) [134], [151], [152]. On the other hand, studies have suggested that CRA usually reports low sensitivity and accuracy [151], [152] and it is based on a subjective chromatic evaluation [149]. Although it is easy to perform, cheap and some parameters have been modified to improve their accuracy [148]. Even though both tests were qualitative, the main difference lies in their specific conditions. As mentioned above, CV in addition to having the same properties in which the

MPs were incubated, a triplicate assay was performed and analyzed by absorbance, while the CRA was based on a binary classification. Therefore, CV test was considered as a possible main indicator of the biofilm production capacity of the isolated samples under the environmental conditions established in this study.

However, it cannot be guaranteed that the samples that tested positive would have the ability to adhere and produce biofilm on the polymers, since the presumptive exopolysaccharides production was detected under specific conditions that differ from the MPs surfaces. Extracellular polymeric substances play a major role in biofilm cohesion and adhesion to surfaces, however, its content in a biofilm may diverge in quantity and character in response to environmental factors [153]. In addition, microorganisms can modify their exopolysaccharides according to the surface properties to which they adhere [153], [154].

5.4.4 Biofilm assay on microplastics

White, yellow, and orange biofilm were found at the bottom of the glass tubes; however, other samples did not show significant growth (Figure 15). As for the samples on the 6-well plate and 96-well plate, the medium was very cloudy, and the biofilm was mainly on top.

Figure 15. PET microplastics incubated with Marsh samples in sterilized glass tubes with M10b medium (5 days).

As mentioned above, the purpose of this assay was to incubate the MPs for subsequent SEM examination, therefore, no further analysis was performed. Nevertheless, it is worth mentioning that while the microbial distribution of biofilms settling on rigid substrates has been well known, there are also floating biofilms, in which microorganisms prefer to colonize

a fluid surface rather than a rigid one [155]. In addition, their accumulation (e.g. air-liquid or liquid-liquid interface) may be influenced by characteristics such as: propulsion (i.e. "pusher or "puller"), cialate and flagella sizes, reenforcing the fact that cell motility is a crucial function in biofilm formation [155].

However, although most of the samples showed agglomerated biofilm at the bottom of the glass tubes, their adhesion to the MPs cannot be guaranteed.

5.5 SEM

SEM was used to observe bacterial colonization or biofilm evidence on PET and PE MPs previously incubated with isolated bacteria from Subtidal and Marsh core sediments.

It was expected that by comparing the samples in both types of MPs, that information would provide a reference on how the polymer surface might affect biofilm development. However, due to factors of time and capacity of use of the SEM, only some samples were run, which were among those that tested positive for biofilm production (M6, M12, M18, M21, M23, S1, S10, S18) or had peculiar appearance after the incubation period (S24, M17 and S4).

Sample S18 showed attached of rod shape bacteria over the entire PE microplastic, in addition to a few small traces of biofilm (Figure 16). The presence of plastic colonizing bacteria on PET was almost zero.

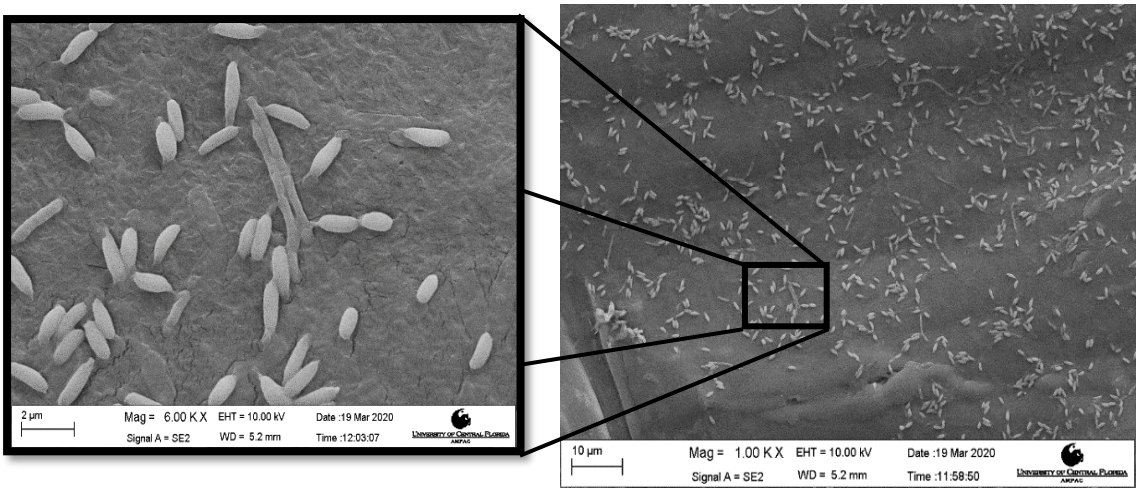

Figure 16. SEM showing bacterial cells attachment on PE microplastic. This image corresponds to a piece of PE incubated with the sample S18 in 96-well microtiter plate at 30°.

The micrographs of sample S10 showed the attachment of different bacterial cell shapes. A group of spherical shape bacteria was observed on PET and PE (Figure 17.a, *see Appendix 11*), which presented biofilm production in both polymers. In addition, rod shape bacteria on PE were determined (Figure 17. b).

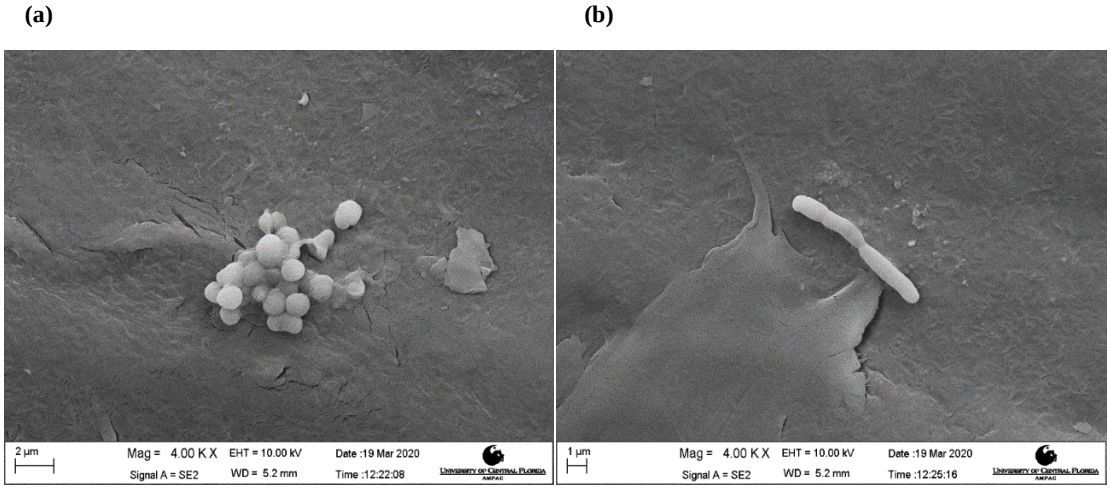

Figure 17. SEM showing bacterial cells attachment on PE microplastic. This image corresponds to a piece of PE incubated with the sample S10 in a 96-well microtiter plate at 30°C. a) Spherical shape bacteria b) Rod shape bacteria.

Micrographs from sample S24 showed rod shape cells distributed over the PET microplastic (Figure 18). In addition, indications of biofilm formation were observed on both PET and PE. However, only a few rod shape bacteria were observed attached to the PE microplastic (Figure 19).

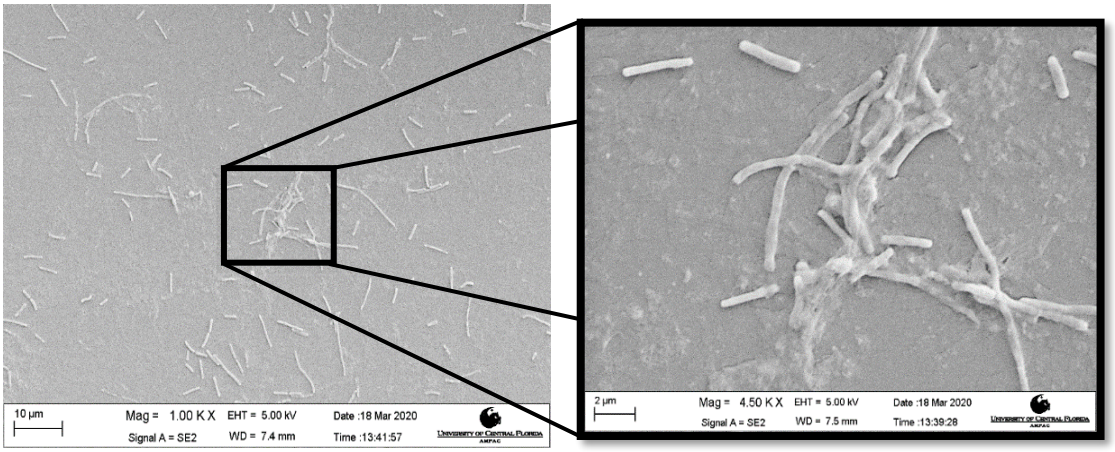

Figure 18. SEM showing bacterial cells attachment on PET microplastic. This image corresponds to a piece of PET incubated with the sample S24 in a glass tube at 30°C. Biofilm indications were observed.

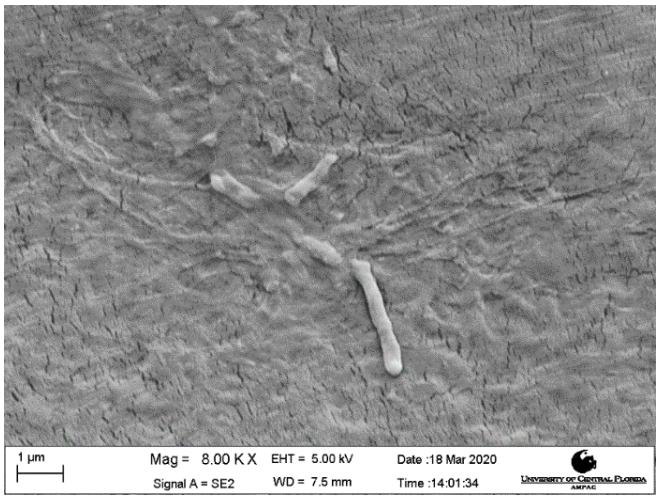

Figure 19. SEM showing bacterial cells attachment on PE microplastic. This image corresponds to a piece of PE incubated with the sample S24 in a 96-well microtiter plate at 30°C. Biofilm indications were observed.

Non-positive results were obtained for M23 sample, which showed the presence of a few bacteria cells on PET and just a single rod shape cell on the PE sample. As for the M12, rod-shaped bacteria on the PET were observed (*see Appendix 12*). However, no cells were found on PE.

From sample M21, only a PET microplastic sample was analyzed, which was significantly colonized by rod-shaped bacteria (Figure 20).

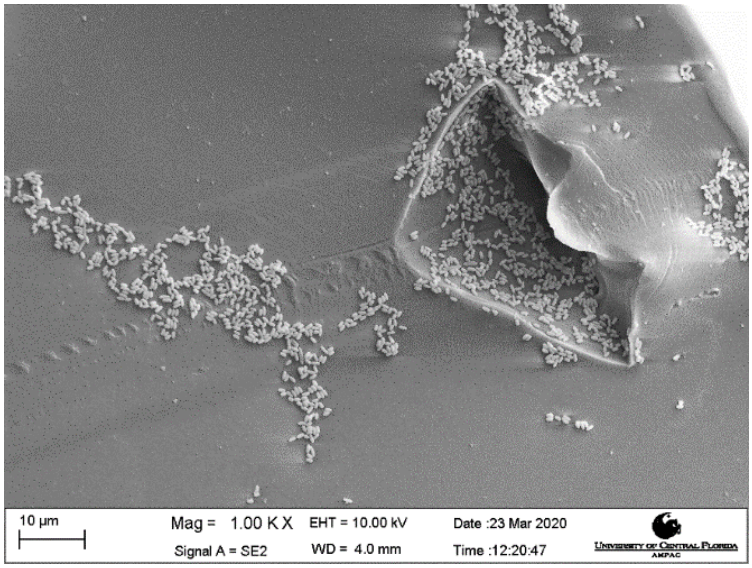

Figure 20. SEM showing bacterial cells attachment on PET microplastic. This image corresponds to a piece of PET incubated with the sample M21 in a glass tube at 30°C.

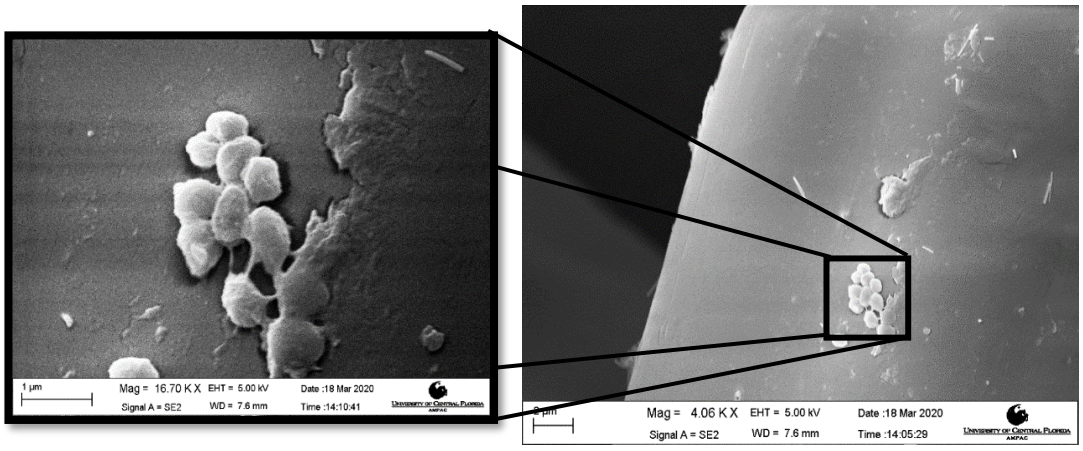

Figure 21. SEM showing bacterial cells attachment on PET fiber microplastic. This image corresponds to a piece of PET incubated with the sample M6 in a 6-well microtiter plate at 30°C.

Moreover, Figure 21 shows a PET fiber (sample M6), with a group of spherical shape cells attached on it. Regarding sample M17, a rod-shaped bacterium was observed, however, this sample consisted of a small amount of the medium, which was analyzed on a sterile paper filter. For sample S4, it showed a peculiar orange growth on the top of the media where it was incubated, therefore, it was analyzed on sterilized filter paper. A voluminous biofilm of spherical shape bacteria was observed.

5.6 DNA SECUENCING

Samples that showed evidence of biofilm production in SEM were analyzed by DNA sequencing (S4, S10, S18, S24, M17 and M21). Nevertheless, as previously mentioned, the growths from the original freezer stocks showed different species, therefore, three-zone streak was made for each unique colony.

Four possible additional species were identified, and the new samples were named according to their colony color. However, Phylogenetic tree based on the 16S rRNA gene was not developed in this study, because some species showed a very close similarity (samples S18-orange and S24-orange), which made them indistinguishable from each other. Therefore, similarity percentages were not specified, and the best matches (highest similarities) were considered as presumptive species. Nevertheless, another gene amplification will have to be performed to verify these results for a subsequent publication.

Table 6. Species identified by DNA sequencing.

Sample	Closest match (sp.)
S4	*B. aquimaris*
S10-white	*B. flexus*
S10-yellow	*Micrococcus yunnanensis*
S18-white	*B. safensis*
S18-orange	*B. vietnamensis / B. aquimaris*
S24-orange	*Fictibacillus arsenicus / Fictibacillus nanhaiensis*
S24-white	*Staphylococcus hominis*
S24-grey	*Virgibacillus dokdonensis*
M17	*Lysinibacillus fusiformis*
M21	*B. pumilus*

The presumptive species are displayed in Table 6. Results from S4 indicated the presence of *B. aquimaris*, which are described as Gram-variable, aerobic, endospore-forming, rod-shaped bacteria. Peritrichous flagella (i.e. flagella all over) are involved in their locomotion, and their colonies are circular or slightly irregular, slightly raised and pale yellow-orange [156]. In addition, as previously mentioned, *B. aquimaris* isolated from oil-contaminated sediment was related to petroleum hydrocarbons degradation [119] and has also showed a great potential to degrade PAHs [133]. However, S4 corresponded to an orange voluminous floating biofilm of spherical shape bacteria and it was not analyzed on the MPs under the SEM. Therefore, adherence to the MPs cannot be assured and S4.

S10-white corresponded to *B. flexus* [157]. This species is considered as Gram-variable, endospore-forming, aerobic, rod-shaped bacteria and it usually produces an opaque [156] milky white colonies [158]. Different strains have shown mobility [159] while others have not [160]. In addition, *B. flexus* strains have demonstrated potential for wastewater biotreatment [160], [161], arsenic bioremediation from water [158], degradation of PP [162] and PVC [70] and biomedical applications [163]. These bacteria can be commonly found in terrestrial and aquatic environment, however, *B. flexus* T6186-2 strain, was also isolated from a deep-subsurface oil reservoir and characterized by its resistance to a variety of antibiotics (e.g. erythromycin, gentamicin and vancomycin) [164]. Since the description of their

colonies and cell shape are consistent with the results obtained, it can be inferred that the rod-shaped bacteria observed on PE correspond to *B. flexus* (*see Figure 17.b*). Although only a couple of cells were present, this evidenced their ability to adhere to PE under controlled marine environmental conditions.

Sample S10-yellow was identified as *M. yunnanensis*. This species is an aerobic, Gram-positive, non-endospore-forming cocci. It has no motility and grows yellow colonies [165]. Moreover, several studies have reported the ability of these bacteria to degrade pharmaceuticals (e.g. ibuprofen) [166], [167], [168] and to adsorb arsenic [169]. As their previous description concurred with the SEM results, it is assumed that the spherical shape bacteria observed on PE (*see Figure 17.a*) corresponded to *M. yunnanensis*. Therefore, it was concluded that this species is also able to adhere and produce biofilm on both incubated polymers.

B. safensis species was identified in sample S18-white. These bacteria are mesophilic, aerobic, chemoheterotrophic, Gram-positive, spore-forming, rod-shaped cells. Their mobility is provided by polar flagella and their colonies are round, undulate and dull white [170]. *B. safensis* was isolated from Mars Odyssey spacecraft and assembly -facility surfaces at the Jet Propulsion Laboratory in California and the Kennedy Space Center in Florida (i.e. at an extremely low biomass environment) [170]. In addition, according to a growth experiment on the International Space Station (ISS), *B. safensis* JPL-MERTA-8-2 was the only strain that showed greater growth in space (60% better) than on Earth [171], which evidences its extreme resilience to oligotrophic environments. Moreover, this species was found on a MP fragment from the North Ocean (i.e. collected MPs were 75% PE) [25]. Furthermore, these bacteria have shown resistance to chromium and arsenic metals, along with the capacity to support plant growth (both in absence or presence of metals), which could represent an option for the remediation of metal-contaminated soils and their planting [172]. However, *B. safensis* has shown a significantly similarity to *B. pumilis* [170], [173]. By comparing the results and the given bacteria description, their capacity to colonize the incubated PE (S18) is suggested (*see Figure 16*).

For S18-orange, *B. vietnamensis* and *B. aquimaris* showed a very close similarity, therefore, could not be differentiated from each other. *B. vietnamensis* are rod-shaped, Gram-positive,

aerobic, endospore-forming bacteria. They possess peritrichous flagella and are described as moderately halotolerant cells [174]. These bacteria have also been related to stainless steel corrosion [175]. However, although the SEM micrographs showed a great bacterial colonization over the PE microplastic (*see Figure 16*), it was not possible to determine with certainty which bacteria were present on the polymer surface. In addition, the possibility of their coexistence with *B. safensis* has been considered, since S18-white and S18-orange were originally isolated from the same sample (S18).

In the same way, *F. arsenicus* and *F. nanhaiensis* were indistinguishable from each other in S24-orange sample. On one side, *F. arsenicus* was fist described as *Bacillus arsenicus* [176], however, it was later reclassified in the genus *Fictibacillus* [177]. This species was considered as strictly aerobic, Gram-positive, motile, endospore-forming, rod-shaped, arsenic-resistant bacterium that produces cream, circular and raised colonies [176], [177]. In addition, due to their resistance these bacteria have been extensively implicated in arsenic bioremediation studies [178], [179]. On the other side, *F. nanhaiensis* was originally described as *Bacillus nanhaiensis* [180], and further reclassified as *F. nanhaiensis* [177]. The last can be categorized as Gram-positive, aerobic, endospore-forming, slightly halophilic, facultatively alkaliphilic, straight rod-shaped bacteria [180]. Growth can occur singularly, in pairs or in short chains and colonies are pale yellow-pigmented. Their mobility is by means a single polar flagellum [180]. However, due to similarity of species it cannot be presumed which was possibly attached to the MPs. Although S24-orange growth showed more similarity to *F. nanhaiensis* colonies.

S24-white was identified as *S. hominis* species. Which was originally described as a Gram-positive, nonmotile, non-spore forming cocci [181]. Their colonies are small, unpigmented (e.g. gray-white) or pigmented (e.g. yellow-orange) and possess a "limited facultatively anaerobic capability" [181]. In addition, *S. hominis* is one of the most common species of staphylococci inhabiting human skin [182] and are also considered as potential pathogens [183], [184]. Nevertheless, due to the nature of these bacteria and the absence of cocci on both analyzed MPs, *S. hominis* was considered as contamination.

Results from S24-grey indicated the presence of *V. dokdonensis*, described as Gram-variable, endospore-forming, rod-shaped bacteria [185]. They are originally classified as anaerobic

cells [185], however, these bacteria have shown growth under aerobic conditions [186]. *V. dokdonensis* are mobilized by peritrichous flagella and their colonies are milky white, irregular, flat, and translucent [185]. Research has been conducted regarding to its great halotolerance properties [186], as well as their capacity for co-production of industrially important enzymes such as α-amylase and protease [187]. Although the color of the colonies did not match completely, it was still considered as a possible option due to more criteria is required to exclude this sequencing result. Therefore, it could be presumed that the biofilm found on S24 (*see Figure 18*) was produced by *F. nanhaiensis*, *F. arsenicus* or *V. dokdonensis* species.

M17 corresponded to *L. fusiformis*. These bacteria are described as Gram-positive, aerobic, endospore-forming, rod-shaped cells [156],[188], [189]. They were first classified within the genus *Bacillus* [188] and subsequently reclassified into *Lysinibacillus* [190]. Studies on their pathogenicity [191] and biotransformation potential (e.g. Isoeugenol to vanilla) [192] have been developed. In addition, *L. fusiformis* have shown a highly chromate resistance [188]. Their ability to use DEHP plasticizer as a carbon source has also been reported [193]. However, M17 was not analyzed on MPs.

Results from M21 sample indicated the presence of *B. pumilus*, described as Gram-positive, aerobic, spore-forming, rod-shaped [189] and motile bacteria [194], [195]. Moreover, UV [196] and hydrogen peroxide (H_2O_2) [197] resistance has been reported, which makes these bacteria very tolerant to common sterilization methods. Also, *B. pumilus* strains have been repeatedly found at the Jet Propulsion Laboratory spacecraft-assembly facility [197]. This could compromise life detection missions, which have very rigorous cleanliness and sterility requirements for spacecraft hardware [197]. Studies have also been conducted on their antibiotic resistance (e.g. streptomycin) [198] and pathogenicity [199]. Although *B. pumilus* is primarily an environmental contaminant, they have also been implicated in clinical infections such as sepsis [199]. In addition, these bacteria have been isolated from oil-contaminated sediment [119], MPs collected from marine environment [105], shrimps and fish [173]. They have also been related to hydrocarbons and LDPE biodegradation [119], [67]. As mentioned above, the M21 sample was only analyzed on PET, and significant

bacterial colonization was observed (*see Figure 20*), however, no biofilms tracks were observed on the plastic surface.

Given that S4 and M17 were not analyzed on polymers under SEM, they were not considered for final findings regarding biofilm on MPs.

5.7 MAIN FINDINGS AND CHARACTERISTICS OF THE BACTERIA IDENTIFIED

One of the main characteristics in common among all the identified bacteria is their spore production capacity, which make them highly resistant to harsh environments [200]. According to the literature, some of these bacteria have shown resistance to arsenic, chromate, antibiotics, UV and H_2O_2. In addition, these microorganisms have been described by their halotolerance, potential for wastewater treatment, degradation of plastics and pharmaceuticals. Curiously, *B. pumilus* was previously isolated from oil-contaminated sediments [119] and *B. flexus* from oil reservoir [164], therefore, the present study reaffirms both findings about their existence in oil-containing environments. Moreover, all species observed in the incubated MPs (except *M. yunnanensis*) are also known to show mobility, which has been related to surfaces attachment and biofilm production [155].

Table 7. Summary of results of the sequenced samples. Considering CRA and CV tests, attachment, and biofilm production on MPs (SEM). Not applicable (NA). Positive results (+). Negative results (-). Indistinguishable from each other by 16S rRNA gene sequence (a and b).

Sample	Closest match	CRA	CV	PE attachment	PET attachment	PE biofilm	PET biofilm
S10	*B. flexus* *M. yunnanensis*	+	-	+	+	+	+
S18	*B. safensis* *B. vietnamensis* [a] *B. aquamaris* [a]	+	+	+	+	+	-
S24	*F. arsenicus* [b] *F. nanhaiensis* [b]	-	+	+	+	+	+
M21	*V. dokdonensis* *B. pumilus*	+	+	NA	+	NA	-

In order to understand all the main findings of this study, Table 7 displays a summary with the results for the sequenced samples. It can be considered that the accuracy of CRA and CV test was similar. CV had a false negative for S10 since the presence of biofilm was evident

in both polymers. In the same way, CRA had a false negative for sample S24. However, both tests had a false positive for biofilm production for samples S18 and M21 on PET. Moreover, both polymers exhibited bacterial adhesion in all 4 samples (except for M21), and PE presented the highest biofilm production according to SEM results. However, S18 and M21 did not produce biofilm on PET.

Nevertheless, it must be noted that bacteria might also need other species to form a biofilm, and there may be some primary colonizing species that will become the foundation of the bacterial community [58]. In addition, it is important to emphasize that the MPs were incubated with the bacteria under specific and controlled conditions that could vary from the natural marine environment.

In this study it was possible to determine for the first time the colonization and biofilm production on PE and PET MPs under controlled marine environment by *M. yunnanensis* and *V. dokdonensis,* in addition to a pair of indistinguishable species: *F. arsenicus* and *F. nanhaiensis.* Moreover, although *B. flexus* was related to PP and PVC biodegradation [162], [61], it was determined they capacity to also produce biofilm on PE and PET MPs. It was also evidenced *B. safensis* ability to attach to PE and PET and produce biofilm on PE, which supports previous findings on PE MPs extracted from sea [25]. Also, even though *B. pumilus* have been associated with LDPE biodegradation [67], [119] it was shown their successful attachment to PET surface. And regarding to another pair of indistinguishable species: *B. aquamaris* and *B. vietnamensis*, it was found their possible capacity to attach both polymers and produce biofilm on PE. And as mentioned above, *B. aquamaris* was also involved in PAHs degradation [133].

Therefore, these new findings can serve as an insight into the possible behavior of these species and their ability to colonize PE and PET under marine environment, which represents a potential risk due to their high persistence and resistance.

6 CONCLUSIONS AND RECOMENDATIONS

A predominance of presumptive *V. parahaemolyticus* was determined in the Subtidal sediment cores (61%), while Marsh cores indicated a majority of presumptive *V. cholera* (64%), which points out a different affinity of these species regarding to the types of sediments.

It was not possible to identify *V. parahaemolyticus* within the isolated bacteria, because *tlh*, *tdh* and *trh* were not detected in the samples.

The ability of some bacteria to only produce biofilm under certain conditions was evidenced by the fact that only 22% of the total samples were positive in both CV and CRA tests. However, Marsh samples showed the highest biofilm production.

Even though CV was firstly considered as a possible main indicator of the biofilm production capacity of the isolated samples, it was concluded according to SEM results that the accuracy of CRA and CV tests were similar in this study.

Both PE and PET MPs exhibited bacterial adhesion in all sequenced samples (except for M21), however, PE presented the highest biofilm production according to SEM results, which could suggest a possible preference for this polymer.

It was determined that all the identified bacteria on MPs possess spore production capacity, which make them highly resistant to harsh environments, in addition to mobility properties (except *M. yunnanensis*), which are related to surfaces attachment and biofilm production.

For the first time, it was possible to determine the biofilm production capacity of *M. yunnanensis*, *V. dokdonensis* and the pair of closely related species: *F. arsenicus* and *F. nanhaiensis*, on PE and PET MPs under controlled marine environment.

In the same way, it was also evidenced: *B. flexus* capacity to produce biofilm on PE and PET MPs, *B. safensis* ability to attach to PE and PET and produce biofilm on PE, a successful attachment of *B. pumilus* to PET and regarding to the other indistinguishable species (*B. aquamaris* and *B. vietnamensis*) it was found their attachment ability to both polymers and biofilm production capacity on PE under controlled marine environment.

The new findings of this study might serve as an insight into the behavior and ability of the identified species to colonize PE and PET or other polymers under marine environment, which may represent a possible risk due to their high persistence and potential pathogenicity.

Multi and interdisciplinarity play a significant role in the proper development of a project like the one presented. An integral overview must be considered when dealing such kind of studies, where environmental engineering use techniques and tools from other disciplines (e.g. microbiology and chemistry) to achieve major goals.

The samples should have been normalized for growth (e.g. OD_{600}) to allow for a quantitative Crystal Violet assay. In addition, different environmental conditions (e.g. temperature) should be tested to give a better insight into how they affect the biofilm production.

Due to *V. parahaemolyticus* were not identified in the samples, it is recommended to perform the three-zone streak method before the TCBS test to guarantee individual colonies (*See Appendix 13*)

It is necessary to develop a Phylogenetic tree based on another gene amplification different from 16S rRNA (e.g. *gyrB* gene) to verify the obtained results for a subsequent study or publication.

Additional studies should be conducted regarding the ability of bacterial spores to adhere to the microplastics.

Conduct future research to analyze the ability of the identified bacteria to biodegrade oil or plastics are also suggested.

Based on everything learned in this study, it is recommended to raise awareness about the currently problem of plastic pollution, as well as to urge the decrease of the use of single-use plastic and to promote the adaptation of a circular economy.

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8 APPENDICES

Appendix 1. PCR primers.

Gene	Primer	Sequence	Reference
tdh	tdh86F	CTGTCCCTTTTCCTGCCCCCG	[201]
	tdh331R	AGCCAGACACCGCTGCCATTG	[201]
trh	trh90F	ACCTTTTCCTTCTCCWGGKTCSG	[201]
	trh500R	CCGCTCTCATATGCYTCGACAKT	[201]
tlh	L-tlh	AAAGCGGATTATGCAGAAGCACTG	[202]
	R-tlh	GCTACTTTCTAGCATTTTCTCTGC	[202]

Appendix 2. Master mix for *Vibrio* spp. identification by PCR.

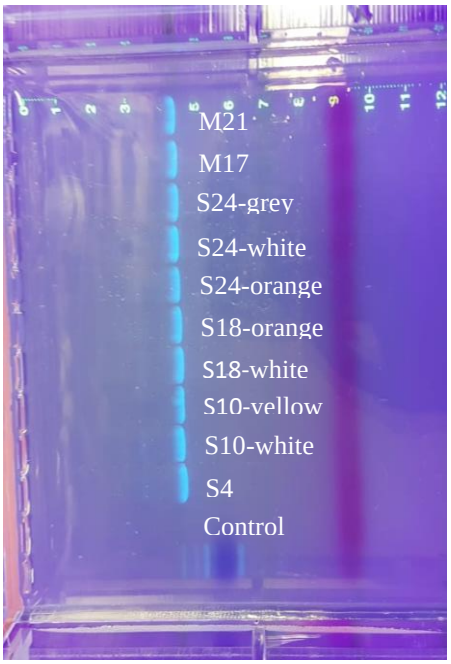
Component	Volume (µl)
H_2O	13.3
Buffer	5.0
dNTPs	2.0
P_F	1.25
P_R	1.25
DNA	2.0
GoTaq®	0.2
Total	25

Appendix 3. Master mix for DNA quality by PCR.

Component	Volume (µl)
H_2O	11.8
Buffer	5.0
dNTPs	2.0
P_F	2.5
P_R	2.5
DNA	1.0
GoTaq®	0.2
Total	25

*Water and DNA volumes were adjusted to have a final DNA concentration of >0.2 (ng/µL)

Appendix 4. DNA bands for sequencing



Appendix 5. Quantification of presumptive Vibrio species colonies from Marsh sediment cores in TCBS plates.

Depths (cm)	*V. cholera* colonies			*V. parahaemolyticus* colonies		
	1M	**2M**	**3M**	**1M**	**2M**	**3M**
0-1	0	1	42	0	4	0
1-2	0	0	50	0	18	0
2-3	1	0	0	12	19	6
3-4	1	0	10	11	11	3
4-6	2	1	1	10	11	1
6-8	7	34	34	2	4	0
8-10	42	20	40	0	1	1
10-12	34	0	0	5	13	3
12-14	30	5	0	2	11	10
Total	**117**	**161**	**177**	**42**	**192**	**24**

Appendix 6. Quantification of presumptive Vibrio species colonies from Subtidal sediment cores in TCBS plates.

Depths (cm)	*V. cholera* colonies			*V. parahaemolyticus* colonies		
	1S	**2S**	**3S**	**1S**	**2S**	**3S**
0-1	29	0	4	6	0	12
1-2	2	0	0	21	9	6
2-3	0	0	0	1	10	1
3-4	1	0	4	16	19	7
4-6	1	0	8	1	0	14
6-8	1	2	7	5	14	8
8-10	0	5	7	2	13	1
10-12	0	3	1	2	19	0
12-14	1	43	0	7	0	0
Total	**35**	**53**	**31**	**61**	**84**	**49**

Appendix 7. The 50 most representative colony samples according to TCBS results. Each isolate number corresponds to a sample, linked to a classification of sediments (1S, 2S, 3S, 1M, 2M or 3M), section/depth (1 -14 cm) and its number corresponding to its position on the patched plate.

SUBTIDAL		**MARSH**	
Isolate number	**Location in sediment and patched plate**	**Isolate number**	**Location in sediment and patched plate**
S1	*1S 1-2 #1*	**M1**	*IM 4-6 #32*
S2	*1S 1-2 #7*	**M2**	*IM 4-6 #33*
S3	*1S 1-2 #7*	**M3**	*2M 2-3 #1*
S4	*1S 1-2 #9*	**M4**	*2M 2-3 #9*
S5	*1S 1-2 #10*	**M5**	*2M 2-3 #19*
S6	*1S 1-2 #11*	**M6**	*2M 2-3 #28*
S7	*1S 1-2 #14*	**M7**	*2M 2-3 #28*
S8	*1S 1-2 #26*	**M8**	*2M 2-3 #40*
S9	*1S 1-2 #33*	**M9**	*2M 2-3 #41*
S10	*1S 1-2 #37*	**M10**	*2M 10-12 #2*
S11	*1S 1-2 #41*	**M11**	*2M 10-12 #27*
S12	*1S 1-2 #46*	**M12**	*2M 10-12 #44*
S13	*1S 1-2 #46*	**M13**	*2M 10-12 #49*
S14	*1S 1-2 #47*	**M14**	*3M 10-12 #33*
S15	*1S 1-2 #48*	**M15**	*3M12-14 #7*
S16	*1S 1-2 #49*	**M16**	*1M 2-3 #15*
S17	*1S 3-4 #13*	**M17**	*1M 2-3 #50*
S18	*1S 3-4 #17*	**M18**	*1M 3-4 #14*
S19	*1S 3-4 #20*	**M19**	*1M 3-4 #15*

S20	*1S 3-4 #4*	**M20**	*1M 3-4 #20*
S21	*1S 3-4 #7*	**M21**	*3M 12-14 #30*
S22	*1S 3-4 #8*	**M22**	*3M 12-14 #47*
S23	*1S 3-4 #25*	**M23**	*2M 1-2 #24*
S24	*1S 3-4 #27*	**M24**	*2M 1-2 #29*
S25	*2S 2-3 #32*	**M25**	*2M 1-2 #38*

Appendix 8. CRA results for Subtidal and Marsh samples. Positive (+) and negative (-) biofilm production.

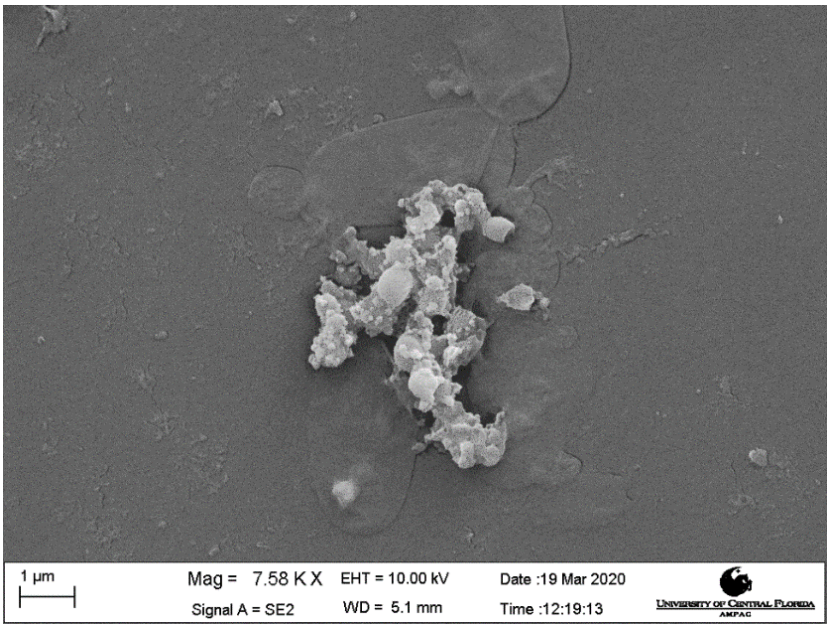
Subtidal		**Marsh**	
Isolate number	**Biofilm (+/-)**	**Isolate number**	**Biofilm (+/-)**
S1	+	M1	+
S2	-	M2	+
S3	-	M3	+
S4	-	M4	-
S5	-	M5	-
S6	-	M6	+
S7	+	M7	-
S8	-	M8	+
S9	+	M9	+
S10	+	M10	+
S11	-	M11	-
S12	+	M12	+
S13	-	M13	+
S14	+	M14	+
S15	+	M15	-
S16	+	M16	-
S17	-	M17	-
S18	+	M18	+
S19	+	M19	-
S20	+	M20	-
S21	+	M21	+
S22	+	M22	-
S23	+	M23	+
S24	-	M24	-
S25	-	M25	+

Appendix 9. Crystal violet results for Subtidal samples. It shows the raw data of the 3 replicates obtained, the average and standard deviation (StDev) of the blanks of each microtiter plate. Two blanks averages were used because the samples were grown in two different 96-well microtiter plate. Rep 1, Rep 2 and Rep 3 correspond to the subtraction of the blanks from each raw replica. It also includes the average of the corrected replicates, their standard deviation and relative deviation.

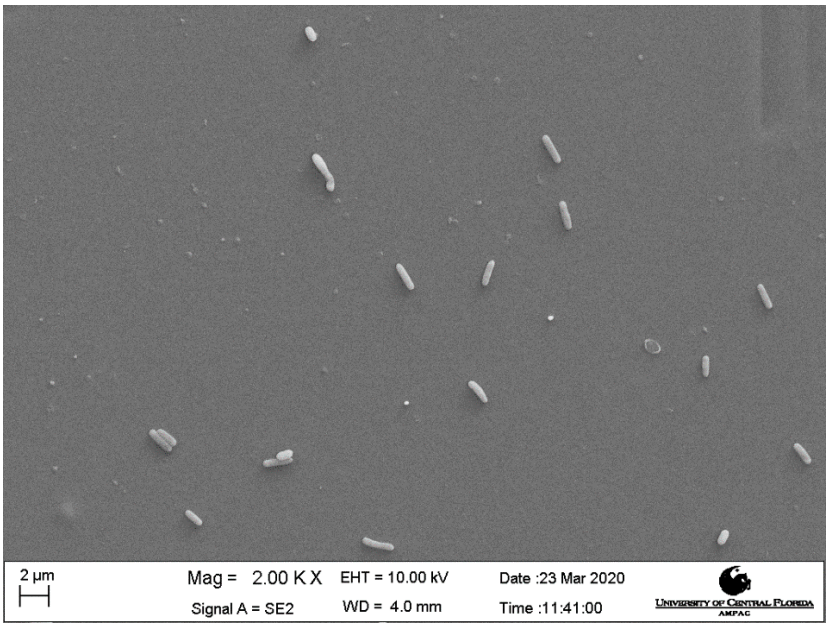
Isolation number	Raw rep 1	Raw rep 2	Raw rep 3	Blank Average	StDev (Blank)	Rep 1	Rep 2	Rep 3	Rep Average	StDev (Rep)	Rel. Dev (%)
S1	1.7080	2.0190	2.0870	0.1428	0.0365	1.565	1.876	1.944	**1.7952**	0.2021	11.2557
S2	0.7390	0.7090	0.3590	0.1428	0.0365	0.596	0.566	0.216	**0.4596**	0.2113	45.9707
S3	0.7870	1.6220	0.7510	0.1428	0.0365	0.644	1.479	0.608	**0.9106**	0.4928	54.1211
S4	3.2500	2.4290	2.1740	0.1428	0.0365	3.107	2.286	2.031	**2.4749**	0.5623	22.7186
S5	0.8190	0.3540	0.4890	0.1428	0.0365	0.676	0.211	0.346	**0.4112**	0.2392	58.1707
S6	0.6740	0.3650	1.0880	0.1428	0.0365	0.531	0.222	0.945	**0.5662**	0.3628	64.0670
S7	0.3940	0.3090	0.2360	0.1428	0.0365	0.251	0.166	0.093	**0.1702**	0.0791	46.4515
S8	0.1830	0.1320	0.1440	0.1428	0.0365	0.040	-0.011	0.001	**0.0102**	0.0267	260.5678
S9	2.1590	2.7020	2.1570	0.1428	0.0365	2.016	2.559	2.014	**2.1966**	0.3141	14.2987
S10	0.3730	0.2030	0.2400	0.1428	0.0365	0.230	0.060	0.097	**0.1292**	0.0894	69.1800
S11	2.1040	0.3140	0.3330	0.1428	0.0365	1.961	0.171	0.190	**0.7742**	1.0280	132.7786
S12	1.0340	0.7320	0.9000	0.1428	0.0365	0.891	0.589	0.757	**0.7459**	0.1513	20.2867
S13	3.2860	0.8780	0.4320	0.0730	0.0196	3.213	0.805	0.359	**1.4590**	1.5353	105.2289
S14	0.2240	0.1650	0.2010	0.0730	0.0196	0.151	0.092	0.128	**0.1237**	0.0297	24.0467
S15	0.2410	0.1970	0.2100	0.0730	0.0196	0.168	0.124	0.137	**0.1430**	0.0226	15.8079
S16	0.3120	0.2590	0.3040	0.0730	0.0196	0.239	0.186	0.231	**0.2187**	0.0286	13.0663
S17	2.3070	2.2920	2.2640	0.0730	0.0196	2.234	2.219	2.191	**2.2147**	0.0218	0.9855
S18	2.5890	2.4400	2.2590	0.0730	0.0196	2.516	2.367	2.186	**2.3563**	0.1653	7.0134
S19	0.3760	0.8320	0.5490	0.0730	0.0196	0.303	0.759	0.476	**0.5127**	0.2302	44.9026
S20	0.3680	0.5050	0.4430	0.0730	0.0196	0.295	0.432	0.370	**0.3657**	0.0686	18.7610
S21	0.4250	0.3780	0.9340	0.0730	0.0196	0.352	0.305	0.861	**0.5060**	0.3083	60.9359
S22	2.1760	0.7250	0.4840	0.0730	0.0196	2.103	0.652	0.411	**1.0553**	0.9153	86.7283
S23	0.9330	0.5260	0.8330	0.0730	0.0196	0.860	0.453	0.760	**0.6910**	0.2121	30.6935
S24	4.0000	4.0000	4.0000	0.0730	0.0196	3.927	3.927	3.927	**3.9270**	0.0000	0.0000
S25	0.4620	0.4530	1.0380	0.0730	0.0196	0.389	0.380	0.965	**0.5780**	0.3352	57.9900

Appendix 10. Crystal violet results for Marsh samples. It shows the raw data of the 3 replicates obtained. the average and standard deviation (StDev) of the blanks of each microtiter plate. Two blanks averages were used because the samples were grown in two different 96-well microtiter plate. Rep 1. Rep 2 and Rep 3 correspond to the subtraction of the blanks from each raw replica. It also includes the average of the corrected replicates. their standard deviation and relative deviation.

Isolation number	Raw rep 1	Raw rep 2	Raw rep 3	Blank Average	St. Dev (Blank)	Rep 1	Rep 2	Rep 3	Rep Average	St. Dev (Rep)	Rel. Dev (%)
M1	0.753	0.359	0.203	0.1593	0.0425	0.594	0.200	0.044	0.2790	0.2835	101.5898
M2	3.009	3.366	3.163	0.1593	0.0425	2.850	3.207	3.004	3.0200	0.1791	5.9291
M3	2.242	1.942	0.708	0.1593	0.0425	2.083	1.783	0.549	1.4713	0.8130	55.2561
M4	0.976	0.735	1.223	0.1593	0.0425	0.817	0.576	1.064	0.8187	0.2440	29.8047
M5	1.603	2.59	2.411	0.1593	0.0425	1.444	2.431	2.252	2.0420	0.5258	25.7512
M6	2.84	2.869	2.941	0.1593	0.0425	2.681	2.710	2.782	2.7240	0.0520	1.9091
M7	0.694	0.595	0.584	0.1593	0.0425	0.535	0.436	0.425	0.4650	0.0606	13.0282
M8	1.904	1.626	1.716	0.1593	0.0425	1.745	1.467	1.557	1.5893	0.1418	8.9250
M9	2.434	2.645	2.573	0.1593	0.0425	2.275	2.486	2.414	2.3913	0.1073	4.4853
M10	0.991	1.63	1.489	0.1593	0.0425	0.832	1.471	1.330	1.2107	0.3357	27.7289
M11	4	3.077	2.863	0.1593	0.0425	3.841	2.918	2.704	3.1540	0.6042	19.1572
M12	1.765	1.677	1.283	0.1593	0.0425	1.606	1.518	1.124	1.4157	0.2567	18.1311
M13	0.971	0.923	1.53	0.0832	0.0188	0.8878	0.8398	1.4468	1.0582	0.3374	31.8900
M14	0.25	0.274	0.168	0.0832	0.0188	0.1668	0.1908	0.0848	0.1475	0.0556	37.6826
M15	4	3.401	3.416	0.0832	0.0188	3.9168	3.3178	3.3328	3.5225	0.3416	9.6972
M16	0.396	0.556	0.353	0.0832	0.0188	0.3128	0.4728	0.2698	0.3518	0.1070	30.4042
M17	0.165	0.134	0.134	0.0832	0.0188	0.0818	0.0508	0.0508	0.0612	0.0179	29.2608
M18	4	4	4	0.0832	0.0188	3.9168	3.9168	3.9168	3.9168	0.0000	0.0000
M19	3.443	3.408	3.292	0.0832	0.0188	3.3598	3.3248	3.2088	3.2978	0.0790	2.3967
M20	2.292	2.517	2.246	0.0832	0.0188	2.2088	2.4338	2.1628	2.2685	0.1450	6.3927
M21	4	4	4	0.0832	0.0188	3.9168	3.9168	3.9168	3.9168	0.0000	0.0000
M22	4	4	4	0.0832	0.0188	3.9168	3.9168	3.9168	3.9168	0.0000	0.0000
M23	3.454	4	4	0.0832	0.0188	3.3708	3.9168	3.9168	3.7348	0.3152	8.4404
M24	2.276	3.221	3.147	0.0832	0.0188	2.1928	3.1378	3.0638	2.7982	0.5255	18.7815
M25	2.246	1.411	2.32	0.0832	0.0188	2.1628	1.3278	2.2368	1.9092	0.5048	26.4412



Appendix 11. Scanning electron micrograph showing bacterial cells attachment and biofilm on PET microplastic. This image corresponded to a piece of PET incubated with the sample S10 at 30°C.



Appendix 12. Scanning electron micrographs showing bacterial cells attachment on PET microplastic. This image corresponded to a piece of PET incubated with the sample M1 at 30°C.

Appendix 13. Three zone streak method used, and correct method suggested.