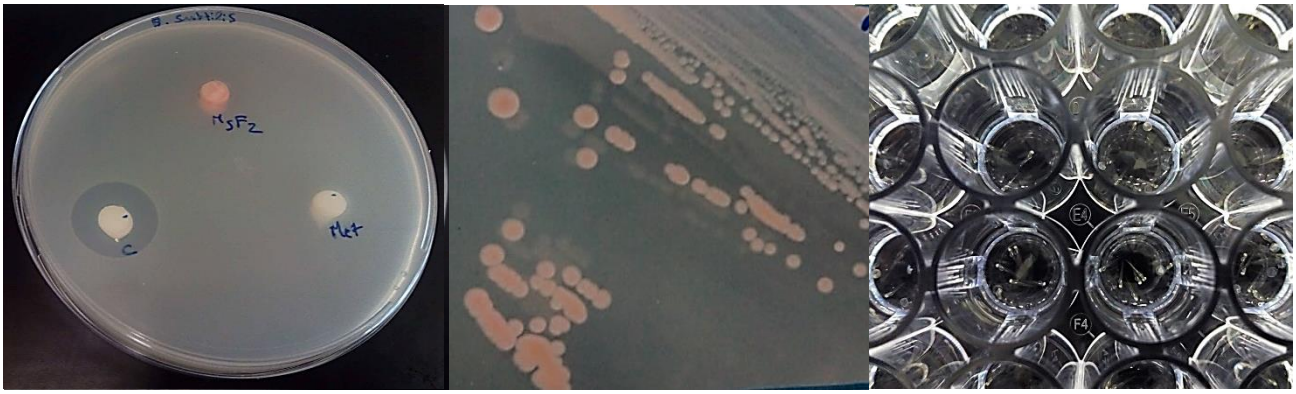

Exploring the secondary metabolite potential of Planctomycetes from marine and freshwater environments

Inês Rosado de Jesus Vitorino

Mestrado em Bioquímica

Departamento de Química e Bioquímica

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Orientador

Olga Maria Lage, Professora Auxiliar, Faculdade de Ciências da Universidade do Porto, Portugal

Coorientador

Tanja Schneider, Associated Professor, Institute for Pharmaceutical Microbiology, Bonn University, Germany

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O Presidente do Júri,

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Resumo

Figurando no topo da lista de preocupações por parte da Organização Mundial de Saúde, a resistência a antibióticos é, na actualidade, um dos maiores entraves à saúde pública. Infecções causadas por estirpes resistentes de bactérias como *Mycobacterium tuberculosis*, *Staphylococcus aureus* e *Streptococcus pneumoniae* são cada vez mais difíceis de tratar e controlar, sendo de extrema urgência encontrar novos compostos que substituam os antibióticos ineficientes e combatam o aumento de super microrganismos. A obesidade é também um dos principais problemas de saúde mundial, sendo considerada uma das doenças metabólicas mais prevalentes. Devido à falta de compostos para tratar esta enfermidade e à ineficiência dos já existentes, novos medicamentos são necessários para combater o aumento exponencial da população obesa.

Numa tentativa de superar esta necessidade de metabolitos inovadores, as atenções estão a ser focadas nos oceanos, uma inesgotável fonte de biodiversidade. Um dos grupos de bactérias menos estudados mas promissor, os Planctomycetes, são um filo que pertence ao super filo PVC. Possuem ciclos de vida complexos, grandes genomas e podem colonizar quase todos os habitats, inclusive já sendo encontrados a viver em associação com organismos marinhos como macroalgas. Estas características, em conjunto com o seu potencial genético para produção de metabolitos secundários, torna-nos em fortes candidatos para a descoberta de novos compostos.

O objectivo deste trabalho foi explorar o potencial dos metabolitos secundários produzidos por várias estirpes aquáticas de Planctomycetes, sendo a maioria isolada a partir do complexo e competitivo biofilme de macroalgas, com enfâse na procura de actividade antimicrobiana contra diferentes microorganismos (B*acillus subtilis*, *Micrococcus luteus* e *Chlamydia trachomatis*) e possível actividade anti obesidade. Paralelamente, pretendeu-se desenvolver e optimizar protocolos para o cultivo de Planctomycetes, preparação de extractos e isolamento de moléculas bioactivas.

Neste estudo, a actividade antimicrobiana foi rastreada usando vários ensaios diferentes e diversos protocolos para preparação das culturas e consequente extracção de metabolitos foram estados. Extractos obtidos de estirpes pertencentes a diversas espécies de Planctomycetes, como *Rhodopirellula baltica* (MsF2 e FF1), *Rhodopirellula rubra* (UC9), *Aquisphaera giovannonii* (OJF8) e *Rubinisphaera*

brasiliensis (Gr7), usando um protocolo de extracção com acetato de etilo, demonstraram atividade antimicrobiana contra *B. subtilis* e *M. luteus*. Os ensaios que avaliaram a actividade contra o agente patogénico *C. trachomatis* e a actividade contra obesidade demonstraram resultados promissores, mas estudos adicionais serão ainda necessários. No entanto, apesar da elevada biodiversidade de rastreios e técnicas de extracção testadas, não foi possível obter um protocolo optimizado que permitisse o isolamento de moléculas bioactivas de Planctomycetes usando cromatografia de permeação em gel.

Este trabalho confirmou a capacidade dos Planctomycetes produzirem metabolitos antimicrobianos efectivos contra bactérias Gram positivo bem como o potencial contra o parasita intracelular *C. trachomatis* e estender o nosso conhecimento a metabolitos com capacidade anti obesidade. Infelizmente, devido a inconsistências de resultados e a adversidades metodológicas, não foi possível o passo subsequente, o do isolamento de moléculas bioativas.

Abstract

Antibiotic resistance is a major actual problem, placed at the top of the list of concerning of world's health by the World Health Organization (WHO). Dangerous bacterial infections caused by organisms like *Mycobacterium tuberculosis, Staphylococcus aureus* and *Streptococcus pneumoniae* are becoming increasingly more difficult to treat, making of extreme urgency to find new compounds that can substitute the inefficient ones and combat the new rising super bugs. Another main health problem is obesity, one of the most prevalent metabolic diseases. The lack of drugs with obesogenic effect and the inefficiency of the existing treatments make it necessary to find new compounds in order to combat the increase of obese population.

To overcome the need of innovative metabolites, attentions are being paid in the endless source of biodiversity: the oceans. One of the less studied and promising group, the bacterial phyla *Planctomycetes*, belongs to the PVC super-phylum. They possess large genomes, complex life cycles and can colonize most habitats, as for instance the biofilm of certain marine organisms, such as macroalgae. These characteristics along with their genetic potential in secondary metabolites point them as good candidates for the discovery of new drugs.

The objective of this study was to explore the secondary metabolite potential of several strains of marine and freshwater Planctomycetes, the majority of them isolated from the complex and competitive biofilm of macroalgae, with emphasis on the search of possible antimicrobials against different targets (*Bacillus subtilis*, *Micrococcus luteus* and *Chlamydia trachomatis*) and possible anti-obesogenic activity. At the same time, it was envisaged to develop and optimize protocols for the culturing of Planctomycetes, extracts preparation and isolation of bioactive molecules.

Various different antimicrobial screening assays and several culturing and extractions preparation protocols were assayed in this study. Bioactivity was observed against *B. subtilis* and *M. luteus* for extracts obtained from different Planctomycetes species, *Rhodopirellula baltica* (MsF2 and FF1), *Rhodopirellula rubra* (UC9), *Aquisphaera giovannonii* (OJF8) and *Rubinisphaera brasiliensis* (Gr7), following a protocol of extraction with ethyl acetate. Assays that evaluated anti-*Chlamydia trachomatis* and anti-obesity activity by Planctomycetes metabolites showed promising results, but additional studies are needed. However, besides the high diversity of screening approaches and extraction techniques assayed, it was not possible to

achieve a good optimized protocol that allowed the bioactive molecules isolation from Planctomycetes through gel permeation chromatography.

This work confirmed the capacity of Planctomycetes to produce antimicrobial metabolites effective against Gram positive bacteria as well as the potential against the intracellular parasite *Chlamydia trachomatis* and extended our knowledge on potential metabolites against obesity. Unfortunately, due to results inconsistencies and methodological adversities we were not able to step forward towards bioactive metabolite isolation.

Key words

Bacteria; Antibiotic resistance; Planctomycetes; Secondary metabolites; Extraction of bioactive compounds; Antimicrobial activity; Anti-obesogenic activity.

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List of abbreviations

º C – Degrees Celsius
%- Percentage sign
®- Registered trademark
µg – Micrograms
µL- Microliters
AR- Antibiotic resistance
B. subtilis- Bacillus subtilis
BMI- body mass index
C. albicans- Candida albicans
C. trachomatis- Chlamydia trachomatis
CIIMAR- Interdisciplinary Centre of Marine and Environmental Research from University of Porto, Portugal
CO_2- Carbon dioxide
DAPI- 4',6-diamidino-2-phenylindole
DMSO- Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
E. coli- Escherichia coli
Fig/ Figs –Figure/ figures
g- Grams
GPC- Gel permeation chromatography
h- Hour/ hours
HCL-Tris- Tris-Hydrochloride
Hep2- Cell line derived from the eukaryotic Human Epidermoid carcinoma
L- Liters
LEMUP- Laboratory of Microbial Ecophysiology from University of Porto
LPS- Chlamydial lipopolysaccharides
m – Minute/minutes
M. luteus- Micrococcus luteus
M607+A- M607 medium supplemented with 1% algae extract
M607+A+*Bacillus*- M607 medium supplemented with 1% algae extract plus 1% of an autoclaved culture of *B. subtilis*
M607+A+coli- M607 medium supplemented with 1% algae extract plus 1% of an autoclaved culture of *E. coli*

mg- Miligrams

mL- Milliliters

MoA- Mode of action/ mechanisms of action

NA medium- Nutrient medium

NAG- N-acetylglucosamine

NB- Nutrient broth

nm- Nanometers

NRPS- Nonribossomal peptide synthetases

OD- Optical density

OV- Overnight

PBS buffer- Phosphate buffered saline

PKS- Polyketide synthases

PTU- Phenylthiocarbamide

RNA- Ribonucleic acid

Rpm- Rotations per minute

SPG buffer- Saccharose-phosphate-glutamate

WHO- World Health Organization

X-Gal- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Introduction

Development of a new drug is an extensive, complex but necessary interdisciplinary process. It involves numerous procedures, beginning with a discovery or synthesis of a new compound and going from steps such as pre-clinical trials with animals to formulation and clinical trials with humans, to serve one propose: human safety and health.

One of the biggest concerns in the current time is antimicrobial resistance (AR). Without an effective antimicrobial agent against several pathogens, some infections can spread quickly through the population and lead to illness and death, but one of the main complications is the compromised safety of numerous medical procedures. Due to infections, several practices like organ transplants, caesarean, cancer chemotherapy and surgeries in general can become more unsafe, needing prolonged and costlier care. Together with antivirals, antifungals and anti-parasites, antibiotics are losing activity due to resistance, normally resulting from the co-evolution between host and pathogen through genetic variations within the population, both facilitated by the small cycles of life that allows bacteria to quickly change. However, these resistances can and are being accentuated by the overuse and/or the incorrect use of the medicines together with the poor sanitary conditions around the globe that help increasing the velocity of the spread. According to the World Health Organization (WHO), the following pathogens (and respective resistances) are of international importance in the current times (WHO, 2014): *Escherichia coli* – resistance to third-generation cephalosporins and to fluoroquinolones; *Klebsiella pneumoniae* – resistance to third-generation cephalosporins and to carbapenems; *Staphylococcus aureus* – resistance to methicillin; *Streptococcus pneumoniae* – resistance (non-susceptibility) to penicillin; Non-typhoidal *Salmonella* – resistance to fluoroquinolones; *Shigella* species – resistance to fluoroquinolones, *Neisseria gonorrhoeae* – decreased susceptibility to third-generation cephalosporins and *Mycobacterium tuberculosis* – resistance to rifampicin. The impact of these microorganisms varies from hospital and community areas to food market and due to the lack of new compounds to substitute the ones there are no longer efficient, research, innovation and inspiration are required in order to fight against these new rising super bugs and help in its dispreads. Sexual transmittable diseases are also of enormous concern. Chlamydial infections (caused by the microorganism *Chlamydia trachomatis*) are one of the most common worldwide.

Patients are mostly asymptomatic (which favors the continuous spreading of the disease) and often develop other types of infections at the same time. Women are more susceptible and even after treatment can gain serious complications as infertility, ectopic pregnancy and chronic pelvic pain. Antibiotics like azithromycin and doxycycline can treat uncomplicated infections (Malhotra *et al*., 2013), but persistent ones are more problematic. Chlamydiae are obligate intracellular bacteria and under stress conditions can change to atypical forms (aberrant bodies): these are non-infectious, have reduced metabolic activity and do not replicate, yet remain alive. This type of forms has a decrease in the lipopolysaccharide (LPS) antigens, making diagnosis difficult, but maintains a high production of chlamydial heat shock protein 60 (hsp60), which induces chronic inflammation. This form also has decreased levels of chlamydial major outer membrane protein (MOMP), which leads to reduced transport of antibiotic across the cell, resulting in failure of treatment in most of the patients with chronic infections (Malhotra *et al*., 2013). Also, there are already evidences of multidrug resistant *C. trachomatis* in women with high bacterial load (Horner 2016), which makes the search for new anti-chlamydia compounds a priority.

Antibiotics normally function by interfering with important pathways within the bacteria and which specific cellular targets are not present in eukaryotic cells, to insure that only these microorganisms are affected. They can act directly against a specific enzyme or by disrupting other important cellular processes through a specific mode of action (Urban *et a*l., 2007). Cell wall, nucleic acids and protein biosynthesis are the most common cellular targets. Cell wall biosynthesis has always been one of the most appellative one, due to the importance of the peptidoglycan layer in bacterial protection and its absence in eukaryotic cells. With this action, B-lactam compounds are an example of one of the most important antibiotics discovered: penicillin (Rang *et al*., 2015). The cell wall as a target is also important for the differentiation between types of antibiotics, according to their range of action. Broad-spectrum antibiotics act against a wide range of bacteria, normally both Gram positive and Gram negative. On the other hand, there are antibiotics more specific for a selected group. This selectivity is often based on the differential cell wall composition between the various types of bacteria. Cell division is also necessary for maintaining and producing new bacterial cells, which makes both nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)) synthesis important targets as well. For example, rifampicin blocks initiation of RNA synthesis by specifically inhibiting bacterial RNA polymerase and it does not interact with mammalian RNA polymerases, making it specific for bacteria. On another hand, quinolones are a key group of antibiotics that specifically interfere with bacterial

topoisomerase II (important cell cycle enzyme) and not with mammalian topoisomerases. Fluoroquinolones are the second-generation quinolones (which include levofloxacin, norfloxacin, and ciprofloxacin) are also an example of compounds that act against this target as well (Rang *et al.,* 2015). Protein synthesis is also a main target, since most important cellular processes need enzymes. Tetracyclines, such as doxycycline, prevent the binding of aminoacyl-tRNA by blocking the A (aminoacyl) site of the 30S ribosome, for example.

Besides antibiotic resistance and non-controlled infections, another main problem against the human health is, currently, obesity. One of the most prevalent metabolic diseases, obesity is the designation of the condition of presenting a body mass index (BMI) above 30 kg/m^2. Individuals who have a high level of body mass (and even more the ones in morbid obesity stage, with BMI above 40 kg/m^2 (WHO, 2000)) are associated with other health complications, as cardiovascular diseases (mainly heart disease and stroke) diabetes, musculoskeletal disorders (especially osteoarthritis – a highly disabling degenerative disease of the joints) and some cancers (including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon), as was observed by the WHO (WHO, 2000). Besides, all patients who suffer this condition need continuous care, making the entire healthcare costlier. Obesity is a complex condition dependent on many factors, tough individual responses may be enough in some cases to decrease the body mass in excess, as lower the food intake, increase the physical activity and eat healthier foods (with lower salt/sugar composition). However, metabolic disturbances may prevent the resolution of the problem, making anti-obesogenic drugs necessary to complement the process. The lack of compounds with this effect is noticed, as only a few medications are currently on the market (Birari *et al*., 2007). Existing treatments work mostly by two different mechanisms of action: appetite suppression and interference with the body's ability to absorb specific nutrients. The drugs most commonly used for this are catecholamines, like amphetamines, and anti-depressants like bupropion (Bray, 1993). On the other hand, orlistat is the drug usually recommended for inhibition of the absorption of fat, being an inhibitor of the enzyme lipase in the intestine (Weibel *et al.*, 1987). However, no treatment is currently entirely efficient, having also frequent gastrointestinal side effects, mostly in infants and adolescents (Viner *et al.*, 2010). Thus, new drugs with obesogenic effect are needed in order to combat the increase of the obese population.

Since the ancient times, the discovery of new drugs has always been a priority for humans, even if in a non-rational way. Nature was the main local to search for

substances that could cure, relieve pain or even increase the wellbeing in general. However, with the advance of technology, the structures of the bioactive compounds were more and more known, changing completely the way that this discipline was seen. Diversity and complexity were the compounds main features, even with some common bases: aromatic rings, heterocyclic structures, carboxylic acids, esters and chirality were the most present characteristics. Also, the elements that constituted those bio metabolites were essentially the same: carbon, oxygen, hydrogen, nitrogen, sulfur and halogenates such as chloride and fluorine. With the knowledge of the structures, semi-synthesis was the subsequent step, with the objective of optimization of production (since the total chemical synthesis of compounds similar to natural ones was almost impossible due to the complexity). However, with advances in chemistry, the search for new compounds in Nature was particularly slightly overlooked in favor of a new era of laboratorial drug synthesis. Improvements of knowledge in Biology and Biochemistry also allowed to do directed and rational search, permitting discoveries based on specific targets or processes, lowering aleatory developments. Currently, with the lack of new drugs, Nature is coming back again as a main source of discovery, thanks to the inexhaustible biodiversity, bioavailability and strength of compounds that it can give us. With this change, the attentions also started to be directed to the less explored marine environments (Bhatnagar & Kim, 2012). The biodiversity of living beings is high in terrestrial habitats and consecutively, the diversity of metabolites produced but the variety in aquatic habitats is enormously higher and much more unexplored. Chordates, Plants, Molluscs, Crustaceans, Insects, Algae, Nematodes, Fungi and Bacteria: they all compete for resources and it is plausible that the production of metabolites is intense and with a large variety of actions. Regardless of the complications of using natural compounds from marine environments (toxicity, high amount of samples needed in several cases and difficulty of extraction), in the past years numerous drugs with origin in aquatic habitats and, more specifically, from microorganisms, which are simpler to cultivate and maintain, have been approved (Desjardine *et al.*, 2007 and Eom *et al*, 2013).

Planctomycetes is a phylum of bacteria that phylogenetically belongs to the PVC super-phylum, along with *Verrucomicrobia*, *Chlamydia* and *Lentisphaerae* (Wagner & Horn, 2016). Considered one of the deepest branches of bacterial phyla, this still unexplored phylum has very particular and uncommon characteristics, like a unique complex system of membranes (Fuerst 2013 and Lage *et al*., 2013) (Fig. 1).

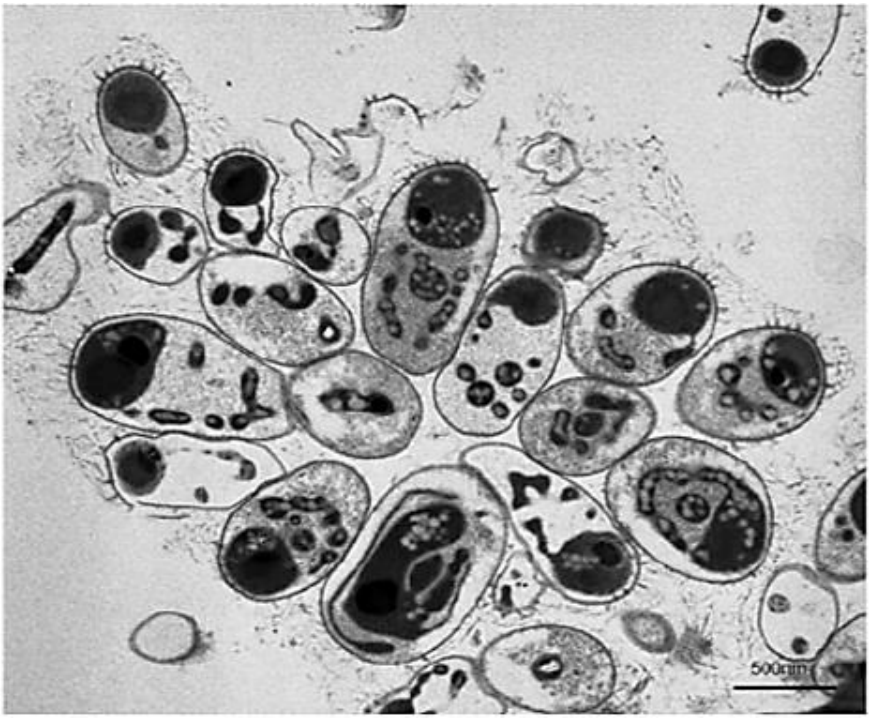

Fig. 1- Ultrathin sections of a strain of *Rhodopirellula rubra* by transmission electron microscopy of cross sections (Lage *et al.,* 2013), showing the unique complex system of membranes of Planctomycetes.

During many years, the cell wall of Planctomycetes was thought to be peptidoglycan free, which placed these bacteria under discussion on a possible eukaryotic cell ancestry (Fuerst & Sagulenko, 2011). Peptidoglycan, or murein, is a complex molecular structure with the function of structural strength and cell protection, a polymer consisting of glycan strands and amino acids. The sugar strands are made up of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues, alternated and cross-linked by short peptides, whose composition is most often l-Ala-γ-d-Glu-meso-A2pm (or l-Lys)-d-Ala-d-Ala (A2pm, 2,6-diaminopimelic acid) (Vollmer *et al.*, 2008). Due to the important role in bacterial cells, murein is also one of the main targets of antibiotics, like the familiar example of penicillin (Rang *et al*., 2015). The absence of this vital compound in cell walls, only observed in a scarce group of bacteria, has always been intriguing, but recent studies have showed clear evidences that the cell wall of Planctomycetes do possess, in fact, peptidoglycan (Jeske *et al*., 2015; Van Teeseling *et al*., 2015). It is located in a layer underneath the outer membrane, which combines with the typical characteristics of Gram-negative bacteria, clarifying the mystery of that supposed differential cell wall composition. Planctomycetes are also resistant to β-lactam antibiotics, due to the production of β-lactamases (Jeske *et al*., 2015), explaining the resistance that was once attributed to

the lack of peptidoglycan. The other phyla belonging to PVC have also been under consideration and the same elucidation about the presence of peptidoglycan was made for bacteria from *Chlamydia,* including with a new metabolic cell-wall labelling method *(*Pilhofer *et al*., 2013; Kåhrström, 2014; Liechti *et al*., 2014). However, one common feature is notice: bacteria from both phyla lack the cytoskeletal protein FtsZ. This protein has been associated universally to cell division in bacteria with walls and classified as an essential peptide but these studies suggest new possible mechanisms of division. Although the constitution of the cell wall of planctomycetes has been elucidated, there are other peculiar details and characteristics remaining to explore and clarify.

In an ecologically view, bacteria from the phylum *Planctomycetes* are found in most of the planet habitats, ranging from terrestrial, freshwater and marine environments (Lage & Bondoso, 2011; Lage & Bondoso, 2014) to more extreme ones, like glacial waters (Zeng *et al*., 2013), locals with a wide range of pollution (with hydrocarbons or others) (Akob *et al*., 2007; Halter *et al*., 2011) and hot springs (Bohorquez *et al*., 2012), demonstrating their capacity of survival and adaptation. They are mostly heterotrophic, aerobic, mesophilic and neutrophilic, with an extra group of anaerobic ammonium oxidation species (or anammox Planctomycetes) which are strictly chemolithotrophic (Fuerst & Sagulenko, 2011), showing also the variety of metabolisms. Some species were also found living associated with other organisms, such as macroalgae (Lage & Bondoso, 2011; Bondoso *et al*., 2014; Lage & Bondoso, 2014), belonging of the three phyla: *Chlorophyta* (green algae), *Rhodophyta* (red algae) and *Heterokontophyta* (brown algae). The strains explored in this study are an example of that association, being previously isolated from the biofilm of several macroalgae from the Portuguese coast. Macroalgae can possibly benefit from the presence of microorganisms thanks to the compounds produced by them and the same in reverse, making a dynamic relationship (Lage & Bondoso, 2014). Diverse algal macromolecules can be nutritive substrates for Planctomycetes, like agar, alginate, cellulose and also sulfated polysaccharides, since the presence of sulfatases was observed and sulfatases genes were already found in *Rhodopirellula baltica* strains (Glockner *et al*., 2003; Wegner *et al*., 2013). On the other hand, molecules produced by Planctomycetes can, possibly, help algae to control of unwanted pathogens as well as reducing competition at the same time.

Besides the uncommon features already exposed, in a first view, Planctomycetes seem to fit in the group of bacteria that possible produce compounds

with interest: (1) their cycle of live is long and complex, which is common among bacteria already studied for this purpose, like Actinobacteria (*Streptomyces)* and *Myxobacteria* (Jeske *et al*., 2013); (2) they possess large genomes; (3) they have a great capacity of adaptation; (4) they belong to an unexplored and independent branch, suggesting new molecules and (5) they live in a competitive and dynamic environmental where they can dominate against other bacteria that have a quicker cycle of live (which suggests that they possible have some strategy). Although some biochemical pathways are still unknown for this relatively understudied phylum, secondary metabolites with interest are starting to be explored. A genomic study about the presence of genes encoding modular polyketide synthases (PKS) and nonribossomal peptide synthetases (NRPS) was firstly performed to a variety of bacteria from different taxa (Donadio *et al*., 2007), in which the only Planctomycete genome available and analyzed corresponded to a strain affiliated with the species *Rhodopirellula baltica*. Its genome possesses genes encoding for two small NRPSs, two monomodular PKSs and a bimodular NRPS/PKS. PKS and NRPS are complex families of enzymes that ultimately lead to most of the bioactive secondary metabolites of interest (Donadio *et al*., 2007). PKS leads to the production of polyketides and NRPS catalyze important peptide products. The presence of genes encoding for these two groups of enzymes is therefore a possible indicator of production of metabolites with biotechnological interest. In Jeske *et al*., (2013), thirteen Planctomycetes genomes were analyzed through genome mining, with the use the software AntiSMASH (for antibiotic and secondary metabolite identification). One hundred and two candidate genes or clusters were found within the analyzed 13 genomes, confirming the genetic potential of this phylum. The potential by Planctomycetes was also confirmed by Jeske *et al*., (2016), where antimicrobial activity was demonstrated against several bacterial targets with extracts from *Rhodopirellula baltica* species. More Planctomycetes were later screened for the presence of genes encoding for NRPS/PKS and for antimicrobial activity with bioactivity studies (Graça *et al*., 2016). This study focused on exploring the potential of marine strains isolated from several macroalgae and affiliated to numerous species such as *Rhodopirellula baltica, Rhodopirellula lusitana, Rhodopirellula rubra, Roseimaritima ulvae* and *Planctomyces brasiliensis.* Almost all strains presented at least one of the genes referred and antimicrobial activity was observed for 43% of the strains against the fungus *Candida albicans* and 54% against the bacterium *Bacillus subtilis*. With the software NaPDos, a bioinformatics tool for the rapid detection and analysis of secondary metabolite genes, some strains were also screened for the potential pathway product that would be formed (Graça *et al*., 2016). Three strains

revealed potential to produce antimicrobial compounds, as myxalamid, bacitracin and pikromycin. This study confirmed the bioactive capacity of Planctomycetes, with emphasis on marine ones, to produce antimicrobial compounds and encouraged further studies envisaging molecule isolation and characterization for the possible discovery of new drugs.

Recent studies also demonstrated that many natural products, including secondary metabolites from plants, cyanobacteria, fungi and phytoplankton, possess anti-obesity activities (Castro *et al*., 2016 and Yun 2010). Therefore, Planctomycetes are also promising sources of novel bioactive molecules with this effect.

Objectives

This Master thesis was performed in the framework of the Masters in Biochemistry of Sciences Faculty from Porto University, Portugal, and it was developed in the Laboratory of Microbial Ecophysiology from University of Porto (LEMUP) and in the Institute for Pharmaceutical Microbiology from University of Bonn, Germany, as an Erasmus^{+} Internship. The objective of this study was to explore the secondary metabolite potential of several strains of marine and freshwater Planctomycetes, with emphasis on the search of possible bioactivity against different targets (antimicrobial and anti-obesity activity). At the same time, it was envisaged to develop and optimize a protocol for culturing of Planctomycetes and preparation of extracts (which comprehended several organic solvents and methodologies) for the obtainment of bioactive molecules. Another objective was the isolation of bio compounds by gel permeation chromatography as well as characterization of their mode of action using *Bacillus subtilis* reporter strains.

Methods

1. Biological material

Thirty-seven strains of several species from the phylum *Planctomycetes* were explored in this thesis, which belong to a collection from the Laboratory of Microbial Ecophysiology from University of Porto (LEMUP). Thirty-five of the strains came from marine environments, which were previously isolated from the biofilm of various marine macroalgae collected along the Portuguese coast (as described in Lage & Bondoso, 2011). On the other hand, the remaining two were isolated from freshwater sediments (Bondoso *et al*., 2011). Besides the origins of the species and the phylogenetic diversity, this panel of strains was also selected by its previously reveled potential to produce metabolites with antimicrobial activity, observed in the studies performed during the internship realized in the Bachelor degree in Biology (unpublished studies) and by Graça *et al*., (2016). This potential was shown by genomic analyses (presence of PKS and/or NRPS genes and prediction pathway product using NaPDos software) and by preliminary bioactivity screenings in liquid medium. The numbering given to the isolates, their affiliation, their origin of isolation, the presence of PKS/NRPS genes and the potential pathway product prediction are all described in Table 1. The appearance of these Planctomycetes is very characteristic and easy to distinguish: in general, they form circular, small, convex and translucent colonies ranging from light pink to almost red in color, which is demonstrated in Fig. 2, where two of the chosen strains are exemplified. One of the strains from the panel, although, is orange pigmented (strain Gr7).

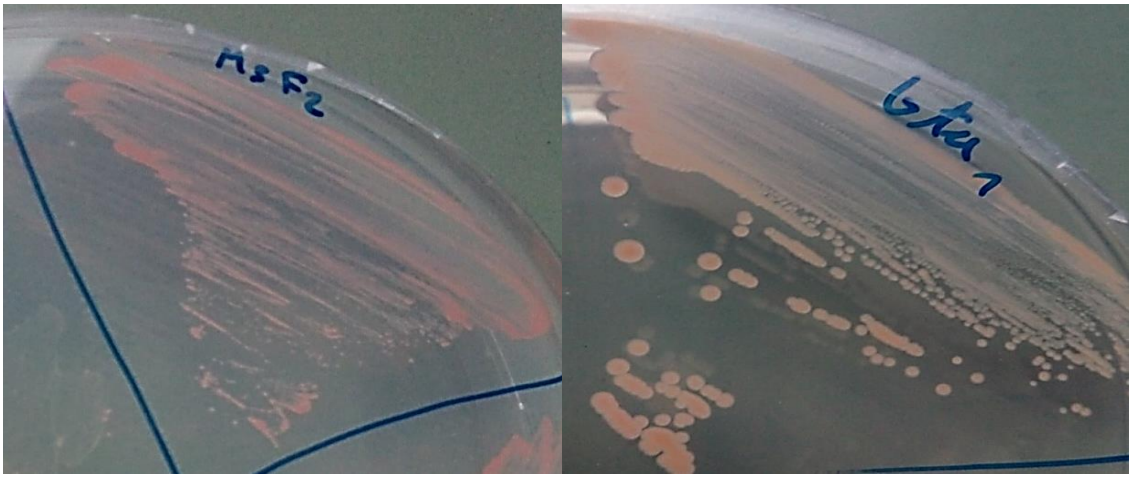

Fig. 2- Colonies of strains MsF2 and Gtu1, respectively, showing the characteristic pink color of colonies of Planctomycetes.

Table 1- Numbering given to the Planctomycetes used in this study, their affiliation, their origin of isolation, the presence of PKS/NRPS genes and the potential pathway product prediction (using NaPDos software).

Strain affiliation	Designation	Isolate origin	Bioactive genes	NaPDoS prediction
Aquisphaera giovannonii	OJF2	Freshwater sediments	PKS/NRPS	ND
Aquisphaera giovannonii	OJF8	Freshwater sediments	ND	ND
Rubinisphaera brasiliensis	Gr7	*Gracilaria bursa-pastoris*	PKS	Myxalamid
Rhodopirellula baltica	UC21	*Ulva* sp.	PKS	ND
Rhodopirellula baltica	UC49.1	*Ulva* sp.	PKS/NRPS	Epothilone
Rhodopirellula baltica	UAP 119	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula baltica	UAP 120	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula baltica	UAP 121	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula baltica	UAP 127	*Ulva* sp.	PKS	ND
Rhodopirellula baltica	UAP 128	*Ulva* sp.	None	ND
Rhodopirellula baltica	PAP 33	*Porphyra dioica*	PKS/NRPS	ND
Rhodopirellula baltica	PAP 34	*Porphyra dioica*	PKS/NRPS	ND
Rhodopirellula baltica	PAP 61	*Porphyra dioica*	PKS/NRPS	ND
Rhodopirellula baltica	PAP 64	*Porphyra dioica*	None	ND
Rhodopirellula baltica	PAP 67	*Porphyra dioica*	PKS	ND
Rhodopirellula baltica	G1	*Gelidium pulchellum*	ND	ND
Rhodopirellula baltica	G3	*Gelidium pulchellum*	ND	ND
Rhodopirellula baltica	MsF2	*Mastocarpus stellatus*	ND	ND
Rhodopirellula baltica	Cor 1	*Corallina* sp.	ND	ND
Rhodopirellula baltica	Gtu1	*Grateloupia turuturu*	ND	ND
Rhodopirellula baltica	FF1	*Fucus spirallis*	ND	ND
Rhodopirellula baltica	FC6	*Fucus spirallis*	ND	ND
Rhodopirellula baltica	Gr17	*Gracilaria bursa pastoris*	ND	ND
Rhodopirellula baltica	SAP 113	*Sargassum muticum*	PKS	ND
Rhodopirellula baltica	SAP 114	*Sargassum muticum*	PKS	ND
Rhodopirellula baltica	SAP 115	*Sargassum muticum*	None	ND
Rhodopirellula lusitana	CcC6	*Condrus crispus*	PKS/NRPS	ND
Rhodopirellula lusitana	CcC8	*Condrus crispus*	PKS/NRPS	Pikromycin
Rhodopirellula lusitana	Sm4	*Sargassum muticum*	PKS/NRPS	ND
Rhodopirellula lusitana	UC13	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula lusitana	UC16	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula lusitana	UF6	*Ulva* sp.	PKS/NRPS	ND
Rhodopirellula rubra	UC9	*Ulva* sp.	PKS/NRPS	Bacitracin
Rhodopirellula sp.	FC 9.2	*Fucus spiralis*	NRPS	Microcystin
Rhodopirellula sp.	FF4	*Fucus spiralis*	PKS/NRPS	ND
Roseimaritima ulvae	UC8	*Ulva* sp.	PKS/NRPS	Myxothiazol
Roseimaritima ulvae	UF3	*Ulva* sp.	PKS	Stigmatellin

ND= not determined

The microorganisms used as targets in the several screenings realized were a clinical isolate of *Candida albicans,* an environmental *Escherichia coli* and *Bacillus subtilis* ATCC 6633, from LEMUP laboratory, or *Micrococcus luteus* DSM 1790 and *Chlamydia trachomatis* D/UW-3/CX, from the Institute for Pharmaceutical Microbiology from University of Bonn. The various bioengineered *Bacillus subtilis* 128 reporter strains used in the mode of action (MoA) assay belonged also to the Institute for Pharmaceutical Microbiology from University of Bonn and their respective vectors/targets are described in Table 2. The host cells needed for maintenance of *C. trachomatis* were from the cell line Hep2, a cell line derived from the eukaryotic Human Epidermoid carcinoma. The adult zebrafish individuals (*Danio rerio*) used for reproduction to obtain the larvae necessary for the anti-obesity assay belonged to the stock of the Interdisciplinary Centre of Marine and Environmental Research from University of Porto, Portugal (CIIMAR) (Fig. 3).

Table 2 - *Bacillus subtilis* 168 reporter strains and their respective targets of main biosynthesis pathways of the bacteria.

Reporter Strain	Vector	Target
B. subtilis 168	pAC6-*Pypu*A	Cell Wall Synthesis
	pAC6-*Pyor*B	DNA Synthesis
	pAC6-*Pyhel*	Protein Synthesis
	pAC6-*Pyvg*S	RNA Synthesis

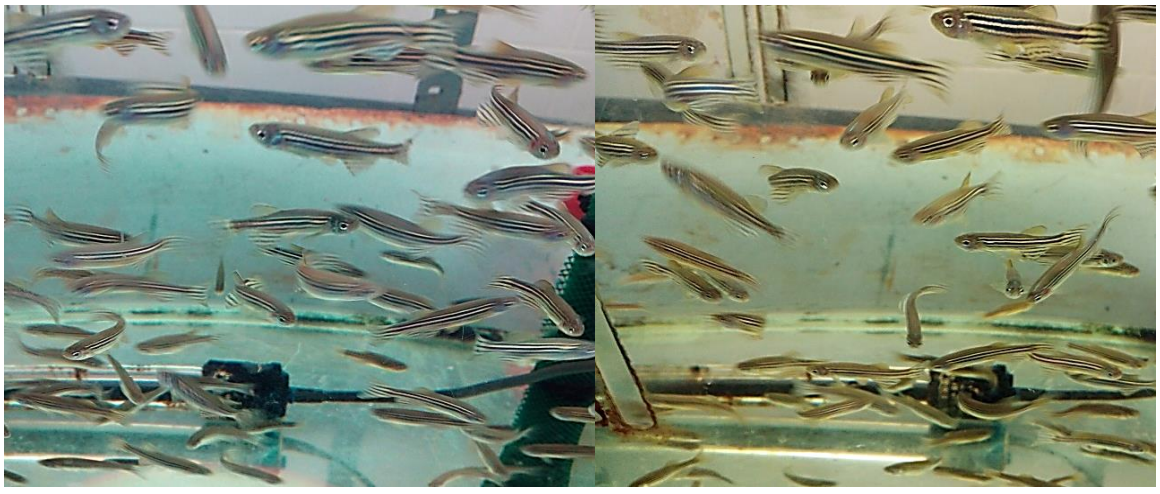

Fig. 3- Adult zebrafish individuals from the stock of CIIMAR, in an aquarium with water settled at 25º C.

2. Media and other material used

2.1. Media for maintenance and incubation of Planctomycetes

For the maintenance and incubation of the freshwater Planctomycetes in pure culture (at 30ºC), PYGV medium was prepared (Table 3). For the incubation and maintenance of the marine Planctomycetes in pure culture (at 25ºC), M607 and M600 medium were also prepared (Table 3). Both media have similar composition but M600 has four times the concentration of the organic carbon sources. This may increase the production of the bioactive compounds of interest, since it creates a more stressful environment for the bacteria. With the objective of stimulating the production of bioactive molecules, other variations of the previously referred M607 medium were additionally prepared: M607 medium with more 15% of salt than the regular one (salt stress); M607 medium supplemented with 1% of an algae extract, as stimulation factor (M607+A); M607 medium supplemented with 1% of the previously mentioned algae extract plus 1% of an autoclaved culture of *E.coli* (M607+A+*coli*) and M607 medium supplemented with 1% of an the same algae extract plus 1% of an autoclaved culture of *B. subtilis* (M607+A+*Bacillus*) (both stimulation factors plus biological stress). The algae solution was prepared with *Ulva* sp., which was one of the macroalgae previously used for isolation of the Planctomycetes belonging to the chosen panel. Pieces of this macroalgae (5 grams) were macerated in sterile water and the mixture sterilized through a 0.22 µm filter. Another variation of M607 medium was also prepared using a solution of 5% of N-acetylglucosamine (NAG) instead of the glucose solution previously mentioned in Table 3. This compound was used in Jeske *et al*., 2016 to substitute glucose as the carbon source in the media for Planctomycetes and it was observed an increase in the production of bioactive molecules when complementing the media with it.

The sea water that was used in the marine media was natural sea water collected in the north coast of Portugal. Due to the unavailability of the same natural water in the experiments performed during the Erasmus^{+} Internship in Bonn, artificial sea water was prepared, with small modifications of the one purposed by Lyman & Fleming (1940), as well as a solution using sea salts (Table 4).

Table 3- Composition of M607, M600 and PYGV media (Lage & Bondoso, 2011; Bondoso *et al*., 2011).

Reagents	Modified M13 agar medium (M607)	Modified M14 agar medium (M600)	PYGV medium
	Units per liter		
Peptone	0.25 g	1 g	0.25 g
Yeast extract	0.25 g	1 g	0.25g
5 mM [1]HCl-Tris, pH 7.5	50 mL	50 mL	----------
Agar	16 g	16 g	16 grams
[2]Filtred natural sea water/[3]Artificial sea water/[4]Sea salts in deionized water	900 mL	880 mL	----------
Deionized water	10 mL	----------	1000 mL
[5,6]Glucose solution (2.5%)	10 mL	40 mL	10 mL
[5,7]Vitamins solution	10 mL	10 mL	10 mL
[5,8]Hutner's solution	20 mL	20 mL	20 mL

[1]Tris-Hydrochloride buffer; [2] Sea water was previously filtered through a 0.45 µm pore filter; [3] Composition provided in Table 4; [4] Sea salts from SIGMA®; [5]These components were sterilized through a 0.22 µm pore filter and added only after medium autoclaving; [6] The solution was prepared with sterilized water; [7] 0.1 µg mL^{-1} cobalamine, 2.0 µg mL^{-1} biotin, 5.0 µg mL^{-1} thiamine-HCl, 5.0 µg mL^{-1} Ca-pantothenate, 2.0 µg mL^{-1} folic acid, 5.0 µg $^{-1}$ riboflavin and 5.0 µg mL^{-1} nicotinamide; [8]Cohen-Bazire *et al*. (1957).

Table 4- Composition of artificial seawater used in the preparation of the media for marine Planctomycetes (modified from Lyman & Fleming, 1940).

Reagents	Units per liter of deionized water	Reagents	Units per liter of deionized water
NaCl	23.477 g	KCl	664.000 mg
$NaSO_4$	3.917 g	KBr	6.000 mg
$MgCl_2$. $2H_2O$	4.981 g	H_3BO_3	26.000 mg
$CaCl_2$	0.832 g	$SrCl_2$	24.000 mg
$NaHCO_3$	192.0 mg	NaF	3.0 mg

2.2. Other media and organic solvents

The microbial targets used for the screening assays were cultured at 37º C in modified Nutrient agar medium (NA medium) (Table 5), and Mueller-Hinton medium from Oxoid®. Mueller-Hinton agar medium was also used for preparation of the plates of the antimicrobial screenings with solid medium (with 1.6 % agar), including the mode of action assay. For diffusion assays, Mueller-Hinton soft agar medium (with only 0.6 % agar instead of the normal 1.6 %) was used. For the co-culture assay, Lysogeny soft agar medium was prepared (Table 5). Dulbecco's Modified Eagle Medium, from ThermoFisher Scienthific, was the medium in which the previously available suspension of Hep2 cells (used as host cells for the chlamydial assay) were cultured. This media was also previously complemented with 5 mL/L of three solutions prepared specifically for this medium, also from ThermoFisher Scienthific: vitamins, fungizone and non-essential aminoacids. Additionality, 50 µg/ml gentamycin and 10% heat inactivated fetal calf serum supplemented the medium. For the maintenance of the zebrafish larvae in petri-dishes, one solution, designed as egg water, was prepared, consisting in Instant Ocean® Sea Salts dissolved in distilled water at a final concentration of 60 µg/ml.

Table 5- Composition of Nutrient agar medium (NA medium) and Lysogeny soft agar medium.

Reagents	**Nutrient agar medium** **Units per liter**	**Lysogeny soft agar medium** **Units per liter**
Peptone	5 g	----------
Yeast extract	1 g	5 g
Agar	16 g	6 g
Tryptone	----------	10 g
NaCl	----------	10 g
Deionized water	1000 mL	1000 mL

Organic solvents used for extraction of compounds and to dissolve extracts were ethyl acetate from PanReac AppliChem and VWR®; acetone from VWR®; dichloromethane from MERCK, dimethyl sulfoxide (DMSO) from VWR® and methanol from MERCK.

3. Extraction of compounds for small scale studies

In order to evaluate antimicrobial activity by the potential compounds synthetized by Planctomycetes, the first step was to extract in small scale those possible metabolites produced for further screening. After normal incubation in M607 agar plates, marine strains were then incubated for 7 days in 20mL of several of the liquid variations of media exposed in section (2.1), at 25ºC and 220 rpm. Each culture was then used to prepare four different types of extracts of compounds: three organic (from the cell pellet (P), from the supernatant of the culture (S) and from the crude culture (C)) and one aqueous, from the filtrate of the culture (F). For the aqueous extract, 2mL of culture were centrifuged at 13.300 rpm. The supernatant was then sterilized thought a 0.22 µm filter. For the organic extracts, an organic mixture consisting in 90% acetone plus 10% DMSO was prepared. To prepare the crude extract, 2mL of the organic mixture were mixed with 2mL of the culture for 1 hour under continuous shaking, at 25ºC. For the pellet and the supernatant extracts, 2mL of culture were centrifuged at 13.300 rpm for 5 minutes and the supernatant and the pellet collected. To each, 2mL of the organic mixture was added followed by incubation for one hour under continuous shaking at 25ºC. After incubation, 2mL of the upper phase of the three organic extracts (P, S and C) were passed to new tubes and dried to about half of the volume (during 12 hours). The respective volumes of the P extracts were then completed until 2 mL using sterile water. All extracts obtained were then stored at -20ºC until use.

4. Extraction of compounds for up-scaled studies

4.1. Liquid-liquid extraction with ethyl acetate

The secondary metabolite production in natural environments is normally very low and subsequently the resultant concentration of products of interest very scarce within the extracted mixture, since more compounds besides the ones pretended are also present at the same time. Based on this fact, an up-scaling of the cultures was made in order to enhance the amount of metabolites with interest. Several of the marine strains were therefore incubated in 500 mL of regular medium M607, for 7 days at 25ºC under continuous shaking (Fig. 4). One of the strains (strain MsF2) was also selected for incubation in 500 mL of two medium variations (see section 2.1): M607

medium supplemented with 1% of a solution of an autoclaved culture of *B. subtilis* plus 1% of algae extract and M607 medium only supplemented with 1% of the same algae extract. The two strains from freshwater environments (OJF2 and OJF8) were incubated in 1L of PYGV medium for 12 days at 30ºC under continuous shaking.

Fig. 4- Cultures of strain MsF2 in regular M607 medium ready for extraction, after 7 days incubating at 25ºC under constant shaking.

Ethyl acetate is a powerful organic solvent and it was used to extract possible compounds of interest produced from the obtained up-scaled cultures. Therefore, after the incubation period of the strains, ethyl acetate was added to the cultures at double of their amount in a separation funnel (Fig. 5A). Since ethyl acetate is not miscible with water (Fig. 5B), two different liquid phases in the mixture were formed. The lower phase was discarded and the upper phase (correspondent to an organic phase with ethyl acetate and possible compounds extracted) transferred to a balloon to be subsequently dried in a rotatory vacuum evaporator (Rotavapor® R-100 equipment from BUCHI). With temperature settled around 30ºC, pressure established around 100 millibar and rotation, ethyl acetate was completely removed by evaporation and 2 mL of DMSO were used to dissolve the solid residues achieved (Fig. 5C). The resultant extracts were then stored at -20º C until use.

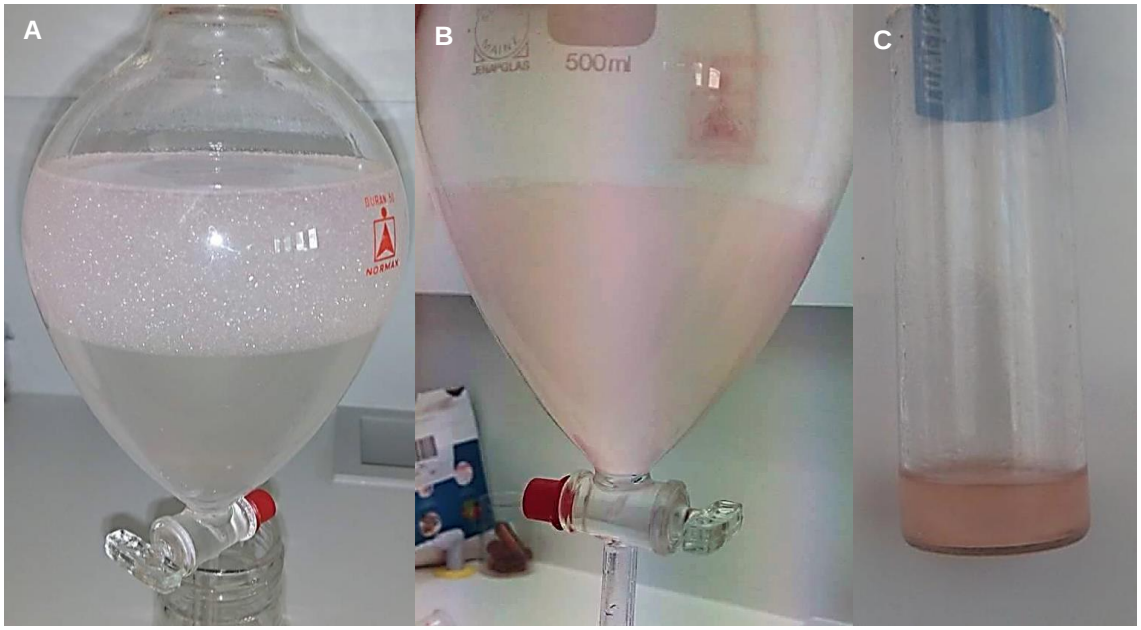

Fig. 5- Sequential phases of strain MsF2 crude extraction with ethyl acetate: A= beginning of the extraction; B= two final different liquid phases clearly distinguish (upper phase= ethyl acetate, lower phase=rest of the culture); C= resultant extract obtained after drying in a rotatory vacuum evaporator, dissolved in 2mL of DMSO.

4.2. Extraction with methanol

Another protocol for culturing and extraction of Planctomycetes was assayed. In order to enlarge even more the volume of culture used, 2L of medium M607 were used to incubate strain MsF2 during 7 days under constant shaking. After this period, cells were collected by centrifugation at 3600 rpm for 5 minutes, in an Eppendorf™ 5810R Centrifuge. To investigate a new type of extraction, methanol was the selected solvent, chosen by its common use for this purpose of extraction of bioactive molecules (Leão *et al*., 2013). This extraction, which was performed at collaboration with CIIMAR, consisted in subjecting the previously pelleted cells to numerous periods (30 minutes each) of incubation with the referred methanol (Fig. 6A). Pellet was then collected, leaving methanol with possible compounds extracted (Fig. 6B). The mixture was subsequently evaporated in a rotatory vacuum evaporator, with temperature settled around 30 ºC, pressure in 100 millibar and rotation. Two mL of methanol were used to dissolve the extract obtained and subsequently transferred to a glass vial. The mixture was then subjected again to evaporation in a rotatory vacuum evaporator under the same conditions and to a final drying in a freeze dryer (Telstar® equipment) (Fig. 6C).

Lastly, the extract was dissolved in 2 mL of DMSO plus 1 mL of methanol and stored at -20º C until use.

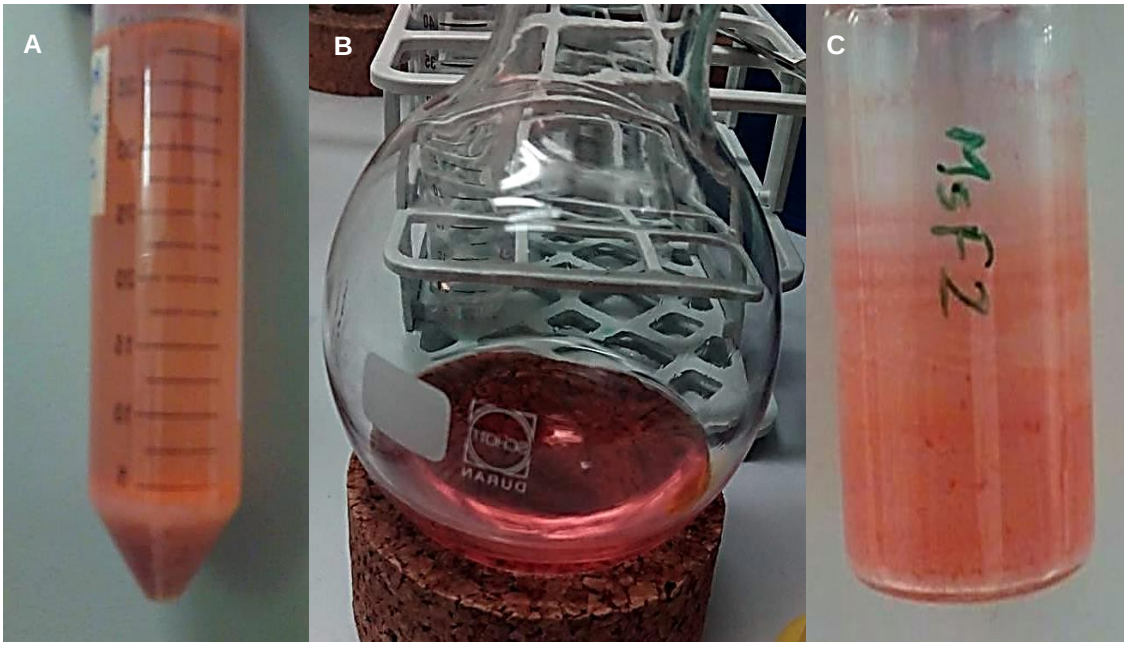

Fig. 6- Extraction of MsF2 cell pellet with methanol: A= incubation with methanol of the pellet obtained from centrifugation of the 2L culture of strain Msf2 in M607 medium; B= Methanol extract after filtration of the previous referred pellet; C= Resultant dried extract.

4.3. Incubation of cultures using XAD resin and extraction with ethyl acetate and acetone

More protocols were tested for incubation and extraction of metabolites from Planctomycetes. A special resin was used to allow the accumulation and further extraction of compounds possible present in the aqueous phase of the cultures. Resin Amberlite® XAD16N (Fig. 7A), from MERCK, is specifically designed to adsorb and hold hydrophobic compounds up to 40,000 MW, being antibiotics the ones with more interest for this thesis. At the same time, another up-scaling was intended. Thus, a 3L culture of strain MsF2 was prepared using medium M607 and incubated for 7 days at 25ºC under continuous shaking. At late of the exponential growth phase (around day 3) 1.5 g/100 mL of resin was added to the cultures (Fig. 7B and C). At the end of the incubation, the resin was separated from the rest of the culture.

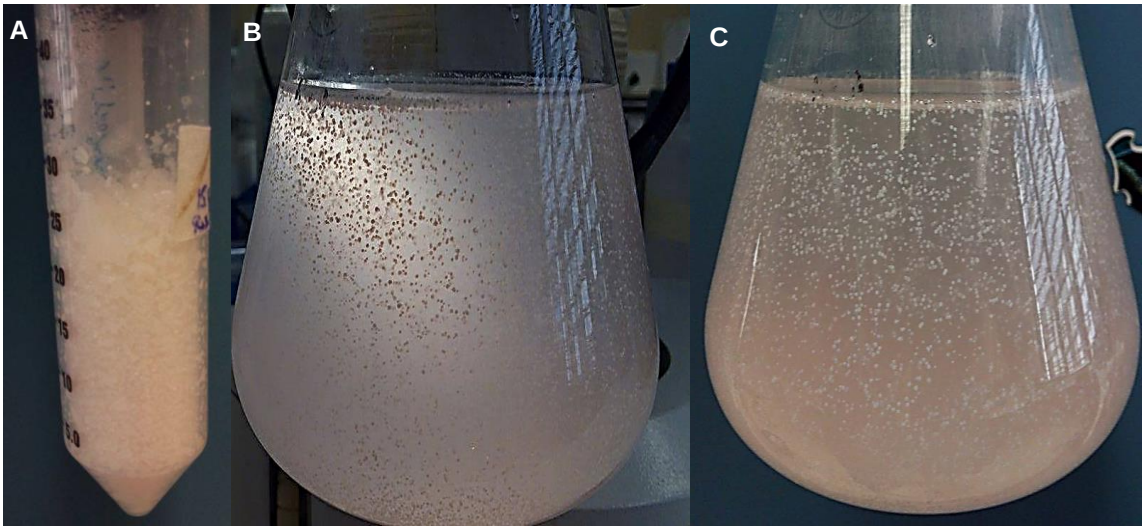

Fig. 7- Incubation of strain MsF2 with resin: A= falcon with the chosen resin XAD16N; B and C= cultures of strain MsF2 in M607 medium incubating with the referred resin.

For secondary metabolite extraction, 270 mL of ethyl acetate were added to the resin and incubated for 1.5 h. The extracted resin was again collected and stored for further use. Ethyl acetate was evaporated in a rotatory vacuum evaporator with pressure settled around 100 millibar, temperature around 30ºC and rotation. The solid residue obtained was dissolved in 5 mL of methanol and stored at -20ºC until use.

The same resin was after used for a second extraction, this time with acetone. This protocol was based on a recent study where the presence of bioactive compounds was demonstrated in Planctomycetes using the same methodology (Jeske *et al*., 2016). Therefore, 750 mL of acetone were added to the resin and incubated 1.5h. The resin was collected and the solvent was removed by evaporation in a rotatory vacuum evaporator with temperature settled around 30º C, pressure around 100 millibar and rotation. Eight mL of methanol were then used to dissolve the residues achieve. The culture (that remained after filtration of the resin) was also subjected to extraction. Thus, the 3L of culture were divided in 50mL falcons and centrifuged at 3600 rpm for 5 minutes. The cell pellets were then collected (Fig. 8A) and subjected to extraction with 100mL of ethyl acetate for 1h, under continuous shaking (Fig. 8B). The mixture was dried in a rotatory vacuum evaporator with temperature settled around 30ºC, pressure in 100 millibar and rotation. The residue obtained was dissolved in 2mL of methanol (Fig. 8C). The extracts obtained from these various extraction techniques were subsequently screened for bioactivity.

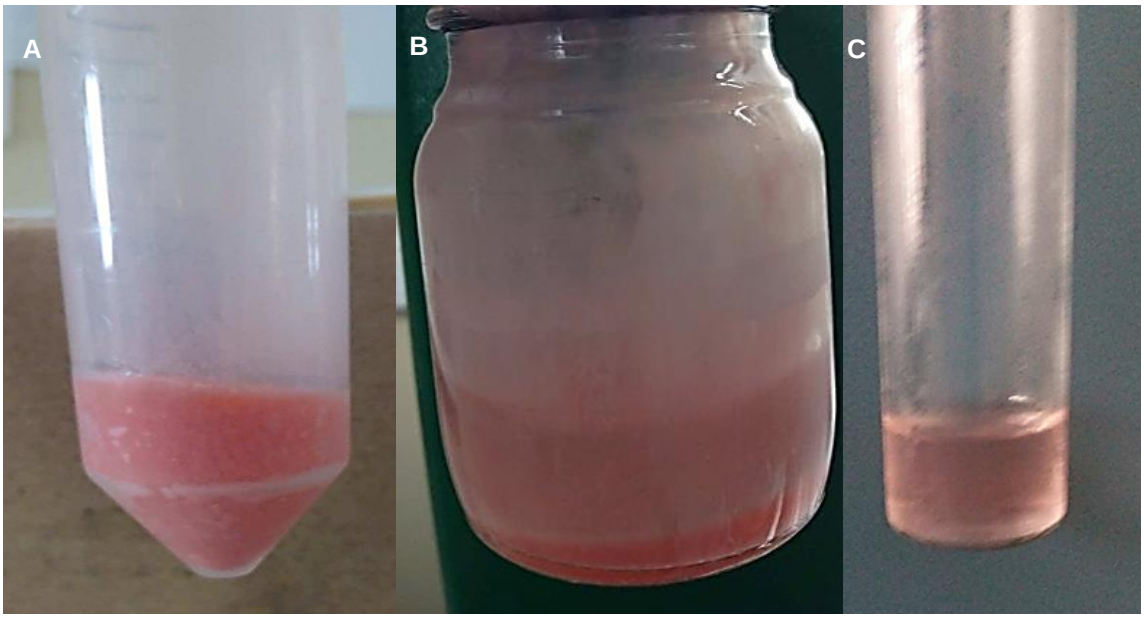

Fig. 8- Collection and extraction of MsF2 cell pellet with ethyl acetate: A= cell pellet collected after centrifugation of the 3L culture of strain MsF2 in M607 medium; B= organic extraction of the referred pellet with ethyl acetate; C= final extract obtained, dissolved in 2mL methanol.

5. Screening assays

5.1. Bioactivity assay in liquid medium

To firstly check if any of the extracts of Planctomycetes obtained from small scaled cultures (section 3) had any effect on the growth of a panel of pathogens, a bioactivity assay in liquid medium, carried out as described by Graça *et al*., (2015; 2016) with small modifications, was made. The targets used were chosen as representatives of the main groups of interest: *Bacillus subtilis* for Gram-positive bacteria, *Escherichia coli* for Gram-negative bacteria and *Candida albicans* for fungi. The cultures used were prepared according to the following procedures: they were incubated overnight in Nutrient broth at 37ºC under continuous shaking (220 rpm). The absorbance of the cultures was measured at 600 nm and the cell concentration adjusted to 2.5×10^5 CFU/mL, as described in more detail in the next paragraph.

To adjust the cell concentration of each target (with the objective of standardized the concentration used), 3 growth curves for each pathogen were initially performed. They were incubated overnight (OV) at 37ºC in Nutrient broth, with continuous shaking. Then, the initial absorbance at 600 nm was measure and the

process was repeated every 30 minutes until the microorganisms entered the stationary phase. In parallel, at every 30 minutes, each culture was used to make serial dilutions (with sterile water) with a factor of ten (from 10^{-2} - 10^{-8}) and 3 drops of 10 µL of each dilution were inoculated in Nutrient agar. The cultures were incubated for 24h at 37 ºC and the colony forming units (CFUs) were then counted. The analysis of the results consisted in the graphic representation of logarithmic data of absorbance or CFUs according to time and delimitation of exponential phase with subsequent determination of the correspondent equation and time of duplication. With the information given by this equation, the concentration of the cultures of the three targets was adjusted for the one that was pretended based on the optical density measured right before use.

The bioactivity assay in liquid medium was performed in 96 well-plates with flat bottom from Orange Scientific™ (Fig. 9). For each extract (assayed in triplicate), 10 µL were incubated with 90 µL of each standardized target culture in the respective well in the plate. Each target was also preferentially assayed in separate plates. Also in triplicate, four different positive controls for each target were used, in which 10 µL together with 90µL of the respective pathogen were incubated: for *Candida albicans*, the positive controls were four concentrations of amphotericin B (0.19 µg.mL^{-1}; 0.39 µg.mL^{-1}; 0.78 µg.mL^{-1} and 1.56 µg.mL^{-1}), for *Bacillus subtilis*, four concentrations of chloramphenicol (3.75 µg.mL^{-1}, 7.5 µg.mL^{-1}, 15 µg.mL^{-1} and 30 µg.mL^{-1}) and for *Escherichia coli,* four concentrations of rifampicin (62.5 µg.mL^{-1}, 125 µg.mL^{-1}, 250 µg.mL^{-1} and 500 µg.mL^{-1}). The three negative controls, also in triplicate, were as follow: 100 µL of Nutrient broth; 100 µL of each target culture and 10 µL of M607 liquid medium plus 90µL of each target culture. Initial absorbance measurement was performed at 600 nm in a Multiskan GO plate reader from Thermo Scientific™ and the plates were then incubated for 24 hours under continuous shaking at 37ºC. After the incubation period, a final absorbance measurement was also performed. Analysis of the results consisted in the calculation of the percentage of inhibition of the growth of the targets for which well, based in the optical density values obtained, using the following equations:

$$OD_R = Abs_F - Abs_I$$

In which OD_R= Optical density of each replica; Abs_f= Final absorbance after 24h; Abs_I= Initial absorbance at 0h.

$$OD_{Ext} = \frac{OD_{R1} + OD_{R2} + OD_{R3}}{3}$$

OD_{Ext}= Optical density of each extract (media of the triplicates).

$$\%_{\text{Inhibition for each extract}} = 100 - \frac{OD_{Ext}}{OD_C} * 100$$

Where OD_c= optical density of the control M607 medium + target culture, after 24h.

Inhibitory values above 20 % were considered as bioactive and having an effect in the growth of the targets.

Fig. 9- Example of a 96-well plate used in the bioactivity liquid assay, already filled with 10µL of the extracts of Planctomycetes/controls plus 90µL of standardized culture of *B. subtilis* in NA broth medium, before incubation.

5.2. Modified Kirby-Bauer assay

To complement the previously described bioactivity assay in liquid medium (96-well plate assay) and to serve as the main antimicrobial screening in this thesis, a protocol with small modifications to the commonly used Kirby-Bauer assay was prepared and followed.

The targets chosen to be tested against in this assay were *Micrococcus luteus* and *Bacillus subtilis*, since they are sensitive and easy bacteria to detect antimicrobial activity. Firstly, an OV culture of each target was made in Mueller-Hinton liquid

medium, at 37º C under continuous shaking. The OD at 600nm was then measured and the exact amount of culture to use (to allow a standardize concentration of 0.5 McFarland) determined using the following formula:

$$\text{Amount of culture to seed} = \frac{\text{Total volume of mixture to seed in each plate [mL]} * 10^7}{\text{OD}_{600\text{nm}} * (2 * 10^9)}$$

The culture was then subsequently incorporated in Mueller-Hinton agar medium and the mixture spread in rectangular Nunc™ OmniTray™ plates, squared petri dishes or regular round petri ones (for the bigger plates, 40 mL of the mixture were spread and for the normal round petri dishes, 20 mL). With adequate space in between, drops of extracts of Planctomycetes with 10 to 50 µL were poured into the surface of the agar (Fig. 10), following incubation of target cultures at 37º C. After 24h, inhibition halos were searched for in the spot of where the extracts were inoculated. The positive control for an example of inhibition was a drop of 10 µL of 0.1 µg/mL ampicillin or 1mg/mL ampicillin. At the same quantity of the extracts, the negative control that was used in each plate variated accordingly to the solvent in which every type of extract was dissolved (DMSO or methanol).

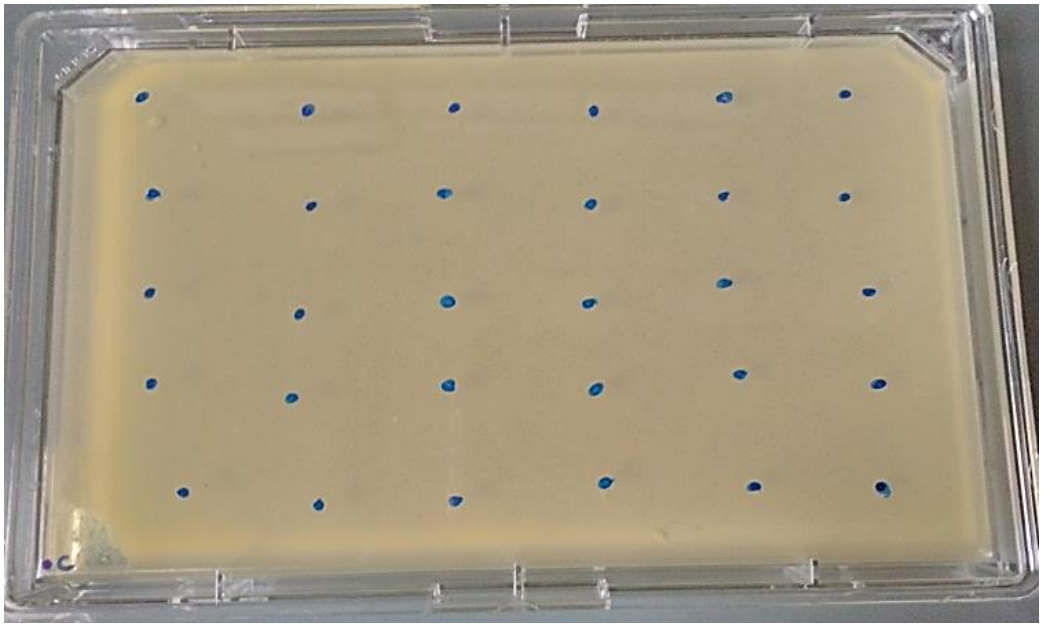

Fig. 10- Example of a NuncTM OmniTrayTM plate used in the modified Kirby-Bauer assay, filled with 40 mL of a standardized culture of *B. subtilis* incorporated in Mueller-Hinton agar. Blue dots correspond to the local were a drop of an extract was placed.

5.3 Well assay

Since the lastly described assay only permitted the use of, in maximum, 50 µL of each extract, another protocol was also used for the screening of antimicrobial activity, with the intent to enlarge the quantity of extract tested. This well assay has similarities with the modified Kirby-Bauer assay, where Mueller-Hinton agar plates were also incorporated with a standardized culture of *B. subtilis* or *M. luteus* (see section 5.2). On the other hand, instead of drops of extracts inoculated into the surface of the plate, wells were made directly into the agar and filled with 100 µL of each extract. Positive controls for an example of inhibition were 10 µL of 0.1µg/mL ampicillin and 1mg/mL ampicillin. At the quantity of 100 µL, the negative control that was used in each plate variated accordingly to the solvent in which every type of extract was dissolved (DMSO or methanol). The cultures were incubated at 37º C for 24h and inhibition halos were searched for near the wells.

5.4 Diffusion assay with filter discs

In order to prevent evaporation or diffusion complications by the compounds possibly present in the extracts that may occur in normal Mueller-Hinton agar cultures (which were used in the two screenings immediately described) a diffusion assay with filter discs was also used to test the antimicrobial activity of extracts of Planctomycetes. Similarly with the last screenings, OV cultures of *B. subtilis* and *M. luteus* were prepared and their concentration standardized (see section 5.2). Two layer plates were then organized: a first layer constituted by regular Mueller-Hinton medium (with 1.6 % agar) and a second layer of Mueller-Hinton soft agar medium (with 0.6 % agar) incorporated with the standardized target culture. Previously sterilized filter discs were then placed above the surface of the agar and filled with 50 or 100µL of each extract. The positive control used to show inhibition was a filter disc filled with 10µL of 1 mg/mL. Solvent controls were filter discs also filled with 50 or 100 µL of the solvent used to dissolve the respective extracts. Cultures were incubated at 37 ºC for 24h and inhibition halos were searched for near the discs.

5.5. Co-culture assay

To observe if live cultures of Planctomycetes were producing bioactive compounds that inhibit the growth of the targets, a co-culture assay was performed. Firstly, Planctomycetes were incubated for 3 days in M607 plates, at 25ºC. An OV culture of *M. luteus* was made in Mueller-Hinton liquid medium, at 37ºC, and the concentration standardized (see section 5.2). This culture was then incorporated in Lysogeny soft agar medium and the mixture poured above the surface of the agar plates with live cultures of Planctomycetes. These cultures were then incubated for 24h at 30ºC. Inhibition of the growth of the colonies of *M. luteus* was searched for near the colonies of Planctomycetes.

5.6. Evaluation of anti-*Chlamydia trachomatis* activity using a fluorescence-microscopy based assay

With the collaboration of Dr. Anna Klöckner in the Institute for Pharmaceutical Microbiology of University of Bonn, it was possible to test the antimicrobial activity of some of the extracts achieved against *Chlamydia trachomatis,* a pathogenic species from phylum *Chlamydiae.* Having in mind that *Chlamydiae* belong to the same super-phylum as *Planctomycetes* and that the usual bioactive producers are from other phylogenetical branches, it was of interest to see if molecules produced by Planctomycetes had any effect in the growth of this pathogen, besides the firstly used targets. On the other hand, chlamydial infections are also a concern, mostly in sub-developed countries. Its difficulty of treatment by its intracellular activity and the appearance of resistant strains make it necessary to search for new antibiotics.

Firstly, 200 µL of an available suspension of host cells (needed for chlamydial maintenance) from eukaryotic line Hep2 (previously grown in complemented Dulbecco's Modified Eagle medium (see section 2.2)) were collected and incubated in 96-well plates for 48h. Then, 50 µL of the supernatant of an available preparation of *Chlamydia trachomatis* D/UW-3/CX (mixed at 1:1 in Saccharose-phosphate-glutamate (SPG) buffer (75 g/L sucrose, 0.52 g/L KH_2PO_4, 1.53 g/L Na_2HPO_4 and 0.72 glutamate)) were added to the host cells, with 1.2 µg/ml cycloheximid. Cycloheximid is an inhibitor of protein biosynthesis in eukaryotic cells and was used to support the

chlamydial infection, which occurred for 48 h at 37 °C with 5 % of carbon dioxide (CO_2). Medium was then removed and renewed with 100 µL of fresh one and the cells exposed to several different treatments: extracts of Planctomycetes dissolved in DMSO within two amounts (2.56 µL and 1.28 µL diluted in 1.28 µL of sterile water), DMSO (also 2.56 µL and 1.28 µL diluted in 1.28 µL of sterile water) and 1µg/mL of the antibiotic ciprofloxacin. Cultures were then incubated for 30 h at 37 °C with 5 % CO_2 and successively fixed with ice cold methanol for 5 min. To evaluate the effects of these treatments in the chlamydial growth, samples were stained using fluorescein-conjugated antibodies specific for chlamydial lipopolysaccharides (LPS): Pathfinder Chlamydia Conformation System kit (from Bio-Rad, Germany) was diluted 1:5 with phosphate buffered saline (PBS) from ThermoFisher Scienthific and 100 µL were added to each well and incubated for 30 min at 37 °C. Evans' blue is a dye that additionally stains the host cell cytoplasm and it was already included in the lastly referred kit. For an extra staining of the host cell DNA, samples were also incubated for 1 min with 3 mg/mL of the fluorescent stain DAPI (4',6-diamidino-2-phenylindole) followed by 2 washing steps with PBS with 10 min each. The samples were then analyzed by fluorescence microscopy. For comparison, more two controls were prepared for this assay: infected cells with no treatments and non-infected cells.

5.7. Mode of action assay

To search for the cellular target of the compounds from Planctomycetes extracts, a mode of action assay was performed. Antibiotics generally function by disturbing the important biosynthetic pathways in bacteria. The type of pathway that is affected is determined by their mode of action. Previously genetically engineered strains of Bacillus *subtilis* 128 (designated as reporter strains) were transformed with pAC6-vector promotor fusions, each one containing one promotor specific for each main biosynthesis pathways in bacteria: cell wall biosynthesis= *PypuA*, DNA biosynthesis= *PyorB*, protein biosynthesis= *PyheI* and RNA biosynthesis= *PyvgS* (Urban *et al*., 2007). To evaluate if an extract has effects in one of these pathways, a LacZ gene copy was also added to the vector. This gene encodes for β-Galactosidase, which, in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is hydrolyzed into two compounds: galactose and the dye 5,5'-dibromine-4,4'-dichlorine, characterized by its deep blue color. As so, if any pathway is stressed by a compound,

the promotor is activated and the LacZ gene transcribed. The formation of the blue residue is the confirmation of this activation (Urban *et al*., 2007). To perform this assay, each reporter strain was incubated OV in liquid Mueller-Hinton complemented with 5 µg/mL chloramphenicol. Then, the concentration of the culture was standardized (see section 5.2) and incorporated in Mueller-Hinton agar, with an extra supplement of 5 µg/mL chloramphenicol and X-Gal (88 µg/mL for DNA, protein and RNA targets and 44 µg/mL for cell wall target). These mixtures were then spread in rectangular plates and drops of 50 µL of extracts from Planctomycetes placed above the surface of the agar, with adequate space in between. At the concentration of 500 µg/mL, four different types of controls were used to show an example of activation of each biosynthetic pathway: ampicillin for cell wall, tetracycline for protein synthesis, rifampicin for RNA and vancomycin for DNA. The cultures were then incubated for 24h at 37ºC. In the end, inhibition halos were searched for in the extracts spots, as well as the visualization of blue color around them (Fig. 11).

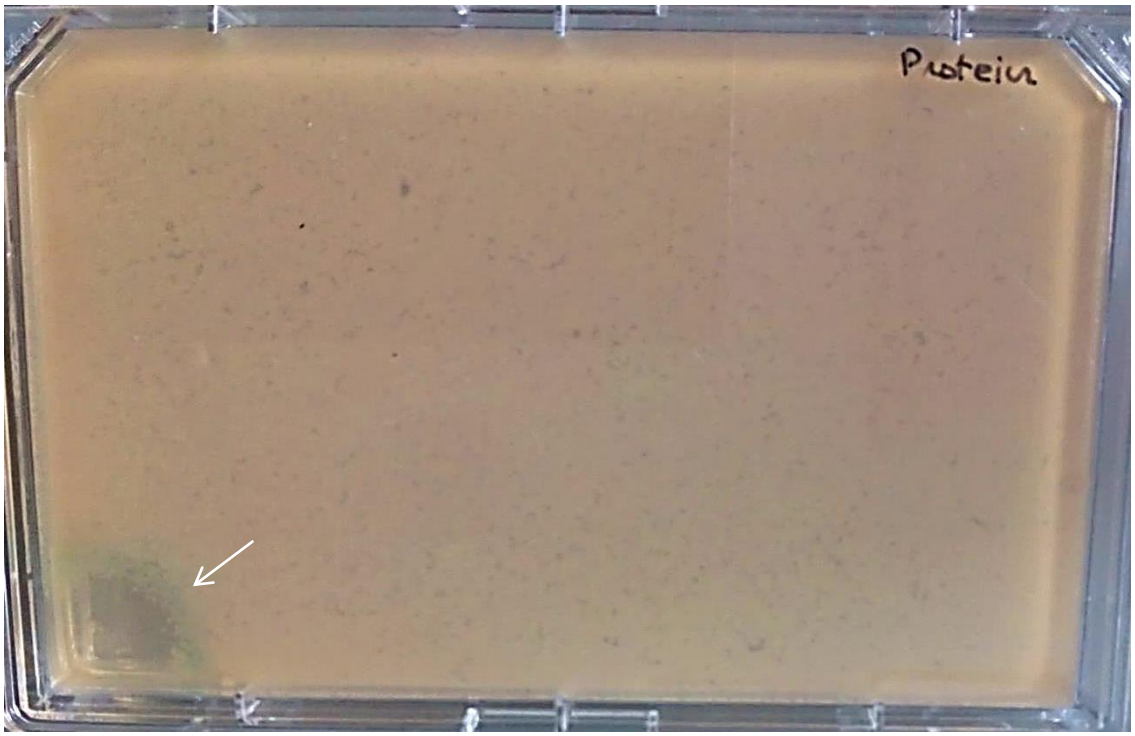

Fig. 11- Mode of action assay with *Bacillus subtilis* 128 transformed with pAC6-vector containing the promotor *PyheI* (for protein target), with an example of the respective promotor activation by 500 µg/mL tetracycline, as visualized by the appearance of blue color around the inhibition halo (arrow).

5.8. Anti-obesity assay using zebrafish larvae with Nile red staining

In collaboration with CIIMAR, it was possible to test extracts from Planctomycetes against other types of aims, such as the production/accumulation of lipids, using larvae of zebrafish as model organism. Unpublished studies with some of the same strains of Planctomycetes have showed that extracts of these bacteria have cytotoxic effects against different cancer cells types. Having this activity, the metabolites produced by these bacteria can possibly affect other targets inside the eukaryotic cells, such as the production of lipids. Zebrafish larvae were the chosen organism as a model for live evaluation of anti-obesogenic activity because of the facility of manipulation and easy visualization of the results, since it is possible to induce transparence into these larvae by adding a solution of phenylthiocarbamide (PTU), which inhibits the melanogenesis, reducing at the same time the necessity of mammalian studies (Noinart *et al*., 2017 and Tingaud-Sequeira *et al*., 2011). Zebrafish larvae also respond to known lipid regulator drugs similarly as humans (Jones *et al*, 2008).

Specifically for this assay, new extracts were obtained. Firstly, medium sized cultures (100mL) were prepared for 9 chosen strains of marine Planctomycetes (UC16, UC9, Rb, CcC6, MsF2, FF1, Gr7, UC 49.1 and UC13) in medium M607 and incubated for 7 days at 25ºC under continuous shaking. These cultures were then used for compounds extracting using ethyl acetate, following the protocol in section (4.1). The solid residues obtained were dissolved in 3 mL of dichloromethane, transferred to glass vials and left under evaporation for one day. The dry weight of the extracts was then calculated and standardized solutions with a concentration of 10 mg/mL were prepared with DMSO and stored at -20º C. For the obtainment of the larvae of zebrafish necessary for this assay, 10 adult males and 10 adult females belonging to *Danio rerio* species were firstly selected by their size (bigger individuals were preferred) and transferred to an aquarium prepared specifically for the reproduction and further collection of eggs (Fig 12A). One day after, eggs were available for harvest. They were firstly cleaned from the debris and fezzes (Fig. 12B) and then left for hatching in an aquarium with water at 25º C. After 72h, larvae were collected to petri dishes with egg water (see section 2.2) (Fig. 12C). They were then sorted by size and transferred in groups of 7 per well to a 48-well plate, along with some egg water (Fig. 13).

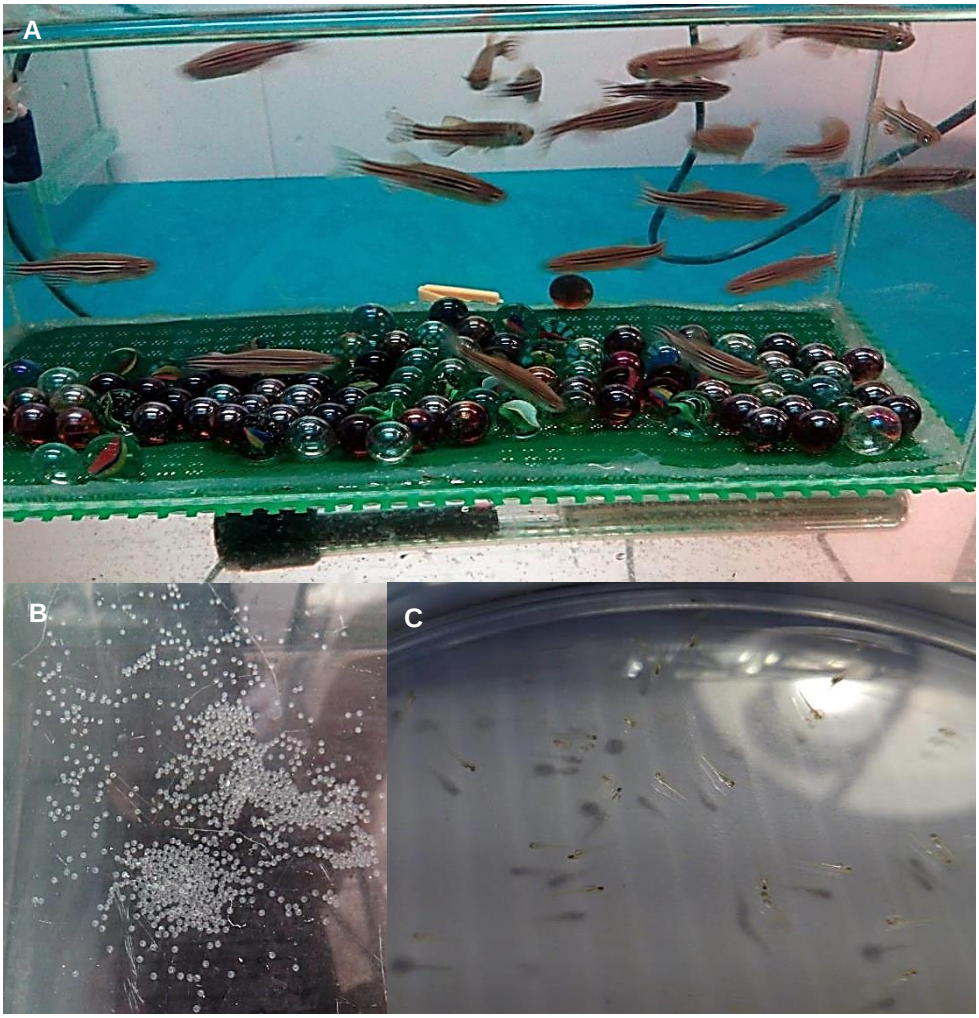

Fig. 12- Obtainment of larvae of zebrafish for use in the anti-lipid production assay: A= Aquarium used for adult zebrafish reproduction. Ten females and ten males were previously selected by their size (bigger individuals were preferred). Temperature of the water was settled at 25ª C; B= Eggs harvested after reproduction; C= Hatched larvae after 72h incubation of the eggs at 25º C.

For the beginning of the exposure to different treatments, in each well, the egg water was firstly withdrawn with a syringe and renewed by 625 µL of a solution of egg water supplemented with 200 µM phenylthiocarbamide (PTU). The several treatments were then added to the respective wells: 0.75 µL of Planctomycetes extracts, 0.75 µL of the solvent/negative control DMSO and 0.75 µL of a previously prepared solution of 11.412 mg/mL resveratrol (positive control to show lipid inhibition) dissolved in DMSO (Fig. 13). After 24h of exposure, the mixture in each well was again renewed accordingly to the amounts previously described. Fifteen microliters of a 500 mg/mL solution of Nile red dissolved in acetone were also added to each well, to allow a fluorescent staining of both membrane and intracellular neutral lipids. After a final

incubation of more 24h, larvae were anesthetized with 3 to 4 drops of a solution of 0.3 % tricaine and the larvae of each well transferred to concave slides in groups of three or four. The fluorescence of Nile Red was induced in a Leica DM6000 microscope (excitation wavelength of 515-560 nm) and images of the larvae and fat accumulation were obtained, to subsequent analysis and calculation of the intensity of fluoresce using the software ImageJ. The mean level of fluorescence intensity obtained for all larvae exposed to the negative control (DMSO) was considered as 100 % lipid production/accumulation and so taken as 0 % activity of lipid production inhibition. The mean percentage of inhibition of lipid production was then calculated for each treatment, using this control to comparison. All data obtained from the ImageJ analyzes to all larvae was also statistically treated using an one-way analysis of variance (ANOVA) test done in Microsoft® Office Excel, with alpha level set at 0.5. Dunnett's test was also performed to evaluate statistic differences between each treatment and the control.

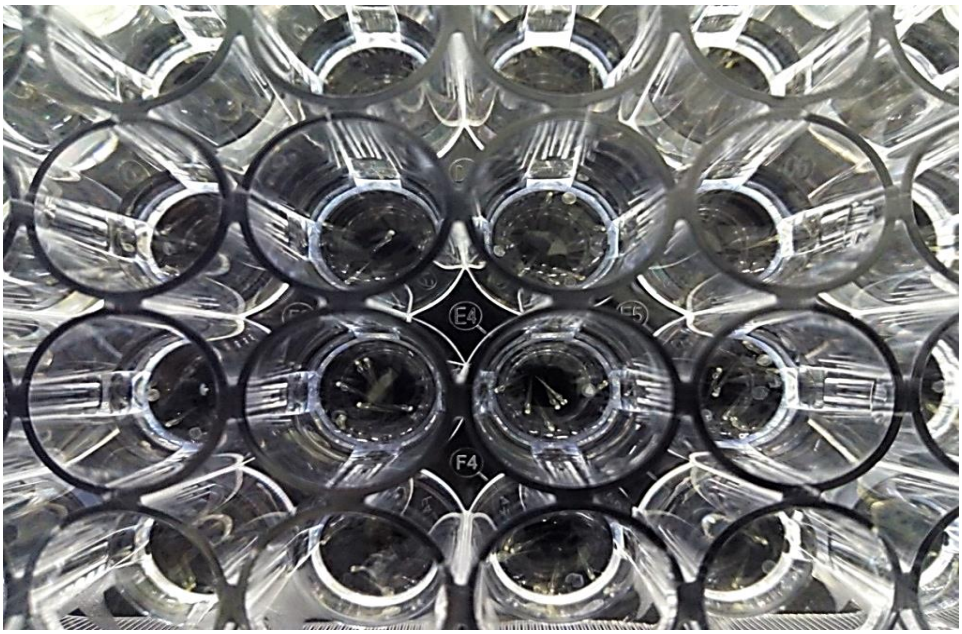

Fig. 13 - 48-well plate prepared for the anti-obesity assay, in the beginning of the exposure: in each well, 7 larvae were initially placed, as well as 625 µL of egg water + 200 µM PTU and 0.75 µL of 10 mg/ml extracts from Planctomycetes.

6. Gel permeation chromatography

In order to begin the isolation of the compound/s of interest from the screened heterogeneous extracts obtained from the cultures of Planctomycetes, gel permeation

chromatography was the technique chosen because it allows the separation of the existing components in mixtures by their molecular size into different fractions.

A fresh culture of strain Gr7 was prepared in 500mL of medium M607 complemented with 1% of algae extract. After 7 days incubation at 25ºC under continuous shaking, this culture was extracted with ethyl acetate, as described in section (4.1). At the end, the solid residue achieved was dissolved in 2mL of methanol, since this is the solvent used in the chromatography. In collaboration with the Department of Chemistry from Sciences Faculty of Porto University, gel permeation chromatography was performed to the obtained extract in a device with a GILSON® pump and a Sephadex LH20 column, chosen by its normal use to separate terpenoids and small weight peptides. This column was previously stabilized overnight with methanol before use. With 2mL of the extract injected into the column at the velocity of separation of 1 mL/minute, fractions were collected every 15 minutes, from minute 0 to minute 225 (Fig. 14). Then, every fraction was dried in a rotatory vacuum evaporator, with temperature settled around 30ºC, pressure in 100 millibar and rotation. The residues obtained from each fraction were dissolved in 2mL methanol and the antimicrobial activity evaluated. The final objective was the search for inhibition halos after 24h of incubation at 37ºC, to see if and which fractions were bioactive to be chosen for further fractioning (aiming at a final isolation of the compound).

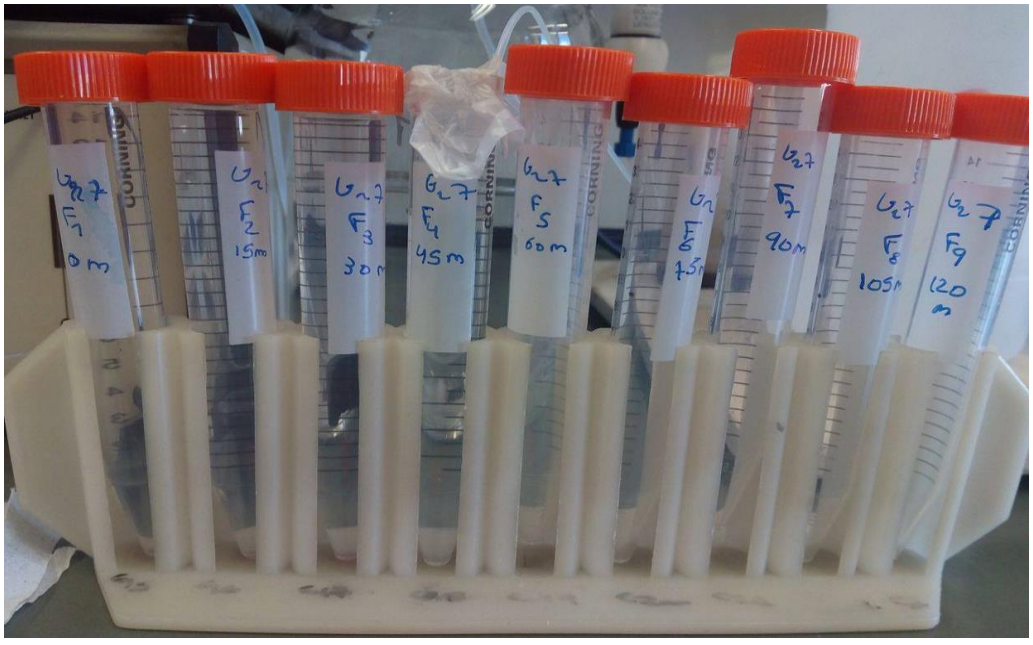

Fig. 14- Collection of fractions obtained from gel permeation chromatography of strain Gr7 extract (from 500mL culture in medium M607+A).

Results

1. Small scale studies

1.1. Preliminary screenings

To achieve assays reproducibility, the preliminary antimicrobial screening performed was based on a certain and constant level of the target strains. Consequently, the growth of the three selected targets (*B. subtilis*, *E. coli* and *C. albicans*) was firstly studied and the time of duplication of each strain calculated. The duplication times obtained were 30.12 minutes for *B. subtilis, 49*.35 minutes for E. coli and 78.92 minutes for *C. albicans*. The relationship between the absorbance reads at 600 nm and the CFUs were also calculated and used for each target culture to standardize its concentration, according to the following equations:

$$\text{For } B.subtilis\text{: } \ln(CFUs) = \frac{0.0251\ln(Culture\ abs\ 600nm) + 0.2210}{0.031} + 7.4049$$

$$\text{For } E.coli\text{: } \ln(CFUs) = \frac{0.0121\ln(Culture\ abs\ 600nm) + 0.0449}{0.013} + 12.152$$

$$\text{For } C.albicans\text{: } \ln(CFUs) = \frac{0.0112\ln(Culture\ abs\ 600nm) + 0.0316}{0.004} + 7.601$$

In the preliminary 96-well plate screening of 12 marine Planctomycetes (UAP119, UAP120, UAP121, UAP127, UAP128, PAP33, PAP34, PAP61, PAP64, PAP67, SAP113 and SAP114), the majority of the 10 µL DMSO+acetone extracts (obtained from 2 mL of cultures) (see section 3 from the methodology) showed to induce inhibition against *B. subtilis* (Fig. 15). Only inhibitions higher than 20% were considered. Although all inhibitions were lower than the positive control of 30 µg/mL chloramphenicol (82 %) several values higher than 60 % were obtained. In general, the most effective extracts were the ones from the cell pellets (mean inhibition values of all extracts = 65 %), followed by the extract from the supernatant (mean value of 48 %). The crude extracts were the ones showing lower inhibitory effects. Strains SAP113 and UAP127 where the ones with extracts that surpass 70 % inhibition. These results indicated that the higher bioactivity was obtained in organic extracts, especially from the cells. The aqueous extracts (F), although all bioactive with levels higher than 30 %

(with the exception of SAP114) were lower than the organic ones (S and P). Why the crude extract was lower than all the others is behind comprehension. These results are consistent with the results obtained by Graça *et al*. (2016), where other marine Planctomycetes were screening with this kind of assay. However, no bioactivity was observed for any extract against the other two targets, the fungus *C. albicans* and the resistant *E. coli*. Graça *et al* (2016) observed bioactivity against *C. albicans*, but also not against *E. coli*.

In a similar assay with the same strains but performed with extracts from cultures using medium M600, measurement of bioactivity was impossible because the negative control (target culture exposed only to medium M600) showed inhibitory effect on the targets growth. This fact is unfortunate, as the higher levels of bioactivity obtained by Graça *et al*., (2016) were in extracts obtained from medium M600.

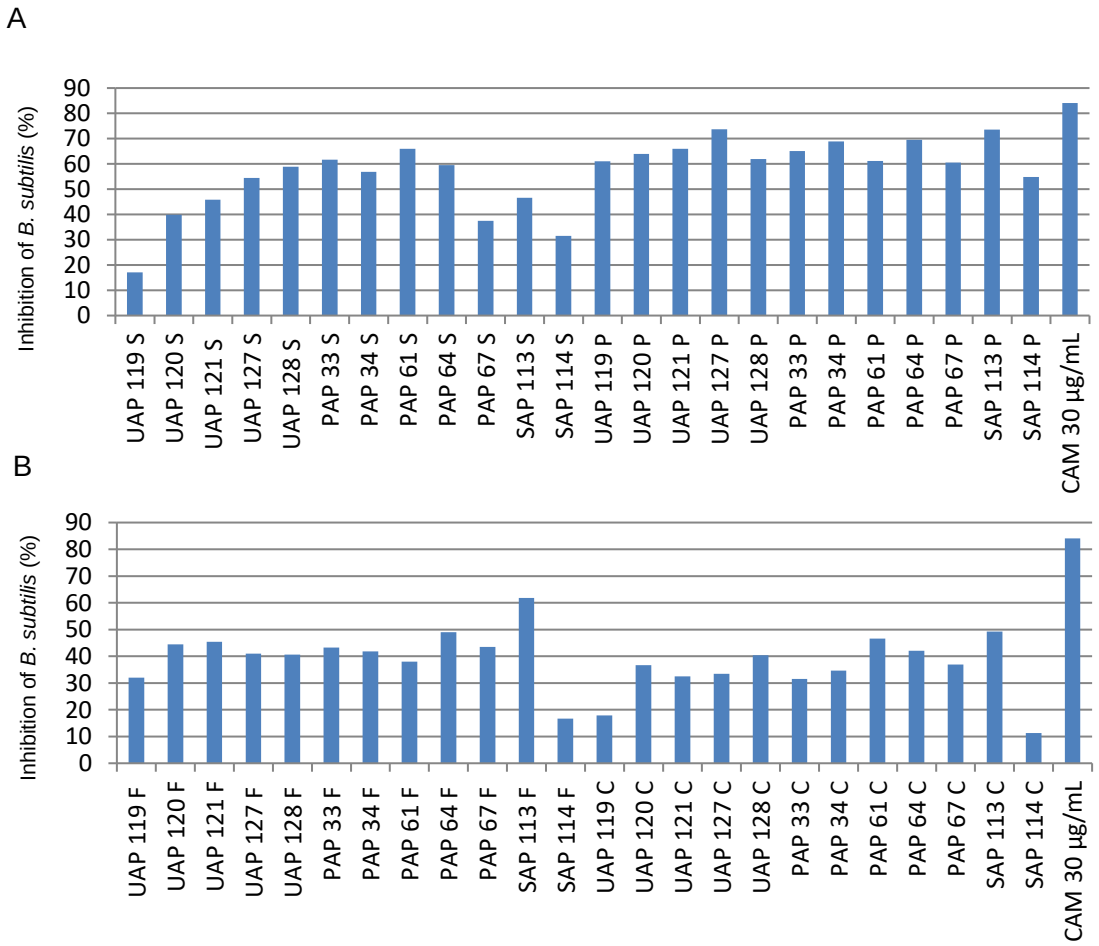

Fig. 15- Inhibition levels of *B. subtilis* after exposure to different extracts of Planctomycetes: A- treatment with the extracts obtained from the supernatant (S) and pellet (P) of centrifuged cultures. B- treatment with the extracts obtained from filtrated cultures (F) and crude cultures (C). 30 µg/mL chloramphenicol (CAM) was the positive control. The levels of inhibition are the ones obtained relative to *B. subtilis* exposed to M607 medium (0% inhibition). Almost all extracts showed inhibition against this target above 20 %, with some reaching more than 60 %, although none as the chloramphenicol positive control.

To complement the previous preliminary screening, a modified Kirby-Bauer assay was performed at the beginning of the internship in Bonn, with an enlarged number of marine Planctomycetes (35 strains) (Table 1) that allowed the screening of a higher diversity. However, the medium M607 for strain cultivation was prepared with artificial sea water, due to the unavailability of natural one. The target strains were *B. subtilis* and *M. luteus* and were also standardized to a similar concentration, for assay reproducibility. In the screening against *M. luteus* with the 10 µL droplets of the DMSO+acetone extract (obtained from 2 mL of cultures), two of them (pellet extracts from strains FF1 and MsF2) showed a slight inhibition halo (Fig. 16). However, against *B. subtilis* no inhibition halo was observed for any extract (Fig. 17).

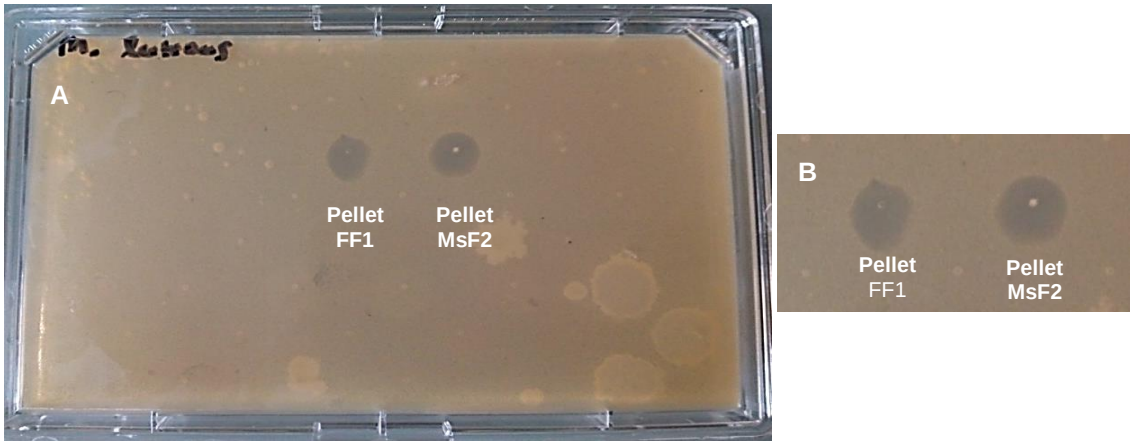

Fig. 16– Modified Kirby-Bauer assay with small-scale extracts of Planctomycetes tested against *M. luteus*, after 24h incubation at 37º C. A= two inhibition halos were visualized for Pellet extract from both strains FF1 and MsF2. B= referred inhibition halos in more detail.

Fig. 17 – Modified Kirby-Bauer assay with small scale extracts of Planctomycetes tested against *B. subtilis*, after 24h incubation at 37º C. The control was 10 µL of 1µg/mL ampicillin (C). No inhibition halos were observed for any extract.

The preliminary antimicrobial screening with the modified Kirby-Bauer assay confirmed, although weakly, the previous referred potential of marine Planctomycetes to produce bioactive compounds (the 96-well assay and the data from the literature (Graça *et al*., 2016)). Therefore, an optimization was required in order to potentiate the bacterial inhibitory capacity.

1.2. Optimization of incubation conditions: organic stress, biological stress, natural environment simulation, chemical stress and NAG as carbon source

Still in a small scale, various modifications to the media used for incubation of the marine planctomycetes with the objective to optimize the production of bioactive compounds were performed. To induce organic stress, strains were incubated in 20mL of medium M600 (which as a 4x fold the organic sources concentration of medium M607). Extraction with DMSO+acetone was made to 2 mL of the 20 mL cultures (see section 3 from the methodology) and the screening was made with the modified Kirby-Bauer assay against *M. luteus,* with 10 µL droplets of extract. However, no inhibition halos were observed for any of the extracts (Fig. 18).

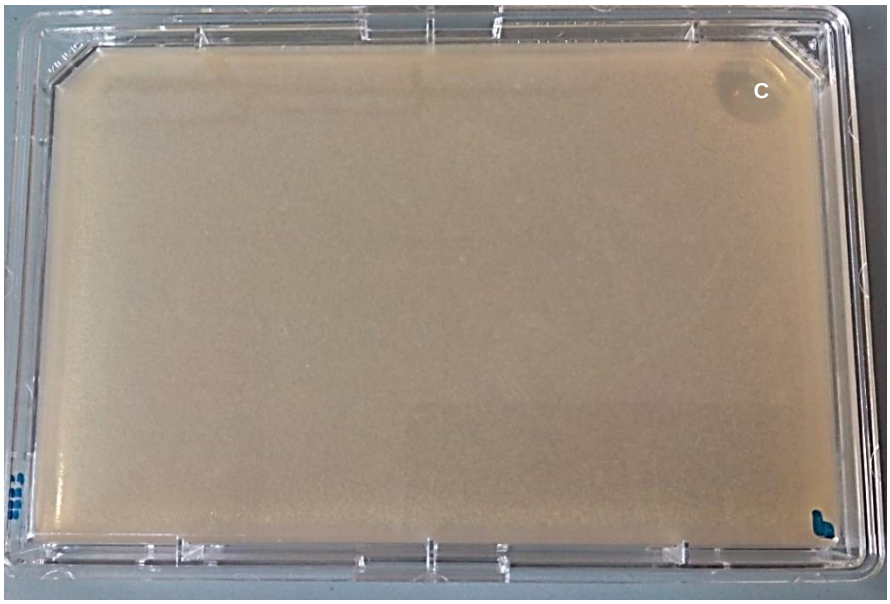

Fig. 18– Modified Kirby-Bauer assay with small scale extracts of Planctomycetes (from M600 cultures) tested against *M. luteus* after 24h incubation at 37º C. The control was 10 µL of 1µg/mL ampicillin (C).No inhibition halos were observed for any extract.

To induce biological stress and to simulate competitive natural environmental conditions, fresh cultures of the marine Planctomycetes were prepared in M607 medium supplemented with 1% of an autoclaved culture of *E. coli* and 1% of a solution of algae extract. These cultures were then extracted and screened in the same way (see sections 3 and 5.2, respectively, from the methodology), against *B. subtilis* and *M. luteus*, using 10 µL drops of extract. However, no inhibition halos were, again, visualized for any extract (Fig. 19).

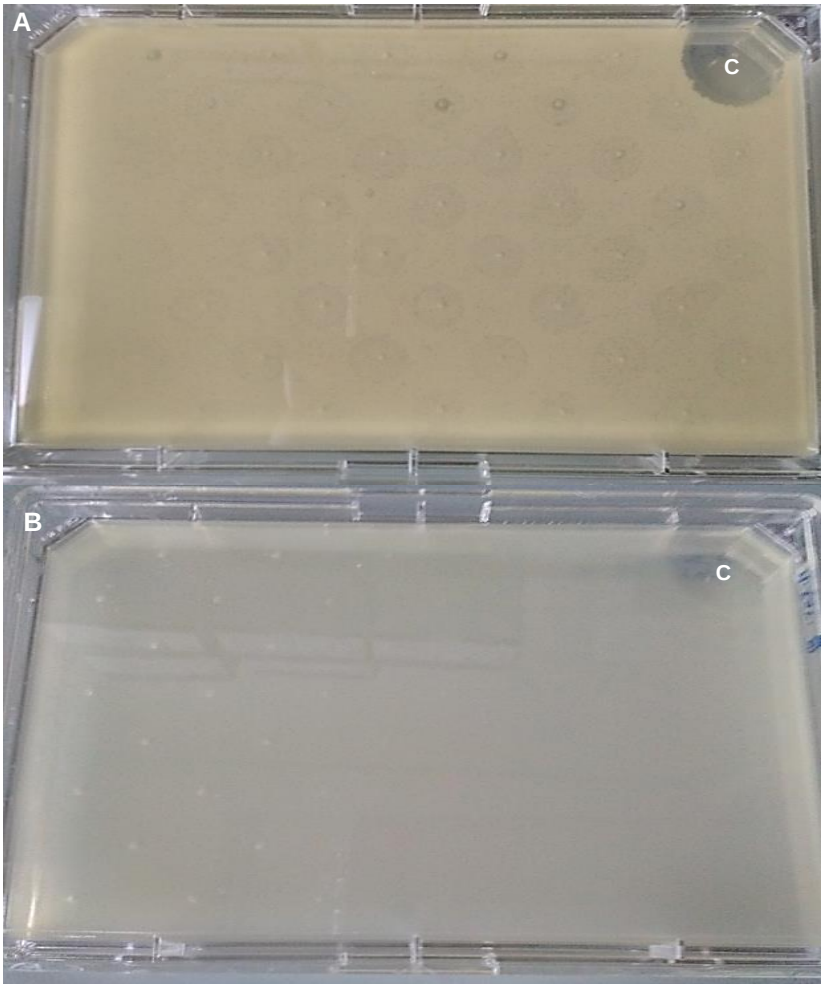

Fig. 19 – Modified Kirby-Bauer assay with extracts of Planctomycetes (from biologically stressed cultures) tested against *B. subtilis* (A) and *M. luteus* (B), after 24h incubation at 37ºC. The control was 10 µL of 1µg/mL ampicillin (C). No inhibition halos were visualized for any extract.

To simulate salt stress (chemical stress), fresh cultures of the marine Planctomycetes were incubated in medium M607 with higher percentage of salt (an increase of 15 %). For comparison, all marine strains were also cultured in the normal medium M607. The extracts were obtained following the same protocol, with 2 mL

different extracts in DMSO+acetone (see section 3 from the methodology) and freeze dried for better preservation of the activity. Before use, they were dissolved in 2mL DMSO and tested against *M. luteus* in the screening described as the well assay, to allow higher concentration of extract tested (100 µL volume drops). However, no inhibition halos were visualized for any extract (Fig. 20).

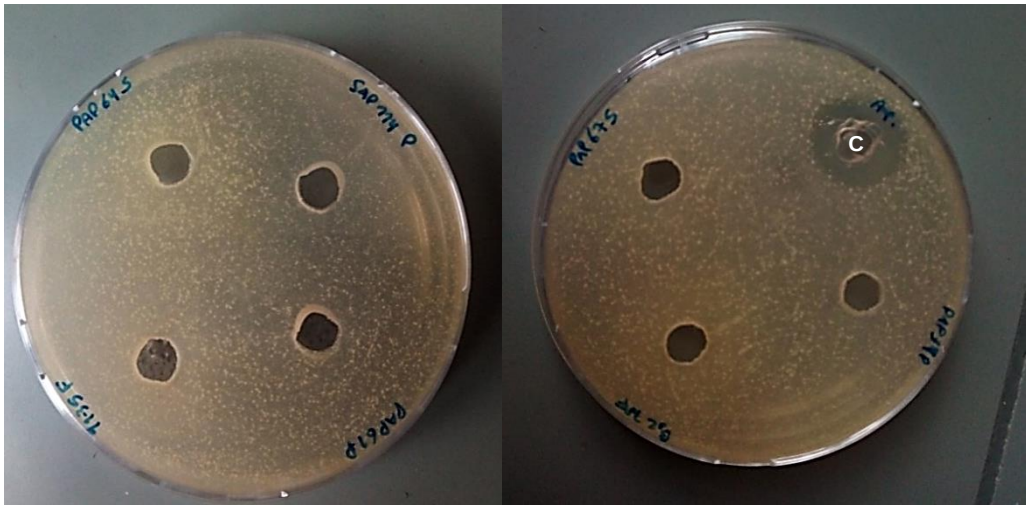

Fig. 20- Well assay where small scale extracts from Planctomycetes (from chemical stressed cultures) were tested against *M. luteus,* after 24h incubation at 37ºC. The control was 10µL of 10µg/mL ampicillin (C). No inhibition halos were observed for any extract, as it is exemplified with these two plates.

Contrarily to what was observed in the preliminary screenings, where MsF2 and FF1 extracts inhibited *M. luteus,* neither the extracts obtained from the various types of stressed cultures nor the extracts from the repeated normal medium cultures presented antimicrobial activity against the chosen targets. Even the use of the well assay to allow 10 fold higher concentration of extract did not enhance the visualization of anti-microbial activity. As in a previous study it was demonstrated that medium supplemented with NAG instead of glucose enhanced the production of secondary metabolite by Planctomycetes (Jeske *et al*, 2016), the two strains that already showed activity, FF1 and MsF2, were assayed under this medium composition. As previously, 2 mL of cultures were extracted in DMSO/acetone (see section 3 from the methodology). To screen for antimicrobial activity, extracts were tested against *M. luteus* in the modified Kirby-Bauer assay, with 10 µL of extract used. However, no activity against this target was visualized in this assay.

1.3. Diffusion assay with filter discs

Since the previous medium alterations used in the incubation of the strains did not potentiate the inhibitory capacity of the extracts, another modification to the antimicrobial screening was made to prevent possible evaporation or diffusion problems by the extracts. MsF2 was the strain chosen, since it was one of the two strains that already revealed antimicrobial activity in the preliminary screening. Thus, a fresh 20mL culture in M607 medium was incubated and 2 mL centrifuged and sterilized through a filter, making the filtrate extract (see section 3 from methodology). The extract was then tested using the diffusion assay against *M. luteus* and *B. subtilis* with filter discs filled with 100 µL. However, no inhibition of the growth of both targets was visualized for the Planctomycete extract (Fig 21).

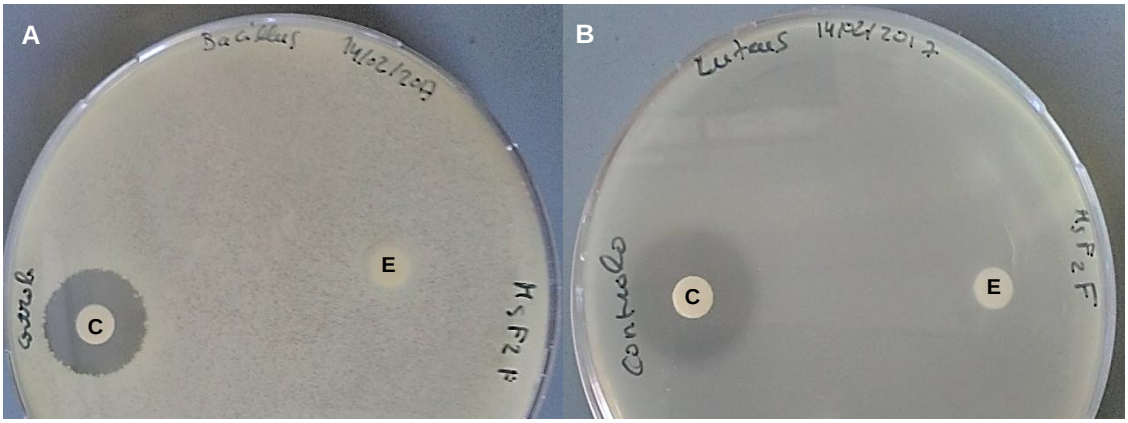

Fig. 21- Diffusion assay with filter discs were MsF2 extract (E) was tested against *B. subtilis* (A) and *M. luteus* (B). The control was a filter disc filled with 10 µL of 0.1µg/mL ampicillin (C). No inhibition was observed near the filter discs filled with the extract.

1.4. Co-culture assay

Due to absence of bioactivity of the extracts screened, it was decided to search for antimicrobial activity directly on live cultures of Planctomycetes. Fresh cultures of all marine Planctomycetes in agar medium M607 were incubated for 3 days and then overlaid with an extra layer of Lysogeny soft agar medium incorporated with a standardized culture of *M. luteus* (see section 5.5 from the methodology). After incubating for 24 at 30º C to allow the growth of *M. luteus*, no inhibition of the target was observed, even not near the colonies of Planctomycetes (Fig 22).

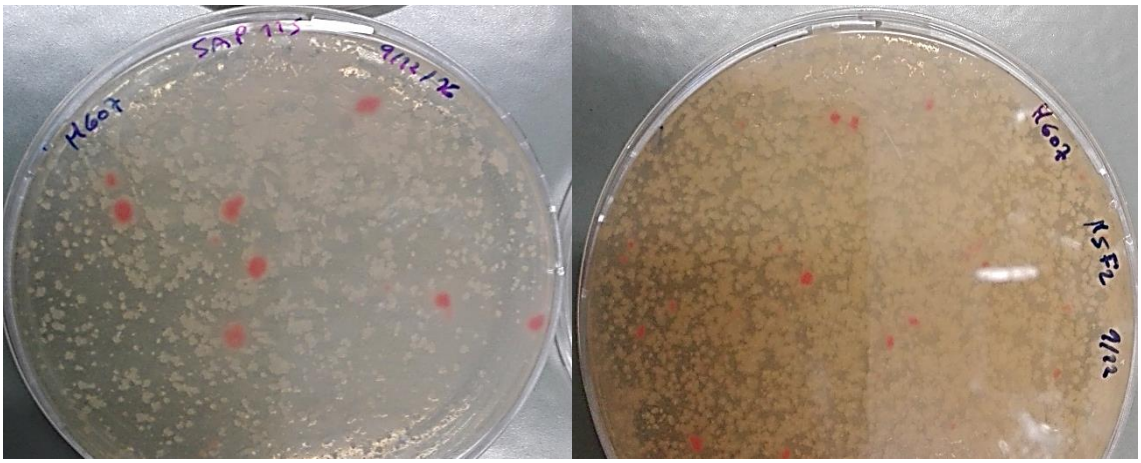

Fig. 22- Co-culture assay with live strains of Planctomycetes in M607 medium against *M. luteus*. No inhibition of the target was observed near the colonies of Planctomycetes (in pink).

2. Up-scaled studies

2.1. Liquid-liquid extraction with ethyl acetate

As almost none of the small-scaled screenings or the co-culture assay allowed the obtainment of bioactivity, up-scaled culturing was assayed. Thus, 500 mL cultures in medium M607 were therefore prepared. Due to the impossibility of making such higher volume cultures with all strains, MsF2, FF1, UC9 and Gr7 were the chosen ones, either because they presented antimicrobial activity in the preliminary screenings or by their previously mentioned potential (Graça *et al*., 2016) (the NaPDoS prediction pathway products for strains UC9 and Gr7 were, respectively, bacitracin and myxalamid, two known antibiotics). Furthermore, MsF2 was also cultured two more times, to try to enhance even more the production of the bioactive molecules: one in 500 mL of medium M607 complemented with 1% of a solution of an autoclaved culture of *Bacillus subtilis* plus 1% of algae extract (M607+A+*Bacillus)* and another in 500 mL of medium M607 only complemented with 1 % of the same algae extract (M607+A). For all these higher volume cultures, artificial sea water was substituted by the sea salts, in preparation of the media. To extract compounds from these up-scaled cultures, another organic reagent was chosen: ethyl acetate. This solvent offers more benefits than DMSO and acetone, such as a higher strength of extraction and immiscibility with water (Siek 1978). Thus, the extracts from the prepared cultures were done with ethyl acetate as referred in the methodology (see section 4.1 from the methodology) and the solid residues obtained dissolved in 2mL DMSO and screened for antimicrobial activity in different assays.

2.1.1. Antimicrobial screening against *B. subtilis* and *M. luteus*

The modified Kirby-Bauer was firstly used to screen antimicrobial activity from the previous referred ethyl acetate extracts obtained from up-scaled cultures of the four Planctomycetes (MsF2, FF1, UC9 and Gr7), against both *B. subtilis* and *M. luteus* (with 50 µL of each extract tested). MsF2 extracts from M607 and M607+A+*Bacillus* showed firstly antimicrobial activity (Fig 23). The rest of the extracts were later screened and the antimicrobial activity was also observed for all of them, including with the first referred two, which were also one more time assayed (Fig. 24). All extracts showed antimicrobial activity against the two targets, although in a more intense way against *B. subtilis* (Fig. 24). Both targets are Gram positive and sensitive bacteria but the antimicrobial action of extracts from these strains against *B. subtilis* was for the first time demonstrated in a solid medium assay, contrarily to *M. luteus*, which already showed susceptibility against Planctomycetes extracts since the preliminary screenings. The extract from MsF2 cultured in M607+A was the one showing the highest bioactivity against *B. subtilis*, compared to the ones obtained from MsF2 cultured in M607 and M607+A+*Bacillus* (Fig. 24A). These results evidenced that the presence of algal material in the medium may influence positively the capacity of MsF2 to produce bioactive antimicrobial compounds. However, when MsF2 was incubated in M607+A+*Bacillus*, the inhibition levels were similar to the ones of MsF2 incubated in normal M607. The solvent control (DMSO) did not present antimicrobial activity.

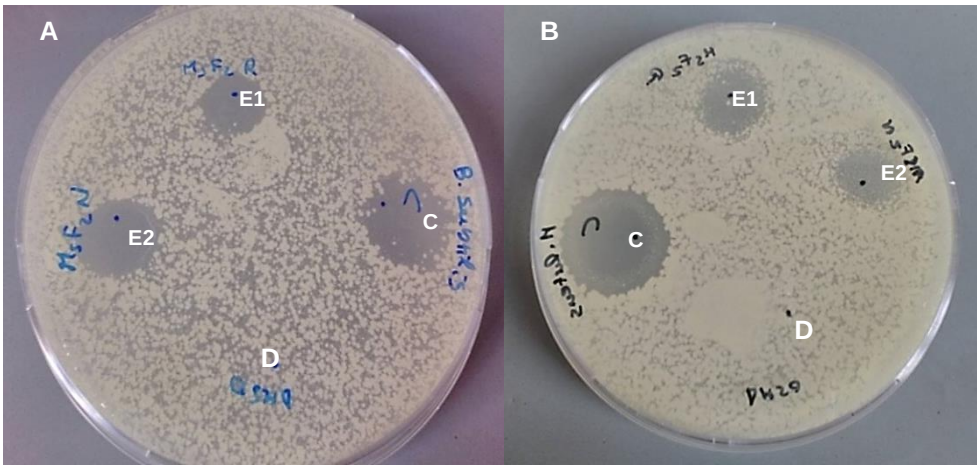

Fig. 23- Modified Kirby-Bauer assay where up-scaled Planctomycetes extracts were tested against *B. subtilis* (A) and *M. luteus* (B), incubated at 37ºC for 24h. E1= ethyl acetate extract from MsF2 cultured in 500mL of medium M607; E2= ethyl acetate extract from MsF2 cultured in 500mL of medium M607+A+*Bacillus*; C= control of 10 µL of 1mg/mL ampicillin; D= control with 10 µL DMSO. Inhibition halos were observed for the two extracts. Control with DMSO did not present antimicrobial activity.

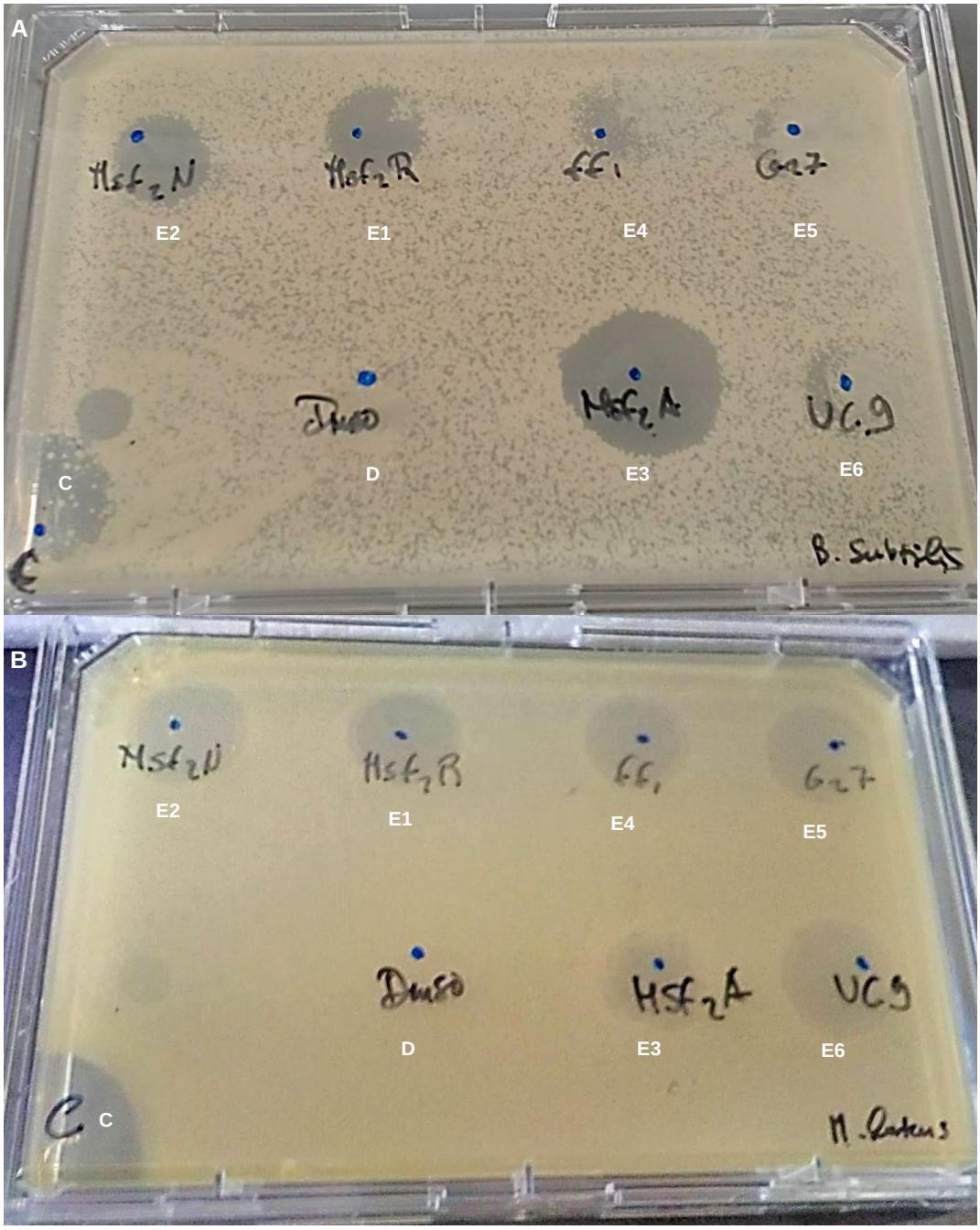

Fig. 24- Modified Kirby-Bauer assay where up-scaled Planctomycetes extracts were tested against *B. subtilis* (A) and *M. luteus* (B), incubated at 37ºC for 24h. E1= ethyl acetate extract from MsF2 cultured in 500mL of medium M607; E2= ethyl acetate extract from MsF2 cultured in 500mL of medium M607+A+*Bacillus*; E3= ethyl acetate extract from MsF2 cultured in 500mL of medium M607+A; E4= ethyl acetate extract from strain FF1 cultured in 500mL of M607 medium; E5= ethyl acetate extract from strain Gr7 cultured in 500 mL of M607 medium; E6= ethyl acetate extract of strain UC9 cultured in 500 mL of M607 medium; C= control of 10 µL of 1mg/mL ampicillin; D= solvent control with 10 µL DMSO. Inhibition halos were observed for all extracts against both targets, although more evident against *B. subtilis*. Against this target, strains FF1, Gr7 and UC9 showed more weakly inhibition halos. Extract from the strain MsF2 cultured in M607+A was clearly the one that presented a higher bioactivity. Control with DMSO did not present antimicrobial activity.

Another Planctomycete, strain UC49.1, was latter screened because it showed anticancer activity in studies performed in our laboratory (unpublished results). This Planctomycete was directly cultured in 500 mL of medium M607+A (prepared with natural sea water), extracted with ethyl acetate (see section 4.1 from methodology) and screened for antimicrobial activity with both modified Kirby-Bauer assay (10 µL of the extract dissolved in DMSO) and diffusion assay with filter discs (50 µL of the extract dissolved in DMSO), against *B. subtilis* and *M. luteus*. However, no inhibitory effect against the two targets was observed, contrarily to the previous 4 strains tested (Fig. 25).

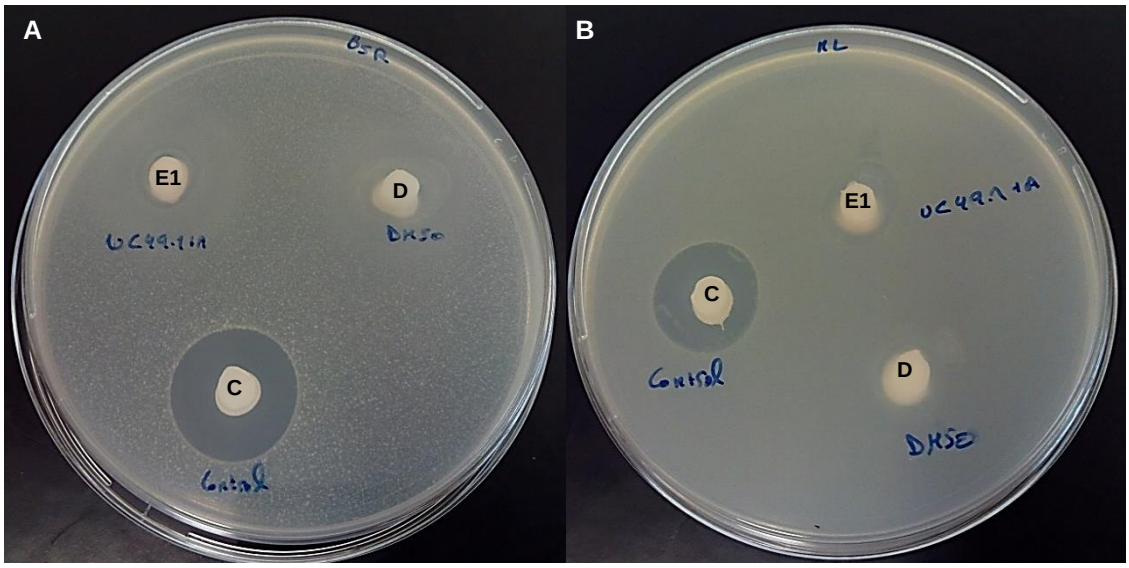

Fig. 25- Diffusion assay with filter discs filled with the UC49.1 up-scaled culture extracted with ethyl acetate (dissolved in DMSO), tested against *B. subtilis* (A) and *M. luteus* (B) and incubated for 24h at 37º C: C= control of 10 µL of 1mg/mL ampicillin; D= control of 50 µL of DMSO; E1= 50 µL of the ethyl acetate extract of strain UC 49.1 cultured in 500mL of M607. A very slightly inhibition halo was observed in both plates, for the extract of this strain.

2.1.2. Antimicrobial screening against *Chlamydia trachomatis*

The antimicrobial screening with the obtained ethyl acetate extracts (dissolved in DMSO) from 4 of the marine strains (MsF2 in M607+A, FF1 in M607, Gr7 in M607 and UC9 M607) was also performed against another target, *Chlamydia trachomatis.* This strain belongs to the phylum *Chlamydiae*, a member of the *Planctomycetes*, *Verrucomicrobia* and *Chlamydiae* (PVC) super-phylum. This is an interesting screening

due to the phylogenetic proximity of these bacteria. On another hand, infections with this pathogen (which causes one of the most prevalent sexual transmittable diseases) are still a concern due to the appearance of resistant strains and need urgent alternative treatments (Horner 2016).

Due to the intracellular activity of *C. trachomatis,* cells from the eukaryotic line Hep2 were firstly infected with this pathogen, exposed to several different treatments (Planctomycetes extracts within two dilutions, positive control of ciprofloxacin and solvent control of DMSO), stained for chlamydial lipopolysaccharides (LPS), host cell DNA and host cell cytoplasm and analyzed through fluorescence microscopy (see section 5.6 from the methodology).

In the untreated control (Figs. 26A and 27A) it was well evident the normal formation of chlamydial inclusions (in green) inside the host Hep2 cells. On the other hand, infected cells treated with the antibiotic ciprofloxacin (Figs. 26B and 27B) revealed a strong decrease of the chlamydial inclusions.

No effect in the chlamydial infection was observed for the less concentrated extracts from Planctomycetes (1.28 µL dilution) as well as for the solvent control (DMSO) at the same quantity (Figs 1 and 2 from the attachments). However, when infected cells were exposed to the more concentrated extracts from Planctomycetes (2.56 µL) (Figs. 26E,F and 27E,F), several levels of chlamydial inhibition were observed, except for strain FF1, for which the infection level was similar to the untreated control (Fig. 26E), where the green color of inclusions is observed inside the host cell. UC9 showed a certain reduction in the size and number of chlamydial inclusions (Fig. 26F). MsF2 and specially Gr7 induced both a great inhibitory activity, with effects similar to the control of cells treated with ciprofloxacin (Figs. 27E and 27F). Effects provoked by strain Gr7 presented even more similarity with the control of non-infected cells (Fig. 27D), as practically no green of chlamydial LPS is observed.

These promising results, however, have to be taken with care since the solvent control of DMSO (at 2.56 µL dilution) also revealed a reaction on the chlamydial inclusions development (Figs. 26C and 27C). Yet, by the comparison of this control and the extract from Gr7 strain, chlamydial action may be present, although no exact distinction can be made between the effects of the Planctomycete extract and the DMSO in which it was dissolved.

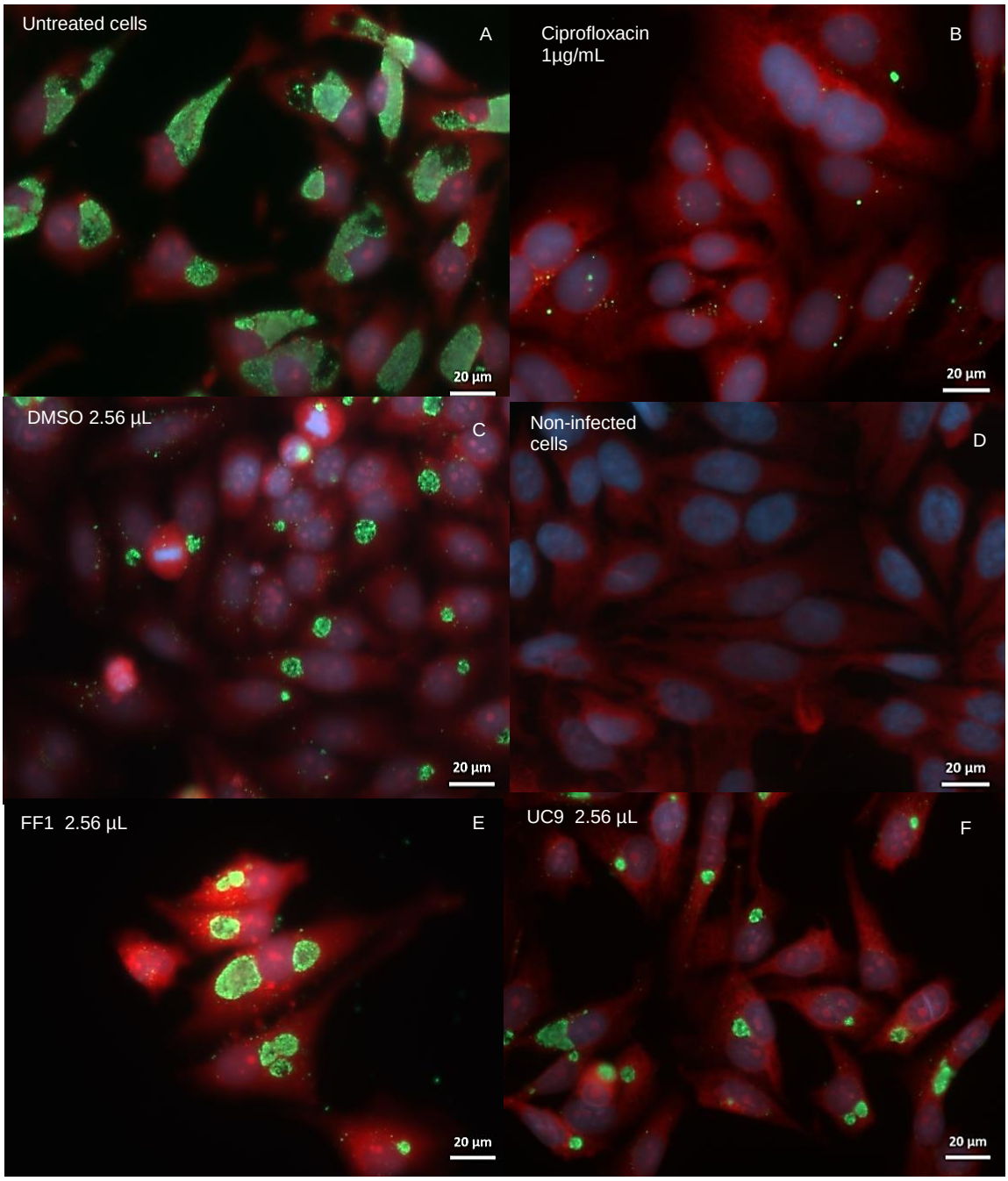

Fig. 26 - Fluorescence microscope images of eukaryotic cells from line Hep2 infected with *Chlamydia trachomatis* and exposed to several treatments, as ciprofloxacin and the 2.56 µL extracts from Planctomycetes strains UC9 and FF1. Blue correspond to DAPI staining of DNA from the host cells, green to chlamydial inclusions and red to host cell cytoplasm. A= control of untreated infected cells; B= control of infected cells treated with 1µg/mL ciprofloxacin; C= infected cells exposed only to 2.56 µL DMSO dissolved in sterile water; D= Control of non-infected cells; E= infected cells exposed to 2.56 µL extract of strain FF1; F= infected cells exposed to 2.56 µL extract of strain UC9. No effect of FF1 extract against the chlamydial infection was visualized at this quantity. On the other hand, cells treated with 2.56 µL of only DMSO presented a diminution in the chlamydial inclusions to a few small round structures. Cells exposed to extract from strain UC9 presented a diminution on the inclusions similar with the control with DMSO, so this anti-microbial activity may be due to the solvent.

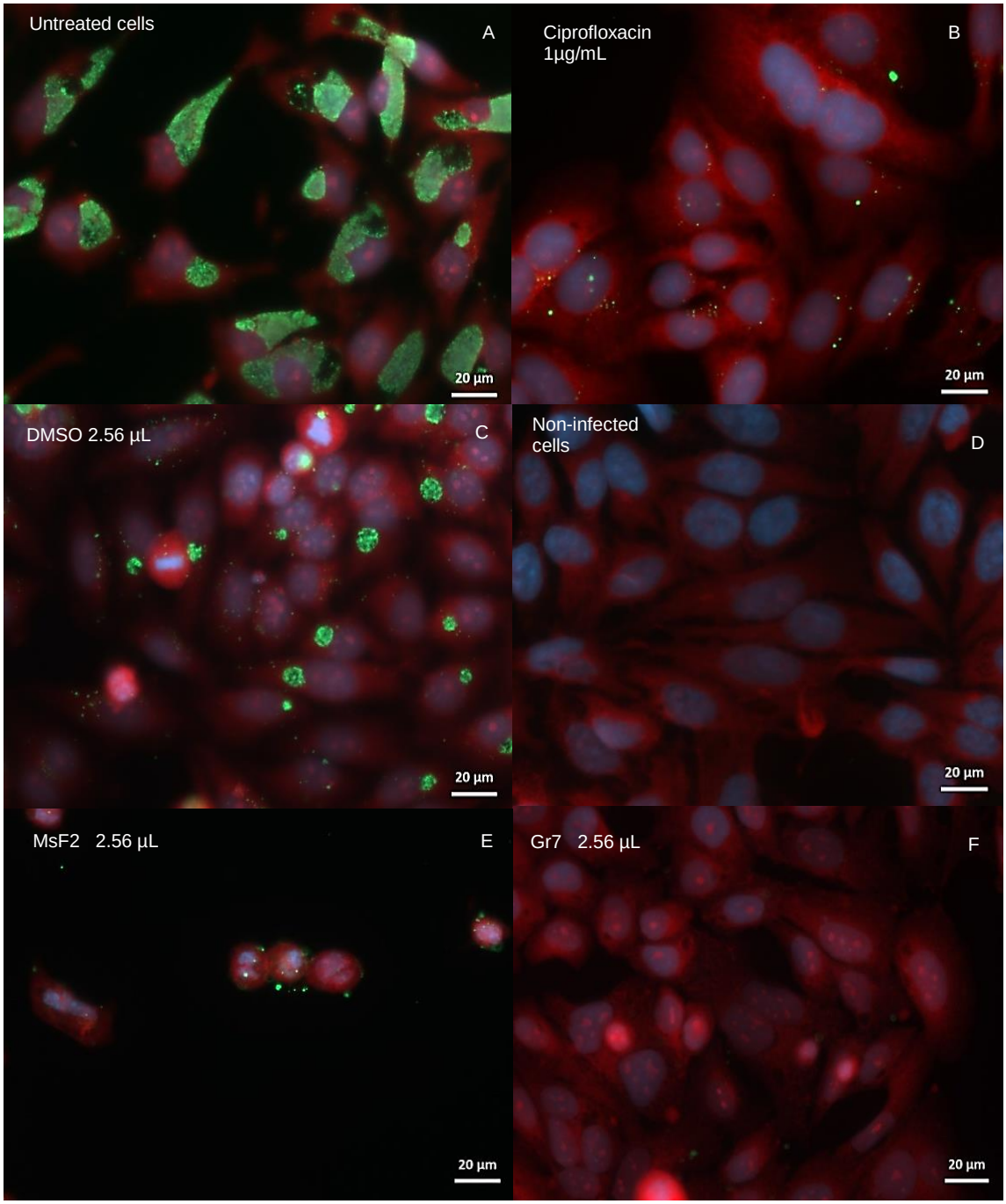

Fig. 27 - Fluorescence microscope images of eukaryotic cells from line Hep2 infected with *Chlamydia trachomatis* and exposed to several treatments, as ciprofloxacin and the 2.56 µL extracts from Planctomycetes strains Gr7 and MsF2. Blue correspond to DAPI staining of DNA from the host cells, green to chlamydial inclusions and red to host cell cytoplasm. A= control of untreated infected cells; B= control of infected cells treated with the antibiotic 1µg/mL ciprofloxacin; C= infected cells exposed only to 2.56 µL DMSO dissolved in sterile water; D= Control of non-infected cells; E= infected cells exposed to 2.56 µL extract of strain MsF2; F= infected cells exposed to 2.56 µL extract of strain Gr7. Cells exposed with the extracts from strains MsF2 and Gr7 showed almost a total effect of a controlled infection, which is demonstrated by their similarity with the cells exposed to the control antibiotic ciprofloxacin. In the case of strain Gr7, the similarity is even bigger with the control of cells with no chlamydial infection, since practically no green is observed inside the cells. However, as the control of DMSO also showed antimicrobial action, the exact effect of the extracts can't be determined.

2.1.3. Mode of action assay

In order to search for the cellular targets of the potential compounds in the Planctomycetes extracts, a mode of action assay was performed. Four different *B. subtilis* reporter strains (designed for the 4 main bacterial targets: cell wall, DNA, RNA and protein synthesis) were exposed to the previous extracts dissolved in DMSO obtained from the extraction of the 500 mL cultures of the 4 marine strains MsF2, FF1, UC9 and Gr7. Inhibition halos were visualized for all extracts of Planctomycetes, confirming the presence of bioactive compounds and showing once more that the extract from strain MsF2 cultured in medium M607+A was the most bioactive one (Figs. 28 and 29). On the other hand, as no blue color appeared around any of the inhibition halos, no conclusion can be drawn on the mode of action behind the compound responsible for the bioactivity. Only the antibiotic tetracycline showed a slight blue coloration against the protein target (Fig. 28), while and no other antibiotic control showed the same effect, as it is exemplified for cell wall and DNA targets (Fig. 29A and B). Since the blue color was also not very robust for the several controls, it is suggested that the concentration of X-Gal was probably too low or that this substance was possibly degraded.

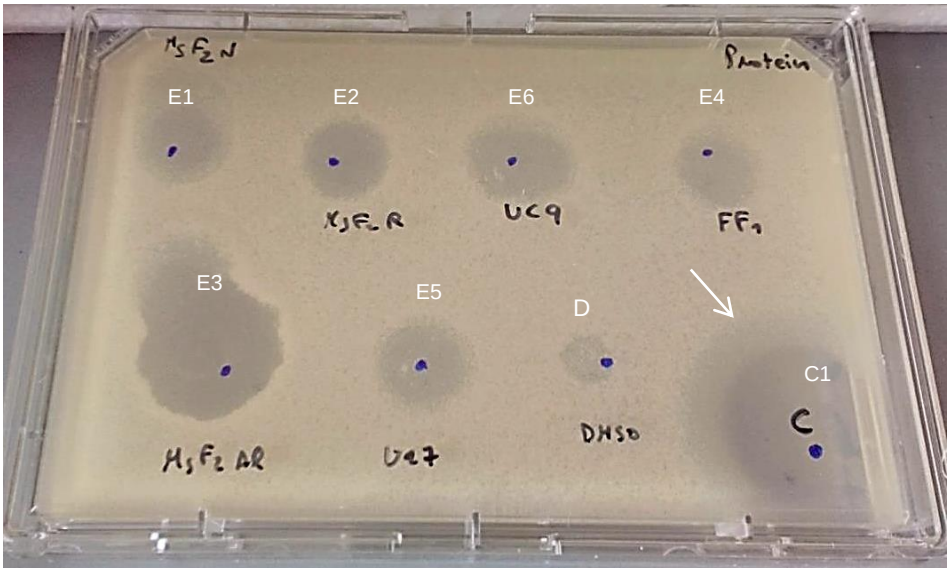

Fig. 28- Mode of action assay where Planctomycetes extracts were tested against *Bacillus subtilis* 128 transformed with pAC6-vector containing the promotor *PyheI* (for protein target), incubated for 24h at 37º C. E1= extract MsF2 cultured in 500mL of M607 medium; E2= extract of MsF2 cultured in 500mL of medium M607+A+*Bacillus*; E3= extract of MsF2 cultured in 500mL of medium M607+A; E4= extract from FF1 cultured in 500mL of M607 medium; E5= extract from strain Gr7 cultured in 500 mL of M607 medium; E6= extract from strain UC9 cultured in 500 mL of M607 medium; C1= control of 500 µg/mL tetracycline; D= control with only DMSO. Inhibition halos were observed for all extracts, with MsF2 incubated in M607+A the most bioactive one. Control with DMSO presented a small effect, however. No blue residues were visualized besides in the halo from the antibiotic control C1 (arrow).

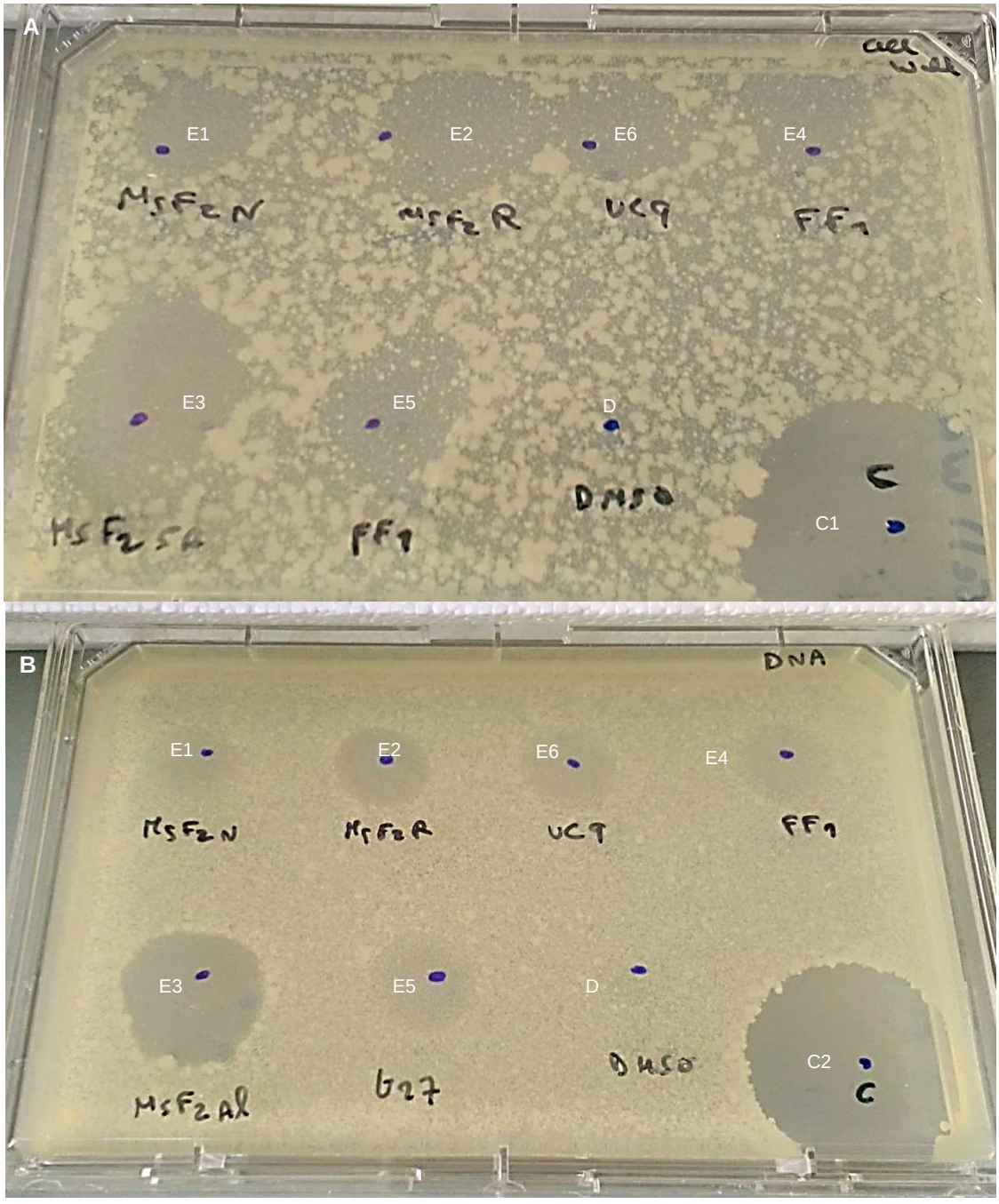

Fig. 29- Mode of action assay where Planctomycetes extracts were tested against *Bacillus subtilis* 128 transformed with pAC6-vector containing the promotor *PypuA* (for cell wall target) (A) or the promotor *PyorB* (for DNA target) (B), incubated for 24h at 37º C. E1= extract MsF2 cultured in 500mL of M607 medium; E2= extract of MsF2 cultured in 500mL of medium M607+A+*Bacillus*; E3= extract of MsF2 cultured in 500mL of medium M607+A; E4= extract from FF1 cultured in 500mL of M607 medium; E5= extract from strain Gr7 cultured in 500 mL of M607 medium; E6= extract from strain UC9 cultured in 500 mL of M607 medium; C2= control of 500 µg/mL ampicillin; C3= control of 500 µg/mL vancomycin; D= control with only DMSO. Inhibition halos were observed for all extracts and extract from the strain MsF2 cultured in M607+A was the most bioactive one. Control with DMSO did not showed bioactivity. No blue residues were visualized.

2.1.4. Gel permeation chromatography

The capacity of Planctomycetes to produce bioactive compounds with antimicrobial activity was observed in the previous assays, confirming their genetic potential previously recognized (Graça *et al*., 2016). Strains MsF2 and Gr7 were therefore chosen for the beginning of compound isolation by gel permeation chromatography (GPC), based on the earlier obtained results (MsF2 was the most consistently bioactive strain and Gr7 showed promising chlamydial activity). Thus, newly cultured MsF2 and Gr7 in medium M607+A (this time, prepared with natural sea water) were extracted with ethyl acetate and dissolved in methanol, since it is the solvent used in the chromatography (see section 6 from methodology). They were then firstly screened for antimicrobial action, before fractionation. As strain Gr7 showed a more pronounced halo than MsF2 (Fig. 30), it was decided to continue for separation with Gr7 extract.

Fig. 30- Modified Kirby-Bauer assay where extracts from strains MsF2 and Gr7 where tested against *B. subtilis*, incubated for 24h at 37º C. E1= extract from MsF2 cultured in 500 mL of medium M607+A; E2= extract from strain Gr7 cultured in 500 mL of M607+A. A more pronounced inhibition halo was observed in the spot of the extract from strain Gr7.

To fractionate the heterogeneous extract obtained from strain Gr7, the column used was Sephadex LH20, chosen by its normal use to separate terpenoids and small weight peptides. Fractions were collected every 15 minutes, from minute 0 to minute 225, dried and at the end screened for antimicrobial activity against *B. subtilis* and *M. luteus* using the well assay, to allow the use of a higher volume of each fraction (100 µL). However no fraction showed bioactivity against the two targets (Fig. 31), which suggests that the concentration of compound extracted was probably too low for isolation and further detection of activity.

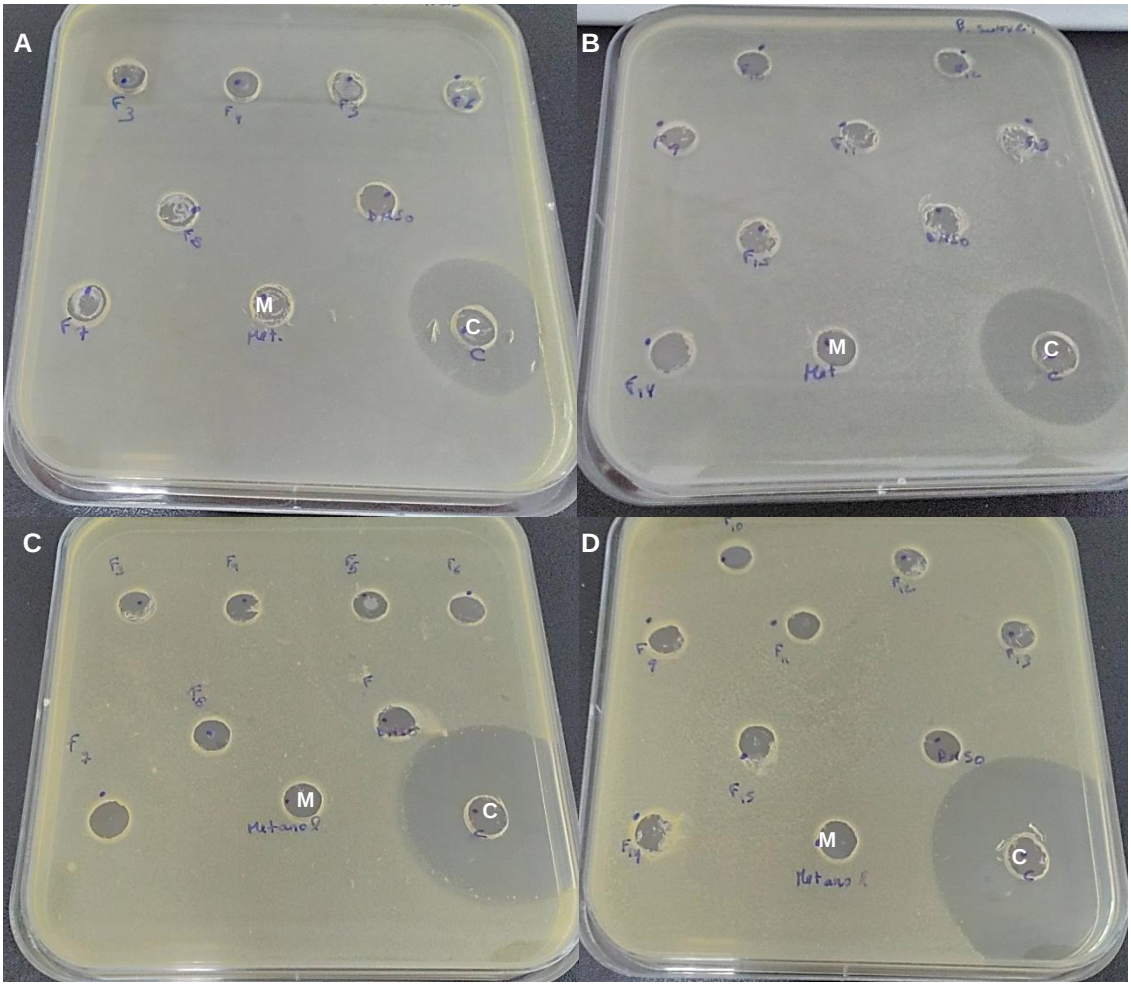

Fig. 31- Well assay where fractions obtained from Gr7 extract subjected to GPC were tested against *B. subtilis* (A and B) and *M. luteus* (C and D), incubated for 24h at 37º C. C= control of 10 µL of 1mg/mL ampicillin; M= control of 100 µL methanol. No inhibition halo was observed for any fraction. Control with methanol did not present any inhibition,

2.2. Extraction with methanol

As the previous separation did not allow the obtainment of a bioactive fraction, enhancement of the compound production levels by Planctomycetes strains was required. Thus, another alternative extraction method was assayed for the strain that in the previous assays showed a higher bioactivity: strain MsF2. Another up-scaling was also intended, so a culture of this strain was prepared in 2L of medium M607. Methanol was the chosen organic solvent to extract possible compounds from the cells of this culture (Leão *et al*., 2013). The extract obtained (Fig. 32A) was subsequently screened for antimicrobial activity with the diffusion assay with filter discs against *B. subtilis*. Unfortunately, no inhibition halo was visualized for this extract (Fig. 32B).

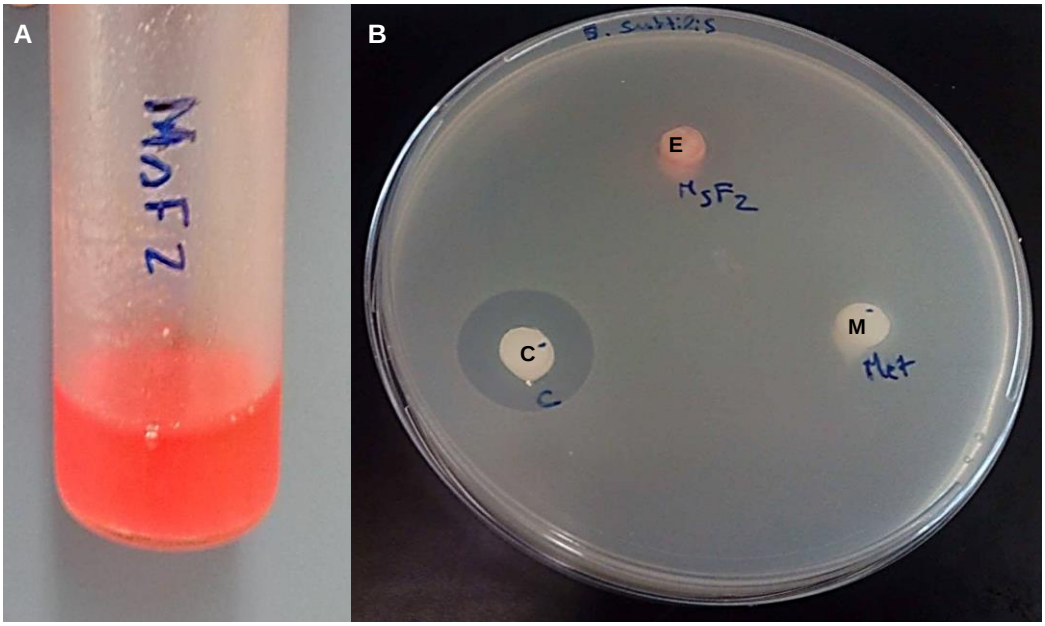

Fig. 32- A= Methanol extract obtained from the 2L culture of strain MsF2 prepared in medium M607, dissolved in 2mL DMSO and 1 mL methanol; B= Diffusion assay with filter discs where MsF2 extract was tested against *B. subtilis,* incubated for 24h at 37º C: C= control of 10 µL of 1mg/mL ampicillin; M= control of 50 µL methanol; E= Filter disc filled with 50 µL of the methanol extract represented in (A). No inhibition halo was visualized in the filter disc with the extract.

2.3. Incubation of cultures with XAD resin and extraction with acetone and ethyl acetate

Since the antimicrobial activity by MsF2 strain was not recovered with the lastly applied protocol, other modifications were tried. One more up-scaling was also done, for which a 3L culture of strain MsF2 was prepared in medium M607. To allow the accumulation and further extraction of compounds possibly present in the aqueous phase of this culture, resin Amberlite® XAD16N was added to it in the late exponential phase. This resin is specifically designed to adsorb and hold hydrophobic compounds up to 40,000 MW such as antibiotics. To extract from this resin, two different protocols were used, one using ethyl acetate and another using acetone, as is described in the methodology (see section 4.3 of the methodology). Acetone was used because it has been also used in a study to extract bio compounds from other strains of Planctomycetes (Jeske *et al*., 2016). The culture (that remained after separation of the resin) was centrifuged and the cells pellet extracted with ethyl acetate. The extraction of the resin with ethyl acetate was the one that resulted in more solid residue after

evaporation (Fig. 33A). The three extracts obtained (Fig. 33B) were consequently screened for antimicrobial activity against *B. subtilis* using the diffusion assay with filter discs (resin extracts) and the modified Kirby-Bauer assay (cell pellet extracts). However, no inhibition halos were observed for any extract (Fig. 34).

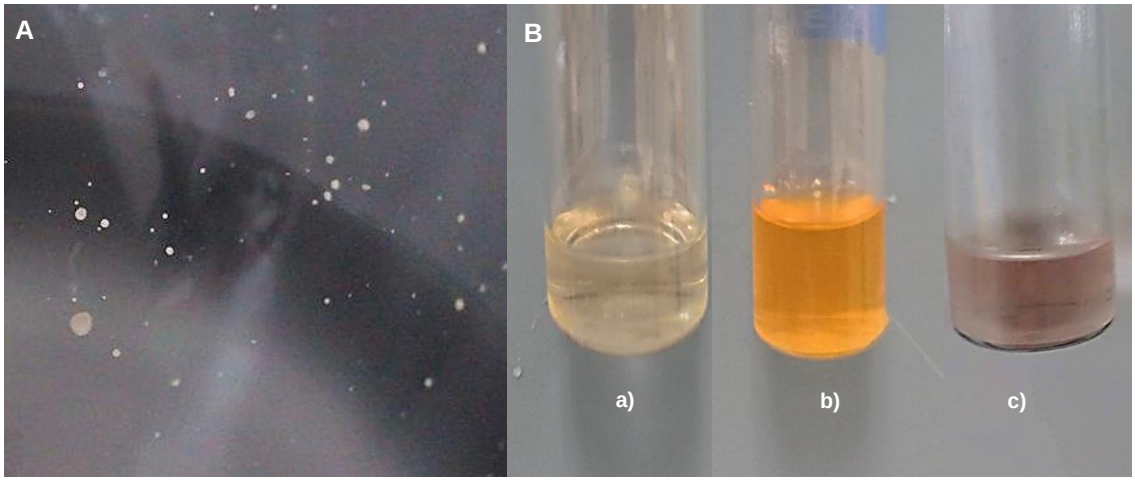

Fig. 33- A= solid residues obtained from the ethyl acetate extraction of the resin, after drying in a rotatory evaporator; B=three different extracts obtained from the 3L culture of strain MsF2 in medium M607 incubated with resin Amberlite® XAD16: a) extract of the resin with ethyl acetate, dissolved in 5 mL of methanol; b) extract of the resin with acetone, dissolved in 8 mL of methanol; c) extract of the cells pellet of the culture with ethyl acetate, dissolved in 2 mL of methanol.

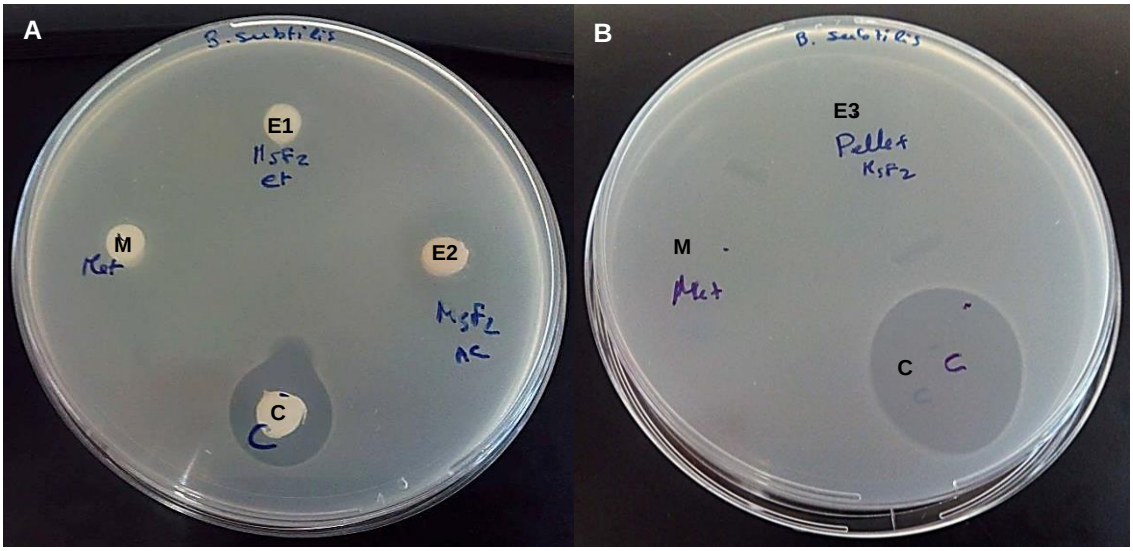

Fig. 34- Diffusion assay with filter discs and modified Kirby-Bauer assay where MsF2 resin (A) and cell pellet (B) extracts were tested against *B. subtilis*, incubated for 24h at 37º C. C= control of 10 µL of 1mg/mL ampicillin; M= control of 50 µL of methanol; E1= Filter disc filled with 50 µL of ethyl acetate extract from the resin incubated with strain MsF2 in 3L of medium M607; E2= Filter disc filled with 50 µL of acetone extract from the resin incubated with strain MsF2 in 3L of medium M607; E3= filter disc filled with 50 µL of the extract from the cells obtained from a 3L culture of strain MsF2 in medium M607. No inhibition halos were visualized for any extract.

2.4. Exploring the potential of freshwater strains

Two strains of Planctomycetes isolated from freshwater sediments were also screened for antimicrobial activity (based on the genetic potential revealed by *Aquisphaera giovannonii* OJF2, as AntiSMASH analyses showed 52 genes clusters for secondary metabolite production in this species). These two strains (OJF2 and OJF8) were directly incubated in an up-scaled culture of 1 L of PYGV medium and extracted using ethyl acetate, since it was the solvent that permitted the visualization of activity with the previous strains. To evaluate activity, the extracts obtained were tested in diffusion assay with filter discs against *B. subtilis* (both OJF8 and OJF2*)* and *M. luteus* (only OJF8). A small inhibition halo was visualized in the extract of strain OJF8 against both targets (Fig. 35A and 35B). A repetition of the assay was made against *B. subtilis* and the activity was once more visualized (Fig. 35C), confirming the potential of this strain. However, the extract from strain OJF2 did not present antimicrobial activity (Fig. 35D).

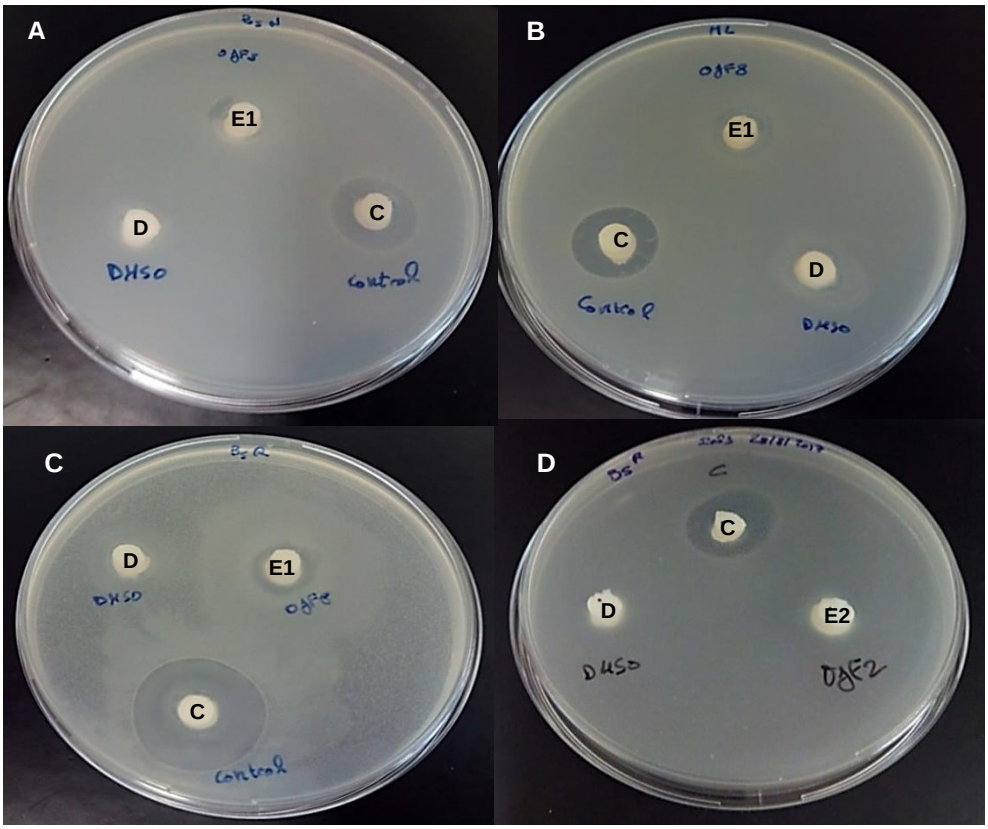

Fig. 35- Diffusion assay with filter discs where freshwater Planctomycetes extracts were tested against *B. subtilis (*A, C and D) and *M. luteus* (B), incubated for 24h at 37º C. E1= Filter disc filled with 100 µL of the extract obtained from strain OJF8 in medium PYGV; E2= Filter disc filled with 100 µL of the extract obtained from strain OJF2 in medium PYGV; C= Filter disc filled with 10 µL of the control of 1 mg/mL ampicillin; D= Filter disc filled with 10 µL of the control of DMSO. Inhibition halos were visualized for OJF8 extract against both targets. However, no inhibition halo was visualized for strain OJF2.

3. Anti-obesity assay with zebrafish larvae

Besides the search for antimicrobial activity focused in this thesis, anti-lipid production activity was also explored for Planctomycetes extracts, having in mind the obesity problematic in current times.

For this assay, 9 marine Planctomycetes were chosen (UC16, UC9, Rb, CcC6, MsF2, FF1, Gr7, UC 49.1 and UC13) and incubated in an intermediate volume in medium M607 (100 mL), due to the impossibility of making higher volume cultures for all strains at the same time. Cultures were then used for extraction with ethyl acetate and the extracts dissolved in DMSO standardized to the 10 mg/mL concentration, to a more precise comparison (see section 5.8 from the methodology). Larvae of zebrafish were also obtained and exposed to several treatments (Planctomycetes extracts, positive control of resveratrol and solvent control of DMSO) in 48-well plates and stained for lipid drops with Nile red. Larvae were then analyzed under fluorescence microscopy and the levels of the inhibitory capacity against lipid production calculated for each treatment, using the mean fluorescence intensity values obtained from the images analyzes with the software ImageJ.

Larvae exposed to the solvent control (DMSO) presented a normal lipid production, since lipid drops stained with Nile red were observed under fluorescence microscopy, mostly under the larvae abdomen (Fig. 37B). DMSO was also chosen as negative control, since this compound does not affect lipid production in zebrafish and is normally used as control in these assays (Jones *et al*., 2008). The mean valor of the inhibition calculated for all larvae exposed to this treatment was therefore considered as 100 % of lipid production/accumulation and subsequently taken as 0 % of inhibitory capacity of lipid production.

On the other hand, larvae exposed to the positive control resveratrol demonstrated no lipid production, since no red staining was observed, as expected (Fig. 37D). This compound can be used as example to show effect on the lipid production in zebrafish (Jones *et al*., 2008) and its action against mice fat was also proven (Lagouge et al., 2006). However, resveratrol seems not to show any effect on humans (Alberdi *et al.*, 2014 and Poulsen *et al*., 2013),

All larvae exposed to Planctomycetes extracts presented different levels of intensity of fluorescence, although red staining of lipid drops was always present

(larvae exposed to strain UC9 exemplified in Fig. 37F), which means that no total inhibition of the lipid production/accumulation occurred. After statistic treatment of the data obtained and calculation of the exact mean percentage of inhibition for each extract exposure (by comparison with the DMSO control), it was confirmed that no Planctomycete extract inhibited in the totality the lipid production, although all presented positive activity (Fig. 36). All treatments were also statistically different from each other ($F_{[8,62\ ]}$= 6.98; $p < 0.05$). Most extracts showed lower values of inhibition of lipid production/accumulation, between 3 % and 20 % (Fig. 36). On the other hand, values of 30 % and 36 % inhibition were obtained, respectively, for extracts from strains CcC6 and MsF2, which are affiliated to *Rhodopirellula lusitana* and *Rhodopirellula baltica* species. These two were the only ones significantly different from the DMSO control (Fig. 36), meaning that their inhibition values showed could be considered. Unfortunately, no larvae exposed to FF1 extract survived the experience (maybe due to the manipulation) and consecutively its results were not considered.

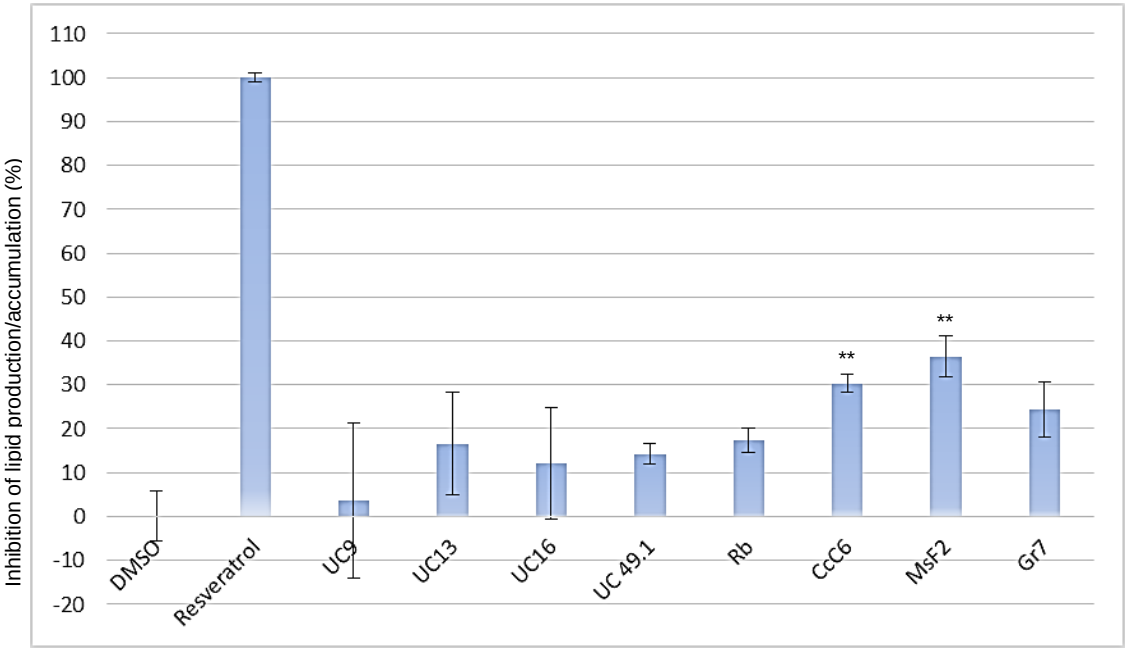

Fig. 36- Inhibition of lipid production/accumulation (%) in larvae of zebrafish exposed for 48h to Planctomycetes extracts, to the control compound resveratrol and to the solvent control DMSO. Error bars were obtained from the calculus of standard error of the mean value. Inhibition values were mostly low, with CcC6 and MsF2 the only ones equal or above 30%. Resveratrol, as expected, produced 100 % of inhibition. **= Dunnett's test performed to the treatments in comparison with the solvent DMSO control showed that CcC6 and MsF2 values were significantly different ($p<0.01$) and their inhibitory values could be considered.

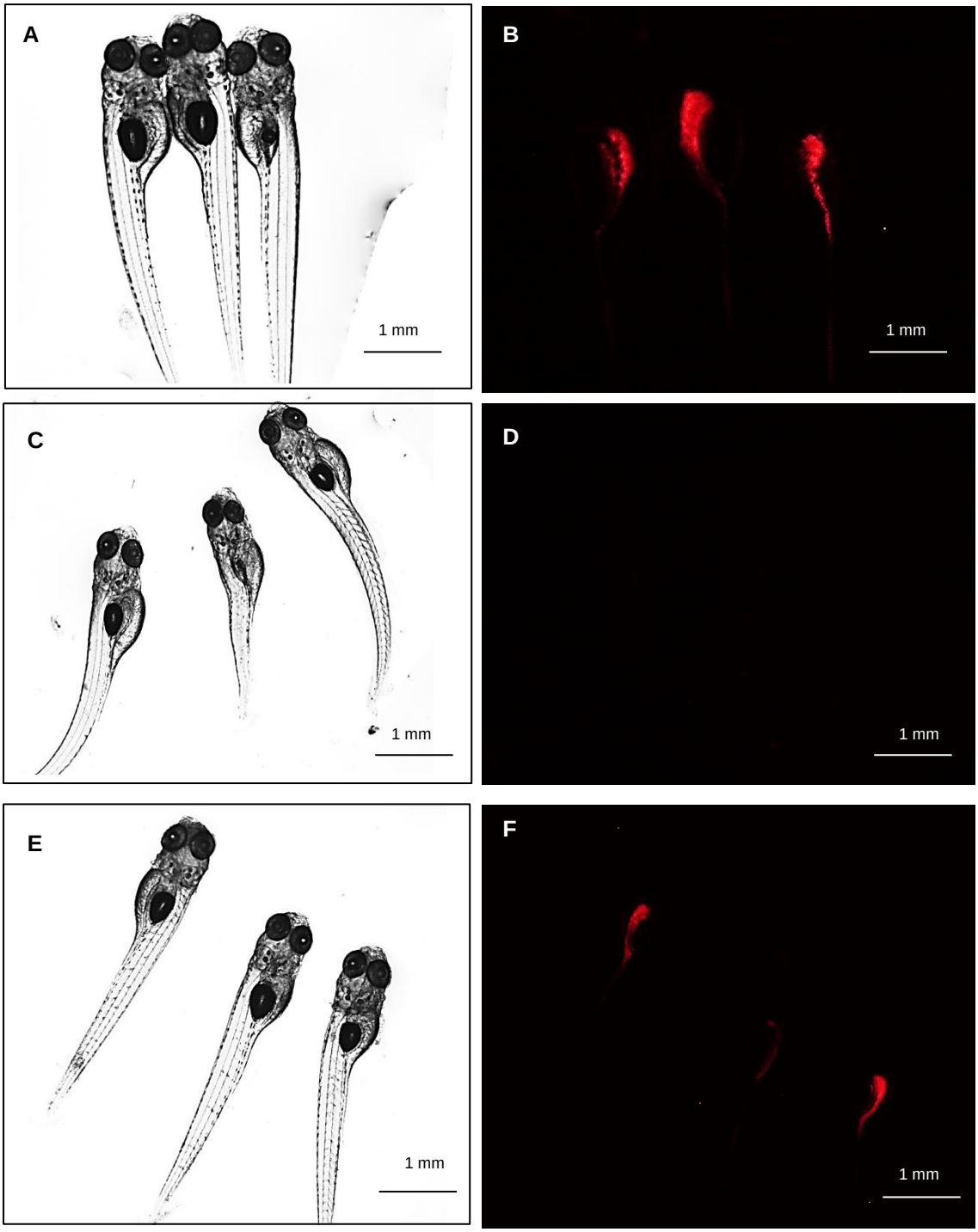

Fig. 37- Contrast phase (A, C and E) and fluorescence (B, D and F) microscope images of larvae of zebrafish exposed to several treatments and stained with 50 µL of 500 ng/mL Nile red (red): A,B= exposure for 48h to 200 µM PTU and to 0.75 µL DMSO; C,D= exposure for 48h to 200 µM PTU and to 0.75 µL of 11.412 mg/mL resveratrol; E,F= exposure for 48h to 200 µM PTU and to 0.75 µL of the extract from strain UC9 dissolved in DMSO (10 mg/mL). For Nile red, excitation wavelength: between 515 and 560 nm and emission wavelength > 590 nm. Control with resveratrol inhibited the lipid production, as is seen by the absence of red color (D). On the other hand, solvent control with DMSO showed total lipid production and accumulation, as is seen by the presence of red residues mostly in the larvae abdomen (B). Extract from strain UC9 did not totally inhibit the lipid production, as is demonstrated by the presence of red coloration (F).

Discussion

Previous works (Graça *et al*., (2016) and Jeske *et al*., (2016)) showed the potential of Planctomycetes to produce antimicrobial compounds. A subsequent step for the characterization of precise molecules behind the activity is the fractionation of extracts for isolation of the compounds. In this work, we aimed to achieve this goal and search for the secondary metabolite potential of several aquatic strains of Planctomycetes.

To do a preliminary screening of the antimicrobial action by Planctomycetes, one assay was initially made in liquid medium (the 96-well assay). This assay was in a small scale, since only 2mL of small cultures (20mL) were used to make extracts, and where metabolites were extracted with DMSO+acetone. The high bioactivity obtained in the screenings against *B. subtilis* with twelve of the marine strains showed consistency with results from Graça *et al*., (2016), where other marine strains of Planctomycetes presented also activity against this target strain. Against the tested *E. coli*, which is a very resistant strain isolated from a high polluted river (Cabral & Marques, 2006), no activity was observed for any of the extracts. Once again, these results are consistent with the ones from the same referred study. However, activity against *C. albicans* was not visualized in the present study, contrarily to the anti-fungal activity demonstrated also by Graça *et al*., (2016). This result may be due to a metabolic alteration of this target strain or to the development of antimicrobial resistance. These first twelve screened strains were isolated from the biofilm of three different macroalgae, however, they are all phylogenetically related to *Rhodopirellula baltica,* which confirms the potential associated to this species. In fact, Jeske *et al*., (2016) also found antimicrobial activity against of *R. baltica* against *B. subtilis*.

In order to complement this initial screening, the modified Kirby-Bauer was used to evaluate the antimicrobial activity of an enlarged panel (35 strains) of Planctomycetes, against two sensitive targets, *B. subtilis* and *M. luteus,* within the same extraction protocol. However, only two inhibition halos were observed against *M. luteus* (FF1 and MsF2 pellet extracts) and none against *B. subtilis*, which is contrary to the activity that was evidenced in the previous assay with the same type of extracts.

The production of secondary metabolites is normally very inconsistent and dependent on many factors, such as the medium composition, stress conditions and the growth phase (Bibb 2005). Since the extracts tested in the modified Kirby-Bauer

assay were prepared after the beginning of the internship in Bonn using artificial sea water for medium preparation, the lack of activity demonstrated may be due: (1) to this modification, (2) to bacterial stress caused by the transportation between the two countries or (3) simply to the differences between the two assays. Several modifications to the medium composition were made, which included biological, chemical and organic stresses as well as natural environment simulation (done with the incorporation of macroalgal extract in the medium). These modifications were ineffective in recover and inducing the secondary metabolite production in small scale, which results are hardly justifiable.

As bioactivity seemed to vanish when extracts were made, co-culture assay was tested with live cultures of Planctomycetes, which avoid extraction. Once again, no activity from Planctomycetes could be observed. However, these results may be due to the short period (3 days) of strains incubation. Normally, Planctomycetes cultures with 3 days growth are in the beginning of the stationary phase and the secondary metabolism could there not yet been activated. The difference in the incubation time in this specific assay was due to time constrains and accessibility of laboratory facilities.

Since the production of compounds by marine Planctomycetes was not enhanced by the previous alterations, new protocols were tested. The culturing and extracting of the bacteria were up-scaled from a small scale (with 2 mL extracted from 20 mL cultures) to 500 mL cultures entirely extracted. Another solvent was also used for extraction to substitute the previously used mixture of DMSO+acetone, ethyl acetate. The four Planctomycetes assayed were chosen either because of their activity already demonstrated (FF1 and MsF2) or their genetic potential (UC9 and Gr7). Both UC9 and Gr7 have been screened by Graça *et al*., (2016) for the presence of PKS/NRPS genes. These genes encode for two major enzyme complexes that are responsible for the majority of the secondary metabolites production (Donadio *et al*., 2007), thus, their presence may be an indicator of possible metabolites of interest. Gr7 and UC9 were also screened for their potential pathway product prediction, using the NaPDos software (which is a bioinformatic tool for the rapid detection and analysis of secondary metabolite genes). The products predicted were, for Gr7 and UC9 respectively, myxalamid (only observed for Myxobacterales) and bacitracin, both known compounds with antimicrobial activity. The analyzes with AntiSMASH software (an antibiotic and secondary metabolite analyses shell) to Gr7 genome showed eight gene clusters for these types of metabolites in which two leaded to potential antimicrobial compounds with biotechnological interest: bacteriocins and lantipepetides

(Yang *et al*., 2014; Field 2015). In these up-scaled tests, where the solvent used for extraction was ethyl acetate, we were able to detect bioactivity for all extracts against *B. subtilis* and *M. luteus*, although more powerfully against *B. subtilis*. It was noted that *M. luteus* developed some resistances since the beginning of its utilization, as it was confirmed by the necessity of using a control of ampicillin with higher concentration. This acquired resistance may be the responsible for that differential strength of inhibition, since both targets are Gram positive bacteria and very sensitive to external compounds.

In an attempt to increase the bioactive potential observed for strain MsF2, the more bioactive strain of the four screened, an up scaled culture in medium variations of M607+A and M607+A+*Bacillus* were assayed. Under these conditions, we were able to have a high degree of bioactivity (especially on MsF2 culture in M607 plus algal extract, which was the higher activity obtained in this work). These results indicate that the use of higher volumes for extraction is an important factor as well as the stimulation of the Planctomycetes by the presence of material from their natural host, the macroalgae (Lage & Bondoso, 2014).

Furthermore, as the previous ethyl acetate extracts obtained showed bioactive potential, they were screened against *C. trachomatis.* Again, MsF2 as well as Gr7 showed anti-chlamydial activity by reducing the formation of chlamydial inclusions inside the host cells. However, the control with the solvent also showed reduction of chlamydial inclusions. Thus, as the exact effect of the DMSO can't be distinguished from the extracts effects, more studies are needed, ideally with a purified compound or extracts dissolved in water, to resolve this solvent problem and confirm the potential activity.

Although the genetic potential revealed by *Aquisphaera giovannonii* OJF2 (AntiSMASH analyses showed 52 genes clusters for secondary metabolite production), the screening with the two strains from this species (OJF2 and OJF8) only revealed a small level of bioactivity for strain OJF8, even though the extraction was made to 1000 mL of culture and using ethyl acetate.

Additional protocols of extraction comprehending different methodologies (more culture up-scaling, methanol as solvent for cell pellet extraction, incubation/extraction with a resin and cell pellet extraction with ethyl acetate) were tested to try to recover the activity once observed by strain MsF2. However, no antimicrobial action was visualized in any of these protocols. Besides the high diversity of screening approaches

and extraction techniques assayed during this study, it was not possible to achieve a good optimized protocol for the subsequent step of bioactive molecules isolation from Planctomycetes using gel permeation chromatography.

Regarding the anti-obesity assay, no final conclusive results can be taken about the capacity of Planctomycetes to inhibit the lipid production and accumulation, since the extracts used where from low volume cultures (100 mL) potentially with low amount of the metabolites of interest within the heterogeneous mixture, in which the pretended action could have been masked. However, strains MsF2 and CcC6 presented under these conditions significative anti-obesogenic activity equal or above 30 %, which is promising. These results encourage further studies, like a repetition of this assay with extracts obtained from an optimized protocol or ideally with purified compounds (Noinart *et al*., 2017), if possible, to more correctly evaluate this capacity and confirm the potential demonstrated for new anti-obesogenic metabolites.

Conclusion

Antimicrobial activity was observed against *B. subtilis* and *M. luteus* for extracts obtained from different Planctomycetes species: *Rhodopirellula baltica*, *Rhodopirellula rubra*, *Aquisphaera giovannonii* and *Rubinisphaera brasiliensis*. Assays that evaluated anti-*Chlamydia trachomatis* and anti-obesity activities by Planctomycetes metabolites showed promising results, but regarding additional studies. Besides the high diversity of screening approaches and extraction techniques assayed, it was not possible to achieve a good optimized protocol for the subsequent step of bioactive molecules isolation from Planctomycetes using gel permeation chromatography.

Future perspectives on this work are based on the optimization of the protocol of culturing and extraction of Planctomycetes, in order to regain the activity of the used strains. The use of revival Planctomycetes strains from the -80ºC collection would be a good practice, since the ones used have already been subcultured for some time, which may imply loss of activity through time. The same is suggested for the target strains, since potential resistance acquisition may be interfering with the antimicrobial detection. A relevant culture up-scaling (10 to 20L) is also proposed, as well as the re-test of sea salts in the medium preparation, since no exact information of the natural sea water composition is known. The use of the resin Amberlite® XAD16N within the Planctomycetes incubation is also suggested because it allows the accumulation of compounds present in the aqueous phase of cultures. For the extraction of compounds, it is proposed to continue the use of ethyl acetate as solvent for crude culture extraction (organic soluble metabolites) and for resin extraction (aqueous soluble metabolites).

Furthermore, compounds isolation and determination of their mode of action are important steps to allow subsequent analyses to their structures and viability to be used in pre-clinical trials.

New and/or optimized anti-*Chlamydia trachomatis* and anti-obesity tests are needed to confirm the obtained results and to step forward to potential use of the bioactive extracts.

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Attachments

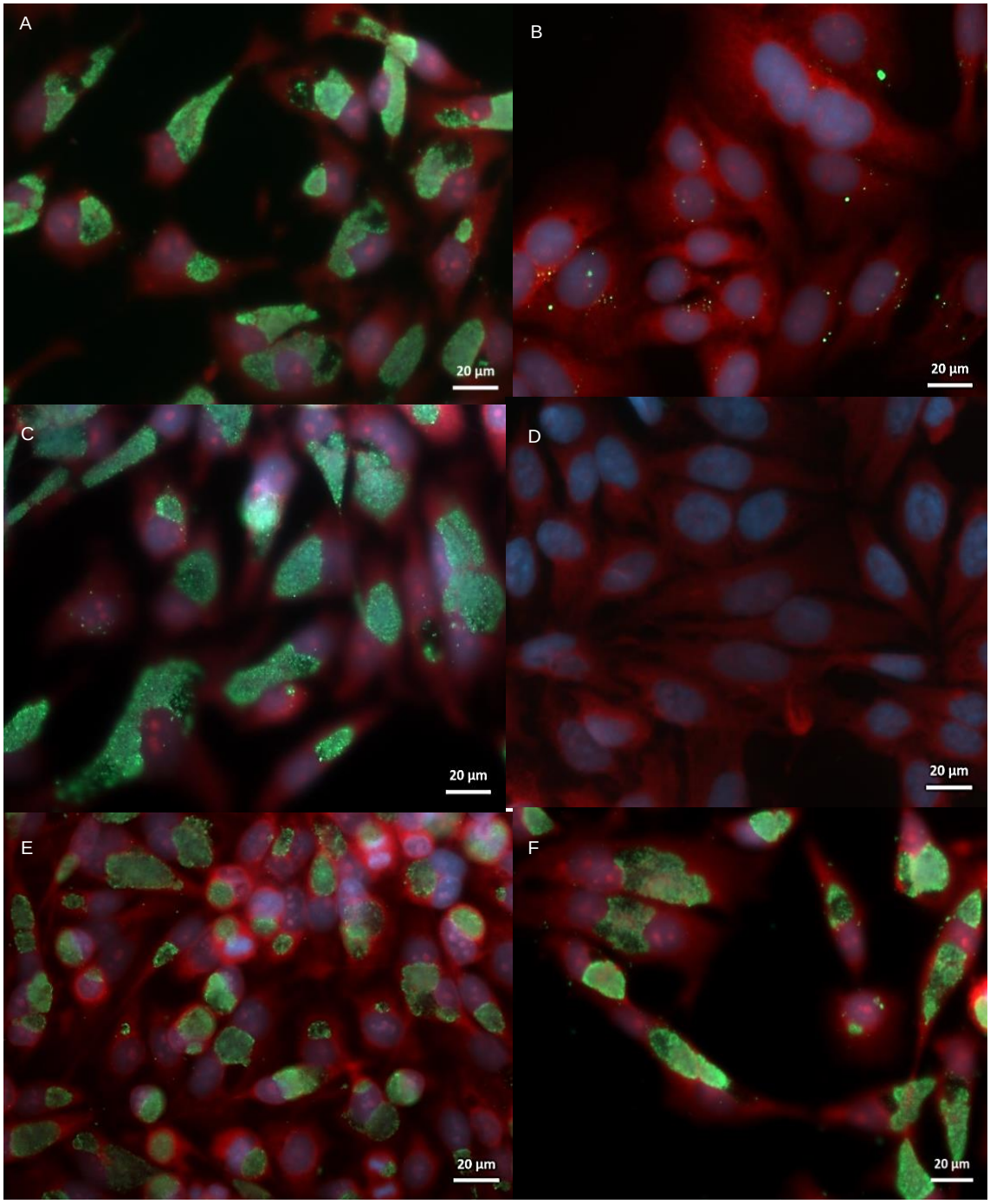

Fig. 1- Fluorescence microscope images of eukaryotic cells from line Hep2 infected with *Chlamydia trachomatis* and exposed to several treatments, as ciprofloxacin and the 1.28 µL extracts from Planctomycetes strains Gr7 andMsF2. Blue color corresponds to the DAPI staining of DNA from the host cells, green to chlamydial inclusions and red to host cell cytoplasm. A= Control of untreated cells; B= Control of cells treated with the antibiotic ciprofloxacin (at 1µg/mL); C= Cells exposed only to 1.28 µL DMSO dissolved in sterile water; D= Control of non-infected cells; E= Cells exposed to 1.28 µL extract of strain Gr7; F= Cells exposed to 1.28 µL extract of strain FF1. No effect of both FF1 and Gr7 extracts in the chlamydial infection was visualized at this quantity, since the aspect of the inclusions (green) is similar with the control of untreated cells (A).

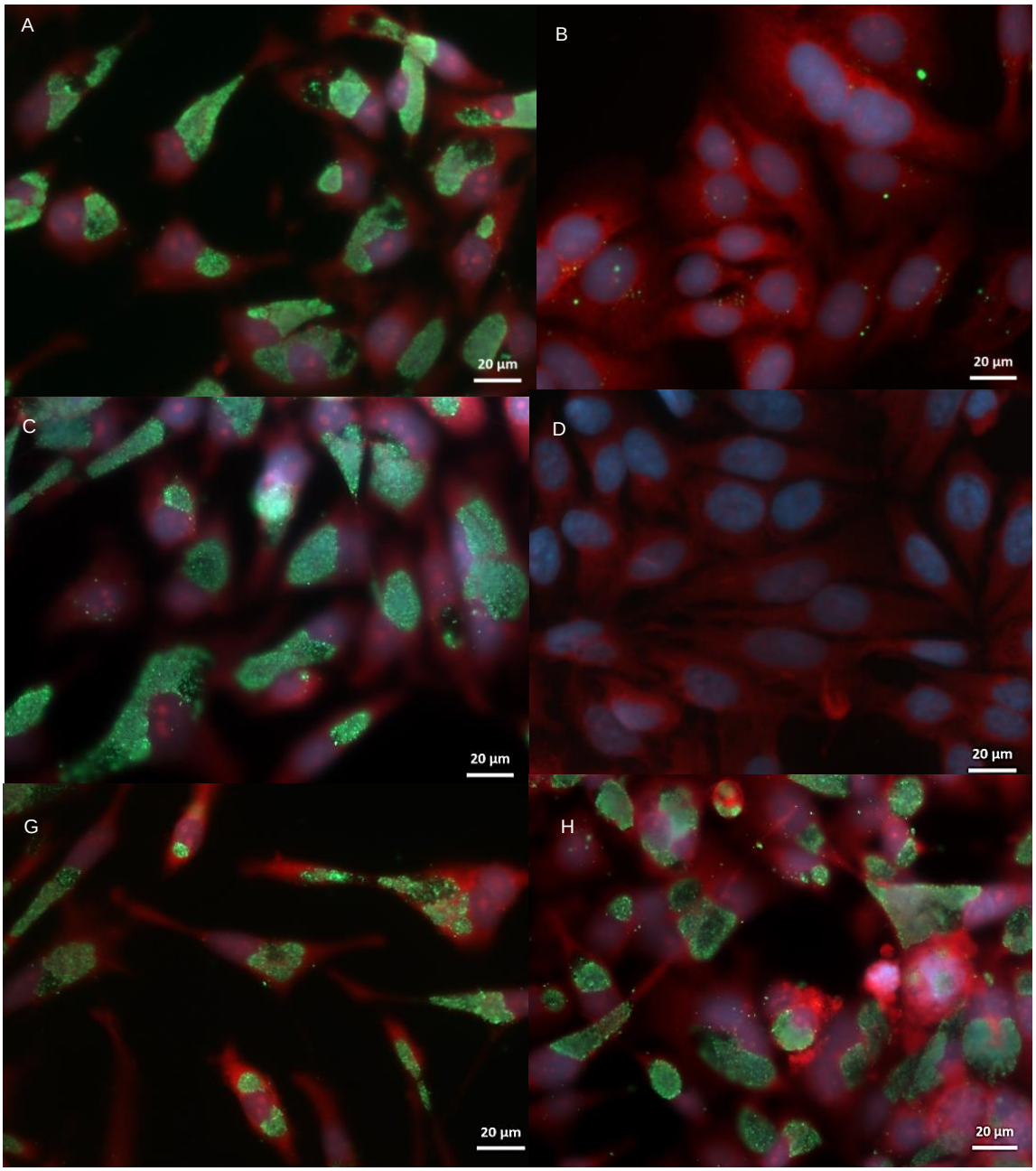

Fig. 2- Fluorescence microscope images of eukaryotic cells from line Hep2 infected with *Chlamydia trachomatis* and exposed to several treatments, as ciprofloxacin and the 1.28 µL extracts from Planctomycetes strains UC9 and FF1. Blue color corresponds to the DAPI staining of DNA from the host cells, green to chlamydial inclusions and red to host cell cytoplasm. A= Control of untreated cells; B= Control of cells treated with the antibiotic ciprofloxacin (at 1µg/mL); C= Cells exposed only to 1.28 µL DMSO dissolved in sterile water; D= Control of non-infected cells; E= Cells exposed to 1.28 µL extract of strain MsF2; F= Cells exposed to 1.28 µL extract of strain UC9. No effect of both UC9 and MsF2 extracts in the chlamydial infection was visualized at this quantity, since the aspect of the inclusions (green) is similar with the control of untreated cells (A).