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Mestrado em Biologia Marinha

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(Stéphanie Birnstiel Falcão Amorim)

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RESUMO

Mais de 5 trilhões de peças de plástico estão presentes no oceano. Seus efeitos sobre os macroorganismos estão bem documentados e sabe-se que podem afetá-los principalmente devido à sua ingestão ou emaranhamento. No entanto, os estudos sobre seus efeitos em microrganismos são menos populares e focados, principalmente, na comunidade de biofilme que pode colonizar a superfície do plástico. Foi demonstrado que os plásticos marinhos são recobertos por matéria orgânica e inorgânica, seguida da colonização bacteriana, que é dominada por Gammaproteobacteria e Alphaproteobacteria, com a possibilidade de a bactéria Bacteroidetes aparecer mais tarde e se tornar abundante. Além disso, os plásticos geralmente contêm aditivos que são adicionados pela indústria para melhorar sua qualidade e desempenho. Esses aditivos e compostos, assim como os blocos monoméricos do plástico, podem ser liberados no meio aquático com consequências para a comunidade microbiana. Verificou-se que as bactérias marinhas absorvem os compostos orgânicos liberados pelo plástico, estimulando seu crescimento. No entanto, quais grupos de bactérias são capazes de usá-los e como isso os afeta ainda é desconhecido. Nossa hipótese é que os lixiviados plásticos podem alterar e afetar a composição e atividade da comunidade de bactérias heterotróficas marinhas quando expostas a diferentes condições ambientais e tipos de plástico. Portanto, o objetivo deste estudo foi caracterizar a comunidade bacteriana e avaliar sua atividade após a exposição aos compostos liberados por diferentes tipos de plásticos. O estudo testou lixiviados de diferentes tipos de plástico comumente encontrados no oceano, como polietileno de baixa densidade (PEBD) e poliestireno (PS), sob uma variedade de condições ambientais, como diferentes temperaturas (15 e 28 °C), exposição à radiação UV e ambiente escuro. Um plástico biodegradável, ácido polilático (PLA), também foi usado para comparar com os lixiviados termoplásticos. Na etapa de fotodegradação, peças plásticas foram adicionadas à água do mar artificial (AMA) em tubos de quartzo, para tratamentos de luz, e em frascos de borosilicato revestidos com folha de alumínio, para tratamentos de escuro. Um experimento comparou PEBD e PLA a 15 °C, enquanto o outro comparou PEBD e PS a 28 °C. As amostras foram incubadas durante 6 dias sob radiação ultravioleta e luz visível e temperatura constante. Na etapa de biodegradação, lixiviados plásticos obtidos no experimento de fotodegradação anterior foram utilizados após a remoção das peças de plástico. Os lixiviados foram inoculados com um inóculo bacteriano natural do Observatório Microbial de Blanes Bay (NO Mediterrâneo). Sua curva de crescimento foi acompanhada até atingirem a fase estacionária.

Em seguida, 72 horas após a inoculação, a comunidade bacteriana que foi capaz de crescer nesses lixiviados plásticos foi caracterizada pelo uso das técnicas de hibridização *in situ* fluorescente de deposição de repórter catalisado (CARD-FISH) e marcação de aminoácidos não canônicos bioortogonal (BONCAT). CARD-FISH é um método que usa sondas oligonucleotídicas marcadas com peroxidase de rábano (HRP) e amplificação do sinal de tiramida, a fim de detectar células com baixo conteúdo ribossômico, que são frequentemente prevalentes em águas oligotróficas. O BONCAT é um método que tem sido usado para caracterizar a atividade de micróbios não cultivados, em seu ambiente de crescimento nativo. Esta abordagem semiquantitativa explora o estado fisiológico das bactérias marinhas, incubando uma amostra bacteriana com um análogo da metionina e usando a química do clique para identificar as células que incorporaram o substrato. Sondas diferentes foram usadas para avaliar a composição da comunidade, como GAM42a, que tem como alvo a maioria das Gammaproteobacterias, CF319a, que tem como alvo muitos membros do grupo Bacteroidetes, EUB338 I-II e –III, que tem como alvo a maioria das bactérias, e Alf968, que tem como alvo as Alphaproteobacterias. Suas abundâncias foram calculadas em relação às suas contribuições para a comunidade total, enquanto sua atividade foi avaliada pelo cálculo de seu valor médio cinza (VMC), que é a soma dos valores de cinza de todos os pixels na célula dividida pelo número de pixels. Um teste-*t* de Student bicaudal foi aplicado a fim de comparar a abundância e a atividade dos diferentes grupos filogenéticos bacterianos. Em ambas as temperaturas, as bactérias começaram a crescer após 24 horas e atingiram a fase exponencial após 72 horas de incubação. Ao final de ambos os experimentos, as amostras de plástico apresentaram maior abundância bacteriana do que os controles sem plástico, exceto para o PS irradiado. Todos os tipos de lixiviados plásticos levaram a uma composição de comunidade microbiana semelhante: elas eram compostas principalmente por Gamma-, Alphaproteobacteria e Bacteroidetes. Ambos os experimentos apresentaram contribuições semelhantes de cada grupo filogenético para a abundância total. Gamma- e Alphaproteobacteria mostraram ser os maiores contribuintes, enquanto Bacteroidetes foi o grupo menos abundante. Os lixiviados plásticos estimularam o crescimento de Gamma- e Alphaproteobacteria nos tratamentos plásticos em relação aos controles sem plásticos. No entanto, o impacto sobre os Bacteroidetes foi mais variável. A irradiação durante a lixiviação de plástico teve resultados contrastantes na abundância bacteriana que dependeu do tipo de plástico e do grupo filogenético. No entanto, lixiviados plásticos previamente irradiados, como os encontrados no oceano, estimularam a síntese de proteínas em bactérias marinhas em relação àquelas não expostas anteriormente à radiação. Portanto, algumas exceções foram capazes de mostrar como diferentes condições e

tipos de plásticos podem ter impactos mistos em cada grupo filogenético e na comunidade bacteriana. Aqui também descobrimos que o plástico biodegradável, PLA, não liberou compostos biodegradáveis que se refletiram em um maior crescimento ou atividade bacteriana. Isso mostra que, na água do mar, o plástico biodegradável como o PLA, nem sempre é biodegradado e seu impacto sobre os microrganismos não difere dos demais termoplásticos. Este estudo foi o primeiro passo para entender como os lixiviados plásticos podem afetar a composição da comunidade microbiana na coluna d'água. Também identificou, pela primeira vez, quais grupos bacterianos são selecionados nos lixiviados plásticos marinhos e o quanto eles são ativos na síntese de proteínas. As sondas aqui utilizadas levaram a uma ampla identificação de microrganismos, em grupos filogenéticos, que incluem muitas espécies diferentes. Portanto, novos experimentos são necessários para identificar os organismos que compõem cada grupo e seu comportamento quando expostos aos lixiviados plásticos em diferentes condições, pois muitos fatores intrínsecos e extrínsecos podem ter diferentes efeitos isolados e combinados sobre as bactérias. Este estudo melhorou nosso conhecimento atual sobre a interação entre a lixiviação de plástico e micróbios marinhos e como isso pode afetar o ambiente, a teia alimentar e o sistema marinhos. Esses resultados fornecem insights cruciais sobre potenciais formas de biodegradação de plástico que podem ser desenvolvidas no futuro.

Palavras-chave: Poluição de plástico, micróbios marinhos, lixiviação de plástico, CARD-FISH, BONCAT

ABSTRACT

Over 5 trillion pieces of plastic are present in the ocean. They usually contain additives that are added to them by the industry in order to improve their quality and performance. These additives and compounds, as well as the monomer blocks of the plastic, can be released into the aquatic media with consequences for the microbial community. It has been found that marine bacteria uptake the organic compounds released by plastic stimulating their growth. However, which bacterial groups are able to use them are still unknown. Therefore, the aim of this study was to characterize for the first time the bacterial community and assess its activity after the exposure to the compounds released by different types of plastics. The study tested the leachates from different types of plastic commonly found in the ocean, such as low-density polyethylene (LDPE) and polystyrene (PS), under different environmental conditions. A biodegradable plastic, polylactic acid (PLA), was also used to compare with the thermoplastic leachates. Then, the bacterial community that was able to grow in these plastic leachates was characterized by using the CARD-FISH and BONCAT techniques. Our results indicate that the bacterial community was mainly composed by Gamma-, Alphaproteobacteria and Bacteroidetes, with the first two being the dominant ones. Overall, plastic leachates increased the growth rates of Gamma- and Alphaproteobacteria in the plastic treatments compared to the controls without plastics. However, the impact on Bacteroidetes was more variable. Irradiation during plastic leaching had contrasting results on the bacterial abundance which depended on the plastic type and the phylogenetic group. On the other hand, plastic leachates that were previously irradiated increased significantly more the activity of marine bacteria compared to the non-irradiated ones. These results provide crucial insights on potential ways of plastic biodegradation that could be developed in the future.

Keywords: Plastic pollution, marine microbes, plastic leaching, CARD-FISH, BONCAT

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1. INTRODUCTION

1.1 MARINE PLASTIC POLLUTION

Plastic has been accumulating in the environment due to its durability and short lifespan, resulting in the presence of over 5 trillion pieces of plastic in the ocean (Eriksen *et al.*, 2014). Over 350 million tons of plastic are produced worldwide every year and over 8 million tons of this debris reaches the oceans (Geyer *et al.*, 2017). Its distribution is not uniform and it is possible to find plastic debris even in places distant from large urban centers, including deep and abyssal waters (Taylor *et al.*, 2016). Its presence in the oceans can result in physical harm to some macroorganisms that live in this environment, such as drowning and suffocation, due to the entrapment in the material, or intoxication and obstructions in the throat and digestive tract, due to their ingestion (Koelmans *et al.*, 2014). When ingested, they are capable of accumulating in the organism, causing physical damage, due to internal clogs and abrasions, and intoxications, which can result in carcinogenesis and deregulate the endocrine system (Wright *et al.*, 2013). Consequently, these internal damages and intoxications end up compromising various activities, such as their breathing, feeding, reproduction, metabolism and molecular and cellular processes (Avio *et al.*, 2015; Taylor *et al.*, 2016).

Most plastic types can be classified as polyethylene terephthalate (PET), polyester (PES), polyethylene (PE), high-density polyethylene (HDPE), polyvinyl chloride (PVC), polypropylene (PP), polyamide (PA) and polystyrene (PS), which are commonly known as soda bottles, polyester clothing, plastic bags, detergent bottles, plumbing pipes, drinking straws, toothbrushes and take-out food containers, respectively (NOAA – Marine Debris Program, 2017). They are made of high molecular weight organic polymers, which, other than carbon and hydrogen, can be composed by oxygen, nitrogen, sulfur, fluorine and chlorine, from synthetic or natural origin. Its molecular composition, proportion and organization dictates the type of plastic. Two main plastic polymer groups are differentiated by their macromolecular structure and temperature-dependent physical properties of the material: the thermoplastics and thermosets. Although the thermoplastics can melt and get soft when exposed to mechanical, thermal and radiation energy, the thermosets never melt and soften (Klein, 2012). Furthermore, there are also biodegradable starch-based plastics including starch blends, such as starch-polyethylene blends, starch-polyester blends and starch-polyvinyl alcohol (PVA) blends, polylactic acid (PLA) polyesters and polybutylene succinate (PBS) polyesters (Shah *et al.*, 2008).

In the ocean, plastics are present in two different forms: as macro- and microplastics. Microplastics are pieces and fragments of plastic smaller than 5 mm (Betts, 2008), which can be introduced indirectly (secondary microplastics), through the degradation of macroplastics (Thompson, 2004; Barnes *et al.*, 2009), or directly (primary microplastics), through the production of plastic granules by the plastic industries or hygiene and personal care products, such as toothpaste and exfoliators (Barboza & Gimenez, 2015), painting and coating of ships, dumping of laundries and dust from urban centers (Boucher & Friot, 2017). The process of degradation of macroplastics into microplastics can happen in several different ways, as a result of biotic and/or abiotic processes. While the abiotic degradation (weathering) is a consequence of chemical and mechanical processes, the biodegradation is driven by microbes (Andrady, 2011). Abiotic degradation is mainly caused due to physical abrasion and photodegradation, which are common and usually occur in coastal regions, where plastics are most vulnerable to wave action and prolonged exposure to UV rays (Barnes *et al.*, 2009). However, biodegradation of plastic is a process that usually occurs after or concomitant with the physical and chemical processes of the abiotic degradation, which weakens the structure of the polymers and manifests as cracks, roughness and molecular changes (İpekogˇlu *et al.*, 2007). Furthermore, these biotic processes result in total or partial conversion of organic carbon into biogas and biomass, which are linked to the activity of different microorganisms capable of using plastic as a carbon source, such as bacteria, fungi, and actinomycetes (Shah *et al.*, 2008).

The 5,300 grades of synthetic polymers produced by the plastic industry are accompanied by a range of chemical additives, such as plasticizers, flame retardants, antioxidants and other stabilizers, pro-oxidants, surfactants, inorganic fillers or pigments (Jacquin *et al.*, 2019), which are added during the plastic manufacture in order to improve their quality and performance (Lusher *et al.*, 2017). Moreover, since plastics have hydrophobic properties, they are capable of adsorbing various types of compounds (Avio *et al.*, 2015), including persistent, bioaccumulative and toxic substances (PBTs), in addition to metals and persistent organic pollutants (POPs) (Mato *et al.*, 2001; Fossi *et al.* 2014; Barboza & Gimenez, 2015). Both additives and pollutants can eventually be released into the surrounding water and within organisms (Engler, 2012; Suhrhoff & Scholz-Böttcher, 2016), representing a major concern for the marine environment and wildlife. The process in which a compound (additive or organic substance derived from polymer degradation) migrates from the plastic to the seawater, is called plastic leaching. The degradation of macroplastics into microplastics (< 5 mm) increases the particle's surface area, leading to additive leaching and interactions with solved compounds (Suhrhoff & Scholz-Böttcher, 2016; Romera-Castillo *et al.*, 2018).

1.2 HOW CAN PLASTIC AND MARINE MICROBES INTERACT?

Plastic’s hydrophobic surface also allows it to function as an artificial reef for microorganisms, as it is compatible with biofilm formation. When colonized by these organisms, the plastic particle can lose its buoyancy and sink into deep waters and eventually settle in sediment (Lobelle & Cunliffe, 2011; Zettler *et al.*, 2013). In addition, this bacterial attachment can also change the particle’s properties, since it can modify its topography, structure and composition, and serve as primary colonizer for other organisms, such as fungi and diatoms (Ghosh *et al.*, 2019). Thus, these particles can act as carriers of pathogens and microorganisms, which can be harmful to other organisms (Vandermeersch *et al.*, 2015), and as a vector to transport invasive species and organic chemicals (Koelmans *et al.*, 2014).

The plastisphere composition is mostly influenced by spatial and seasonal factors, in addition to the polymer type, surface properties and size, which result in a complex interaction (Jacquin *et al.*, 2019). Furthermore, hydrophobicity and other substratum properties (crystallinity and crystal structure, roughness, glass transition temperature, melting temperature, modulus of elasticity) can also affect the selection of the bacterial community in the beginning of the colonization (Pompilio *et al.*, 2008). Although these characteristics can determine what microorganisms will attach, communities from different substrates can converge over time as their biofilms mature (Amaral-Zettler *et al.*, 2020). First, marine plastics are covered by inorganic and organic matter, called the “conditioning film”, followed by the bacterial colonization, which is dominated by Gammaproteobacteria and Alphaproteobacteria (Oberbeckmann *et al.*, 2015). At last, Bacteroidetes bacteria can appear later on and become abundant (Lee *et al.*, 2008).

As it is known, phytoplankton’s primary production represents the main source of dissolved organic carbon (DOC) in the ocean. Besides the phytoplanktonic activity, bacteria can also contribute to the marine DOC through the release of dissolved organic compounds (Hansell & Carlson, 1998), in addition to the death and lysis of phytoplankton cells (Agustí *et al.* 1998). On the other hand, the heterotrophic respiration (bacterial uptake) represents its main sink, with most of the DOC being decomposed by bacterioplankton in the surface (Kirchman *et al.*, 1991; Druffel *et al.*, 1992). Therefore, microorganisms not only uptake plastic or its additives during their colonization, but also use the compounds once they leach into the water column (Romera-Castillo *et al.*, 2018). Although the DOC released from plastic into the ambient seawater can stimulate the activity of heterotrophic microbes (Romera-Castillo *et al.*, 2018), plastic leachates showed an opposite effect on autotrophic bacteria, reducing their capacity to grow, photosynthesise or produce oxygen (Tetu *et al.*, 2019). However, although many experiments have been conducted to test the effects of isolated plastic components on marine microbes using monocultures (Zhang *et al.*, 2013; Boll *et al.*, 2020), no information is available regarding the composition of a natural bacterial community growing in plastic leachates.

We hypothesize that plastic leachates may alter and affect the composition and activity of marine heterotrophic bacteria when exposed to different environmental conditions and types of plastic.

1.3 MICROBIAL IDENTIFICATION AND ACTIVITY

1.3.1 CARD-FISH

Fluorescent *in Situ* Hybridization (FISH) is a method used to identify microbial cells to the species, phyla or domain level. Most FISH target 16S ribosomal RNA (rRNA) with oligonucleotide probes, which can be labelled with fluorescent dyes or enzyme horseradish peroxidase, followed by the cell identification through the fluorescence *in situ* hybridization (Amann & Fuchs, 2008). Its main steps consist on the cell fixation, incubation with a probe, washing and, finally, identification and quantification by epifluorescence microscopy or flow cytometry (Pernthaler & Pernthaler, 2007).

The Catalyzed Reporter Deposition Fluorescent *in Situ* Hybridization (CARD-FISH) is a method that uses horseradish peroxidase (HRP)-labeled oligonucleotide probes and tyramide signal amplification, in order to detect cells with low ribosomal content, which are often prevalent in oligotrophic waters (Pernthaler *et al.*, 2002). This method improves the FISH sensitivity and signal, by combining the horseradish peroxidase-labelled probes with a catalyzed reported deposition (CARD) of fluorescently labelled tyramides. The radicalization of multiple tyramide molecules by a single horseradish peroxidase amplify the signal and the activated tyramides can permanently bind to the cells, resulting in a stronger fluorescent signal (Amann & Fuchs, 2008). The CARD-FISH protocol is detailed in Pernthaler *et al.* (2002).

1.3.2 BONCAT

The BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) is a method that has been used to characterize the activity of uncultured microbes, in their native growth environment. This semi-quantitative approach explores the physiological status of marine bacteria, by incubating a bacterial sample with an analogue of methionine and using click-chemistry to identify the cells that have incorporated the substrate (Hatzenpichler *et al.*, 2014; Hatzenpichler & Orphan, 2015). After the cell uptake, the methionine analogue exploits the low specificity of methionyl-tRNA synthetases and is incorporated into newly synthesized proteins. Then, the translationally active cells can be identified through a click reaction, where a fluorophore is covalently ligated to the analogue. Finally, a fluorescently labeled population of translationally active cells can be further analyzed by different microscopy and analytical methods (Valentini *et al.*, 2020). The BONCAT protocol used is detailed in Leizeaga *et al.* (2017).

2. OBJECTIVES

2.1 GENERAL OBJECTIVES

The aim of this study was to characterize the bacterial community growing in the leachates from the most commonly plastic types found in the ocean: low-density polyethylene (LDPE) and polystyrene (PS). Moreover, a biodegradable plastic, such as polylactic acid (PLA), was used to compare with thermoplastics. The leachates were produced at two different temperatures to study the effect of temperature during leaching on microbial communities growing in plastic leachates. The information obtained will increase our current, limited understanding, on the interaction between plastic and marine microbes and how it could be affecting the marine environment, food-web and system.

2.2 SPECIFIC OBJECTIVES

1) To identify the bacterial groups growing in leachates from different types of plastics using the CARD-FISH technique.

2) To characterize the activity of the identified microbial communities using the BONCAT technique.
3) To study the effect of leachates from irradiated plastic on the structure and activity of microbial communities compared to non-previously irradiated plastic.
4) To study the effect of temperature during the plastic leaching on the structure and activity of microbial communities.

3. MATERIALS & METHODS

3.1 EXPERIMENTAL SETUP

Experiments involving 3 types of plastic exposed to different conditions were conducted. Plastics were provided by the *Goodfellow* company in a granular form, with sizes that varied from 3 to 5 mm: 3, 3.5 and 5 mm diameters for PLA, PS and LDPE, respectively. One experiment compared LDPE and PS at 28 °C (Exp_LDPE_PS), while the other compared LDPE and PLA at 15 °C (Exp_LDPE_PLA). Both experiments included 3 treatments (2 types of plastic and a control without plastic) under light and dark conditions. All treatments were performed in triplicates.

The artificial seawater (ASW) was prepared by diluting 45 grams of salt (Sigma-Aldrich Sea Salt) per 1 litre of MiliQ water, in order to obtain a salinity of 37 PSU. Afterwards, in order to sterilize the solution, the ASW was placed in an autoclave (Presoclave II 75 L) at 121 °C for 20 minutes and then left to cool down until the next day. In order to avoid contamination of the samples, before the experiment, all the glass-ware was cleaned with Acid (HCl:MiliQ) and MiliQ water and combusted at 450 °C for 5 hours. All the experiment preparation was performed in a sterilized fume hood to avoid bacterial contamination.

3.2 ABIOTIC STAGE: PHOTODEGRADATION EXPERIMENT

Plastic pieces (144 granules) were added with the help of tweezers to 300 mL of ASW in 500 mL quartz tubes, for light treatments, and in 500 mL borosilicate bottles covered with aluminium foil, for dark treatments. Controls without plastic contained only ASW (Figure 3.1A). Before the beginning of the experiment, plastics were rinsed 3 times by adding MiliQ water. The tubes and bottles were incubated in a bath connected to a continuous water flow which kept constant temperatures of 28 and 15 °C, for the Exp_LDPE_PS and the

Exp_LDPE_PLA, respectively. Samples were incubated during 6 days under UV and Visible light radiation (Figure 3.1B). Artificial photosynthetic active radiation (PAR) were provided by a HQI-T Powerstar lamp (250 W, Osram) and UV radiation by 2 Philips TL100W/10 R fluorescent tubes. Bacterial abundance was quantified by flow cytometry at the end of the incubation to ensure that bacterial growth did not occur during the process (section 3.4.1).

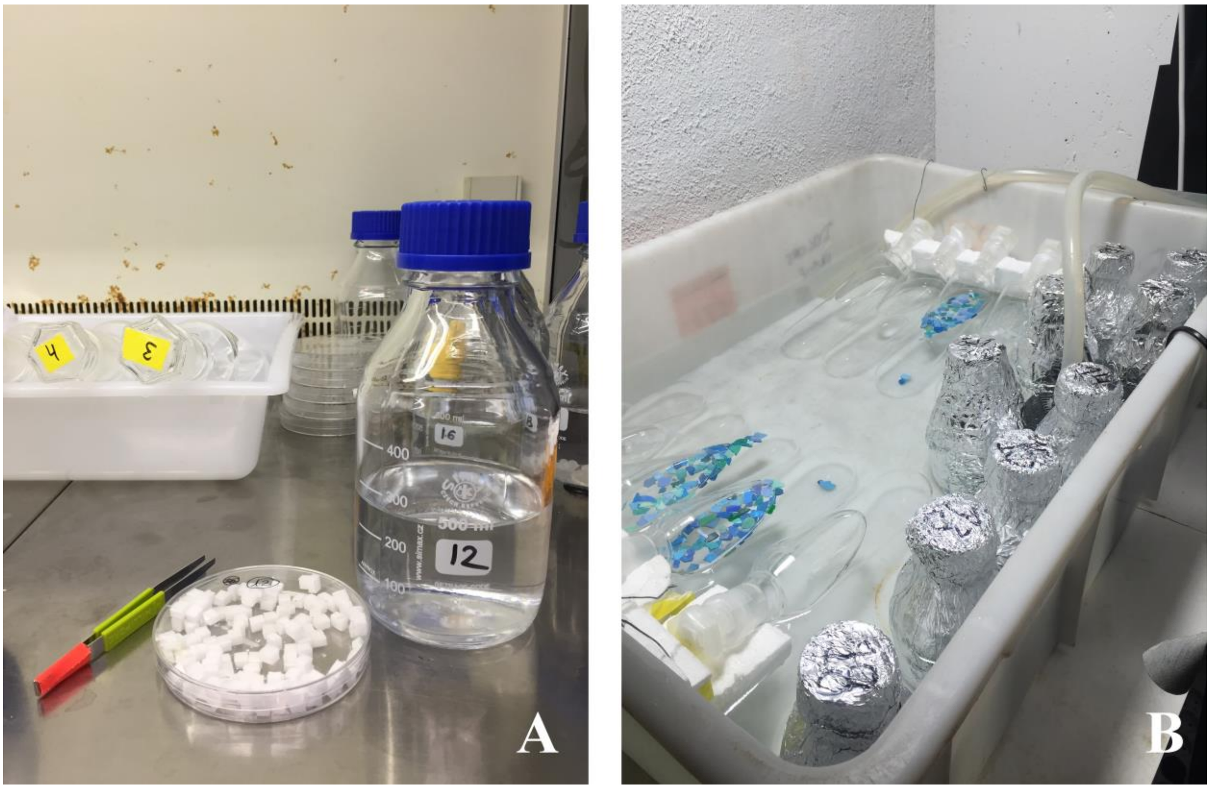

Figure 3.1. Plastic particles ready to be added to the ASW (A) and vials from the light and dark treatments incubated in a bath during the photodegradation experiment (B).

3.3 BIOTIC STAGE: BIODEGRADATION EXPERIMENT

Plastic leachates obtained in the previous photodegradation experiment were used after removing the plastic pieces. Leachates were inoculated with a natural bacterial inoculum from Blanes Bay Microbial Observatory (BBMO; NW Mediterranean; 41°40'13.5"N, 2°48'00.6"E) in a ratio 9:1 (leachate:inoculum), collected on the 14th of May and 11th of June 2019, for the Exp_LDPE_PS and Exp_LDPE_PLA experiments, respectively. Seawater inoculum was previously filtered through a 0.8 μm polycarbonate filter in order to avoid predators. In order to prevent nutrient limitation of bacterial growth, nitrogen and phosphorus were added to the samples, by adding NH_4Cl and NaH_2PO_4 to a final concentration of 10 and 2 μM, respectively. The samples were incubated in the dark at 22 ºC until bacteria stopped growing. Bacterial abundance was assessed every 24 hours through flow cytometry (section 3.4.1), in order to follow the growth curve of each sample until they reached the stationary phase. Samples for CARD-FISH and BONCAT (9 mL) were collected after 72 hours of incubation, at the end of the exponential phase (section 3.4.2).

3.4 BACTERIAL ANALYSIS

3.4.1 Bacterial abundance

Aliquots of 1.8 mL from each bottle were transferred to cryovials and fixed with 180 µL of a “P+G” mixture (10% formaldehyde and 0.5% glutaraldehyde), stored in the dark for 10 minutes at room temperature and frozen at – 80 °C until further analysis. Microbial DNA was stained with 2 µL of SyberGreen and 10 µL of fluorescent beads (Fluoresbrite carboxylate microspheres, 1 µm; Polysciences Inc., Warrington, PA) were added as a reference. Finally, bacteria were detected using a FACSCalibur flow cytometer (Becton Dickinson) relying on their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence) on the Paint-A-Gate software.

3.4.2 Bacterial identification and activity (CARD-FISH and BONCAT)

3.4.2.1 Cell incubation and fixation

From the biodegradation experiments, just before bacterial cells reached the stationary phase (after 72 hours of incubation), 9 mL of each treatment were collected. Samples were incubated by adding 45 µL of 200 µM L-Homopropargylglycine (HPG) to each tube (final concentration 1 µM) and left in the dark for 2.5 hours. Then, they were fixed by the addition of 1 mL of 0.2 µm-filtered paraformaldehyde 10% (PFA), with a final concentration of fixative of 1% [v/v] and left at 4 °C for 24 hours.

3.4.2.2 Cell filtration

The 10 mL solution (sample + PFA) was then filtered in a fume hood, with the help of a Millipore Vacuum Pump and a Carbon 14 Centralen filtration equipment. In order to improve the distribution of the cells, a 0.2 µm pore size polycarbonate filter was placed on top of a 0.8

µm cellulose acetate filter. Each sample (10 mL solution) was filtered in a cylinder. Then, the filters were rinsed with 5 mL of a 0.2 µm-filtered MiliQ water and left to dry on paper. Each filter was labelled and stored in a petri dish at – 80 °C until further processing. The cylinders were rinsed with MiliQ water between every sample.

3.4.2.3 Cell attachment and permeabilization of the filters

After thawing, the filters were dipped in a previously boiled 0.1% (w/w) agarose solution and placed on a glass plate covered on with parafilm in order to attach the cells to the filter and prevent cell loss during permeabilization and downstream procedures. Then, they were left to dry in a hybridization oven for 30 minutes and permeabilized with lysozyme and achromopeptidase. Thus, the filters were incubated at 37 °C for 1 hour in 10 mL of a fresh lysozyme solution (1 mL EDTA 0.5 M, 1 mL 1M Tris HCl, pH 8, 8 mL MiliQ, 100 mg lysozyme) and for 30 minutes in a fresh achromopeptidase solution, containing 10 mL of achromopeptidase buffer (100 µL NaCl 5 M, 500 µL 1M Tris HCl, pH 8, 50 mL MiliQ) and 20 µL of achromopeptidase. Finally, they were washed with MiliQ water and Ethanol 70%, left on paper to dry and stored at – 80 °C.

3.4.2.4 Hybridization

Before doing the hybridization, each filter was cut into 4 triangular slices (one for each probe) with an Albion blade, labelled with a pencil and stored at – 20 °C. The filter sections were hybridized with the following horseradish peroxidase (HRP)-labelled probes: GAM42a together with its unlabeled competitor probe, which targets most Gammaproteobacteria (Manz *et al.*, 1992), CF319a, which targets many members of the Bacteroidetes group (Manz *et al.*, 1996; Acinas *et al.*, 2015), EUB338 I-II and –III, which targets most Bacteria (Daims *et al.*, 1999) and Alf968, which targets Alphaproteobacteria (Neef, 1997). For the hybridization, the sections were added to an Eppendorf tube containing a mixture of 900 µl of Hybridization Buffer (HB; 3.6 mL NaCl 5M, 0.4 mL 1M Tris HCl, 50 µL SDS 10%, 3 mL water, 11 mL formamide, 2 mL blocking reagent) and the (HRP)-labelled oligonucleotide probe (0.16 ng µl^{-1} final concentration). Specific hybridization conditions were established by addition of formamide to the HBs (45% formamide for the Alf968 probe and 55% for the other probes). Hybridization was performed by leaving the Eppendorf tubes overnight in the oven at 35 °C. In addition, 4 Falcon tubes containing ~ 45 mL of MiliQ water were left overnight in the oven at 37 °C. The following day, in order to prepare the washing buffer (WB), 0.5 mL EDTA, 1 mL Tris HCl and 50 µl SDS (10%) were added to each of the overnight Falcon tubes and the ones corresponding to the 55% formamide received 30 µl NaCl and the one corresponding to 45% formamide received 160 µl NaCl. The tube was filled up with MiliQ water until 50 mL and left in the oven at 37 °C for 15 minutes. The filter sections were transferred to their respective WBs and placed in the oven at 37 °C for 15 minutes. The sections were removed from the tube with a Büchner funnel, transferred to a petri dish containing phosphate-buffered saline (PBS) 1X and left in the dark at room temperature for 15 minutes.

3.4.2.5 Catalyzed reporter deposition

A fresh stock of H_2O_2 was prepared by mixing 200 µl of PBS 1X with 1 µl 30% H_2O_2. The mixture was vortexed and 10 µl of it, combined with 4 µl of Alexa488, was transferred to the Eppendorf tube containing 1 mL of the amplification buffer (AB; 2 mL PBS 20X, 0.4 mL blocking reagent, 16 mL NaCl, MiliQ water until 40 mL, 4 g dextran sulfate). The sections were transferred to the Eppendorf tube and left in the oven at 46 °C for 15 minutes. Then, they were placed in a petri dish containing PBS 1X in the dark at room temperature for 15 minutes. At last, the sections were washed with PBS 1X and Ethanol 70%, left on paper to dry and stored at – 20 °C.

3.4.2.6 Cu-catalyzed click chemistry

Fresh stocks of sodium ascorbate (100 mM; Sigma-Aldrich) and aminoguanidine hydrochloride (100 mM; Sigma-Aldrich) were prepared: 99 mg of sodium ascorbate and 55 mg of aminoguanidine hydrochloride were separately added to 0.5 mL of PBS 1X. Then, each mixture was diluted in 1:10 for the working stock. For the click reaction, the dye premix was prepared by mixing 10 µL of copper sulfate ($CuSO_4.5H_2O$; 20 mM), 8 µL of Alexa594 and 20 µL of Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA; 50 mM) and leaving it to react at room temperature for 3 minutes in the dark. Meanwhile, a solution containing 1.77 mL of PBS 1X, 100 µL of the previously 1:10 diluted sodium ascorbate and aminoguanidine hydrochloride was prepared. Then, the filter sections were placed in an empty Eppendorf tube, the dye premix was added to the PBS-ascorbate-aminoguanidine mix, mixed gently by inverting the tube twice and carefully added to the Eppendorf containing the filter sections avoiding bubbles. Before closing the Eppendorf, Parafilm was placed between the top and the lid in order to minimize exposure to oxygenated conditions and avoid bubbles, so a reducing condition was maintained, which is necessary for the click reaction. The tube with the mix was left at room temperature for 30 minutes in the dark and, after the click reaction, the sections were washed three times for 3 minutes each in PBS-filled petri dishes and by incubating them for 1 hour in a 1:1 solution of PBS:Ethanol.

3.4.2.7 Visualization on the microscope

After the sections were air dried, they were stained with a solution containing 20 μL of 4',6-diamidino-2-phenylindole (DAPI) and 980 μL of oil mix (10 mL glycerol, 2 mL Vectashield, 1 mL PBS 20X), yielding a final concentration of 1 μg mL^{-1} of DAPI. The oil solution was spread on a microscope slide and the sections (6 per slide) and a coverslip were placed on top. The slides were, then, placed on the Zeiss Axio Imager Z2 Microscope (Figure 3.2A), which was set up to use UV Light (385 nm) for DAPI, blue light (470 nm) for Alexa488 (CARD-FISH) and red light (590 nm) for Alexa594 (BONCAT). The automatic cell count was conducted by using the AxionVision and ACMEtool softwares. The AxionVision software was set up to acquire the images by scanning each slide (Figure 3.2A) and 55 pictures (fields of view; FOV) were taken from each filter section (Figure 3.2B), with the exposure times shown below:

Table 3.1. Exposure time in milliseconds (ms) for each of the probes.

	GAM42a	**EUB(I-III)**	**CF319a**	**Alf968**
DAPI	100	100	100	100
CARD-FISH	50	50	150	50
BONCAT	120	120	120	120

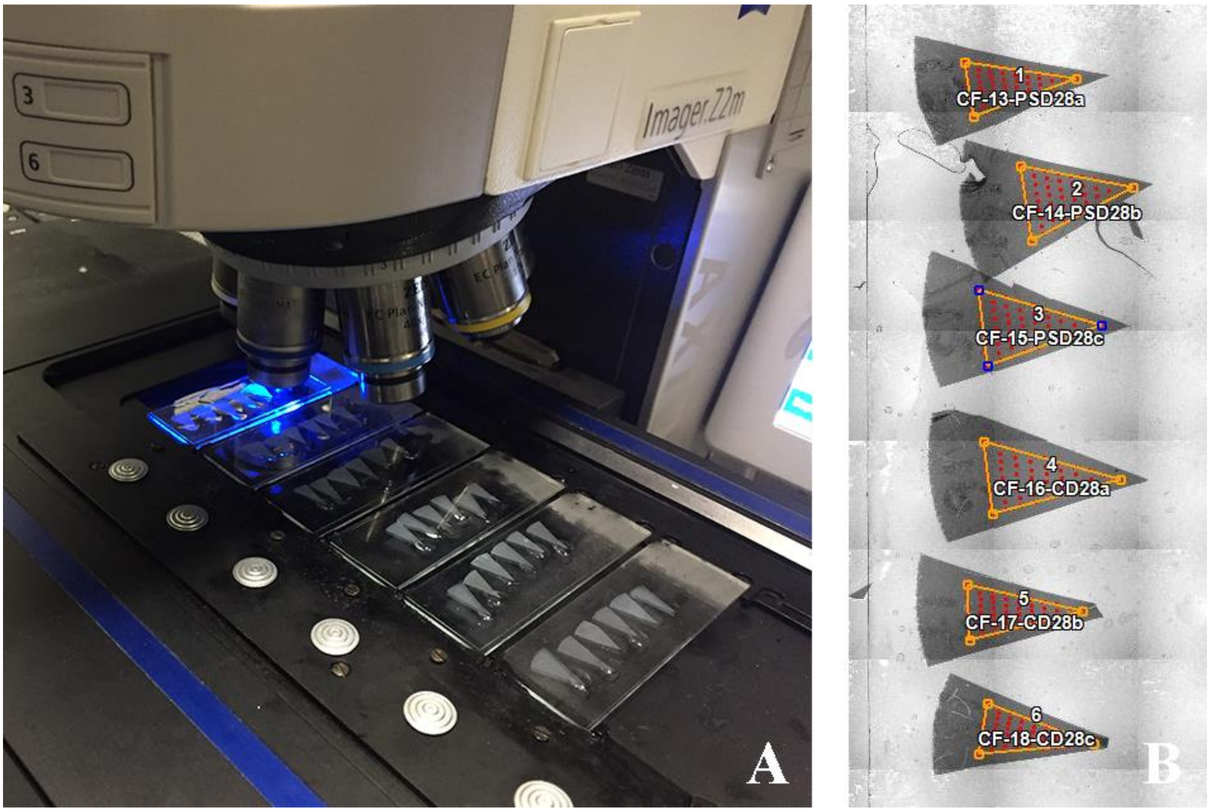

Figure 3.2. Zeiss Axio Imager Z2 Microscope taking pictures of the filter sections on the slides (A) and a scan of the filter sections on a slide (B), with each red dot representing a field of view (FOV) where a picture was taken.

The software was left to take the pictures overnight and, then, all the images were analysed on the ACMEtool (Automated Cell Measuring and Enumeration Tool) software. After all the pictures were imported to the software, the images that were out of focus or in low quality were manually removed, decreasing the number of FOVs of each filter section. The metadata file was created by using the channels below:

Table 3.2. Order of channels used to create the metadata file.

Reference image	Image processing methods	Colour
DAPI	DAPI	Light blue
BONCAT	DAPI	Red
CARD-FISH	DAPI	Green
DAPI2	DAPI	Dark Blue

Finally, all the BONCAT-positve (active), CARD-FISH (hybridized) and CARD-FISH active (hybridized bacteria that were labelled for BONCAT) bacteria were quantified and a sample report was created on Excel with all the cell counts.

3.5 DATA ANALYSIS

3.5.1 CARD-FISH and BONCAT cell counts

First, the analysis of the CARD-FISH counts was done based on the percentage of each CARD-FISH positive cell in relation to the total number of cells stained by DAPI. The contribution of each bacterial group to the activity was then calculated as the percentage of CARD-FISH positive cells that were also labelled with BONCAT in relation to the total number of BONCAT+ cells.

The CARD-FISH analysis included the Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes counts, "other bacteria" (estimated from the difference between the general probe for Eubacteria (EUB) and the sum of Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes counts), and the difference between 100% and the EUB counts ("other DAPI"). Given the low proportion of Archaea in surface Mediterranean waters (< 5%; Alonso-Sáez *et al.*, 2007), cells not detected by the EUB probe likely represent cells that did not have enough ribosomes to be detected by CARD-FISH. In addition, the BONCAT analysis also included the Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes counts and the difference between 100% and the sum of Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes counts, which represent the "other active bacteria" that were not targeted with the probes tested. Since the EUB probe targets most bacteria (Daims *et al.*, 1999), it was mainly used for the calculation of "other bacteria" in the samples. However, whenever the sum of Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes was greater than the EUB value, the sum of these probes was used instead of EUB.

Secondly, in order to obtain the contribution of each probe in cells/mL, the CARD-FISH and BONCAT results were recalculated taking into consideration the percentages obtained in the first calculation and the flow cytometry values.

3.5.2 BONCAT+ intensity assessment

Since the intensity of the BONCAT signal is proportional to the activity (Leizeaga *et al.*, 2017), this intensity was used to calculate the contribution of each phylogenetic group to the community activity. The intensity of the BONCAT+ cells was assessed using the mean gray value (MGV), which is the sum of the gray values of all the pixels in the cell divided by the number of pixels (Sebastián *et al.*, 2019). The most active cells were characterized as the brighter and whiter particles, while the least active ones were characterized as the darker and grayer ones.

In order to obtain the contribution of each group (Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes) to the total MGV, for each sample, the sum of each group's MGV was divided by the sum of the total MGVs. So, the percentage of contribution was calculated and the difference between 100% and the sum of the groups represented the "other bacteria" that could be contributing to the total MGV (i.e. activity). The total activity of a sample was obtained by summing all the MGVs obtained in that sample normalized by the number of FOVs taken. The average MGV of each sample was also calculated in order to obtain the average activity of the cells in each sample.

Finally, in order to assess the degree of intensity of the cells per treatment, the intensities of individual cells were rank-ordered to obtain the maximum and minimum values and the intensity range was then equally divided into three groups: high intensity (top third), intermediate intensity (middle third) and low intensity cells (bottom third).

3.6 STATISTICAL ANALYSIS

In order to check whether there were significant differences in the structure and activity of the microbial communities between treatments, a series of two-tailed Student's *t*-tests were applied to either the abundance or the activity of the bacterial phylogenetic groups to compare them regarding the effects of plastic leachates, light, temperature during leaching and the differences between plastics.

The calculation was conducted on Excel, by using the *t*-test formula. Since 3 replicates were used for each treatment, 2 degrees of freedom were used, $t(2) = 2.92$. Therefore, following the Student's *t*-test table, the absolute values greater than 2.92 indicated that there was a significant difference between the treatments.

4. RESULTS

4.1 BACTERIAL ABUNDANCE

In both experiments, bacteria started to grow after 24 hours and reached the exponential phase after 72 hours of incubation (Figure 4.1). At the end of both experiments, plastic samples presented a higher bacterial abundance than controls without plastics, except for irradiated PS.

Exp_LDPE_PS

Effect of plastic leachates. After 72 hours, coinciding with the sampling for CARD-FISH and BONCAT, LDPE treatments presented a significantly higher (*t*-test, $p < 0.05$) cell abundance than the controls in both light ($3.59\cdot10^{6}$ cells$\cdot$mL^{-1} ± $3.05\cdot10^{5}$) and dark ($2.97\cdot10^{6}$ cells$\cdot$mL^{-1} ± $1.56\cdot10^{4}$) treatments (Figure 4.1A). PS also presented a significantly higher (*t*-test, $p < 0.05$) bacterial abundance in the dark samples ($2.02\cdot10^{6}$ cells$\cdot$mL^{-1} ± $2.67\cdot10^{4}$) than in the dark control without plastic ($1.72\cdot10^{6}$ cells$\cdot$mL^{-1} ± $8.39\cdot10^{4}$). For the irradiated samples, PS showed lower bacterial abundance ($1.69\cdot10^{6}$ cells$\cdot$mL^{-1} ± $1.20\cdot10^{5}$; *t*-test, $p < 0.05$) than the control ($1.99\cdot10^{6}$ cells$\cdot$mL^{-1} ± $2.30\cdot10^{5}$; Figure 4.1B).

Effect of light. At 72 and 96 hours, both LDPE and control presented a higher bacterial abundance in the light treatments, while PS showed an opposite behavior, showing a higher abundance in the dark. However, the difference was only significant (*t*-test, $p < 0.05$) for LDPE at 72 hours and PS at 96 hours, while control showed a significant difference (*t*-test, $p < 0.05$) at both time points.

Differences between plastic types. At the end of the experiment, bacteria reached higher abundances in LDPE leachates, in both light and dark, than in the PS ones. However, the difference was not significant (*t*-test, $p > 0.05$) in the irradiated treatments after 72 hours of incubation, only at 96 hours (*t*-test, $p < 0.05$).

Exp_LDPE_PLA

Effect of plastic leachates. Both plastic treatments showed a significantly (*t*-test, $p < 0.05$) higher bacterial abundance than the controls without plastic for irradiated and dark samples at the end of the experiment. At 72 hours, LDPE reached values of $3.52\cdot10^{6}$ cells$\cdot$mL^{-1} ± $1.87\cdot10^{5}$ and $3.26\cdot10^{6}$ cells$\cdot$mL^{-1} ± 0.00, for both light and dark, respectively (Figure 4.1C). While the control treatments were $2.04\cdot10^{6}$ cells$\cdot$mL^{-1} ± $8.76\cdot10^{4}$ for the irradiated samples and $1.96\cdot10^{6}$ cells$\cdot$mL^{-1} ± $8.71\cdot10^{4}$ for the dark ones. PLA reached a bacterial abundance of $3.01\cdot10^{6}$ cells$\cdot$mL^{-1} ± $2.62\cdot10^{5}$ and $3.22\cdot10^{6}$ cells$\cdot$mL^{-1} ± $3.88\cdot10^{5}$ in light and dark treatments, respectively, after 72 hours of growth (Figure 4.1D).

Effect of light. At 72 and 96 hours, both LDPE and control presented a higher bacterial abundance in the light treatments, while PLA showed an opposite behavior, showing a higher abundance in the dark. However, the difference was only significant (*t*-test, $p < 0.05$) for LDPE at 96 hours.

***Differences between plastic types*.** Bacteria also reached higher abundance in LDPE leachates regarding PLA ones, in both light and dark, but the difference was only significant in the light at 96 hours (*t*-test, $p < 0.05$).

Effect of temperature during leaching

When comparing the temperatures from the photodegradation stage, LDPE leachates and control without plastics in the dark presented a significantly (*t*-test, $p < 0.05$) lower bacterial abundance in the Exp_LDPE_PS than in the Exp_LDPE_PLA, at both 72 and 96 hours. Irradiated LDPE and control did not present a significant difference (*t*-test, $p > 0.05$) between temperatures at both 72 and 96 hours.

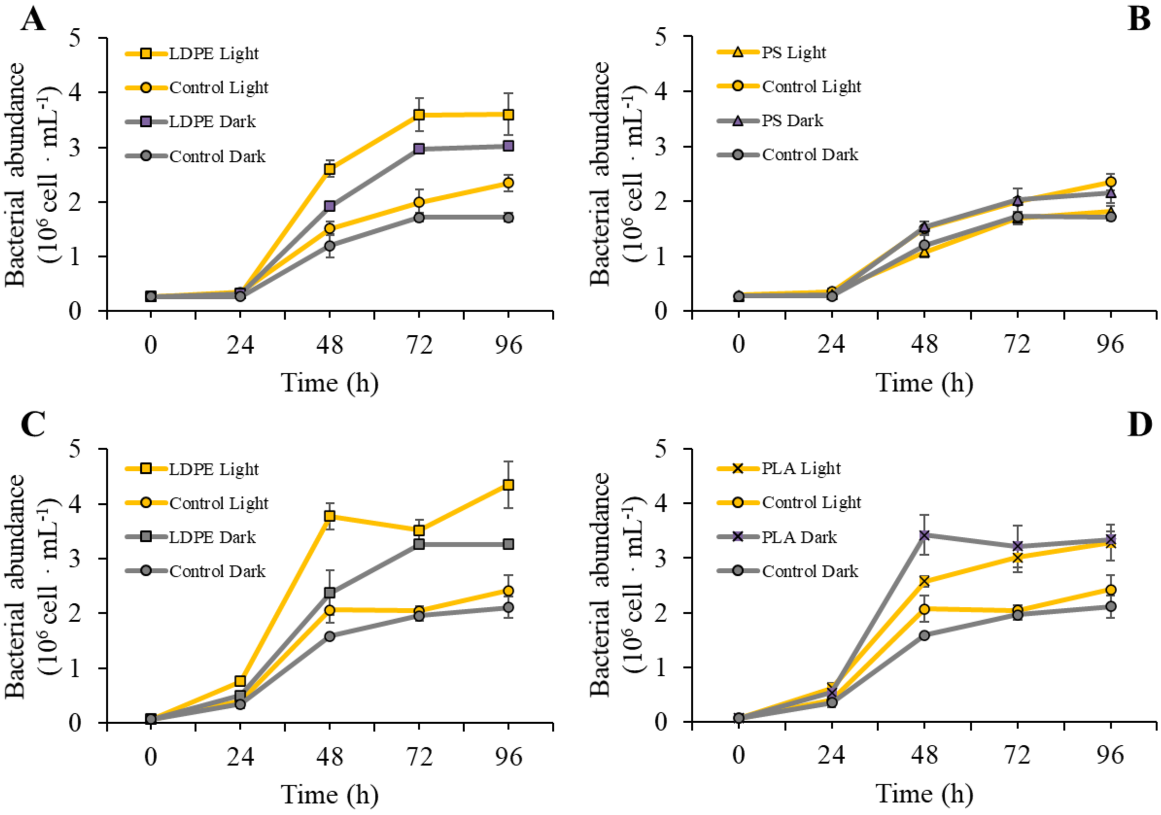

Figure 4.1. Bacterial growth during 96 hours of incubation in (A) LDPE and Control in the Exp_LDPE_PS, (B) PS and Control in the Exp_LDPE_PS, (C) LDPE and Control in the Exp_LDPE_PLA and (D) PLA and Control in the Exp_LDPE_PLA.

4.2 CARD-FISH AND BONCAT CELL COUNTS

All plastic types leachates regardless temperature and light conditions during the photodegradation stage led to a similar microbial community composition: they were mainly composed by Gamma-, Alphaproteobacteria and Bacteroidetes. Both experiments presented similar contribution of each phylogenetic group to the total abundance: Gamma- and Alphaproteobacteria showed to be the greatest contributors, while Bacteroidetes was the least abundant group. “Other DAPI” and “other bacteria” were not present in any of the treatments in both experiments.

Exp_LDPE_PS

Effect of plastic leachates. Gamma- and Alphaproteobacteria cell abundance in plastic leachates was significantly higher (*t*-test, $p < 0.05$) than the controls without plastic for both irradiated and dark treatments. Only in irradiated PS samples, Alphaproteobacteria was not significantly different (*t*-test, $p > 0.05$) than the light control without plastic. In irradiated LDPE samples, Gamma- and Alphaproteobacteria cell abundances reached $2.33 \cdot 10^6$ cells·mL^{-1} ± $1.99 \cdot 10^5$ and $1.57 \cdot 10^6$ cells·mL^{-1} ± $9.28 \cdot 10^3$, respectively. On the other hand, both phylogenetic groups presented similar values in light PS ($1.21 \cdot 10^6$ cells·mL^{-1} ± $1.58 \cdot 10^4$ and $1.04 \cdot 10^6$ cells·mL^{-1} ± $1.41 \cdot 10^4$) and control treatments ($1.08 \cdot 10^6$ cells·mL^{-1} ± $2.97 \cdot 10^4$ and $1.19 \cdot 10^6$ cells·mL^{-1} ± $2.10 \cdot 10^5$). Alphaproteobacteria in the dark plastic treatments ranged between $1.11 \cdot 10^6$ cells·mL^{-1} ± $1.03 \cdot 10^5$ (PS) and $1.34 \cdot 10^6$ cells·mL^{-1} ± $1.43 \cdot 10^4$ (LDPE), being higher than the dark control ($6.82 \cdot 10^5$ cells·mL^{-1} ± $1.02 \cdot 10^5$; *t*-test, $p < 0.05$). In dark LDPE and PS leachates, Gammaproteobacteria reached $1.84 \cdot 10^6$ cells·mL^{-1} ± 14.0 and $1.15 \cdot 10^6$ cells·mL^{-1} ± $3.74 \cdot 10^4$, respectively (Figure 4.2A), which were also higher than the dark control ($8.88 \cdot 10^5$ cells·mL^{-1} ± $3.34 \cdot 10^4$; *t*-test, $p < 0.05$).

Bacteroidetes cell abundance growing in irradiated LDPE leachates ($6.30 \cdot 10^3$ cells·mL^{-1} ± $7.02 \cdot 10^3$) was significantly lower (*t*-test, $p < 0.05$) than in the control without plastic ($2.56 \cdot 10^4$ cells·mL^{-1} ± $6.24 \cdot 10^3$). This bacterial group showed the highest values in the irradiated PS leachates ($3.62 \cdot 10^4$ cells·mL^{-1} ± $1.38 \cdot 10^3$). Bacteroidetes did not show significant differences (*t*-test, $p > 0.05$) in the dark between plastic and no plastic treatments, with values ranging between $1.51 \cdot 10^4$ cells·mL^{-1} ± $2.17 \cdot 10^2$, for PS, and $1.73 \cdot 10^4$ cells·mL^{-1} ± $1.23 \cdot 10^3$, for LDPE (Figure 4.2B).

Effect of light. Gamma- and Alphaproteobacteria growing in LDPE leachates reached higher values (*t*-test, $p < 0.05$) in the irradiated samples than in the dark. The same trend was observed in the control without plastics (*t*-test, $p < 0.05$) but with smaller differences between light and dark than those shown by the LDPE samples. However, Gamma- and Alphaproteobacteria cell abundances in PS leachates was similar (*t*-test, $p > 0.05$) independently if the samples were previously irradiated or not (Figure 4.2A). Bacteroidetes abundance did not show any significant difference between the irradiated treatments and their corresponding dark samples (*t*-test, $p > 0.05$), except for PS leachates, which showed a higher abundance after irradiation (*t*-test, $p < 0.05$; Figure 4.2B).

Differences between plastic types. In general, Gamma- and Alphaproteobacteria grew more in LDPE than in PS leachates (*t*-test, $p < 0.05$) but the differences between them were smaller in the dark (*t*-test, $p < 0.05$; Figure 4.2A). On the other hand, Bacteroidetes grew more in PS than in LDPE light leachates (*t*-test, $p < 0.05$), while LDPE showed a higher abundance than PS in the dark (*t*-test, $p < 0.05$; Figure 4.2B).

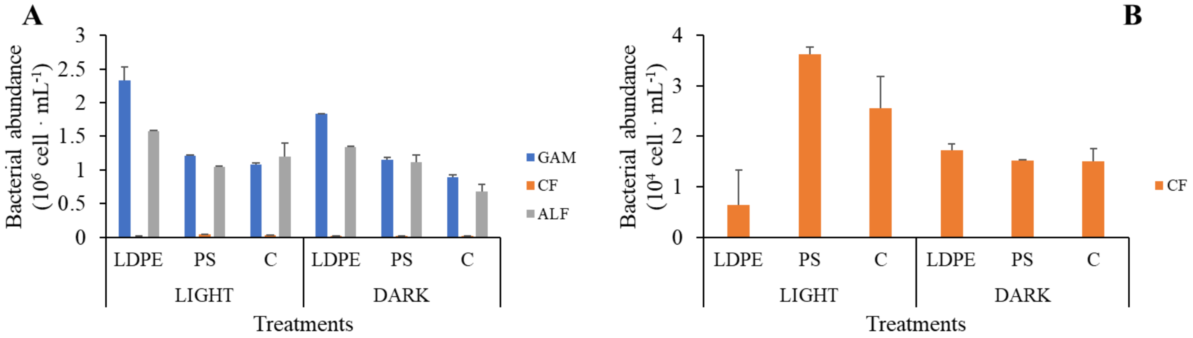

Figure 4.2. Bacterial abundance of the different CARD-FISH targeted bacterial groups (cell · mL-1; ± standard deviation) per treatment in the Exp_LDPE_PS. GAM: Gammaproteobacteria, CF: Bacteroidetes, ALF: Alphaproteobacteria. Panel B displays the CF+ counts, note the change in the Y-axis scale.

Exp_LDPE_PLA

Effect of plastic leachates. In irradiated LDPE and PLA samples, Gamma- and Alphaproteobacteria cell abundances presented similar values, reaching $2.48\cdot10^6$ cells·mL^{-1} ± $6.66\cdot10^4$ and $2.46\cdot10^6$ cells·mL^{-1} ± $2.70\cdot10^4$ in LDPE and $1.77\cdot10^6$ cells·mL^{-1} ± $4.72\cdot10^4$ and $1.73\cdot10^6$ cells·mL^{-1} ± $2.58\cdot10^5$ in PLA, respectively. In irradiated treatments, Gamma- and Alphaproteobacteria cell abundances were significantly higher (*t*-test, $p < 0.05$) in the plastic treatments than in the controls without plastics ($1.29\cdot10^6$ cells·mL^{-1} ± $2.66\cdot10^4$ and $1.40\cdot10^6$ cells·mL^{-1} ± $4.50\cdot10^4$, respectively). Only Alphaproteobacteria did not show significant differences (*t*-test, $p > 0.05$) between PLA and control without plastic after irradiation. Alphaproteobacteria in the dark plastic treatments ranged between $1.21\cdot10^6$ cells·mL^{-1} ± $1.19\cdot10^5$, for PLA, and $1.56\cdot10^6$ cells·mL^{-1} ± $1.85\cdot10^5$, for LDPE, with only LDPE cell abundance being significantly higher (*t*-test, $p < 0.05$) than the dark control ($1.02 \cdot 10^6$ cells·mL^{-1} ± $2.03 \cdot 10^4$). In dark LDPE and PLA leachates, Gammaproteobacteria reached $1.39 \cdot 10^6$ cells·mL^{-1} ± $1.17 \cdot 10^5$ and $2.50 \cdot 10^6$ cells·mL^{-1} ± $8.91 \cdot 10^4$, respectively, with only PLA abundance being significantly higher (*t*-test, $p < 0.05$) than the dark control ($1.23 \cdot 10^6$ cells·mL^{-1} ± $1.72 \cdot 10^4$; Figure 4.3A).

Bacteroidetes cell abundance growing in plastic leachates was significantly higher (*t*-test, $p < 0.05$) in the irradiated PLA ($5.70 \cdot 10^4$ cells·mL^{-1} ± $2.07 \cdot 10^4$) than in the control without plastic ($1.21 \cdot 10^4$ cells·mL^{-1} ± $2.60 \cdot 10^3$), while irradiated LDPE ($1.45 \cdot 10^4$ cells·mL^{-1} ± $9.08 \cdot 10^3$) showed a similar value to the control (*t*-test, $p > 0.05$). In the dark, Bacteroidetes was significantly higher (*t*-test, $p < 0.05$) in the plastic treatments than in the control ($9.69 \cdot 10^3$ cells·mL^{-1} ± $3.18 \cdot 10^3$), with values ranging between $2.67 \cdot 10^4$ cells·mL^{-1} ± $2.03 \cdot 10^3$ in LDPE and $3.11 \cdot 10^4$ cells·mL^{-1} ± $8.97 \cdot 10^2$ in PLA (Figure 4.3B).

Effect of light. Gamma- and Alphaproteobacteria growing in LDPE leachates reached higher values (*t*-test, $p < 0.05$) in the irradiated samples than in the dark. The same trend was observed in the control without plastics (*t*-test, $p < 0.05$), but with smaller differences between light and dark than those shown by the LDPE samples. However, Gamma- and Alphaproteobacteria cell abundances in PLA leachates presented opposite behaviors, with Gammaproteobacteria cell abundance being greater in the dark (*t*-test, $p < 0.05$) and Alphaproteobacteria abundance being greater in the light (*t*-test, $p < 0.05$; Figure 4.3A). On the other hand, Bacteroidetes abundance did not show a significant difference (*t*-test, $p > 0.05$) between the light and dark for any of the treatments (Figure 4.3B).

Differences between plastic types. In general, Gamma- and Alphaproteobacteria grew more (*t*-test, $p < 0.05$) in LDPE than in PLA leachates, except for Alphaproteobacteria in the dark (*t*-test, $p > 0.05$). Gammaproteobacteria, however, was significantly more abundant (*t*-test, $p < 0.05$) in PLA than in LDPE leachates in the non-irradiated treatments (Figure 4.3A). On the other hand, Bacteroidetes grew more in PLA than in LDPE leachates (*t*-test, $p < 0.05$) in both irradiated and dark treatments (Figure 4.3B).

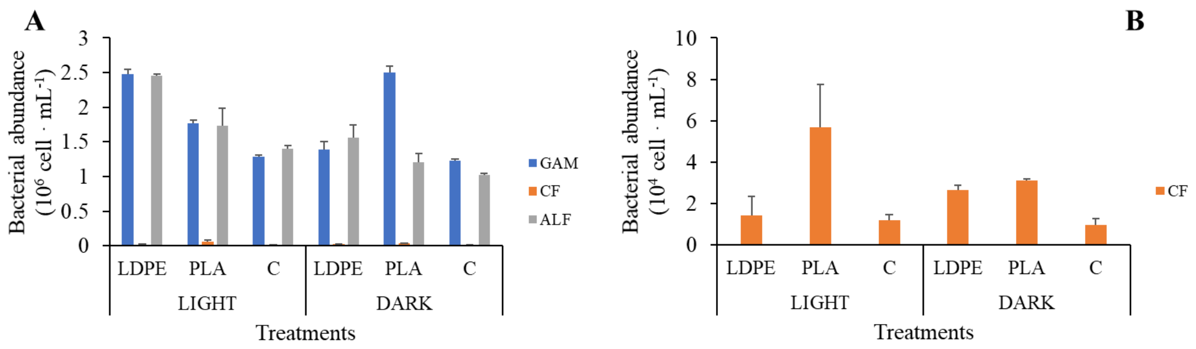

Figure 4.3. Bacterial abundance of the different CARD-FISH targeted bacterial groups (cell · mL-1; ± standard deviation) per treatment in the Exp_LDPE_PLA. GAM: Gammaproteobacteria, CF: Bacteroidetes, ALF: Alphaproteobacteria. Panel B displays the CF+ counts, note the change in the Y-axis scale.

Effect of temperature during leaching

When comparing the temperatures from the photodegradation stage, the phylogenetic groups from irradiated LDPE showed to be more abundant in the Exp_LDPE_PLA, but only Alphaproteobacteria showed a significant difference between temperatures (*t*-test, $p < 0.05$). Alphaproteobacteria and Bacteroidetes in non-irradiated LDPE were also more abundant in the Exp_LDPE_PLA, with only Bacteroidetes showing a significant difference (*t*-test, $p < 0.05$). Gammaproteobacteria, however, was significantly more abundant (*t*-test, $p < 0.05$) in the Exp_LDPE_PS.

On the other hand, in the controls without plastics, Gammaproteobacteria was more abundant in the Exp_LDPE_PLA in both light and dark (*t*-test, $p < 0.05$). Alphaproteobacteria showed the same trend, however, it only showed a significant difference for the dark control (*t*-test, $p < 0.05$). Conversely, Bacteroidetes showed to be less abundant in the Exp_LDPE_PLA control treatments, only presenting a significantly lower abundance for the irradiated control (*t*-test, $p < 0.05$).

4.3 BONCAT+ INTENSITY ASSESSMENT

Exp_LDPE_PS

Effect of plastic leachates. The total mean cell activity in plastic leachates was significantly higher (*t*-test, $p < 0.05$) than the control without plastics (100.70 MGV ± 3.61) for the irradiated treatments, with LDPE reaching 119.58 MGV ± 2.30 and PS reaching 118.58 MGV ± 2.26. Non-irradiated treatments followed the same trend, with LDPE cells (77.48 MGV ± 0.10) and PS cells (89.83 MGV ± 0.46) being more active than the control (76.29 MGV ±

2.75), although only PS activity was significantly different (*t*-test, $p < 0.05$) than the dark control (Figure 4.4A).

In irradiated treatments, the mean cell activities of all phylogenetic groups in both LDPE and PS were significantly higher (*t*-test, $p < 0.05$) than the control without plastics. Only Gamma- and Alphaproteobacteria from LDPE did not show significant differences (*t*-test, $p > 0.05$) regarding the control without plastic. In the dark, bacterial groups from all plastic treatments did not show a significant difference (*t*-test, $p > 0.05$) regarding the controls, except for Alphaproteobacteria in PS leachates (118.49 MGV ± 11.76), that presented a significantly higher activity (*t*-test, $p < 0.05$) than the control without plastics (93.80 MGV ± 5.07; Figure 4.4B).

Regarding the distribution of the intensities, communities grown in both irradiated LDPE and PS leachates contained more high activity and less low activity (*t*-test, $p < 0.05$) bacteria than the control in the light. LDPE leachates presented the greatest percentage of high activity cells (21.63% ± 2.54), while PS reached intermediate values and control was mostly dominated by low intensity cells (67.45% ± 1.94). In the dark, however, all the treatments showed a similar behavior and were dominated by low activity bacteria (75-80%; *t*-test, $p > 0.05$; Figure 4.6A).

Effect of light. All light treatments showed a significantly higher mean cell activity than the dark ones (*t*-test, $p < 0.05$) for all the plastic treatments (Figure 4.4A) and phylogenetic groups (Figure 4.4B). For the control without plastics the differences were smaller (*t*-test, $p < 0.05$) between light and dark than those shown by the LDPE and PS samples. Regarding the distribution of the intensities, all treatments showed a higher percentage (*t*-test, $p < 0.05$) of low activity bacteria in the non-irradiated treatments compared to their irradiated counterparts, with LDPE and PS leachates going from less than 50% of the community to almost 80% in dark treatments (Figure 4.6A).

Differences between plastic types. Although the plastic treatments in the light showed very similar total mean cell activities (*t*-test, $p > 0.05$) among them, PS total cell activity was significantly higher than LDPE only in the dark (*t*-test, $p < 0.05$; Figure 4.4A). In the light treatments, both Gamma- and Alphaproteobacteria cells were more active in PS leachates (*t*-test, $p < 0.05$). On the other hand, Bacteroidetes showed an opposite behavior, being more active in LDPE leachates (*t*-test, $p < 0.05$). In the non-irradiated treatments, all phylogenetic groups followed the same trend, with cells showing a significantly higher activity (*t*-test, $p < 0.05$) in PS than in LDPE, except for Bacteroidetes (*t*-test, $p > 0.05$; 4.4B). Regarding the distribution of the intensities, although PS showed to have less (*t*-test, $p < 0.05$) high activity bacteria than LDPE in the light, in the dark their compositions were almost identical (Figure 4.6A).

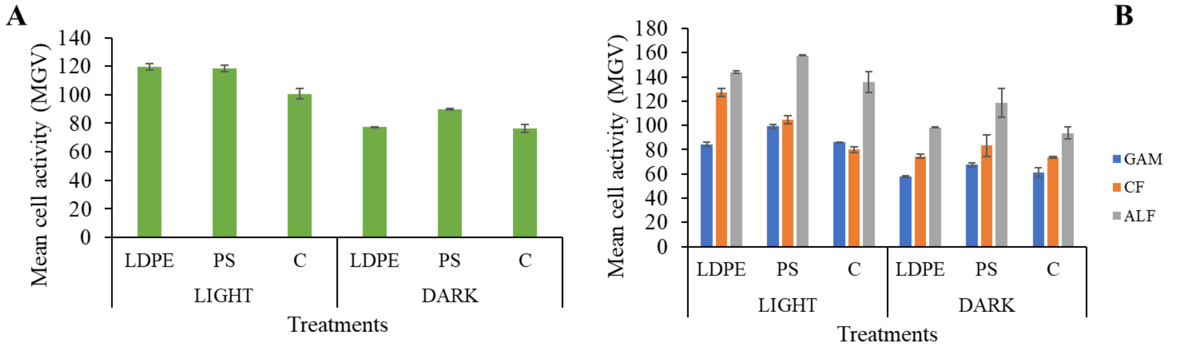

Figure 4.4. Mean cell activity (MGV; ± standard deviation) (A) per treatment (total) and (B) per each phylogenetic group in the Exp_LDPE_PS.

Exp_LDPE_PLA

Effect of plastic leachates. The total mean cell activity in plastic leachates was significantly lower (*t*-test, $p < 0.05$) than the control without plastic (140.53 MGV ± 1.81) for the irradiated treatments, with LDPE reaching 132.04 MGV ± 2.99 and PLA reaching 106.62 ± 0.11 MGV. Although in the dark LDPE mean activity (103.50 MGV ± 5.22) presented a higher value than the control (95.01 MGV ± 2.08) and PLA presented a similar value (95.15 MGV ± 1.28) to the control, the mean cell activities from both plastics were not significantly different than the control in the non-irradiated treatments (*t*-test, $p > 0.05$; Figure 4.5A).

In irradiated treatments, the mean cell activities of all phylogenetic groups in both LDPE and PS were significantly different (*t*-test, $p < 0.05$) than the control without plastics. Bacterial groups belonging to both light plastic treatments presented a lower mean cell activity than the light control (*t*-test, $p < 0.05$). Only Alphaproteobacteria from irradiated LDPE showed a higher cell activity regarding the control (*t*-test, $p < 0.05$). On the other hand, the non-irradiated plastic treatments did not show any significant difference regarding the dark control (*t*-test, $p > 0.05$). Only Alphaproteobacteria in dark LDPE (122.80 MGV ± 1.35) showed to be significantly more active (*t*-test, $p < 0.05$) than the non-irradiated control without plastics (101.97 MGV ± 7.40; Figure 4.5B).

Regarding the distribution of the intensities, the microbial community in the control contained more high activity bacteria (40.47% ± 2.27) than both LDPE (32.35% ± 5.25) and PLA (16.24 ± 1.15) in the light, showing a significant difference (*t*-test, $p < 0.05$) only regarding PLA. PLA presented the highest value for low activity bacteria (50.43% ± 4.48) in the irradiated treatments. In the dark, however, all the treatments showed a similar behavior and were dominated by low activity bacteria (60-70%; Figure 4.6B).

Effect of light. All light treatments showed a significantly higher total mean cell activity than the dark ones (*t*-test, $p < 0.05$; Figure 4.5A). Gamma- and Alphaproteobacteria growing in LDPE and PLA leachates reached significantly higher mean cell activities (*t*-test, $p < 0.05$) in the irradiated treatments than in the dark, except for Gammaproteobacteria in PLA (*t*-test, $p > 0.05$). Furthermore, Bacteroidetes also presented a significantly higher cell activity (*t*-test, $p < 0.05$) in the irradiated treatments than in the dark ones. Irradiated control without plastics followed the same trend, presenting a higher activity (*t*-test, $p < 0.05$) in the light than in the dark for all the bacterial groups (Figure 4.5B). Regarding the distribution of the intensities, all treatments showed a higher (*t*-test, $p < 0.05$) percentage of low activity bacteria in the non-irradiated treatments, with LDPE and PLA going from up to 50% of the community to up to 70% (Figure 4.6B).

Differences between plastic types. At last, LDPE cell activity showed to be higher than PLA activity in both light and dark, with LDPE being significantly different than PLA only in the irradiated treatments (*t*-test, $p < 0.05$; Figure 4.5A). The phylogenetic groups in LDPE leachates were significantly more active (*t*-test, $p < 0.05$) than in PLA in both light and dark treatments. Only the non-irradiated plastic treatments showed no significant difference regarding the Gammaproteobacteria mean cell activity (*t*-test, $p > 0.05$; Figure 4.5B). Regarding the distribution of the intensities, LDPE showed to have more (*t*-test, $p < 0.05$) high activity bacteria than PLA in both light and dark (Figure 4.6B).

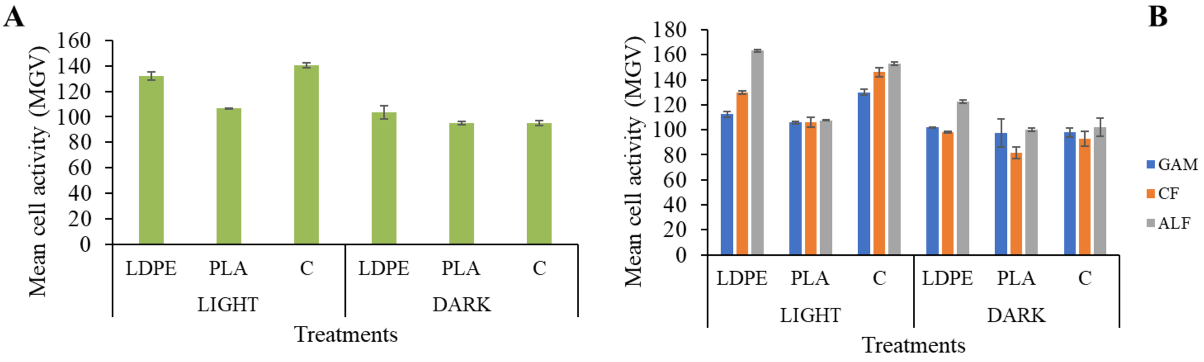

Figure 4.5. Mean cell activity (MGV; ± standard deviation) (A) per treatment (total) and (B) per each phylogenetic group in the Exp_LDPE_PLA.

Effect of temperature during leaching

When comparing the temperatures from the photodegradation stage, the total mean cell activity in LDPE leachates obtained in the Exp_LDPE_PLA was significantly higher (*t*-test, $p < 0.05$) in both light and dark, than that in leachates incubated in the Exp_LDPE_PS, reaching its greatest mean cell activity in the light in the Exp_LDPE_PLA (132.04 MGV ± 2.99). The control without plastics followed the same trend between the temperatures, with greater values (*t*-test, $p < 0.05$) in the leachates from the Exp_LDPE_PLA.

The mean cell activities of all phylogenetic groups were significantly higher (*t*-test, $p < 0.05$) in the Exp_LDPE_PLA for all treatments when compared to the Exp_LDPE_PS treatments. Only Bacteroidetes in irradiated LDPE and Alphaproteaobacteria in non-irradiated control did not show a significant difference (*t*-test, $p > 0.05$) regarding the different temperatures from the photodegradation stage.

Regarding the distribution of the intensities, Exp_LDPE_PLA presented a significantly higher (*t*-test, $p < 0.05$) percentage of high activity cells in both LDPE and control treatments.

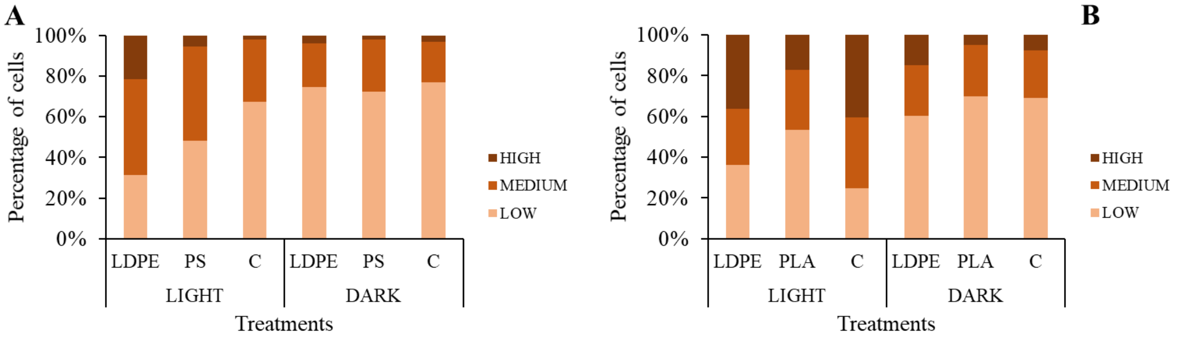

Figure 4.6. Percentage of cells with low, medium and high activity per treatment in (A) Exp_LDPE_PS and (B) Exp_LDPE_PLA.

5. DISCUSSION

This study has characterized the microbial community composition, including its abundance and activity, in leachates from different plastic types under irradiated and dark conditions. The microbial community was composed of Gamma-, Alphaproteobacteria and Bacteroidetes in all treatments. In general, plastic leachates stimulated the growth of Gamma- and Alphaproteobacteria regarding the controls without plastics. However, the impact on Bacteroidetes was more variable. Irradiation during plastic leaching had contrasting results on the bacterial abundance which depended on the plastic type and the phylogenetic group.

However, plastic leachates previously irradiated, as those found in the ocean, stimulated the synthesis of proteins in marine bacteria regarding those not previously exposed to radiation.

In this work, bacterial abundance ranged between 1.5 and $4 \cdot 10^6$ cells·mL^{-1} at 72 hours, which falls within the range observed in previous studies with similar experiments (Romera-Castillo *et al.*, 2018; Zhu *et al.*, 2020). Gamma-, Alphaproteobacteria and Bacteroidetes dominated the bacterial groups growing not only in the plastic leachates, but also in the controls without them. These are also the most abundant bacterial groups found in Blanes Bay Observatory (NW Mediterranean) waters, where the inoculum was collected from (Alonso-Sáez *et al.*, 2007; Castillo, 2017). Although all the experiments involved different conditions and types of plastic, they achieved similar community compositions. Gammaproteobacteria and Alphaproteobacteria were not only the most abundant, but also the main contributors to the total activity (total BONCAT+ cells) in all the treatments. Bacterial community growing in the leachates followed the same pattern as the plastic biofilm formation, with the bacterial colonization being dominated by Gamma- and Alphaproteobacteria (Oberbeckmann *et al.*, 2015). Although Bacteroidetes are known for having the capacity of degrading polymers (Fernández-Gómez *et al.*, 2013), they were the least abundant type of bacteria in all treatments. On the other hand, if they also follow the biofilm behaviour, as the Gamma- and Alphaproteobacteria, they could become more abundant with time (Lee *et al.*, 2008).

In general, plastic leachates stimulated the growth of Gamma- and Alphaproteobacteria in the plastic treatments regarding the control without plastics in both irradiated and non-irradiated samples with some exceptions in the dark ones from the Exp_LDPE_PLA. Conversely, Bacteroidetes followed the same trend in the Exp_LDPE_PLA and showed an opposite or no difference in the Exp_LDPE_PS. The fact that most phylogenetic groups presented a greater abundance in the plastic leachates than in the controls without plastics indicate that the presence of plastic leachates positively affected the microbial growth. This agrees with previous studies (Romera-Castillo *et al.*, 2018; Zhu *et al.*, 2020) which found that plastic leachates were releasing an extra DOC which stimulated bacterial growth. In addition, most groups belonging to the irradiated plastic treatments in the Exp_LDPE_PS showed a significantly higher mean cell activity than the controls, while in the Exp_LDPE_PLA, they showed an opposite behavior. However, at both experiments, most bacterial groups from the non-irradiated plastic treatments did not show any difference in the mean cell activity regarding the control. This means that light could be affecting the dissolved organic matter (DOM) leached from plastics, leading to a bacterial growth inhibition or stimulation. For instance, it is known that UV radiation can transform (photodegrade) the organic matter and make it more labile (Lønborg *et al.*, 2020) which will stimulate microbial growth.

The effect of the irradiation during plastic leaching on the microbial abundance changed depending on the plastic type used. Total bacterial abundance was higher in the irradiated LDPE than in the dark controls. This contrasts with a previous experiment where no differences were found in total bacterial abundance between previously irradiated LDPE plastic treatments and their dark controls (Romera-Castillo *et al.*, 2018). Moreover, in such study, some dark treatments presented higher bacterial abundance than those previously irradiated. The differences could be due to the different LDPE used. It is known that plastic present additives and they change from one plastic to another. The LDPE used here, in pellets, probably have different additives than the one used by Romera-Castillo *et al.* (2018) which was in form of film. In our experiment, irradiation during LDPE leaching stimulated the bacterial abundance of Gamma- and Alphaproteobacteria but did not affect Bacteroidetes. In PS leachates, instead, no difference between the irradiated and non-irradiated treatments were observed in Gamma- and Alphaproteobacteria abundances, meaning that PS irradiation had no effect on those two phylogenetic groups. However, Bacteroidetes was favored by the irradiation of PS during leaching. PLA leachates showed contrasting results depending on the phylogenetic group with higher Gammaproteobacteria in the dark and higher Alphaproteobacteria in the light and no effect on Bacteroidetes.

These results show that the effect of radiation during plastic leaching have contrasting results on the bacterial abundance, which depend on the plastic type and phylogenetic group. Although the UV exposure of the plastic treatments can lead to an increase of the leaching, releasing more DOC and increasing the stimulation of the bacterial activity, photodegradation can also produce free radicals (Gewert *et al.*, 2015), potentially inhibiting bacterial growth (Anesio *et al.*, 2005). Thus, although DOC released from most plastics are readily utilized by marine bacteria, they can also release organics or co-leachates that inhibit microbial growth, making leachates formed during plastic photodegradation have mixed impacts on marine microbes (Zhu *et al.*, 2020). Interestingly, although the phylogenetic groups presented contrasting results in the microbial community abundances regarding the light and dark, all treatments in both experiments presented a significantly higher mean cell activity in the light. Non-irradiated plastics not only presented very similar abundances and community compositions as the control in both experiments, but also a lower mean cell activity than the light treatments and a dominance of low activity bacteria. This means that plastic leachates previously irradiated, as those found in the ocean, stimulated marine bacterial abundance as well as their synthesis of proteins.

Although Bacteroidetes was the least abundant phylogenetic group in all the treatments, it showed an intermediate mean cell activity regarding the other groups, most times even higher than the Gammaproteobacteria. Gammaproteobacteria, however, although shown to be very abundant, it was the least active (mean cell activity) phylogenetic group. Overall, Alphaproteobacteria was abundant and more active (mean cell activity) than the other groups. Bacteroidetes are known for being major utilizers of high-molecular-weight DOM in marine ecosystems (Cottrell & Kirchman, 2000) and plastic leachates are mainly composed by low-molecular-weight DOM (Lee *et al.*, 2020), which could explain their lower abundance in all treatments. However, the possibility of Bacteroidetes being the least abundant bacterial group from the beginning, in the inoculum, should also not be discarded.

The mixed impacts of different substrates on different phylogenetic groups are also shown in Alonso-Sáez & Gasol (2007), which analyzed the uptake patterns of three components (glucose, amino acids and ATP) of DOM in Blanes Bay (NW Mediterranean). It was found that most cells from Gamma-, Alphaproteobacteria and Bacteroidetes actively took up ATP, while Gammaproteobacteria and Bacteroidetes did not show a high activity when uptaking glucose compared to Alphaproteobacteria. Then, Alphaproteobacteria and Gammaproteobacteria considerably took up dissolved free amino acids, but not Bacteroidetes. Moreover, the activities of different phylogenetic groups showed to vary throughout the year, especially during the summer (nutrient-limited season). However, their individual contributions to total marine carbon heterotrophic processing is still unknown. Therefore, this reinforces our observations that different substrates released by different plastic types could lead to different dominances and activity levels in each phylogenetic group.

It has been shown that plastic leachates can be composed by both additives, such as bisphenol A, phthalates, citrates and Irgafos® 168 phosphate, and oligomers (Suhrhoff & Scholz-Böttcher, 2016). On the other hand, it is difficult to precisely identify what is affecting the microbial community and activity, as leachates from a single item may contain very large numbers of different organic substances and their breakdown products (Tetu *et al.*, 2020). Thus, which plastic components could be inhibiting or stimulating the growth of the different bacterial groups and their activity is still unknown. However, it is estimated that 23.600 MT of DOC is released by marine plastic every year, making up to 10% of the DOC in the surface microlayer in highly contaminated areas, which can stimulate the activity of heterotrophic microbes (Romera-Castillo *et al.*, 2018). So, this additional DOC could disrupt bacterial processes and alter the community, as heterotrophic production (bacterial uptake) represents the main sink of DOC in the ocean, with most of it being decomposed by bacterioplankton in the surface (Kirchman *et al.* 1991; Druffel *et al.* 1992).

Most phylogenetic groups were more abundant in the Exp_LDPE_PLA than in the Exp_LDPE_PS in both LDPE leachates and control, with just Gammaproteobacteria (dark) and Bacteroidetes (light) being more abundant in the Exp_LDPE_PS. Furthermore, almost all the phylogenetic groups showed a significantly higher mean cell activity in LDPE and control in the Exp_LDPE_PLA, when compared with the Exp_LDPE_PS treatments. Also, treatments from the Exp_LDPE_PLA were dominated by high activity bacteria. These differences found in abundance and activity between experiments could be due to two factors: 1) the temperature during leaching could affect the compounds that are released; 2) the initial bacterial community was different in each experiment. Different temperatures may affect the plastic degradation rate, by inhibiting or accelerating the chemical reactions (O'Brine & Thompson, 2010), with elevated temperatures enhancing degradation (Ho *et al.*, 1999). This matches with our higher release of DOC during incubation at 28 ºC (Exp_LDPE_PS), almost doubling that at 15 ºC (Exp_LDPE_PLA; data not shown). Therefore, a higher abundance and mean cell activity of the different phylogenetic groups in the Exp_LDPE_PS would be expected. Since our results showed an opposite trend, this could indicate that more inhibitory compounds could be released from plastics incubated at 28 ºC regarding that incubated at colder temperature. However, the fact that the same trend was found in the controls without plastic, suggests that the differences are probably not due to the different temperatures, but to the different microbial community composition in the inoculum. Therefore, temperature during the leaching period did not seem to play a role in altering the bacterial community composition and behavior.

Here we also found that biodegradable plastic, PLA, did not release biodegradable compounds that were reflected in a higher bacterial growth or activity. This shows that, in seawater, biodegradable plastic such as PLA, is not always biodegraded and its impact on microorganisms did not differ from the other thermoplastics. Since PLA is classified as a biodegradable plastic and, thus, as a more sustainable option (Sin *et al.*, 2012), it could be expected that its leachates would lead to a higher biodegradation and bacterial abundance. However, here we found that PLA leaching products were not affecting microbial growth regarding the thermoplastics. This agrees with the fact that PLA is only biodegraded in compost plants at temperatures higher than 50 ºC, under industrial conditions, which do not occur in the ocean (Tokiwa & Calabia, 2006). PLA is often blended with starch to increase its biodegradability and reduce costs, which makes it become fragile. To solve this issue, different low molecular weight plasticizers, such as glycerol, sorbitol and triethyl citrate, are added to it (Shah *et al.*, 2008). Thus, this biodegradable plastic also contains additives and chemicals, which can eventually leach and affect the microbial community, leading to similar results as the thermoplastics. Special care must be taken regarding biodegradable plastics, since not all of them are biodegradable at any condition.

When it comes to experiments involving plastics and marine microbes, data usually rely on culture-based approaches in laboratory conditions. Since the bacteria that can be cultured represent less than 1% of the number of bacteria in nature (Hugenholtz *et al.*, 2009), it is difficult to study the behavior of bacterial communities under natural conditions. However, by using a natural inoculum, it was possible to obtain results that resemble their behaviour in the natural environment. On the other hand, in the natural environment, the plastic leaching could have different effects on the bacterioplankton community and activity, as the plastic would probably be colonized by other microorganisms and this could lead to an additional leaching (İpekogˇlu *et al.*, 2007; Kroukamp & Wolfaardt, 2009). Therefore, further experiments need to be conducted to investigate the effects of this additional leaching.

Since it is known that the Gammaproteobacteria class includes many paradigmatic bacterium and pathogens (Williams *et al.*, 2010), plastic leaching could be stimulating the growth and activity of this type of bacteria. It has already been shown that many microbial groups that colonize and attach to plastic debris include members of the genus *Vibrio* and other potentially pathogenic microorganisms, with the possibility of *Vibrio* bacteria dominating the plastic microbial community (Zettler *et al.*, 2013). *Vibrio* can cause disease not only in wild and cultured organisms, but also in humans. Since fish, crustaceans and mollusks are its main targets, it has become the most common pathogen in fish and shellfish aquaculture (Lafferty *et al.*, 2015). In addition, it can also cause human gastrointestinal infections (McCormick *et al.*, 2014). Thus, a narrower identification is needed to better understand which specific groups are being favored by the plastic leaching.

6. CONCLUSION

This study was able to characterise, for the first time, the composition of the microbial community growing in plastic leachates as well as its activity. It was the first step into understanding how plastic leachates might affect the microbial community in the water column. It also identified, for the first time, which bacterial groups are selected in marine plastic leachates and how much active they are regarding protein synthesis. The identification of the phylogenetic groups that are actively uptaking the compounds released by plastics and being benefited by them can contribute to the future development of plastic biodegradation methods. Moreover, the results of this study improved our current knowledge on the interaction between plastic debris and marine microbes and how it could be affecting the marine environment, food-web and system. However, future research is needed in order to identify the organisms composing each phylogenetic group. The probes used here led to a broad identification of microorganisms, in phylogenetic groups, which include many different species. Thus, the next step would be the characterization of the involved species through DNA sequencing techniques. This would allow us to reach more accurate results identifying specific bacteria, which could even represent a threat to humans, marine life and the environment. Thus, further investigation is needed in order to better understand the behaviour of each phylogenetic group when exposed to plastic leachates under different conditions, as many intrinsic and extrinsic factors can have different isolated and combined effects on bacteria.

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