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Building novel bioaugmentation consortia for degradation of polycyclic aromatic hydrocarbons: from selection to metabolic and genetic characterization of adaptive evolved microbial strains

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant compounds considered as priority pollutants in soil, water and the atmosphere. They can have natural origin (fires, volcanic eruptions, among others) or anthropogenic origin by the incomplete combustion of organic matter (coal, oil, wood). This is not only an environmental problem but also impacts on public health, since studies that indicate the toxicity and mutagenic/carcinogenic effects of PAHs are reported.

Many attempts have been made to develop strategies to eliminate these compounds. Bioremediation has emerged as a possible solution to the problem using microorganisms and taking advantage of their degrading capacity to reduce or eliminate the presence of PAHs. The presence of PAHs on planet Earth through the evolution of life shaped the evolution of metabolic pathways allowing microorganisms to use these compounds as carbon and energy sources.

A screening of forty-seven strains (Biotask Bioremediation Culture collection) from adaptive evolution experiments was performed. The initial inoculum of the experiments was from a wastewater treatment plant. In order to evaluate the degradative potential of these strains, growth assays were performed with naphthalene, anthracene and phenanthrene as the only source of carbon and energy. Directly, an HPLC method was developed and optimized that allowed the quantification of PAHs present in the biological samples. Indirectly, a cell viability study was performed through the most probable numbers. The most recent cycle of adaptive evolution revealed the best results as expected.

The selection of the best degraders was performed through a principal component analysis. In addition, an environmental contaminant revealed an interesting degradative behaviour and was included in the BBC collection.

Sequencing of the bacterial 16S rRNA gene, allowed the four strains to be presumptively identified as belonging to the genus *Pseudomonas*, *Acinetobacter* and *Paraburkholderia*.

Characterization of the strains involved three phases. Initially, the growth of the microorganisms was monitored having the PAHs as the carbon source for 15 days, both individually and in a consortium. The strain belonging to the genus *Pseudomonas* present the best results and possibly a bi-phasic growth. The consortium also revealed that strains together yield better results than individual growth. Then, the production of biosurfactants was evaluated qualitatively through the observation of an emulsion and quantitatively through the measurement of surface tension. The strain belonging to the genus *Acinetobacter* was the only one that apparently synthesizes a compound with surfactant activity. Finally, the presence of genes associated with PAH catabolism and growth having the metabolic intermediate phthalate as the sole source of carbon was evaluated. The strains belonging to the genera *Pseudomonas* and *Paraburkholderia* were the ones that presented greater metabolic plasticity.

In summary, it can be concluded that the objective of the work was achieved, and the most promising strains with higher potential for degradation of PAHs were selected and studied in metabolic and genetic terms. The growth of the strains in the consortium was successfully achieved and some characteristics that may explain strains degradative behaviour were revealed. More studies will be needed to elucidate more clearly the whole process in order to build an effective consortium for the bioremediation of contaminated sites.

Key-Words: Bioremediation, Bioaugmentation, Metabolism, Polycyclic Aromatic Hydrocarbons, Consortium

Resumo

Os hidrocarbonetos aromáticos policíclicos (HAP) são poluentes ambientais encontrados na água, no solo e na atmosfera. Estes compostos são de elevada importância devido ao perigo que representam em termos de saúde humana e em termos ambientais, existindo uma lista de 16 HAP considerados poluentes prioritários, apresentando propriedades tóxicas, mutagénicas e/ou carcinogénicas.

Por um lado, podem ser gerados naturalmente através de fogos florestais, atividade vulcânica e derramamentos de petróleo. Por outro lado, podem ter origem em atividades antropogénicas durante a combustão incompleta de materiais orgânicos, como o óleo, o petróleo ou a madeira. O facto de serem insolúveis em água dificulta a sua remoção e consequentemente leva a que se tornem recalcitrantes no meio ambiente, persistindo por muitos anos.

Várias tecnologias de remediação têm sido testadas com o objetivo de remover estes contaminantes ambientais. Muitas das abordagens têm uma natureza química, o que além de se traduzir em elevados custos económicos também está associado à geração de subprodutos também de natureza tóxica. Recentemente, a biorremediação têm sido alvo de atenção já que o uso de microrganismos para remoção de HAP tem sido vista como uma opção mais segura e com menos custos. Vários géneros de bactérias Gram positivas e Gram negativas, bem como fungos e algas capazes de utilizar estes compostos como fonte de carbono e energia têm sido isolados e caraterizados. Na natureza, são raras as vezes que estes microrganismos com capacidade de degradar poluentes se encontram individualmente. Geralmente, existem em consórcio, ou seja, associações naturais de vários microrganismos diferentes que interagem sinergicamente entre si, aumentando a eficiência de degradação. Esta cooperação resulta por exemplo em interações onde os produtos metabólicos de um microrganismo podem ser o substrato para outro microrganismo.

Nas estações de tratamento de águas residuais, o objetivo é obter um efluente que possa retornar ao ciclo da água com o mínimo impacto no ambiente. Neste contexto ecológico, os microrganismos geralmente realizam metabolismo aeróbio, tendo o oxigénio como aceitador final de eletrões. Catabolicamente, as enzimas com atividade de dioxigenase têm um papel fundamental na ativação do HAP para degradação. Um aspeto do metabolismo aeróbio é a formação de intermediários metabólicos como o salicilato, o catecol, o gentisato e o ftalato que após reações enzimáticas originaram metabolitos que compõem o ciclo dos ácidos tricarboxílicos. Estas proteínas com atividade catalítica estão amplamente distribuídas por diferentes microrganismos, considerando-se mesmo a existência de diferentes famílias de enzimas associadas a determinados taxa microbianos.

Além disso, verifica-se uma plasticidade metabólica no que diz respeito à degradação destes compostos podendo ocorrer também degradação em condições de anaerobiose, tendo compostos inorgânicos como o sulfato, o nitrato e o ferro como aceitadores de eletrões. No entanto, ainda existe muito por revelar sobre este metabolismo.

O presente trabalho tem como objetivo a seleção e caracterização metabólica e genética das melhores estirpes degradadoras presentes na coleção Biotask Bioremediation Culture, com vista à construção de um consórcio para degradação de HAPs, nomeadamente naftaleno, antraceno e fenantreno.

Quarenta e sete estirpes microbianas provenientes de quatro experiências de evolução adaptativa e de vários ciclos dessas mesmas experiências tendo antraceno, fenantreno, óleo mineral e tristearina

como única fonte de carbono e energia foram estudados. O inóculo inicial das experiências de evolução adaptativa foi proveniente de uma estação de tratamento de águas residuais.

Após sete dias de crescimento das quarenta e sete estirpes tendo naftaleno, antraceno e fenantreno como única fonte de carbono e energia, foi realizada uma triagem das quarenta e sete estirpes através de uma avaliação direta e uma avaliação indireta da eficiência de degradação. Diretamente, foi desenvolvido e otimizado um método de quantificação por cromatografia líquida de elevada performance. Através da construção das curvas de calibração para naftaleno, antraceno e fenantreno foi possível dosear a quantidade desses poluentes presentes em amostras biológicas provenientes do crescimento. Indiretamente, foram realizados ensaios que permitiram concluir sobre a viabilidade celular através do método dos números mais prováveis dos microrganismos em estudo após os sete dias de crescimento. Desta triagem inicial, foi possível concluir que os melhores resultados foram obtidos para as estirpes provenientes do ciclo de evolução adaptativa mais recente, ou seja, foi corroborado o sucesso das experiências de evolução adaptativa. Surgiram também evidências da volatilidade e da toxicidade do naftaleno ser um fator que justifica a sua baixa concentração quantificada cromatograficamente e a baixa viabilidade dos microrganismos na presença deste HAP.

Com vista a selecionar as estirpes com maior potencial degradativo foi realizada uma análise multivariada que integra os dados quantitativos da cromatografia líquida de elevada performance e os dados de viabilidade celular resultantes dos números mais prováveis. A análise em componentes principais permitiu escolher as estirpes que melhor se posicionavam no espaço formado pelas novas variáveis – componentes principais. Também esta análise permitiu observar que as estirpes pertencentes aos ciclos de evolução adaptativa mais recentes e que se esperam exibirem um melhor comportamento degradativo estavam localizadas na zona do gráfico onde seria expectável encontrar os melhores degradadores.

Além das três estirpes selecionadas pela análise de componentes principais, num ensaio de crescimento surgiu um novo microrganismo como contaminante ambiental que foi adicionado à coleção já que exibia um comportamento degradativo em relação aos HAPs e dada a dificuldade em encontrar ao acaso microrganismos com essas caraterísticas (geralmente são isolados em locais já contaminados pelos poluentes), pensou-se ser interessante explorá-lo melhor e incluí-lo nos estudos futuros.

Em seguida, os quatro microrganismos selecionados foram identificados presuntivamente através de sequenciação do gene bacteriano que codifica para o RNA ribossomal 16S como pertencentes aos géneros *Pseudomonas* (dois microrganismos), *Acinetobacter* e *Paraburkholderia*.

Com o propósito de investigar o metabolismo degradativo dos HAPs por parte destas bactérias, foram realizados estudos de caraterização metabólica e genética.

Em primeiro lugar, foi monitorizado durante quinze dias o crescimento destes microrganismos tendo naftaleno, antraceno ou fenantreno como única fonte de carbono e energia. As estirpes foram estudadas isoladamente e em conjunto, constituindo um consórcio bacteriano. Observou-se aparentemente um crescimento bifásico e a estirpe pertencente ao género *Pseudomonas* destacou-se, apresentando o melhor comportamento degradativo. O consórcio também revelou que as estirpes em conjunto apresentam resultados superiores do que se estiverem a crescer individualmente.

Em segundo lugar, foi avaliada a produção de biosurfactantes como estratégia de solubilização destes compostos poluentes. Após uma pré-seleção com um teste qualitativo onde se observou a formação de uma emulsão estável em duas estirpes, foi realizado um ensaio quantitativo através da medição da tensão superficial. Foi possível concluir que a estirpe pertence ao género *Acinetobacter* aparentemente sintetiza algum composto com atividade surfactante.

Em terceiro lugar, foi realizada uma pesquisa de genes que estão reportados como associados ao catabolismo dos PAHs através de polimerase chain reaction (PCR), tendo como alvo enzimas chave para o clivagem de intermediários metabólicos. Além disso, foi também monitorizado o crescimento tendo ftalato como única fonte de carbono e energia. Foi encontrada evidência que a estirpe pertencente ao género *Acinetobacter* possui genes que lhe permitem optar por um metabolismo aeróbio com clivagem *orto* do metabolito intermediário catecol. Quanto às estirpes pertences ao género *Pseudomonas* e ao género *Paraburkholderia* foi observada a presença de genes que lhe permitem seguir a via de degradação *meta* do catecol e ainda a via do gentisato. Em adição, a estirpe pertencente ao género *Paraburkolderia* revelou ser a única que claramente é capaz de crescer tendo ftalato como única fonte de carbono, o que indica que pode também seguir esta via degradativa. Relativamente ao outro microrganismo pertencente também ao género *Pseudomonas* não foi detetada nenhuma amplificação nem crescimento em ftalato.

Em suma, pode concluir-se que o objetivo do trabalho foi atingido, tendo sido selecionadas e estudadas em termos metabólicos e genéticos as estirpes mais promissoras em termos de potencial de degradação de HAPs. Foi verificado o sucesso do crescimento das estirpes no consórcio e relevadas algumas características que podem explicar o seu comportamento degradativo. Mais estudos serão necessários para elucidar mais claramente quais as vias metabólicas do catabolismo destes compostos e consequentemente ter acesso ao máximo do seu potencial de degradação, com vista à construção de um consórcio eficaz para a biorremediação de locais contaminados.

Palavras-Chave: Bioremediação, Bioaumentação, Metabolismo, Hidrocarbonetos Aromáticos Policíclicos, Consórcio

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Abreviation List

PAH	Polycyclic Aromatic Hydrocarbons
EU	European Union
SCF	EU Scientific Committee for Food
USEPA	United States Environmental Protection Agency (USEPA)
WWTP	Wastewater Treatment Plant
TCA	Tricarboxylic Acid
RDH	Ring Hidroxylating Dioxygenase
ISP	Iron Sulfur Protein
NADH	Nicotinamide Adenine Nucleotide
NADPH	Nicotinamide Adenine Phosphate Nucleotide
NAPL	Non-Aqueous Phase Liquid
CMC	-Critical Micellar Concentration
BBC	Biotask Bioremediation Culture Collection
AE	Adaptive Evolution
ANT	Anthracene
NAP	Naphthalene
PHE	Phenanthrene
MiO	Mineral Oil
GTS	Glyceryl Tristearate
HPLC	High Performance Liquid Chromatography
LLE	Liquid-Liquid Extraction
MPN	Most Probable Numbers
TSB	Tryptic Soy Broth
CFU	Colony Forming Units
PCA	Principal Component Analysis
PC	Principal Component
ATP	Adenosine Triphosphate
AUC	Area Under the Curve

CHAPTER I – Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules composed only by carbon and hydrogen atoms. Structurally, they have a core formed by two or more fused benzene rings with a linear, angular or clustered geometry and a pair of carbon atoms shared between these aromatic rings (Parab and Phadke, 2017). These compounds are categorized as low-molecular weight if they comprise up to three fused aromatic rings or high-molecular weight if containing more than three aromatic rings (Cravo-Laureau, 2017).

PAHs are considered ubiquitous since they can be found in water, soil and atmosphere, being environmentally persistent with numerous different structures and varied toxicity. These compounds are present in priority pollution list of European Union (EU), EU Scientific Committee for Food (SCF) and the United States Environmental Protection Agency (USEPA) because they are considered environmental pollutants which cause deleterious effects on flora and fauna of contaminated sites (USEPA, 2008). In addition, PAHs can cause health problems and genetic defects in human beings, having been reported mutagenic and carcinogenic effects (Armstrong *et al.*, 2004). The mechanism of toxicity is related to interferences with cellular membrane function as well as with enzyme machinery (Abdel-Shafy and Mansour, 2016).

PAHs can be formed naturally through biological processes of natural petroleum formation as products of incomplete combustion from natural combustion sources such as forest fires or volcanic eruptions. However, the major source is anthropogenic with incomplete combustion in incinerators and industrial processes being a large point source. Other anthropogenic sources are automotive emissions, cigar smoke, barbecues, petroleum product spills and sewage sludge, among others (Abdel-Shafy and Mansour, 2016).

1.1 Bioremediation

PAHs are difficult to remove from nature. Until recently, to eliminate these pollutants physicochemical treatments (steam distillation, solidification, chemical precipitation and incineration) have been implemented. However, besides being more expensive and generate toxic sub products that require additional treatment, the methods described tend to transport contaminant compounds from one place to another rather than really eliminate them (Blanco-Enríquez *et al.*, 2018).

Bioremediation is an approach to convert the toxic compounds to less hazardous/non-hazardous forms with less input of chemicals, energy and time. This is a process to remove pollutants in an eco-friendly manner and have low cost operation costs. PAH-contaminated sites can be remediated via individual microorganisms – algae, bacteria and fungi - or in combination – microbial consortia (Waigi *et al.*, 2015). Bacterial genera that are known to degrade PAHs are present in Appendix A (Seo *et al.*, 2009). Employing microbial consortia leads to large-scale production since pure cultures are not often economically practicable due to high production costs, substrates and biomass recovery. In addition, microorganisms are rarely isolated as pure cultures but are naturally part of consortia in contaminated sites (Blanco-Enríquez *et al.*, 2018).

In wastewater treatment plants (WWTP), the biodegradation of pollutants by taking advantage of microorganisms present in activated sludge is one of the most important strategies to remove organic

contaminants from wastewater. Nevertheless, this approach has limitations since many contaminants are not efficiently eliminated. To counterweight the limitations, the concept of bioaugmentation has emerged by the introduction of specific and efficient pollutant-degrading microorganisms (natural or genetically engineered) into a microbial community to enhance their hydrocarbon-oxidizing potential (Nzila *et al.*, 2016).

1.2 Bacterial metabolism of PAHs

A wide variety of bacterial, fungal and algal species have the potential to degrade PAHs. However, this introduction will only focus on the bacterial metabolism of PAHs degradation.

1.2.1 Aerobic degradation

The initial step in the metabolic aerobic degradation process of PAHs involve their chemo-, regio- and stereospecific hydroxylation by dioxygenases, which catalyse the incorporation of two oxygen atoms from molecular oxygen into the aromatic ring. From this reaction, cis-dihydrodiols are formed and targeted by dehydrogenases to form a limited number of central aromatic acid intermediates, such as the monoaromatic compounds o-phthalate, salicylate and gentisate. The aromatic ring of the oxyfunctionalized metabolites produced is then cleaved at the ortho or meta position by ring cleaving dioxygenases such as C-C breaking oxygenases, yielding intermediates which can enter in central metabolic routes for example the tricarboxylic acid cycle (TCA) or the β-oxidation cycle (Ghosal *et al.*, 2016) (Bruno and Schmid, 2004).

The degradation scheme described (Figure 1) is called “metabolic funnel” because it suggests that microorganisms increased the range of substrates by developing highly diverse upper pathways including enzymes with large substrate spectra able to turn a variety of initial substrates into one of the central metabolites. The oxygenases involved in the metabolism of PAHs belong to different families of enzymes and are distinguished by the type of reaction they catalyse, in particular the range of substrates they can transform (Ghosal *et al.*, 2016). The location and geometry of the active site are critical in enzyme specificity because they influence substrate access and orientation. Depending on this selectivity, different dihydrodiols are formed, which determines the subsequent steps of the degradation pathway (Ferraro *et al.*, 2006). Consequently, this variability contribute to the flexibility of the microbial machinery for degrading PAHs (Bruno and Schmid, 2004).

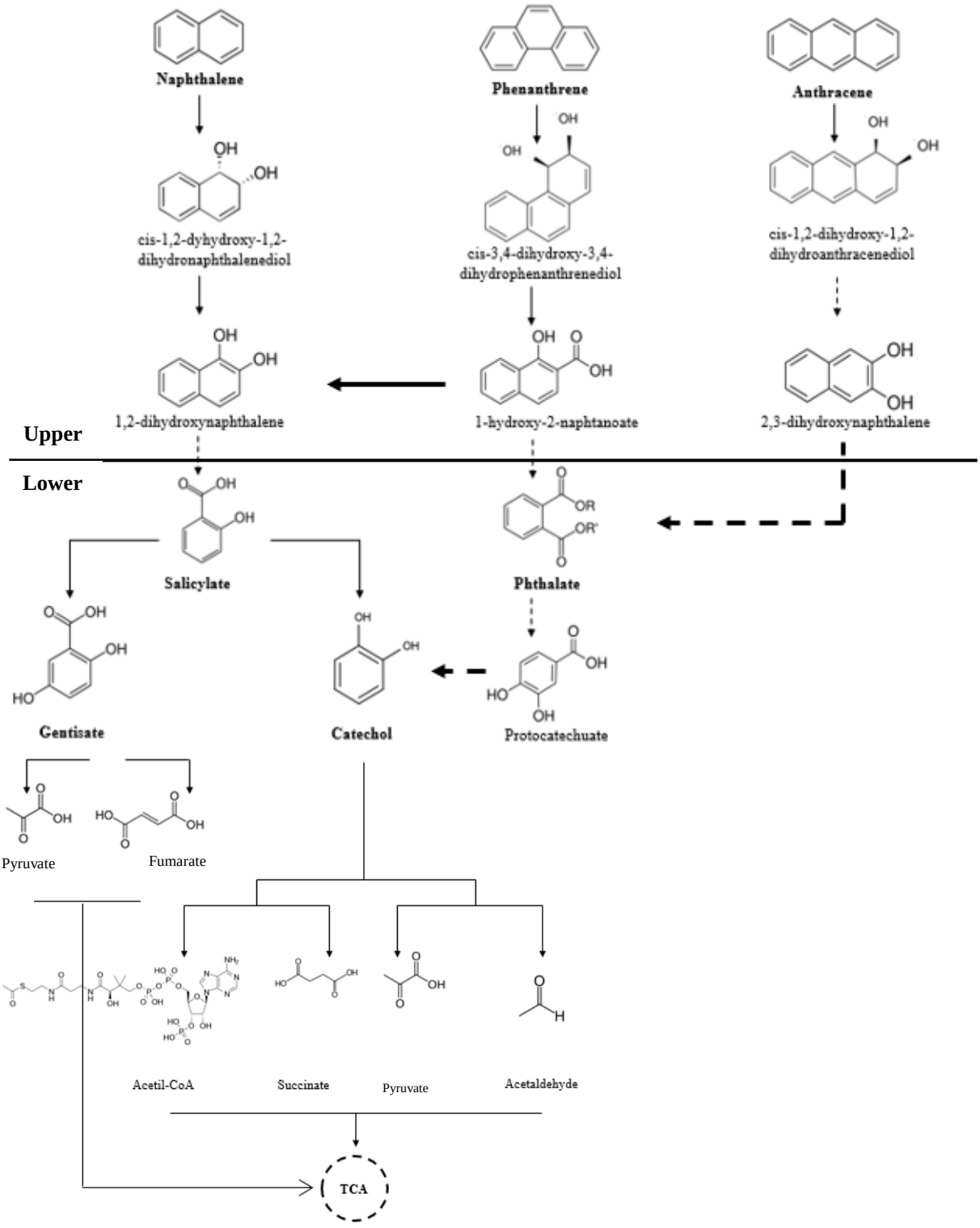

Figure 1 Representative scheme of the main upper and lower aerobic degradation pathways of PAHs under study. Dashed lines indicate the omission of intermediate enzymatic steps (information adapted from KEGG Pathway Database).

The Rieske non-heme iron dioxygenase called ring hidroxylating dioxygenase (RHD) (Bugg and Ramaswamy, 2008) is the essential enzyme for initiating PAH catabolism by ring hydroxylation. It consists of a nicotinamide adenine nucleotide (NADH) reductase, a ferredoxin [2Fe-2S] and a terminal oxygenase. This last component is formed by a larger α-subunit with the iron-sulfur proteins (ISP), with Fe (II) as cofactor in the active centre of the enzyme which confers the designation of extradiol dioxygenase (Lipscomb, 2009) and a smaller β-subunit with structural functions. The reductase and ferredoxin transport electrons to the α-subunit, which inserts molecular oxygen into the substrate to give cis-dihydrodiols, which are subsequently transformed to diols by a dehydrogenase (Cravo-Laureau, 2017) (Figure 2).

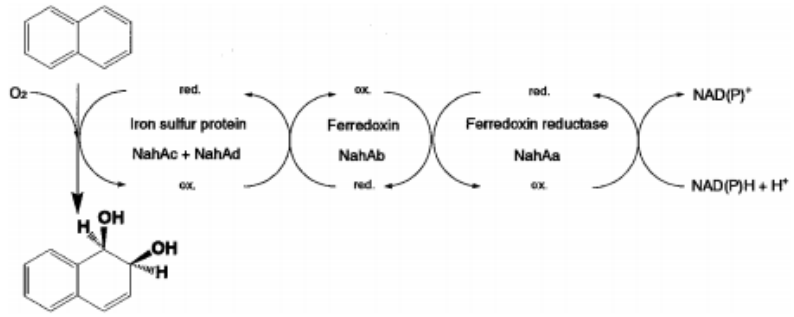

Figure 2 Initial oxidation of naphthalene to cis-1,2-dihydroxy-1,2-dihydronaphthalenediol by naphthalene dioxygenase (extracted from Habe and Omori, 2003).

PAH-RDHs are largely distributed enzymes across microbial taxa and comparisons of similarities in amino acid sequence have revealed distinct PAH-RDH families, the phylogenies of which generally follow those of the host (see Appendix B). Proteobacterial RDH are generally most active with low molecular weight PAHs such as naphthalene, anthracene and phenanthrene than actinobacterial RDH (Cravo-Laureau, 2017). The variability in gene clusters and the promiscuity of the enzymes involved in PAHs catabolism provide microorganisms with the capacity to degrade a great range of compounds.

1.2.2 Anaerobic degradation

In the absence of oxygen, it is known that naphthalene and two-ring PAHs can be used as the sole carbon source of carbon and energy. Whether three or more ring PAHs are used as growth substrate or co-metabolized in anaerobic condition remains unclear (Meckenstock *et al.*, 2004).

It was reported the success of sulfate-reducing naphthalene-degrading cultures from different PAHs contaminated anoxic aquifers, pointing that the capacity to degrade naphthalene anaerobically with sulfate as terminal electron acceptor is widespread in nature (Kümmel *et al.*, 2015). Other studies indicate that anaerobic PAH-degradation can also be performed by iron-reducing microorganisms (Kleemann and Meckenstock, 2011). There were also reports on degradation of PAHs with nitrate as electron acceptor but it has been difficult to reproduced them (Eriksson *et al.*, 2003).

1.3 Microbial uptake of PAHs

The bioavailability of the pollutants is influenced by polarity, hydrophobicity and water solubility. As PAHs are hydrophobic and practically insoluble in water and present high octanol-water partition coefficient they remain adsorbed in the non-aqueous phase liquid (NAPL). Besides that, PAHs strongly bind to soil and sediment organic matter. However, since the degradative enzymes essential for the metabolism of PAHs have an intracellular location, they must be solubilized so that they can enter the cytoplasm and be metabolized. Two strategies can be implemented to enable the access of microorganisms to the compounds and consequently their biodegradation: either the target compound is dissolved in the aqueous phase or the microorganism adheres directly to the NAPL (Schluep *et al.*, 2001)..

PAH's properties limit their bioavailability and thus the effectiveness of a bioremediation process. Driven by the solubilization capacity of surfactants - synthetic and natural - for hydrophobic compounds, surfactant-mediated remediation has been a research focus in recent years (Makkar and Rockne, 2003; Li and Chen, 2009)

A surfactant is an amphiphilic molecule composed of a hydrophilic group and a hydrophobic group that can accumulate at the liquid-liquid interfaces and reduce both surface tension and interfacial tension. In addition, surfactants increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds, such as PAHs. When the surfactant concentration exceeds a certain level, called critical micellar concentration (CMC), the molecules aggregate and micelles are formed. In this condition, hydrophobic molecules, depending on their polarity, are incorporated into the micelle core or the micelle/water interface, and their solubilization occurs (Li and Chen, 2009).

Surfactants can enhance bioremediation in three ways (Figure 3). First, the microorganism can take up the pollutant from the direct interaction of the micelle with cell membrane. Second, microorganisms use only the pollutant molecules that are dissolved in the aqueous phase and the role of the surfactant is thereby to increase the mass transfer from NAPL to aqueous phase. Third, the presence of surfactants changes cell hydrophobicity, facilitating the direct contact with cells (Schippers *et al.*, 2000).

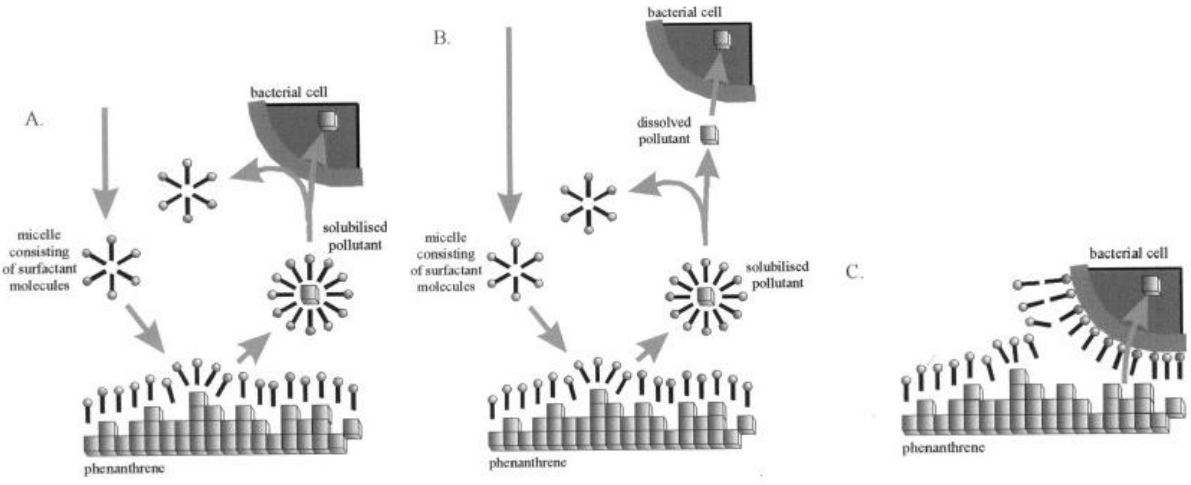

Figure 3 Explanatory approaches for the surfactant mediated incorporation of pollutants (extracted from Schippers et al., 2000).

Possibly, a more promising answer for bioavailability enhancement may be the use of surfactants produced naturally by microorganisms – biosurfactants - whose primary function is to facilitate microbial life in hydrophilic-hydrophobic environments. Over the last years, interest in biosurfactants increased due to their several advantages over their synthetic counterparts. For example, biosurfactants are easily biodegradable and consequently pose no extra pollution threat. In addition, most studies indicate that they are not toxic to microorganisms and therefore not likely to inhibit biodegradation of PAHs. Biosurfactant production is also potentially more cost-effective than synthetic surfactants and can be easily achieved *in situ* at contaminated sites using inexpensive raw materials. In contrast, synthetic surfactants can be toxic, be degraded instead of the pollutant and their production is expensive since they derived only from synthetic stocks, which make the remediation difficult to achieve on site (Makkar and Rockne, 2003).

Biosurfactants can be classified based on their compound structure as glycolipids, lipopeptides and lipoprotein, fatty acids, phospholipids and neutral lipids, polymeric surfactants and particulate biosurfactant (Shekhar *et al.*, 2015). Detailed information about the microorganism and associated biosurfactant are present in Appendix C.

Since PAHs exhibit low solubility in water and a high octanol-water partition coefficient, they tend to partition in cell wall structures generally through passive transport driven by the concentration gradient between the environment and the cell. The bilayer of phospholipids that constitutes bacterial membranes creates a hydrophobic region in the interior of the membrane that can act as a reservoir for accumulation of hydrophobic compounds (Bugg *et al.*, 2000). Thus, both the concentration of the pollutant and its bioavailability are crucial for the transport process. The higher the concentration and bioavailability of PAHs, the greater is their transport to the cell, always considering the saturation limit of the transporters. On one side, microorganisms can use this passive diffusion to reach rapid degradation of PAHs, with consequent growth. On the other side, the rapid accumulation of PAHs may lead to cell membrane disruption or inhibition of the membrane proteins etc., causing cell death.(Sikkema *et al.*, 1995) When PAHs concentration and bioavailability is low, the transport may not occur, decreasing the efficiency of microbial degradation and therefore the bioremediation process. However, some microorganisms seem to have adapted to that conditions and exhibit mechanisms such as direct contact with solid-phase PAHs and biosurfactant excretion to facilitate desorption and solubilization (as referred earlier) (Schluep *et al.*, 2001).

Some studies have shown the presence of an active efflux mechanism of phenanthrene, fluoranthene and anthracene in *Pseudomonas fluorescens* LP6a (Bugg *et al.*, 2000). It has also been proposed the involvement of a specific energy-dependent carrier for the incorporation of naphthalene into *Pseudomonas fluorescens* Uper-1 (Whitman, 1998). In *Mycobacterium* sp. strain RJGII-135 was suggested the existence of passive and active transport for the use of phenanthrene (Miyata *et al.*, 2004). Although limited research has been performed on cellular binding and transport of PAHs, several experiments suggest that passive and active transport systems can be exploited to increase the efficiency of bioremediation.

1.4 Genes associated with catabolism of PAHs

***nah*-like genes**

Genetically, naphthalene catabolism has been extensively studied using the *Pseudomonas putida* G7 strain as a model, since a transmissible metabolic plasmid that carries genetic information enabling its host to use naphthalene as a sole source of carbon and energy was isolated (Early, 1973). In NAH7 plasmid, naphthalene oxidation genes are organized in two operons: the upper catabolic pathway operon includes genes *nahABCDEF* encoding the enzymes involved in the conversion of naphthalene to salicylate and the lower catabolic pathway operon includes genes *nahGHIJK*, coding the enzymes involved in the salicylate to TCA cycle intermediates pyruvate and acetaldehyde via catechol meta-cleavage pathway. It is implied that the enzymes required for the catechol ortho cleavage pathway originating the TCA cycle intermediates succinyl CoA and acetyl CoA are encoded in genes located on the chromosome (Yent and Gunsalus, 1985). Both upper (*nah*) and lower (*sal*) catabolic pathway operons are regulated by a trans-acting positive control regulator coded by the *nahR* gene, located between the two operons (Schell, 1985). The *nahR* gene encodes a LysR-type transcriptional repressor-activator that binds to promoters upstream of both the upper and lower catabolic pathway operons and represses transcription allowing only a basal level of expression. Binding of salicylate to NahR at the promoters induces a conformational change in DNA-bound NahR that enables transcription to occur (Cravo-Laureau, 2017). This regulatory protein is needed for the high-level expression of the *nah* genes (Habe and Omori, 2003).

Nucleotide sequences of genes encoding naphthalene upper-catabolic enzymes from several *Pseudomonas* spp. strains were described. These genes were named according to the strains's feature of substrate use: *nah* for naphthalene degradation from *P. putida* strain G7 and *P. stutzeri* strain AN10 (Simon *et al.*, 1993; Moore, Bosch and Garcı, 1999), *ndo* for naphthalene dioxygenation from *P. putida* strain NCIB9816 (Palva and Teeri, 1988) (equivalent to *nah*), *dox* for dibenzothiophene oxidation from *Pseudomonas* sp. strain C18 (Denome *et al.*, 1993), and *pah* for polycyclic aromatic hydrocarbon (phenanthrene) degradation from *P. putida* strain OUS82 and *P. aeruginosa* strain PaK1 (Wang and Road, 1999) (later, all of these strains were found to grow on naphthalene). The gene organization and sequence similarity (about 90%) among the upper catabolic pathways genes of these strains were similar to those of the *nah* genes from the NAH7 plasmid of strain G7. Consequently, these genes are called "classical *nah*-like genes". In the upper catabolic pathway, the genes coding for NDO ferredoxin reductase (*nahAa*), NDO ferredoxin (*nahAb*), the a subunit of NDO (*nahAc*), the b subunit of NDO (*nahAd*), naphthalene cis-dihydrodiol dehydrogenase (*nahB*), salicylaldehyde dehydrogenase (*nahF*), 1,2-dihydroxynaphthalene dioxygenase (*nahC*), an unknown ORF (*nahQ*), trans-ohydroxybenzylidenepyruvate hydratase-aldolase (*nahE*), and 2-hydroxychromene-2-carboxylate isomerase (*nahD*), are arranged in this order (*nahAaAbAcAdBFCQED*) (Habe and Omori, 2003).

For the lower catabolic pathway, the reported genes are present on *Pseudomonas stutzeri* strain AN10 and encode the salicylate hydroxylase (*nahG*), chloroplast ferredoxin-like protein (*nahT*), catechol 2,3-dioxygenase (*nahH*), hydroxymuconic semialdehyde dehydrogenase (*nahI*), hydroxymuconic semialdehyde hydrolase (*nahN*), 2-oxopent-4-enoate hydratase (*nahL*), acetaldehyde dehydrogenase (*nahO*), 2-oxo-4-hydroxypentanoate aldolase (*nahM*), 4-oxalocrotonate decarboxylase (*nahK*), and 4-oxalocrotonate isomerase (*nahJ*), being arranged in this order (*nahGTHINLOMKJ*) (Moore *et al.*, 2000).

***phd* genes**

Comamonas teststeroni strains (GZ38A, GZ39 and GZ42) were isolated from river sediment and exhibited the ability to grow on naphthalene as well as phenanthrene except for strain GZ38A which grows on phenanthrene and anthracene. The catabolic genes present on the three strains had no genetic relatedness to the *nah*-like genes from *P. putida* strain NCIB 9816-4 discussed above (Zylstra, 1996). In strain GZ39, the genes responsible for the initial conversion of naphthalene and phenanthrene were named *phd* genes. The results indicated that the genes for phenanthrene degradation in strain GZ38A are similar (but not identical) to those from strain GZ39, but that strain GZ42 did not have any genes similar to the *phd* genes from strain GZ39. This make evident that these *C. testosteroni* strains represent at least two new classes of genes involved in PAH degradation. It also indicates that at least three non-homologous families of genes responsible for phenanthrene degradation can be found in the same environmental sample (Zylstra, 1996; Zylstra, 1997)

In strain GZ39, the genes coding for ferredoxin (*phdAb*), ferredoxin reductase (*phdAa*), cis-dihydrodiol dehydrogenase (*phdB*), the α subunit of ISP (*phdAc*), the b subunit of ISP (*phdAd*), isomerase (*phdD*), an unknown ORF, glutathione-S-transferase, and hydratase-aldolase (*phdE*) were arranged in this order (*phdAbAaBAcAdDE*). This gene organization was relatively diferent from that of the *nah*-like genes, and several genes such as extradiol dioxygenase (*nahC* analogue) were not within the cluster (Zylstra, 1997) The *phd* genes were compared to genes known from PAH catabolism and the α subunit sequence of ISP (PhdAc) was found to fit within the naphthalene family of dioxygenases although they are not closely related. In contrast, the sequences of the β subunit of ISP (PhdAd) and ferredoxin (PhdAb) showed little similarity to proteins having the same function in other aromatic ring dioxygenases (Habe and Omori, 2003).

It is important to mention that although strain GZ42 has novel genes for phenanthrene degradation, they are divergent relatives of those found in *P. putida* NCIB 9816. However, the nucleotide structure of the genes reveals some very important differences between the two strains. For example, the amino acid sequences of the enzymes are much closely related than the nucleotide sequence of each analogous gene, revealing genetic drift with conservation of enzyme structure. More significant is the evidence that, although the gene order in strain GZ42 is identical to *P. putida* NCIB 9816, there are two new genes between the genes encoding ferredoxin reductase (*nahAa*) and ferredoxin (*nahAb*). These genes were designated *nahAc2* and *nahAd2* because they were very similar to those of the α and β subunits, respectively, of another aromatic-ring dioxygenases ISP (Zylstra, 1997). But these genes have been thought to be non-functional in strain GZ42 (Habe and Omori, 2003).

***nag* genes**

A naphthalene catabolic operon with the same gene organization and similarity to the *nah* operon of the *C. testeroni* strain GZ42 was found in *Ralstonia* sp. strain U2 and characterized. The naphthalene dioxygenase genes (*nag* genes) coding for ferredoxin reductase (*nagAa*), ferredoxin (*nagAb*), the a subunit of ISP (*nagAc*), the b subunit of ISP (*nagAd*), cis-dihydrodiol dehydrogenase (*nagB*), and aldehyde dehydrogenase (*nagF*) were arranged in this order and two open reading frames named *nagG* and *nagH* (analogous to *nahAc2* and *nahAd2* of strain GZ42) were inserted between *nagAa* and *nagAb*. The *nagG* product aligns with the α subunit of other aromatic-ring dioxygenases, but the *nagH* product exhibited limited homology with the β subunit of other aromatic-ring dioxygenases. It was suggested that the hydroxylation of salicylate occurs through NagGH acting as a monooxygenase, with an associated electron transport chain composed by a ferredoxin and a ferredoxin redutase providing the electrons from a reduced nicotinamide cofactor, like NAD or NADP. This electron

transport chain for salicylate 5-hydroxylase is probably formed by NagAa and NagAb given the proximity between the genes coding for these proteins (*nagAa* and *nagAb*) and the *nagG* and *nagH* genes (Fuenmayor *et al.*, 1998). The genes for conversion of naphthalene to gentisate (*nagAaGHAbAcAdBFCQED*) are ordered in the same way as the genes in the classic *nah*-like operon of *Pseudomonas* spp. strains, with the exeption of the *nagGH* insertion. Furthermore, there is another difference between the *nag* operon and *nah* operon which is related to the regulatory gene *nagR* being located upstream the genes of upper catabolic pathway whereas its analogue *nahR* is located downstream (Zhou *et al.*, 2001).

Another gene cluster (*nagJIKLMN*) was found and the *nagI*, *nagK*, and *nagL* genes encoded the enzymes involved in the further catabolism of gentisate to fumarate-pyruvate: gentisate 1,2-dioxygenase, fumarylpyruvate hydrolase, and maleylpyruvate isomerase, respectively (Zhou *et al.*, 2001).

Nucleotide sequence comparisons suggest that the novel gene order *nagAa-nagG-nagH-nagAb-nagAc-nagAd-nagB-nagF* represents the model for naphthalene strains (like U2 and GZ42) that metabolize salicylate through the gentisate pathway instead of the catechol meta cleavage pathway (Fuenmayor *et al.*, 1998).

***phn* genes**

A divergent set of PAH catabolic genes named *phn* genes were described and these genes can be divided in two families, who are significantly different in sequence and gene order from the other classic genes involved in PAH degradation. For example, *phn*-like genes, although isofunctional, have little homology with the *nah*-like genes (Laurie and Zealand, 1999). The $PhnAc_{AFK2}$ family was first identified in *Alcaligenes faecalis* AFK2 and the Phn term is used to indicate that microorganisms with this genotype are characteristically limited to phenanthrene as sole carbon and energy source to support growth. The ring hydroxylating dioxygenase encoded in these genes leads to the formation of phthalate as an central intermediate. Although the name bears some resemblance, the $PhnAc_{RP007}$ genotype identified in *Burkholderia* sp. strain RP007 differs from the $PhnAc_{AFK2}$ family because it allows the utilization of naphthalene and phenanthrene to support growth and it leads to the production of salicylate from the upper pathway rather than phthalate (Cravo-Laureau, 2017).

Regarding *Alcaligenes faecalis* strain AFK2, the *phn* genes have a novel operon structure, and the genes coding for ferredoxin (*phnAb*), ferredoxin reductase (*phnAa*), cis-dihydrodiol dehydrogenase (*phnB*), the α subunit of NDO (*phnAc*), the β subunit of NDO (*phnAd*), putative 2-hydroxychromene-2-carboxylate isomerase (*phnD*), glutathione-S-transferase (*gst*), trans-2-carboxybenzalpyruvate hydratase-aldolase (*phnH*), 1-hydroxy-2-naphthoate dioxygenase (*phnG*),2-carboxybenzaldehyde dehydrogenase (*phnI*), 3,4-dihydroxyphenanthrene dioxygenase (*phnC*)), 1-hydroxy-2-naphthoaldehyde dehydrogenase (*phnF*), and putative transo-hydroxy benzylidenepyruvate hydratase-aldolase (*phnE*) are arranged in this order (*phnAbAaBAcAdDHGICFE*) (Kiyohara *et al.*, 1982)

Also, *Burkholderia* sp. strain RP007 presents a *phn* operon distinct from those reported so far. The naphthalene and phenanthrene can be degraded through a common upper pathway via salicylate (Habe and Omori, 2003). pathway These *phn* genes encode regulatory proteins (*phnR* and *phnS*), aldehyde dehydrogenase (*phnF*), hydratase-aldolase (*phnE*), extradiol dioxygenase (*phnC*), isomerase (*phnD*), ISP α subunit of initial dioxygenase (*phnAc*), ISP β subunit of initial dioxygenase (*phnAd*), dihydrodiol dehydrogenase (*phnB*), arranged in this order (*phnRSFECDAcAdB*). Some particularities are noted, such as the fact that the locus has ISP σ and β subunits of PAH-initial dioxygenase (*phnAcAd*)

but lacks both the ferredoxin and reductase components. In addition, the *phnB* gene encoding cis-diol dehydrogenase is more closely related to the corresponding genes from biphenyl catabolic pathways than to those found in the classical *nahB*-like genes. Furthermore, *phnC* gene encodes a novel extradiol dioxygenase with phylogenetic relationships with known PAH and biphenyl dioxygenases (Laurie and Zealand, 1999). Also two catechol 2,3-dioxygenase genes involved in the lower pathways (meta-cleavage yielding acethaldeyde and piruvate) were characterized (Laurie and Lloyd-jones, 1999). Upstream of the *phn* catabolic genes, there are two putative regulatory genes, *phnR* and *phnS*, being PhnS a LysR-type transcriptional activator and PhnR is a member of the s54-dependent family of positive transcriptional regulators (Laurie and Zealand, 1999).

***bph* genes**

Sphingomonads (composed of *Sphingomonas*, *Sphingobium*, *Sphingopyxis* and *Novosphingobium* genera) are capable of degrading PAHs like naphthalene, phenanthrene and anthracene by complete mineralization via similar pathways and enzymes found in other Gram-negative bacteria (Waigi *et al.*, 2015). It was identified and characterized the complete sequence of a catabolic plasmid named pNL1 from *Novosphingobium aromaticivorans* strain F199 (Romine *et al.*, 1999). This nucleotide sequence revealed a complex arrangement of genes for catabolism of all PAHs that are metabolized by the strain, wherein at least 13 gene clusters were predicted to encode enzymes associated with degradation of these compounds. Genes associated with degradation of naphthalene and byphenyl to catechol are predicted to take place in at least six different gene clusters. Seven different three-component oxygenases are expected from sequence analysis and seemed to interact with only one set of ferredoxin and reductase components in pNL1, acting as the only electron transport system for multiple dioxygenases (Romine, Fredrickson and Li, 1999). Only single copies of *bphA3* and *bphA4*, coding for ferredoxin and ferredoxin reductase, have been detected in strain F199. The *bphA1f* gene in strain F199 is likely to encode the α subunit of the naphthalene initial dioxygenase, even though its enzymatic activity has not been confirmed. In addition, it was verified the presence of at least six *bphA1* and *bphA2* dioxygenase gene homologs spread throughout the plasmid. These dioxygenases may be associated with upper pathways generating either salicylate or phthalate (Waigi *et al.*, 2015). Therefore, the key adaptation of most PAH-degrading sphingomonad strains is the multiple genes encoding catabolically versatile enzymes, specifically dioxygenases that can degrade different PAHs.

The gene organization and regulation of degradative pathways in sphingomonads are very complex comparatively to non-sphingomonads (such as pseudomonads), which reveals an evolutionary deviation. This adaptability mechanism is named “flexible” gene organization (*i.e.* different combinations of conserved gene clusters), which benefits in quick and efficient adjustment to new compounds in contaminated sites (Basta *et al.*, 2017).

***nar* genes**

A novel catalytic component of a naphthalene dioxygenase composed by a Rieske [2Fe-2S] centre was reported from *Rhodococcus* sp. strain NCBIMB12038 which is capable of utilizing naphthalene as its exclusive carbon and energy source. The nucleotide sequences of *narAa* and *narAb* genes encode the α and β subunits of ISP, respectively. However, these genes are not homologous to their previously characterized counterparts in *Pseudomonas* (Larkin *et al.*, 1999). It was also described a *narB* gene coding for cis-naphthalene dihydrodiol dehydrogenase. Once again this nucleotide sequence shows low overall similarity with *nahB* from *P. putida* strain G7 (Kulakov *et al.*, 2000). Nonetheless, the organization of naphthalene catabolic genes in strain NCIMV12038 (*narAaAbB*) is identical to the *nah* genes from *Pseudomonas* species (Habe and Omori, 2003). This dioxygenase coded by the *nar*

genes are generally associated with upper pathways that lead to phthalate formation (Cravo-Laureau, 2017).

***phd* genes**

The *phd* genes of *Nocardioides* sp. strain KP7 are the most studied PAH-catabolic genes in Gram-positive bacteria, and belong to a new class of PAH-catabolic genes because of differences in gene organization and sequence similarity (Habe and Omori, 2003). This strain is able to grow on phenanthrene (but not on naphthalene) and the degradation occurs via the phthalate pathway. The genes responsible for degradation of phenanthrene to phthalate (*phd*) are located on the chromosome. The *phdA*, *phdB*, *phdC* and *phdD* genes, which encode the α and β subunits of the oxygenase component, a ferredoxin, and a ferredoxin reductase, respectively, of phenanthrene dioxygenase were identified. Both subunits showed low sequence identity to the α and β subunits of other aromatic ring dioxygenases. Relatively to *phdC* gene, it codes for a ferredoxin which presents more similarity to the [3Fe-4S] or [4Fe-4S] type of ferredoxin, but not to the [2Fe-2S] type of ferredoxin found in most PAH dioxygenases. Contrarily, the ferredoxin reductase coded by *phdD* shows moderate sequence identity to the ferredoxin reductase of other isofunctional enzymes. All three components (PhdABCD) are indispensable for the effective dioxygenase activity that converts phenanthrene to his cis-diol compound (Harayama, 2000). There are other genes in the cluster (*phdEFABGHCD*) encoding enzymes responsible for the transformation of phenanthrene to 1-hydroxy-2-naphthanoate, such as dihydridiol dehydrogenase (*phdE*), extradiol dioxygenase (*phdF*), hydratase-aldolase (*phdG*) and aldehyde dehydrogenase (*phdH*). This gene cluster did not contain the ORF encoding isomerase, found in all PAH catabolic genes from Gram-negative bacteria. In adition, the catabolic genes for the conversion of 1-hydroxy-2-naphthanoate to phthalate encode 1-hydroxy-2-naphthoate dioxygenase (*phdI*), trans-2'-carboxybenzalpyruvate hydratase-aldolase (*phdJ*), and 2-carboxybenzaldehyde dehydrogenase (*phdK*) (Habe and Omori, 2003).

1.5 Objectives

Focusing on the negative impact of PAHs in environment and human health and in the context of a current research project of Lab Bugworkers|M&B-BioISI with Biotask Company, adaptive evolution experiments using microbial consortia from activated sludge and PAHs as selection pressure have previously been set up to obtain more efficient strains that can be used as novel bioaugmentation inocula, leading to a culture collection with over 300 microorganisms.

The goal of this MSc thesis is to select, identify and characterize at metabolic and genetic levels the best PAHs degraders in a sub-set of 47 evolved strains from this previous work in order to develop a novel bioaugmentation consortium.

CHAPTER II - Materials and Methods

2.1 Microbial strains

The strains used in this work belong to the Biotask Bioremediation Culture Collection (BBC) and have been isolated from adaptive evolution (AE) experiments carried by Pedro Teixeira, in the scope of his PhD thesis.

The adaptive evolution experiments were performed in mineral culture medium with the recalcitrant compounds anthracene (ANT), phenanthrene (PHE), glyceryl tristearate (GTS) and mineral oil (MiO) as sole carbon source for selective pressure. A WWTP sample was used as initial inoculum and kept for over 50 cycles, each consisting of a 7-day incubation with continuous shaking and addition of 1% inoculum from previous cycle. At different AE cycles along the experiments, aliquots were plated in different solid medium (Figure 4) and morphologically distinct types of colonies were isolated as pure cultures and genomically characterized by M13 PCR-fingerprinting (P. Teixeira, personal communication).

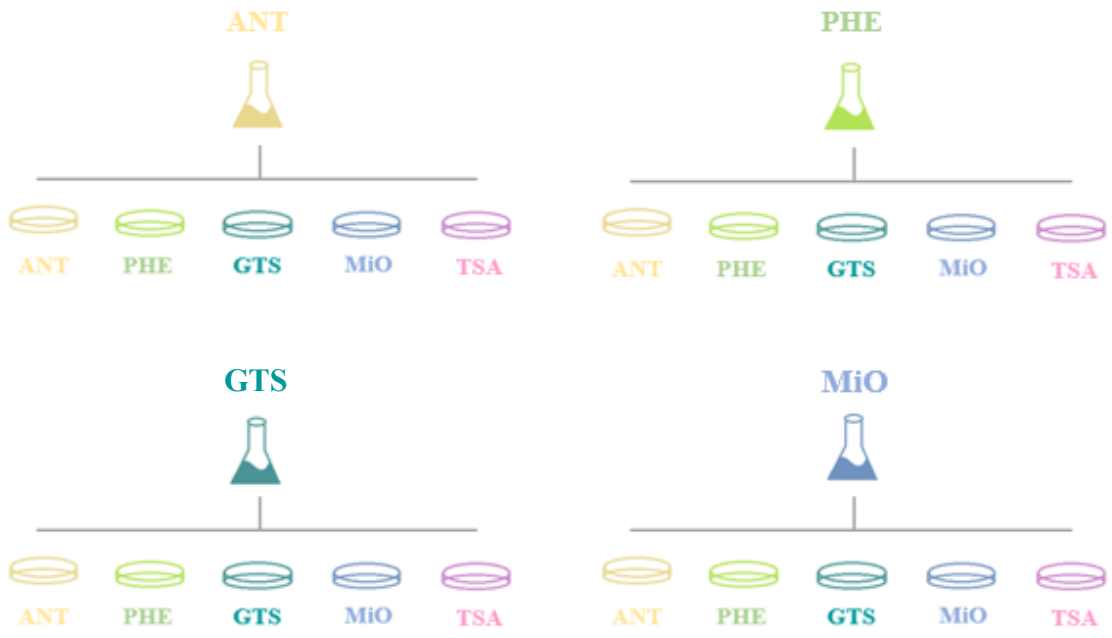

Figure 4 Simplified scheme of the previous work carried by Pedro Teixeira, in the scope of his PhD thesis where the 4 experiments of adaptive evolution are represented, from which the microorganisms were isolated in different isolation medium. In all cases, the indicated carbon source is the only carbon source of the experiment (ANT- anthracene; PHE – phenanthrene; GTS – glyceryl tristearate; MiO – mineral oil; TSA – tryptic soy agar).

The 43 bacterial and 4 yeast strains used in the work described in this MSc thesis (Table 1) were selected as representatives of the genomic diversity of the collection of BBC isolates from these AE experiments (see Appendix D).

Table 1 Origin of the strains used in the present work - experiment and cycle of adaptive evolution and isolation medium (P. Teixeira, personal communication).

Adaptive Evolution Experiment	Isolation Medium	Cycle	Strains
Anthracene (ANT)	TSA	12	BBC|216
		21	BBC|253; BBC|256
	ANT	29	BBC|331; BBC|332 BBC|333; BBC|336 BBC|338
	PHE	29	BBC|342; BBC|344
	UO	29	BBC|354; BBC|355
Phenanthrene (PHE)	PHE	5	BBC|290; BBC|291 BBC|292; BBC|293 BBC|294
		16	BBC|295; BBC|297 BBC|300; BBC|302
		20	BBC|311; BBC|312 BBC|314
		29	BBC|360; BBC|361 BBC|362; BBC|364 BBC|365; BBC|368 BBC|369
	UO	29	BBC|378; BBC|379 BBC|380; BBC|381
Glyceryl tristearate (GTS)	TSA	12	BBC|236
		21	BBC|279
	GTS	16	BBC|309
	UO	29	BBC|390; BBC|392
Lubricant Oil (UO)	TSA	21	BBC|284
	ANT	29	BBC|395; BBC|396 BBC|398; BBC|401
	GTS	20	BBC|318
	UO	29	BBC|409

2.2 PAH-degradation experiments

PAH-degradation experiments were carried out using glass tubes with screw stopper (Duran) containing 5 mL of M9 mineral medium (see composition in Appendix E) and PAHs were added with a final concentration of 2000 mg/L as sole carbon and energy source. The PAHs used in this test were naphthalene, anthracene and phenanthrene (Sigma Aldrich), studied separately. The inoculum was prepared by growing each of the 47 strains in TSA at 28°C overnight. Subsequently, the cells were recollected to a 0.8% sodium chloride (NaCl) solution. The optical density of cells was adjusted by using a UV-visible spectrophotometer (Unicam) at 600 nm to OD of 0.01 (1 unit of absorbance corresponds to 10^8 bacterial cells.ml-1 and 10^7 yeast cells.ml-1) (software Vision). The cell inoculum corresponding to 8 ×106 cells.ml-1 was added to each glass tube. The culture was shaken and incubated under aerobic conditions in a shaker (Thermo Scientific) at 28°C and at a speed of 150 rpm during 7 days. Abiotic control was performed by adding PAH into M9 without inoculum. After 7 days, the remaining PAH concentration was analysed using high performance liquid chromatography (HPLC) method. The assay was performed in duplicate.

Optimization tests were performed in order to define the concentration parameters of the calibration standards, considering that HPLC's quantification and detection limits are different for each compound. The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample (these concentrations included) for which has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. For naphthalene, the standard concentrations (ng/µL) used were 10.0, 50.0, 100.0, 200.0, 300.0 and 400.0. For anthracene, the standard concentrations (ng/µL) used were 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0. For phenanthrene, the standard concentrations (ng/µL) used were 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0.

2.3 Extraction and quantification of PAHs

After a liquid-liquid extraction (LLE) with 5 mL of chloroform (Carlo Erba) and manual agitation, the residual PAHs were analysed by HPLC (Beckman Coulter) with C18 reverse phase (5 µm particle size, Length × Internal Diameter: 25 cm × 4.6 mm, Supelco) column under gradient condition using acetonitrile (Carlo Erba): water (40:60) (v/v) as mobile phase for 5 minutes and then with linear increase of acetonitrile (100:0) (v/v) for 25 minutes. A fluorescence detector (Jasco FP-2020 Plus) equipped with a data acquisition system (32 Karat) were used at 280 nm for excitation wavelength and 355 nm for emission wavelength. The system operating pressure and flow rate were 2000-3000 psi and 1.0 mL/ min, respectively.

In order to validate the HPLC method for quantification of PAHs, the following parameters were taken in account.

(i) Selectivity/Specificity

Specificity is the capacity of an analytical method to separate and quantify the analyte in the presence of other compounds in the sample like impurities, degradants or matrix. It's an important parameter in a quantification assay because it should provide an exact result that allows an accurate statement on the content of the analyte in a sample (ICH, 2005).

In liquid chromatography, selectivity is achieved by choosing optimal columns and setting chromatographic conditions (mobile phase composition, column temperature and detector wavelength). To provide unique selectivity for PAH's separation, reversed-phase liquid chromatography on chemically bonded C18 phases was used (Wise *et al.*, 1981). In addition, the specificity of the method was determined by analysing a sample containing all the three PAHs – naphthalene, anthracene and phenanthrene - under study, and a good resolution between two peaks (retention times) without overlaps was observed.

For chromatographic procedures, representative chromatograms should be presented to demonstrate specificity and individual components should be appropriately labelled (see Appendix F).

(ii) Accuracy

The accuracy of an analytical method expresses the closeness of agreement between an accepted reference value and the value found (ICH, 2005).

(iii) Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (ICH, 2005).

(iv) Limit of detection

The detection limit of an individual analytical method is defined as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (Shrivastava and Gupta, 2011). It is calculated based on standard deviation of response (σ) and slope (s) expressed as:

$$\text{LOD} = \frac{3.3\sigma}{s}$$

The slope may be estimated from the calibration curve. The standard deviation is calculated through the standard deviation of y-intercepts of regression lines.

(v) Limit of quantification

The quantification limit of an individual analytical method is as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy value (Shrivastava and Gupta, 2011). It is calculated based on standard deviation of response (σ) and slope (s) expressed as:

$$\text{LOQ} = \frac{10\sigma}{s}$$

The slope may be estimated from the calibration curve. The standard deviation is calculated through the standard deviation of y-intercepts of regression lines.

(vi) Calibration range and linearity

For quantification by HPLC, a good calibration is essential to ensure reliable results and it requires a serie of standards of increasing concentration in order to create a calibration curve. The linearity of an analytical procedure is the capacity (within a given range) to obtain test results which are directly proportional to the concentration of analyte present in the sample.

2.4 Microbial cell viability

Assessment of the most probable number (MPN) of viable microbial cells was performed in 96 microwell plates, using 290 µL of tryptic soy broth (TSB) (Sigma Aldrich), 10 µL of inoculum from each 10-fold serial dilution (1 mL final volume). Incubation was performed at 28 °C for 48h.

For determination of characteristic number of microorganisms capable of PAHs degradation, two criteria were used. The positive wells criteria consist of using the number of positive wells (wells that shows turbidity resulting from growth) in the least concentrated dilution in which all or the highest number of wells were growth-positive and the numbers of the growth-positive wells in the following two higher dilutions were used. The negative wells criterion was also used (no growth is observed) where the lowest dilution where all the wells are negative and the numbers corresponding to the positive wells of the next two more concentrated dilutions are considered. The characteristic number obtained is then multiplied by the dilution factor in order to obtain the MPN values, based on 10-fold dilutions and 3 wells per dilution (n=3), were derived from published McCrady statistical tables (Marques-Pinto and Galhardo, 1983).

2.5 Principal component analysis

The results from the HPLC quantification and MPN determination were gathered in a matrix in order to perform a Principal Component Analysis (PCA) using NTSYSpc 2.2 software. The quantitative data was standardized by subtracting the average and dividing by the standard deviation. The similarity was calculated applying Pearson's correlation coefficient to the standardized matrix. Analysis of the explanatory variables for PCs can be found in Appendix G and H.

2.6 Molecular identification of strains

Amplification of 16S rRNA gene was performed in a final volume of 50 µl with 1x PCR reaction buffer (Invitrogen), 2 mM MgCl2, 50 pmol of each primer PA (5'AGAGTTTGATCCTGGCT CAG3') (Massol-Deya *et al.*, 1995) and 1392R (5'ACGGGCGGTGTGTRC3') (Baker *et al.*, 2003), 0.2 mM of dNTP's mix, 1 U of Taq polymerase and 1 µl of template DNA.

Amplifications reactions were performed in a UNO II Thermal Cycler (Biometra, Germany), with the following conditions: 3 minutes of initial denaturation at 94°C, followed by 35 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C and 1 minute of extension at 72°C, and by a final elongation step of 3 minutes at 72°C.

PCR products were visualized by gel electrophoresis in a 1.2% (w/v) agarose gel in 0.5x TBE buffer (40 mM Tris; 45 mM Boric acid; 1 mM EDTA; pH 8.3) at 90V for 1h. After, the gel was stained in an 0.5 µg/mL ethidium bromide solution and photographed in an Alliance 4.7 UV Transilluminator (UVItec, Cambridge), using Alliance software version 15.15 (Uvitec, Cambridge). PCR products which amplified correctly were provided to Eurofins Genomics to be sequenced through Sanger sequencing. Results obtained were BLASTed against NCBI database to find the most probable identification.

2.7 Growth curves

Experiment

To evaluate the biodegradative potential of the selected PAH degrading microorganisms, growth curves were performed for single strains and a consortium (a combination of all isolates), using 15 mL of M9 medium and 10 mg of one PAH in Erlenmeyer flasks. Pre-inoculum from each isolate and the consortium was added to a flask in order to have 9 x 10^6 cells/mL, using a UV-visible spectrophotometer (Unicam) at 600 nm for the adjustment (software Vision). Each experiment was made in duplicate. The experiment was carried out in a shaking incubator (Thermo Scientific) at 28 °C and 160 rpm for 15 days. All flasks were capped with cotton wool and covered with aluminium foil. During the 15 days incubation, samples were collected aseptically for bacteria enumeration every 24 hours for the first 5 days and every 48 hours on the remaining days. For each sample, viable bacterial number was determined by MPN (see section 2.4). The MPN method was validated by counting colony forming units (CFU). Three strains were randomly chosen to validate each one of the three PAHs and triplicates were made, using TSA medium and 100µL as inoculum which was spread with spheres and incubated at 28 °C for 24h. For comparison of strains growth behaviour, the area under the curve (AUC) was used. Using the "Analyse" tool from GraphPad Prims software,

Traceability and Confirmation

During the growth curve assay, the presence of a contaminant in samples was detected. In order to trace their origin, samples were taken from all flasks and spread on plates with TSA medium. After 48h, colonies were observed with a stereomicroscope and the colonies that appeared to be different were inoculated in a new plate. Subsequently, DNA from these colonies was extracted by boiling method and isolates were differentiated by M13 PCR-fingerprinting. The assay was repeated and the results presented in this work refer to this second assay. At the end of the 15-day assay, samples were also taken from each flask, the microorganisms were grown on plates with TSA medium, DNA from the colonies observed after 48h was extracted by boiling method and the isolates were differentiated by M13 PCR-fingerprinting to confirm the maintenance of isolates of interest at the end of the assay.

DNA was extracted through boiling method, adapted from Sambrook et al. (1989). Biomass was collected into a microtube with 50 µl of TE buffer (10 mM Tris; 1 mM EDTA; pH 8). Tubes were heated to 100 °C for 10 minutes in a heating block, in order to lysate the cells and release DNA to the solution. Tubes were then centrifuged at 14 000 rpm for 10 minutes and supernatant was collected into a new tube.

M13 PCR-fingerprinting was performed in a total volume of 25 µl, with 1x PCR buffer (Invitrogen), 3 mM MgCl2, 25 pmol of primer csM13 (5'GAGGGTGGCGGTTCT3') (Meyer et al., 1993), 0.2 mM of dNTP's mix, 1 U of Taq Polymerase and 1 µl of template DNA. Amplification was performed in a UNO II Thermal Cycler (Biometra, Germany), with the following conditions: 5 minutes of initial denaturation at 95°C, followed by 40 cycles of 1 minute of denaturation at 95° C, 2 minutes of annealing at 50 °C and 2 minutes of extension at 72°C, and by a final elongation step of 5 minutes at 72 °C.

PCR products were separated by gel electrophoresis, together with 1 kb plus DNA ladder, in a 1.2% (w/v) agarose gel in 0.5x TBE buffer (40 mM Tris; 45 mM Boric acid; 1 mM EDTA; pH 8.3). Electrophoresis was run at 90V for 3h, after which gel was stained in an 0.5 µg/mL ethidium bromide solution and photographed in an Alliance 4.7 UV Transilluminator (Uvitec, Cambridge), using Alliance software version 15.15 (Uvitec, Cambridge).

2.8 Determination of biosurfactant activity

After the 15 days of growth experiment, cells and solid carbon source were removed from the broth by centrifugation at 4000 g during 10 minutes and the supernatant of each sample was harvested for use in the biosurfactant activity assay.

Qualitative assay - Emulsification activity

The emulsification test was carried out using 1 mL of supernatant with 0.5 mL of *n*-hexadecane placed in a microtube and vortexed for 30s. The samples which produced a stable third layer were chosen to take part in a larger test using a final volume of 3 mL of supernatant and 3 mL of *n*-hexadecane. This suspension was vortexed for 2 minutes and the height of the emulsion layer was measured after 2h.

Quantitative assay - Surface tension measurement

The surface tension of the supernatant was determined using a tensiometer (Kruss Force Tensiometer K100) at 25°C by the Du Noüy method (Du Noüy, 1925) (see Appendix I). Tensiometers calculate the surface tension with aid of an optimally wettable ring suspended from a precision balance. The liquid is raised until it contacts with the surface is registered. The sample is then lowered again with the purpose of the liquid film produced beneath the ring is stretched. As the film is stretched, a maximum force is experienced and that force is measured, allowing the surface tension calculation[2]. The data was collected and analysed by the software Kruss Laboratory Desktop [K100 Surface and Interfacial Tension].

2.9 PCR approach to search for genes involved in PAH degradation

The search for genes involved in PAH catabolic pathways was performed through PCR analysis, using DNA extracts from the genomic DNA bank of BBC collection (kindly provided by P. Teixeira). Details about the selected primers can be found in Table 2.

Table 2 PCR primers used to search for genes involved in PAHs catabolism.

Enzyme target	Size of gene fragment (bp)	Primer	Sequence (5' - 3')	Reference
Ring-hidroxylating dioxygenase	306	PAH-RHD GN F	GAGATGCATACCACGTKGGTTGGA	Cébron *et al*, (2008)
		PAH-RHD GN R	AGCTGTTGTTCGGGAAGAYWGTGCMGTT	
catechol 1,2 - dioxygenase	282	C12Of	GCCAACGTCGACGTCTGGCA	Sei *et al*, (1999)
		C12Or	CGCCTTCAAAGTTGATCTGCGTGGT	
catechol 2,3 - dioxygenase	380	C23Of	AAGAGGCATGGGGGCGCACCGGTTCGATCA	Sei *et al*, (1999)
		C23Or	CCAGCAAACACCTCGTTGCGGTTGCC	
salicylate 5-hydroxylase	920	sgp319F	TGCCCCTAYCAYCARTGG	Izmalkova *et al*, (2013)
		1238R	CGCCAGTABTBGTACATGCC	

Ring-hydroxylating dioxygenase (Gram negative bacteria)

PCR reactions were performed in 50 µL reaction volumes containing 1x PCR buffer (Invitrogen) supplemented with 1.5mM $MgCl_2$ (Invitrogen), 200 µM of each dNTPs (Invitrogen), 0.2 µM of each primer, 1.2 U of Taq Polymerase (Invitrogen) and 1 µL of DNA template.

The amplifications were carried out in a thermocycler (Biometra Uno II) with the following conditions: step one for initial denaturation at 95°C (5 min), followed by 30 cycles of 3 steps with 30 s of denaturation at 95°C, 30 s at the primers specific annealing temperature 57°C and 30 s of elongation at 72°C. The final step for final elongation consisted of 7 min at 72°C.

Aliquots (10 µL) of the PCR products were analysed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide.

Catechol dioxygenases

PCR reactions were performed in 50 µL reaction volumes containing 1x PCR buffer (Invitrogen) supplemented with 2mM $MgCl_2$ (Invitrogen), 200 µM of each dNTPs (Invitrogen), 0.5 µM of each primer, 1 U of Taq Polymerase (Invitrogen) and 1 µL of DNA template.

The amplifications were carried out in a thermocycler (Biometra Uno II) and PCR was conducted for 40 cycles with initial denaturation at 95°C (5 min), followed by 3 steps with 30 s of denaturation at 94°C, 30 s at the annealing temperature and 30 s of elongation at 72°C. Considering that each C12O or C23O target sequence has a different homology score with the primers, a touch-down method was employed. For C120, the annealing temperature was 61°C in the first 10 cycles followed by a step down to 59°C in the next 15 cycles and 57°C in the last 15 cycles while for C23O, the annealing temperature was 59°C in the first 10 cycles followed by a step down to 57°C in the next 15 cycles and 55°C in the last 15 cycles The final step for final elongation consisted of 7 min at 72°C.

For optimization purposes, amplifications were also carried out under the same conditions to the exception of the annealing temperature which was 63°C during 40 cycles.

Aliquots (10 µL) of the PCR products were analysed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide.

Salicylate hidroxylase

PCR reactions were performed in 50 µL reaction volumes containing 1x PCR buffer (Invitrogen) supplemented with 2mM MgCl2 (Invitrogen), 200 µM of each dNTPs (Invitrogen), 0.5 µM of each primer, 1 U of Taq Polymerase (Invitrogen) and 1 µL of DNA template.

The amplifications were carried out in a thermocycler (Biometra Uno II) with the following conditions: step one for initial denaturation at 95°C (5 min), followed by 30 cycles of 3 steps with 45 s of denaturation at 94°C, 30 s at the primers specific annealing temperature 55°C and 90 s of elongation at 72°C. The final step for final elongation consisted of 7 min at 72°C.

For optimization purposes, amplifications were also carried out under the same conditions to the exception of the annealing temperature which was 59°C during 30 cycles.

Aliquots (10 µL) of the PCR products were analysed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide.

CHAPTER III - Results and Discussion

3.1 Screening of PAHs degradation potential of adaptive evolved microbial strains

In order to evaluate the PAHs degradation potential of 47 strains isolated from AE experiments a direct evaluation and an indirect evaluation were carried out. Direct evaluation consists in quantify the degradation efficiency through HPLC by obtaining the calibration curves of the compounds naphthalene, anthracene and phenanthrene and dosing biological samples after growth with the compounds of interest as single carbon source. This implies a development of the HPLC method with parameter optimization and validation. Indirect evaluation corresponds to obtaining the cellular viability in the biological samples through MPN, also after growth with the compounds of interest as single carbon source.

3.1.1 HPLC – Calibration curves with simple linear regression

Calibration curves of one analyte were generated by plotting peak areas *versus* the standard concentrations (Figure 5A, 5C, 5E). For each concentration, triplicates were performed.

The calibration curves were created using simple linear regression as statistical method because it allows the study of relationships between two quantitative variables. In this case, the *x* variable, regarded as the predictor, explanatory or independent variable, is the range of standard concentrations (ng/µL) of each PAH whereas the *y* variable, regarded as the response, outcome or dependent variable is the peak area quantified by HPLC.

In order to find the best fitting line for this data set, the least squares criterion was applied where the residual error (or prediction error), *i.e*. the difference between the value predicted by the regression line and the observed value, is as small as possible.

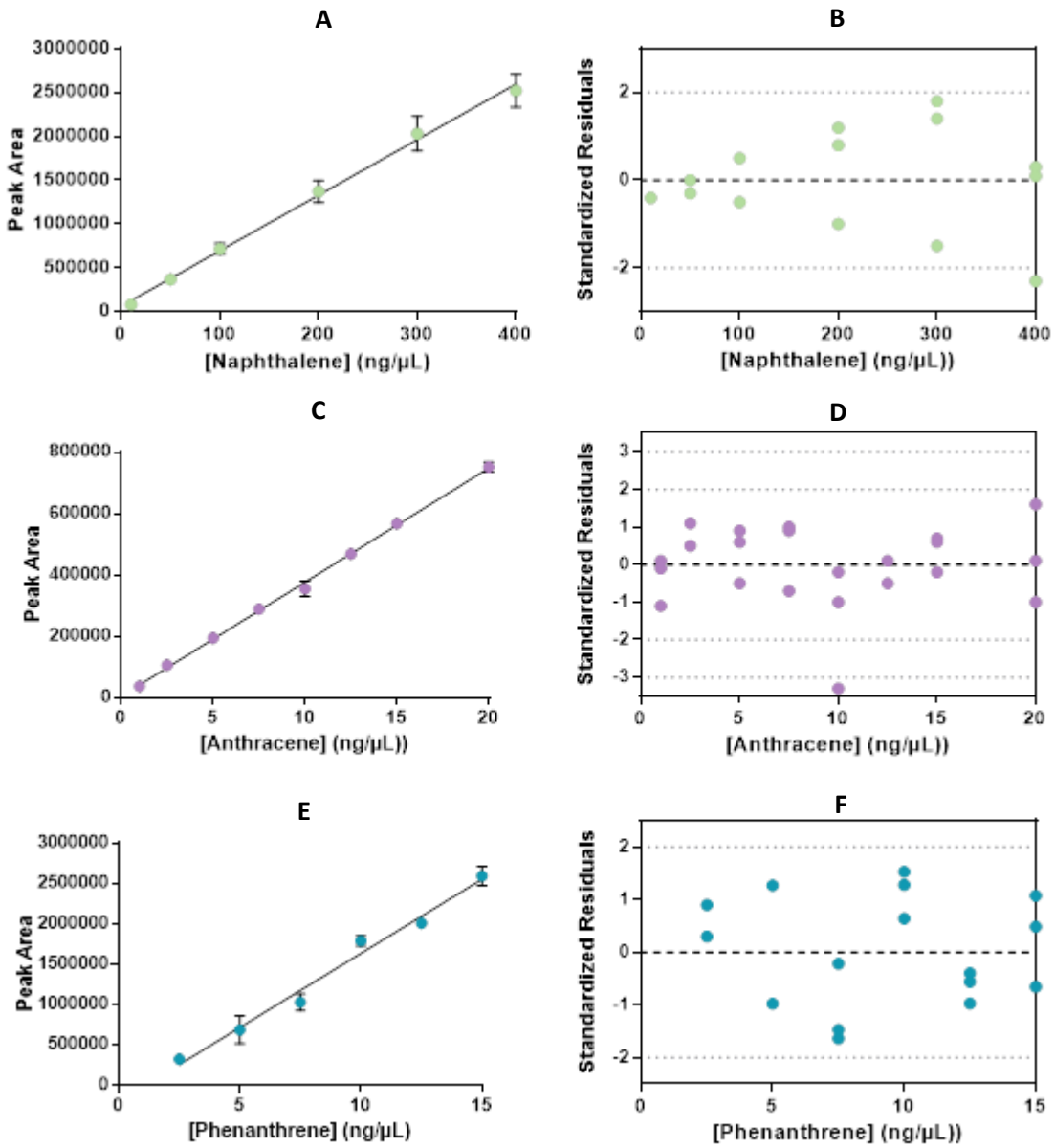

Figure 5 Calibration curves with linear regression (A, C, E) and residual plot (B, D, F) for naphthalene, anthracene and phenanthrene. In the linear regression, the points represent a mean of the measures with the respective standard deviation.

The regression equation $y = b1x + b0$, that makes the sum of the squared residual error the smallest it can be, and other important regression analysis parameters such as retention times (R_T) for naphthalene, anthracene and phenanthrene are summarized in Table 3.

Table 3 Linear regression validation parameters for naphthalene (NAP), anthracene (ANT) and phenanthrene (PHE).

PAH	R_T (min)	Range (ng/µL)	Regression Equation	R^2	LOD (ng/µL)	LOQ (ng/µL)	Accuracy %	Precision %
NAP	9.5	10 - 400	Y = 6563.4x + 42102	0.99	0.015	0.045	100 ± 10.8	93.74
ANT	15.3	1 - 20	Y = 37285 + 6027.8	0.9984	0.00006	0.00017	99,9 ± 9.6	94.73
PHE	14.5	2,5 - 15	Y = 183665x + 199422	0.9734	0.00003	0.00008	65 ± 23.2	90.01

In order to support the assumptions of linearity in regression and independence, normality and equal variances of the error, a residual analysis was made by plotting the residuals *versus* the standard concentration of PAHs (Figure 5B, 5D, 5F). Residuals were transformed in standardized residuals by dividing them by their standard deviation, making them unitless and directly comparable. It can be observed that the standardized residuals are randomly distributed on the plot and they form a roughly horizontal band around the residual zero line. However, a data point on ANT's plot and another data point on NAP's plot stand out from the random pattern of the other residuals. It is known that, when data is normally distributed, 95% of the measurements will present standardized residuals values higher than -2 and smaller than 2. Therefore, any observations greater than 2 or smaller than 2 should be flagged as being an outlier. This happened for one of the 400 ng/µL NAP triplicates. When standardized residuals are greater than 3 or smaller than 3, they are considered as extreme outliers, which is the case of one of the 10 ng/µL ANT triplicates. Because the data sets are small, the points who were identified as outliers where excluded from the regression line (Zar, 2010).

For the three compounds, the R^2 value - coefficient of determination - is greater than 95% which indicates a strong linear relationship between the response y and the x predictor. This means that in the case of naphthalene, anthracene and phenanthrene, respectively 99%, 99.84% and 97.34% of the peak area total variation is explained by the variation in the standard concentration and the rest of the variation is due to random error.

Both naphthalene and phenanthrene calibration presented a high accuracy ($\geq$ 99.9%) confirming that the regression line provides estimated concentration values coinciding with the known standard concentrations. Yet, the anthracene calibration has a higher precision that translates into a lower standard deviation compared to naphthalene precision, which means that for it presents more coincident values in a series of concentration measurements taken from same sample by multiple injections. The phenanthrene calibration, although displaying the lowest percentage of accuracy and precision .shows an acceptable linearity.

3.1.2 HPLC – Strains degradation efficiency

In a previous work, adaptive evolution experiments were performed in mineral medium M9 with different pollutants as sole carbon source for selective pressure. The WWTP inoculum was kept for over 50 cycles and this strategy preserves the dominant microbial strains, leading to wash-out of the weakest ones. The 47 strains in the present work were obtained from different experiments at several cycles and from selective solid media (ANT, PHE, GTS and MiO) and represent the diversity present on BBC collection.

The residual PAH resulting from the microbial degradation was quantified by HPLC. Results are presented according to the cycle of adaptive evolution corresponding to strain's isolation in order to assess the effectiveness of microbial adaptation (Figure 6). The degradation efficiency presented is relative considering the maximum and minimum degradation of each of the PAHs, for the set of strains.

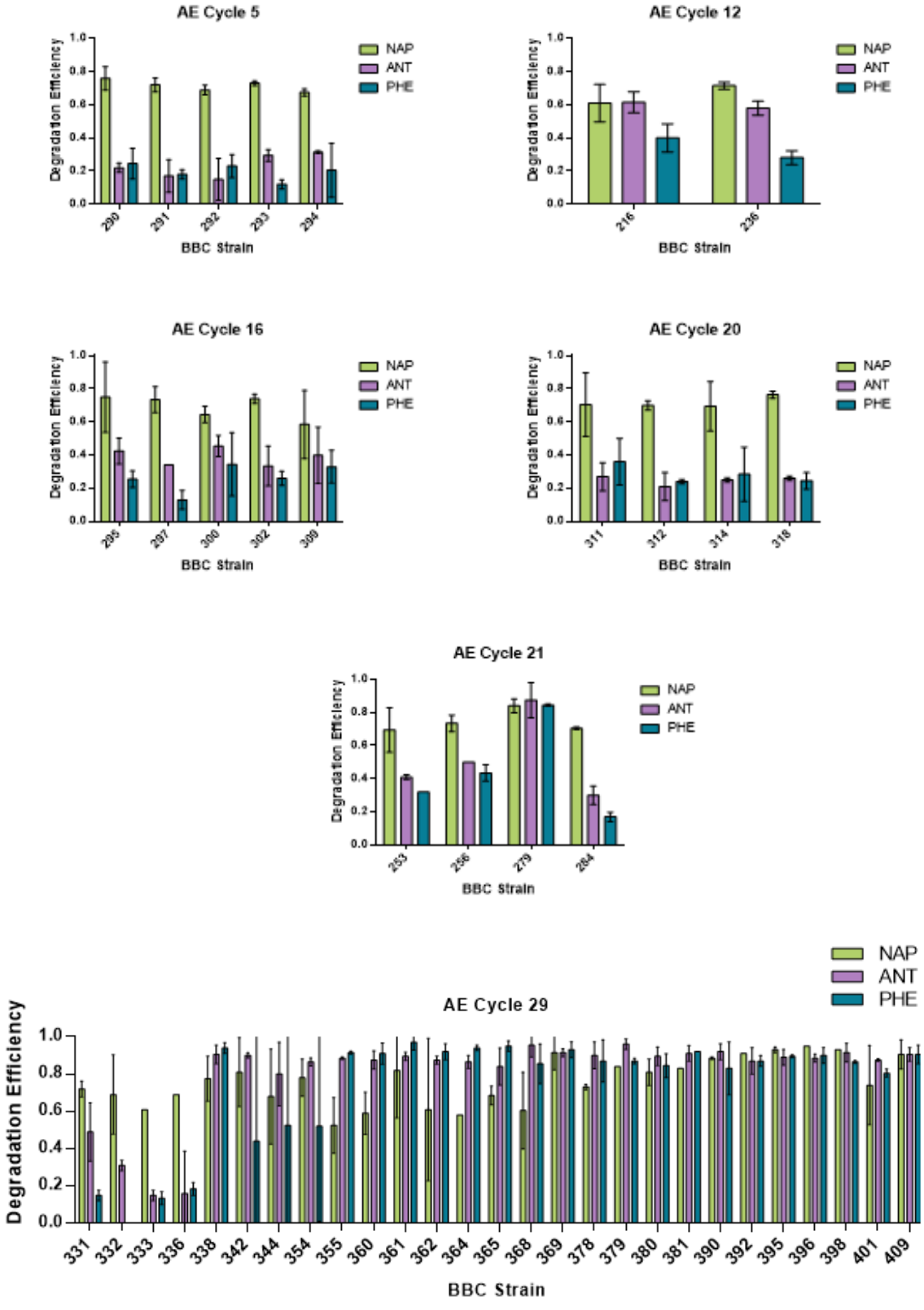

Figure 6 Efficiency of degradation of 47 strains relative to the maximum and minimum value of degradation obtained in each of the PAHs after 15 days growth experiment. The efficiency values represented here range from 0 to 1, where the worst degrader correspond to 0 and the best degrader correspond to 1. The graphs are organized by the 6 cycles of adaptive evolution from which the 47 strains under study come from. The bars refer to the mean ± standard deviation.

The results showed that 90% of the 20 strains belonging to cycles 5, 12, 16, 20 and 21 degraded more easily naphthalene compared to anthracene and phenanthrene. This is expected due to the chemical structure of the compounds since naphthalene has two benzene rings compared to anthracene and phenanthrene which have three, revealing a greater ability of the microorganisms to open the naphthalene's rings, since it involves fewer catabolic enzymatic steps (Figure 1). This corroborates that the degradation efficiency of the microorganisms decreases with increasing structural complexity of PAH (LJS, 2016).

Another factor that may justify the percentages of degradation efficiency is the different solubilities of the compounds in aqueous medium (M9), expressed in moles of solute per liter of solution. Although PAHs non-polar structures prevent dissolution in water, these compounds are not completely insoluble, particularly the low molecular weight PAHs (Abdel-Shafy and Mansour, 2016). For example, naphthalene's aqueous solubility at 25°C is 249 µmol/L, a value that represents low water solubility but is much higher than anthracene and phenanthrene's solubilities, which correspond to 0.37 µmol/L and 7.2 µmol/L at 25°C, respectively (Pearlman et al., 1984). Therefore, small amounts of PAHs do dissolve in water, where they become bioavailable. As naphthalene presents the highest water solubility, then it is the PAH with greater availability for microbial degradation.

However, it's important to mention a relevant naphthalene's property that can also explain its higher percentage of degradation efficiency. Naphthalene is a solid that sublimes at atmospheric temperature and pressure (Carrol, 2014). Thus, if naphthalene is able to go directly from the solid to the vapor phase, the reduction in naphthalene concentration may be due to loss of the compound to the air, instead of microbial degradation. However, the experiment conditions were the same for all strains in terms of amount of naphthalene and the volume of M9 medium, so it is expected that the losses were equally distributed and the differences observed between strains should be explained by their metabolism.

About the three-ring PAHs, an overall tendency for a higher anthracene degradation efficiency is present, except for cycle 20.

The standard deviations do not seem to be related to HPLC sensitivity because there are duplicates that have exactly the same value which translates into very low standard deviations. The differences can be explained by the fact that these are biological replicates where factors such as the different accessibility of the microorganisms to the solid substrate in the different samples, the microbial metabolism itself and adaptation to the medium with the complex carbon source as the only source of carbon and energy have influence on degradative behaviour.

Interestingly, it is in cycle 29 that the highest values of degradation efficiency are observed. This result corroborates the success of the adaptive evolution experiment since the most evolved strains are the ones with the highest degradation efficiency. This may be due to the fact that over time these have been selected by the natural pressure of having only naphthalene, anthracene or phenanthrene as the sole carbon source, which translates into a greater metabolic, physiological and genomic adaptation in terms of gene expression and protein synthesis as well as in the production of biosurfactants or biofilms. Accordingly, these adaptations may translate into greater ease in using these compounds in their metabolism.

3.1.3 MPN– Strains viability

In order to analyse indirectly the degradation of PAHs, assessment of the viability of strains at the end of the degradation assays were performed by MPN. This method estimates the concentration (cells/mL) of viable microorganisms in a sample by replicate liquid broth growth in ten-fold dilutions. MPN was used for the viability experiment because the substrates under study – naphthalene, anthracene and phenanthrene - are solid at strain's growth temperature (28ºC) and therefore they would interfere in methods that would use the optical density at 600 nm as a monitoring parameter.

It is observed in Figure 7 that 93,62% of strains showed growth above the concentration of cells / ml from the initial inoculum. This result is expected because these microorganisms have been selected from adaptive evolution experiments for being interesting for their degradation capacity.

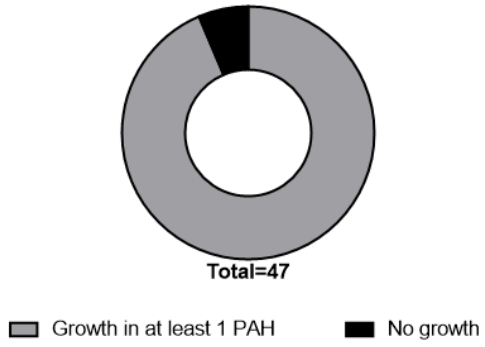

Figure 7 Representation of the distribution of the 47 strains under study in terms of absence /presence of viability at the end of the 15 days of degradation experiment.

Considering that for all MPN's tests, the initial inoculum concentration used was 8 x 10^6 cells.mL^{-1} and strains have the same amount of available PAH as the sole carbon source, the observed differences in viability results present on Figure are due to lower or higher degradation efficiency.

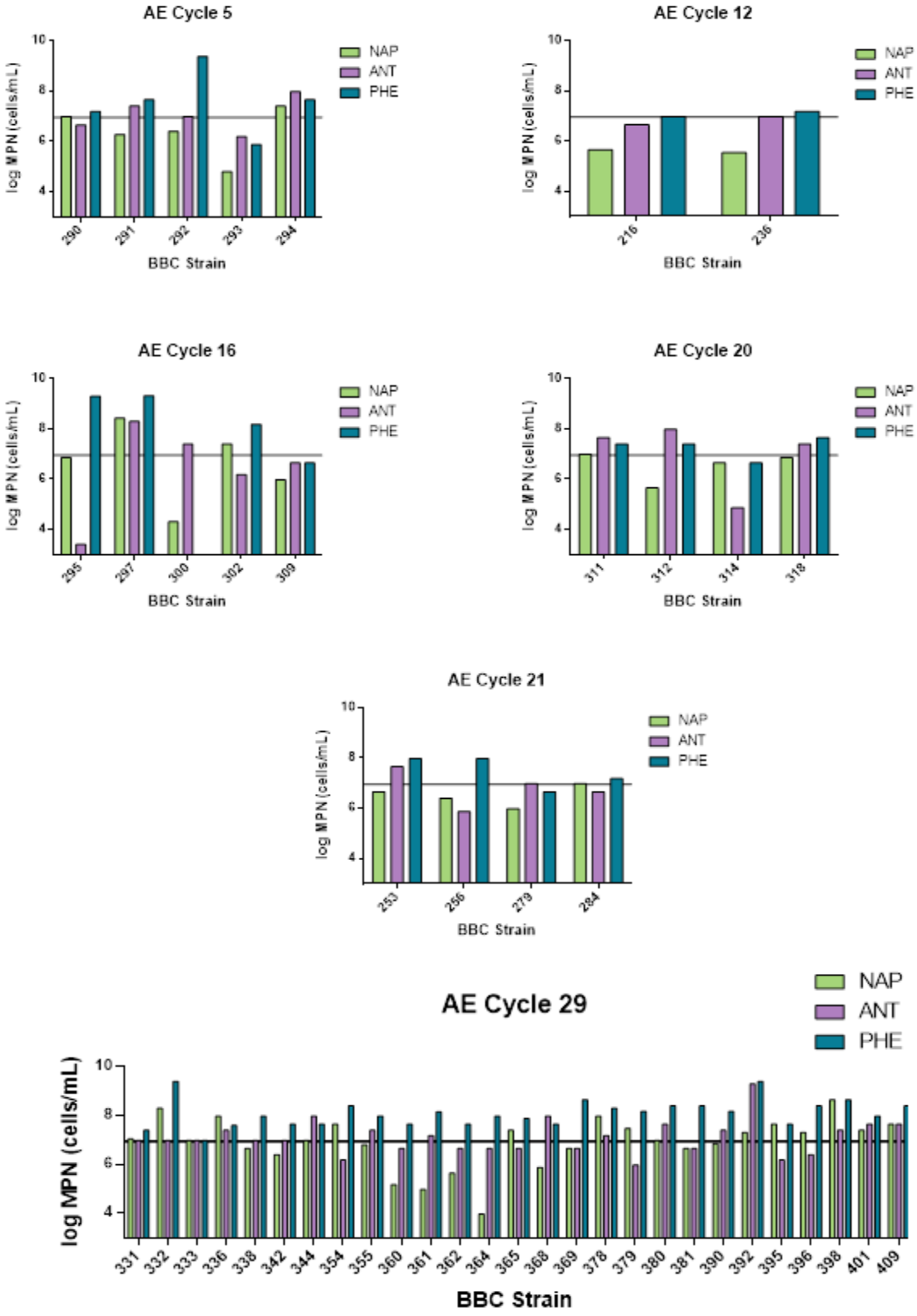

Figure 8 Cell viability expressed in log of cells per mL of the 47 strains which grow for 15 days with PAHs under study as sole carbon source. The line in the graphs corresponds to reference value 6.90 corresponding to the 8 x 10^6 cells that were inoculated first.

For strains BBC|293, BBC|309 and BBC|314 that exhibited a cells/mL value lower than the initial inoculum, it can be concluded that there was a loss of viability. This phenomenon could be due to an inability of the strain to metabolize the compounds naphthalene, anthracene and phenanthrene although this is not expected since they were selected from experiments where they had pollutants as the only source of carbon. Strain BBC|293 of cycle 5 and strain BBC|314 of cycle 20 are derived from the experience of adaptive evolution in phenanthrene and was also isolated in solid medium with phenanthrene. Strain BBC|309 of cycle 16 comes from the experience in glyceryl tristearate and was also isolated in solid medium containing glyceryl tristearate. It should be noted that in all three cases, the experience of adaptive evolution and the medium in which the strains were isolated contains the same compound as the sole carbon source, which does not give evidence of a high metabolic plasticity.

Strains BBC|293, BBC|309 and BBC|314 demonstrated less viability in naphthalene relative to anthracene and phenanthrene. Compared to HPLC results (Figure 5), there is no consistency in this case because the degradation efficiency of naphthalene is higher and the reasons for this have already been enumerated above. In the other compounds, for strain BBC|293 there is concordance since for the anthracene presented higher viability and degradation efficiency than for phenanthrene, although its experience of adaptive evolution and its isolation contained phenanthrene as a single carbon source. As to strain BBC|309, it presents equal results in terms of cell viability and a greater efficiency of anthracene degradation. This bacteria was presumptively identified in the scope of Pedro Teixeira's work as belonging to the genus *Shinella*. A species belonging to the genus *Shinella* described as capable of achieving greater degradation of anthracene was isolated from activated sludge bioreactor treating municipal wastewater (Ntougias *et al.*, 2015). It is important to mention that this strain was once again isolated in cycle 20 of adaptive evolution in glyceryl tristearate, reinforcing the idea of its ability to survive having the pollutant as the only source of carbon. For strain BBC|314, the HPLC results show a higher efficiency in phenanthrene degradation which is consistent with its origin in an experience of adaptive evolution and isolation in phenanthrene. However, the lowest viability was also found for that compound. As for naphthalene and anthracene, the strain had the same viability value for both.

Considering the dendrogram constructed from the profiles obtained by PCR-fingerprinting (using primers M13, Ph, GTG5, Appendix D), it should be noted that strain BBC|293 and strain BBC|314 are phylogenetically close with a similarity higher than 90%. In addition, the fact that they respectively belong to evolution cycle 5 and 20 indicates that the most recent strain may be the result of the evolution of the older strain.

Nevertheless, the absence of concordance between HPLC and MPN can be explained not by strains degradation low efficiency but by the growth curve characteristics. The final phase of growth is the death phase where a net loss of culturable cells is observed. During this phase, total count of microorganisms may remain constant but the viable count decreases by various reasons, such as depletion of nutrients. In some cases, death is accompanied by actual cell lysis which results in accumulation of toxic products in addition to the metabolites already produced during growth.

Once again, it can be seen that the highest values of cell viability are observed in cycle 29. This result also corroborates the success of the adaptive evolution experiment since the most evolved strains are the ones with the greatest growth. This may be due to the fact that over time these have been selected by the natural pressure of having only naphthalene, anthracene or phenanthrene as the sole source of carbon, which translates into greater metabolic, physiological and genomic adaptation and consequently greater ease to grow in environments with these pollutants.

3.2 Selection of best PAH degraders

In order to evaluate the PAHs degradation potential of 47 isolates, a degradation assay was carried out, having the hydrocarbons naphthalene, anthracene and phenanthrene as the only source of carbon and energy. Further quantification of residual substrate was performed using HPLC. In addition, the MPN method was also applied to evaluate the viability of the isolates at the end of the assay.

These results presented values in mg/mL of residual substrate for naphthalene (HPLC|NAP), anthracene (HPLC|ANT) and phenanthrene (HPLC|PHE) and values of cells/mL of the isolates grown in naphthalene (MNP|NAP), anthracene (MNP|ANT) and phenanthrene (MNP|PHE). A univariate analysis of these two data sets composed of six variables is limited because it does not consider the possible correlations between the amount of residual substrate resulting from degradation and viability of microorganisms in presence of that same substrate. In this case, multivariate analysis can be used to assess the best PAH degraders by possibly forming groups based on the degradation capability of each isolate, reducing the number of variables involved in the study (Fraga *et al.*, 2016).

There are numerous approaches in the area of multivariate analysis that allow joint analysis of different traits. Principal component analysis (PCA) is a statistic method that uses an orthogonal transformation to reduce a set of correlated variables into a set of uncorrelated new variables, called principal components (PCs), with minimal loss of information (Hongyu *et al.*, 2016).

PCs are calculated through linear combinations of the original variables with eigenvectors. The absolute value of an eigenvector determines the importance of the traits in a principal component. Each eigenvector is calculated from an eigenvalue of the correlation matrix of the data, which is related to the variance of each principal component. The first principal component (PC1) explains the highest percentage of the total variance, the second principal component (PC2) explains the second most, and so on, until all of the variance is explained (Fraga *et al.*, 2016). To choose principal components that explained most of the variation in the data set, generally the Kaiser criterion is used, where PCs with eigenvalues greater than unity ($\lambda i > 1$) are selected (Kaiser, 1958).

The mean results from the measurements of the six variables from the 47 isolates were gathered in a matrix in order to perform PCA. The data were standardized by subtracting the average and dividing by the standard deviation; similarity was calculated applying Pearson's correlation coefficient to the standardized matrix.

PC1, PC2 and PC3 were selected because they explain 82% (>75%) of the variance of the results (Table 4). Analysis of the correlation of original variables with the PCs (Table 5), revealed that PC1 represents two kinds of responses along the axis: strain's ability to grow on phenanthrene according to MPN and strain's degradation efficiency for naphthalene, anthracene and phenanthrene consistent with HPLC results; PC2 represents a gradient in strain's ability to grow on naphthalene according to MPN and PC3 represented a gradient in strain's ability to grow on anthracene according to MPN. A detailed analysis of the explanatory variables for PC1, PC2, PC3, PC4, PC5 and PC6 can be found in Appendix G and H.

Table 4 Eigenvalues and respective variance (%) explained by the new variables – principal components.

Principal Component (PC)	Eigenvalue (ʎi)	% Variance	% Variance (accumulated)
PC1	2,57	42,78	42,78
PC2	1,38	22,98	65,77
PC3	0,99	16,43	82,20

Table 5 PC loadings (correlation coefficients of original variables and PCs).

Original Variable	PC1	PC2	PC3
MPN|ANT	0,16	0,27	0,94
MPN|NAP	0,60	0,69	-0,04
MPN|PHE	0,71	0,32	-0,13
HPLC|ANT	-0,80	0,57	-0,07
HPLC|NAP	-0,62	-0,35	0,26
HPLC|PHE	-0,82	0,53	-0,089

A PCA of this dataset was carried out with the aim of obtain a clearer perception of the strain's performance, as each strain's position in this analysis reflecting its global response in terms of the degradability and subsequent growth on three compounds studied. This analysis revealed mostly a distribution according to their respective isolation medium and adaptive evolution cycle, as evidenced in Figure 9 and Figure 10, respectively.

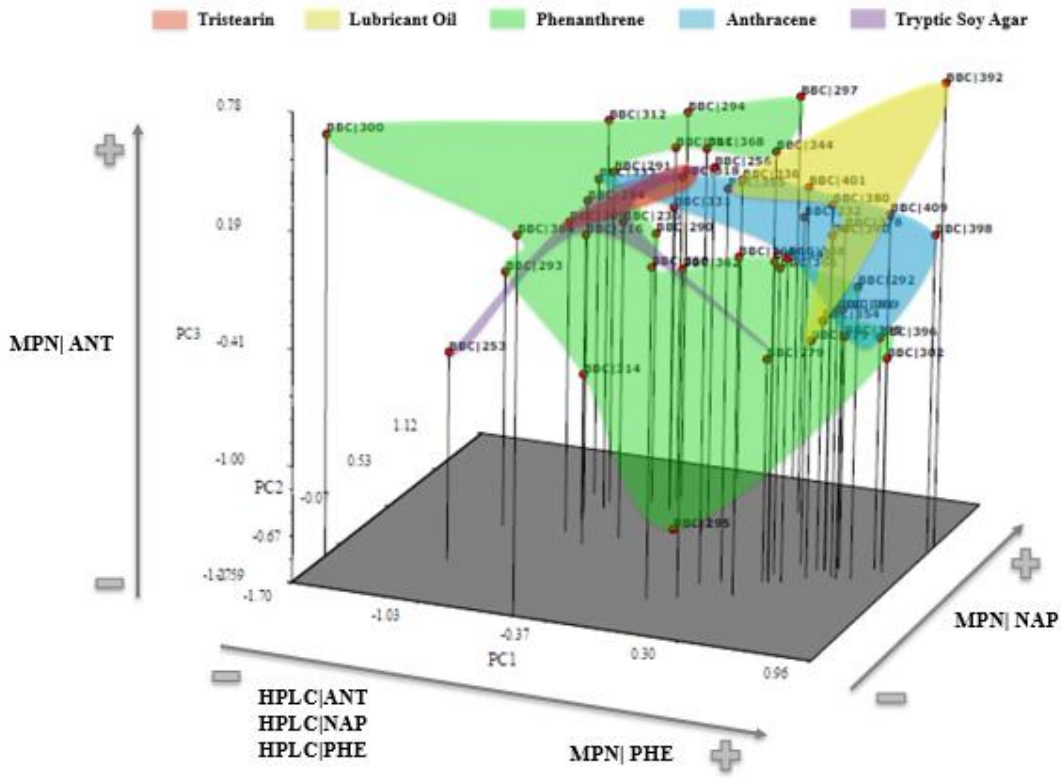

Figure 9 Spatial distribution of the strains considering the three-dimensional space formed by the 3 new variables – principal components. The selective medium from which the strains were isolated is evidenced by different colors.

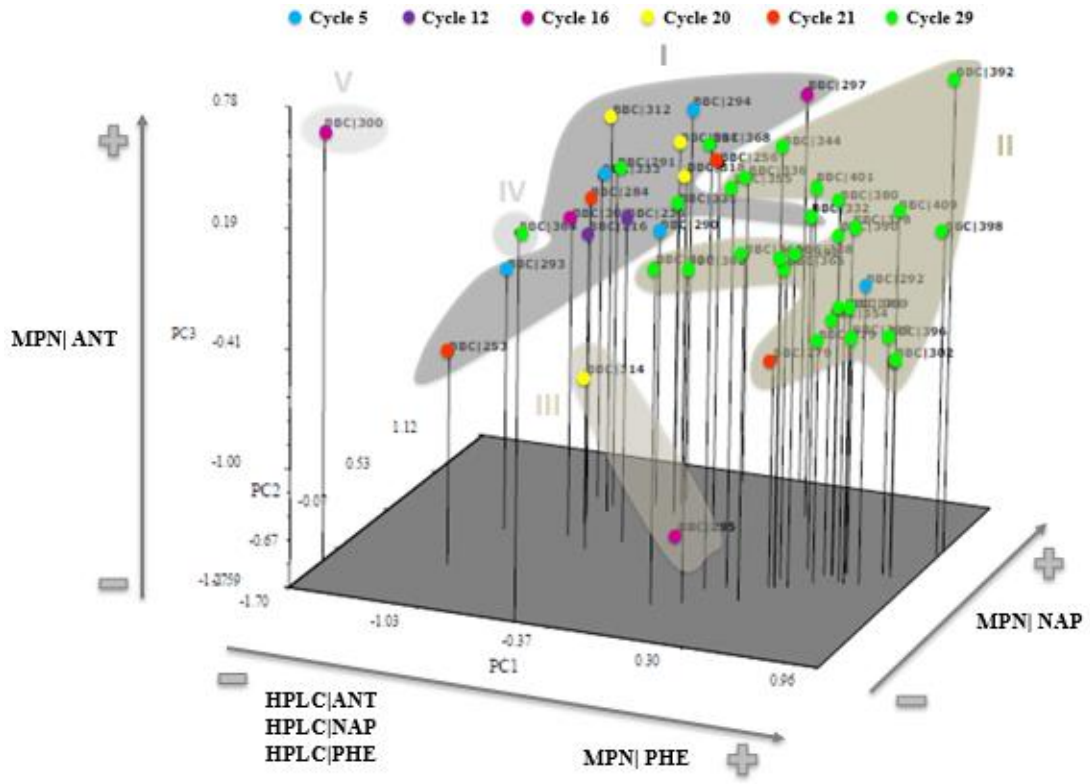

Figure 10 Spatial distribution of the strains considering the three-dimensional space formed by the 3 new variables – principal components. The adaptive evolution cycle from which the strains were isolated is evidenced by different colors.

The distribution of the strains by the space formed by the PCs can be observed in Figure 9 where the isolation medium is highlighted through different colours.

The phenanthrene isolation medium is the one containing the largest number of strains, which are distributed widely across the space. In this medium, is verified the existence of strains that present the greatest discrepancies between MPN and HPLC, especially with anthracene as the only source of carbon. For example, the BBC|300 strain, derived from the experience of adaptive evolution in phenanthrene and isolated in phenanthrene in cycle 16, shows high viability, but did not show a high degradation efficiency. In the case of the BBC|295 strain, originating from the experience of adaptive evolution in phenanthrene and isolated in phenanthrene in cycle 16, the opposite is found with an intermediate degradation efficiency and very low viability. As regards to naphthalene as the sole source of carbon, both a highly viable strain (BBC|297 from the experience of adaptive evolution with phenanthrene and isolated in phenanthrene at cycle 16) and a strain with the lowest viability (BBC|364 originating from the experience of adaptive evolution with phenanthrene and isolated in phenanthrene in cycle 29) are present.

Apparently, strains isolated in phenanthrene are able to degrade and grow in phenanthrene efficiently. However, for naphthalene and anthracene, there is a great variability in the response of the different strains in terms of efficiency and viability, reflecting a specialization in the degradation of phenanthrene as a source of complex carbon.

As for strains isolated in anthracene, it can be observed that they are concentrated in the zone corresponding to high values of degradation efficiency and viability. Note that the BBC|398 strain comes from an adaptive evolution experiment in lubricating oil and has been isolated in anthracene. Interestingly, this strain has the best position in terms of the use of phenanthrene as the only available carbon source (in combination with BBC|392). Thus, it can be concluded that it presents metabolic plasticity in terms of the use of different degradation pathways for different carbon sources.

The strains isolated in lubricating oil should also be emphasised because they are located in the zone of the space corresponding to the best positions regarding the use of naphthalene, anthracene and phenanthrene. This is the case of the promising BBC|392 strain, derived from the experience of adaptive evolution in glyceryl tristearate and isolated in lubricating oil in cycle 29, which shows the best results for anthracene and phenanthrene, being also relatively well positioned for naphthalene.

The strains belonging to the glyceryl tristearate and tryptic soy agar media are located in the central zone of the graph, not revealing an interesting degradative potential with respect to the three carbon sources.

In addition, from the distribution of the strains in the space of the PCs presented in Figure 10, the cycle of adaptive evolution to which they belong were highlighted with different colours. Furthermore, a dendrogram was constructed from the coordinates of the strains in the space of the PCs (using the euclidean distance and average linkage) and the different clusters – I to IV – are also highlighted in grey.

It is noteworthy that the strains belonging to cycle 29 constitute the majority of cluster II which is located in the zone where the best PAH degraders are expected to be. This may be considered as evidence of the success of the adaptive evolution experience.

The strains of cycles 5, 12, 16, 20 and 21 are mostly located in cluster I that occupies the central zone of the graph with an intermediate degradability that does not show much interest for the objective of this work.

Note that it is in cycle 16 that the greatest variability between HPLC and MPN is found as already explained above for the strains BBC|300 and BBC|295.

Clusters IV and V formed by strains BBC|368 and BBC|300, respectively, have the most extreme and discrepant values. Cluster III also does not have strains that exhibit high degradation efficiencies.

By analysing the graph of principal components and considering the adaptive evolution cycle and the isolation medium, strains BBC|297, BBC|392 and BBC|398 were selected for further study:

(i) Strain BBC|297 was selected because it showed high growth in naphthalene, anthracene and phenanthrene. Although it belongs to an intermediate cycle of adaptive evolution (cycle 16) it was thought that it would be interesting to compare it with strains from recent cycles that are expected to be more evolved.

(ii) Strain BBC|392 has the best positioning on the graph in terms of HPLC and NMP results for all the three carbon sources.

(iii) Strain BBC|398 presents the best results for naphthalene and phenanthrene.

In addition to the strains selected by the PCA, at that time another strain (BBC|652) was added to the BBC collection, identified and studied simultaneously. After PCA selection and during the first growth experiment, a contaminant capable of growth in the abiotic controls of naphthalene, anthracene and phenanthrene was identified. PCR fingerprinting was performed with DNA extracted from all the apparently distinct colonies in all the samples of the experiment. It was concluded that the microorganism did not belong to the collection, possibly referring to an environmental contaminant whose origin goes back to one of the solutions used for make the M9 mineral medium. A decision was made to include in future studies due to the fact that strain BBC|652 presents a degradative behaviour as its viability increased over time in the presence of the complex carbon sources under study. Note that after contaminant detection, the growth experiment was terminated and subsequently repeated successfully.

3.3 Identification of best PAH degraders

Strains BBC|297, BBC|392 and BBC|398 were selected for future studies and their identification is essential. The bacterial16S rRNA gene sequencing results allowed to presumptively identify strains BBC|297 and BBC|398 as belonging to the genus *Pseudomonas* and strain BBC|392 as a member of the genus *Acinetobacter*. This is consistent with the genomic relatedness of the three strains (Appendix D) where it was possible to conclude that strains BBC|297 and BBC|398 are in the same cluster and that strain BBC|392 is located in a more distant cluster.

Table 6 Results regarding the partial sequencing of bacterial 16S rRNA gene.

Strain	Hit Taxonomy[1]	Query Length (nt)	Cover %	Identity %
BBC|297	Bacteria Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*	1176	99	99
BBC|392	Bacteria Proteobacteria Gammaproteobacteria Pseudomonadales Moraxellaceae *Acinetobacter*	1079	99	99
BBC|398	Bacteria Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*	1146	99	99
BBC|652	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Burkholderiaceae *Paraburkholderia*	1169	99	99

[1] The ranks of domain, phylum, class, order, family ang genus are presented.

3.4 Characterization of best PAH degraders

Following the identification of selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - different studies were carried out to gather as much information as possible about the microorganisms that may explain their degradative capacity. It is important to mention that the consortium was only composed of BBC|297, BBC|392 and BBC|398 strains - the evolved strains.

Initially, the aim was to understand and differentiate the performance of the strains over time in terms of viability with naphthalene, anthracene or phenanthrene as the only carbon source through the establishment of growth curves.

Subsequently, in the metabolic terms, the possible production of biosurfactants by the strains was investigated as a strategy to solubilize the pollutant compounds and, consequently, to increase their degradation efficiency.

Finally, a search for functional genes associated with PAH catabolism and growth on catabolism intermediate substrate was made in order to unveil the possible metabolic pathways of degradation that could explain the degradative behaviour of each strain.

3.4.1 Comparison of growth curves

In order to monitor the ability of the selected strains to grow of selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - in the presence of different carbon sources, growth curves were carried out for 15 days, being the evaluation parameter the cell viability by MPN. The results are plotted in Figure 11.

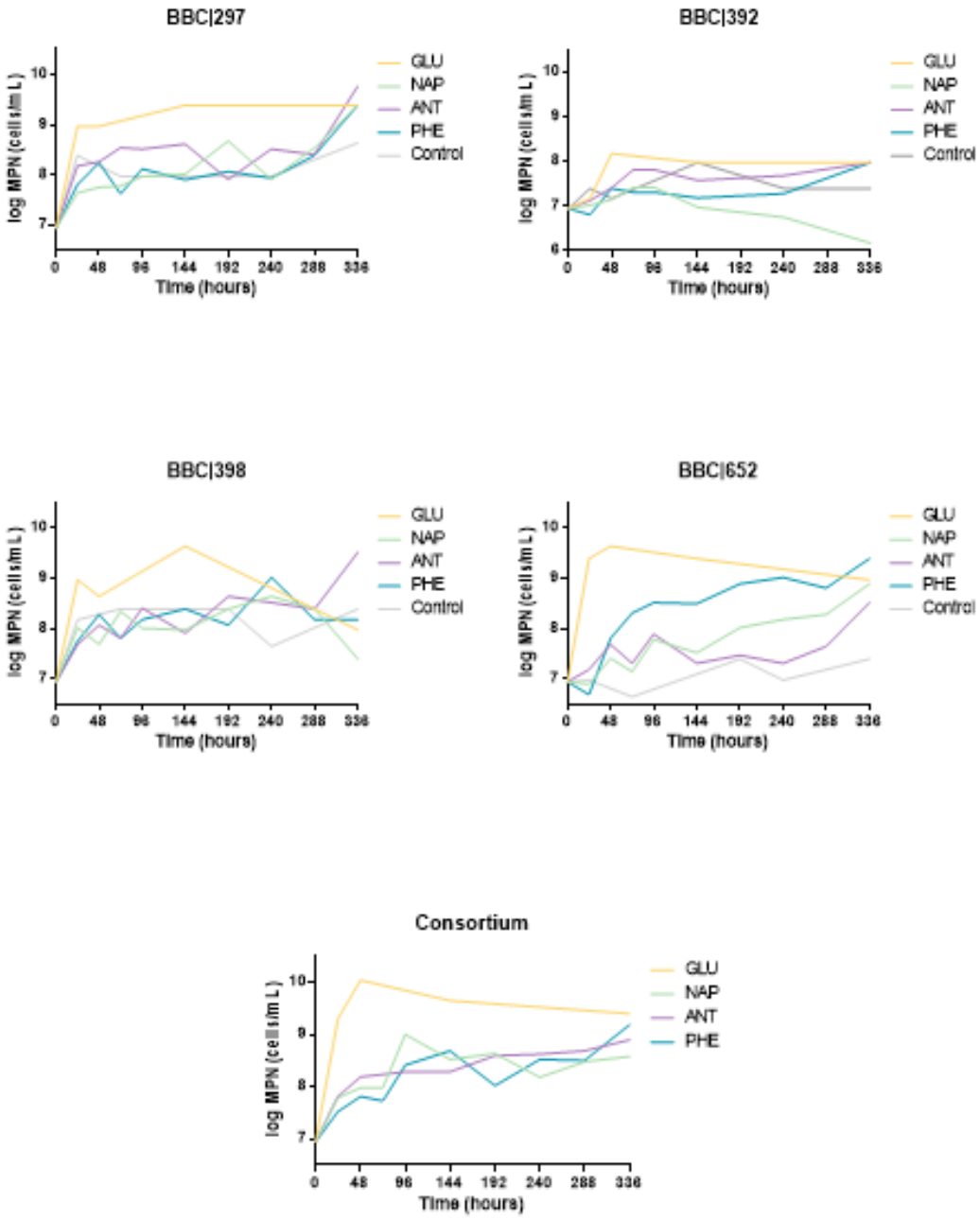

Figure 11 Growth curves for the selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - having naphthalene, anthracene, phenanthrene and glucose as the carbon source for 15 days. In gray is also depicted the biotic control of the strain without any carbon source.

Growth experiments were performed having naphthalene, anthracene, phenanthrene or glucose as the sole source of carbon. The use of glucose aims to observe the behaviour in the presence of a simple and easily metabolized carbon source compared to the PAHs, which are complex carbon sources. It can be seen in Figure 11 that the greatest viability values over time are obtained having glucose as the sole carbon source in all strains.

All experiments started with the same initial inoculum. For strains BBC|297 and BBC|398 it is found that during the first 24 hours there is an increase of approximately 1-log step and 2-log steps in the presence of the PAHs or glucose as the sole carbon source, respectively. In the case of strain BBC|297 a stabilization phase is observed after this increase in all growth curves up to 288 hours. Thereafter, the strain maintains the viability value for glucose but for the three PAHs there is a substantial increase in the viability value suggesting the possibility of bi-phasic growth. The biphasic growth occurs when bacteria have distinct carbon sources, one of which is completely consumed before the bacterial synthetic machinery begins to consume the second carbon source (Cossio *et al.*, 2012). In this case, the strains are supplemented with only one carbon source. However, when observing the control that does not have any carbon source can be observed that during the 336 hours it maintains some viability. This suggests that these strains are able to remain viable even without addition of a carbon source, entering a latency state or using products resulting from the autolysis of microorganisms and products of population metabolism as carbon source. This phenomenon observed here for all strains may be of interest in the production of commercial liquid inoculum which maintains viability for several days (at least 15 days). When the cells adapt to the medium with the complex carbon source and activate the genetic and enzymatic machinery necessary for its catabolism, then the second phase of growth enable higher viability values. For the strain BBC|398, a stabilization phase is also present in all growth curves up to approximately 288h. From this, what appears to be a two-phase growth in anthracene, while for naphthalene and phenanthrene there is a decrease in growth.

Strain BBC|392 achieves the increase of 1-log step in the presence of glucose at 48 hours. Growth with the 3 PAHs remains approximately stable at a viability value close to the initial inoculum value from the start up to 240 hours. Thereafter an increase in viability for anthracene and phenanthrene is achieved up to the viability value obtained in the presence of glucose. Relative to naphthalene, there is a decrease in viability. The discrepancies between the high initial screening results and the low viability results in this experiment may be due to the fact that a different starting amount of inoculum could have been used since this bacteria tends to aggregate.

As for strain BBC|652, the increase of 2-log step in the presence of glucose at 24 hours is observed. This strain exhibits different growth behaviours with the three complex carbon sources. Despite presenting a small initial decrease possibly due to adaptation to the complex carbon source, elevated and increasing viability values were observed after 24 hours in the presence of phenanthrene, reaching a value higher than the value of registered viability after 336 hours in the presence of glucose. For the naphthalene it presents an intermediate and increasing growth from the 144 hours reaching the viability value registered in the presence of glucose at 336 hours. In the presence of anthracene, the strain has the lowest viability values, noting an increase from 240 hours, but not reaching the viability values recorded in the presence of glucose.

The consortium presents initially an increase of 3-log step in the presence of glucose and a 1-log step in the presence of each of the three PAHs. The viability values remain relatively stable during the 336 hours never reaching the viability values recorded in the presence of glucose. From 288 hours a slight increase in the three growth curves corresponding to the three PAHs is observed, being this increase more evident in the case of phenanthrene.

From the growth curves, it can be concluded that in all cases in the presence of PAHs there is an adaptation phase corresponding to a plateau in the graph, that represents the adaptation of the microorganisms to a new culture medium and the exposure to complex carbon sources. The initial growth observed in some cases may be due, for example, to the fact that the bacteria present in the initial inoculum were growing in the general medium TSA and presented high metabolic rates and consequently a high fitness, that is, the reproduction rate was high. It is further noted that at 240 to 288 hours corresponding to days 10 and 12 of the experiment, a substantial growth in the complex carbon sources is generally observed, suggesting the existence of an initial phase of growth and a secondary phase where the microorganisms access the substrate and are able to metabolize it more efficiently.

In order to compare the strains with each other, the area under the curve (AUC) was calculated and the percentages of AUC in each condition relative to the maximum AUC in glucose was determined for each strain. The AUC value in glucose from the consortium, being the maximum value, was used as the reference value.

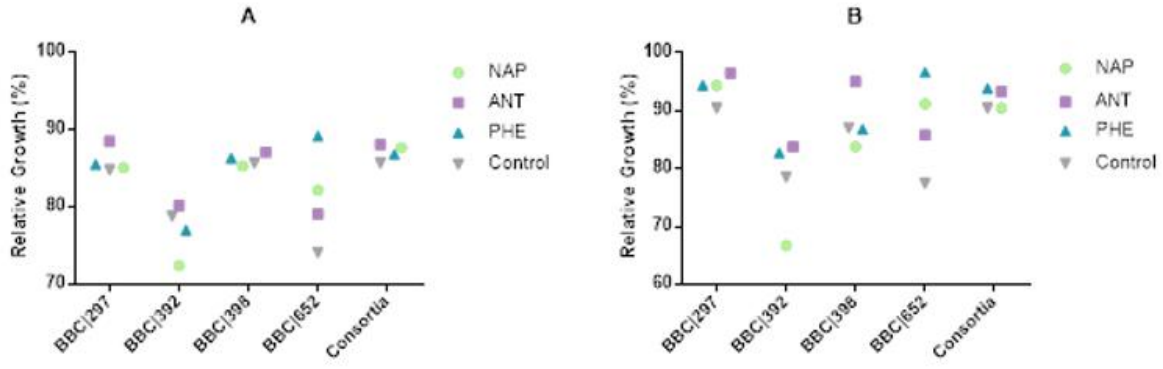

Figure 12 Representation of the relative growths (%) of each strain in each PAH and its abiotic control. All growth values are relative to the reference value which is the viability value of glucose for each strain. (A) Relative growths are organized by strain and correspond to 15 days of experience. (B) Relative growths are organized by strain and correspond to the last 48h of the experiment.

Graph A of Figure 12 shows the distribution of percentages of relative growth in each PAH organized by the strain and referring to the 15 days of growth. Overall, most of the values are between 80% and 90%. It should be noted that the values found below 80% refer only to the BBC|392 and BBC|652 strains, which again reinforces the idea of being less efficient PAH degraders. In fact, BBC|392 strain exhibits the worst result as most growth percentages are below 80%, with naphthalene being the carbon source with the lowest value, followed by phenanthrene having both PAHs lower percentages than the control. This may be due to the inability of to deal with the toxicity inherent to naphthalene and the greater complexity of phenanthrene as a carbon source. Strain BBC|652 exhibits a very disperse behaviour with a high range of percentages of relative growth in different PAHs. Interestingly, this strain has a greater facility in degrading phenanthrene, the more complex carbon source. Furthermore, strain BBC|652 also shows the greatest discrepancy between growth percentages without carbon source - control - and in the presence of complex carbon sources. The highest relative growth percentages occur for the BBC|297 and BBC|398 strains, with the former apparently more easily

degrading anthracene. Concerning the consortium, it is interesting to note that the percentages are relatively close and high, with no particular carbon source being highlighted.

Graph B of Figure 12 also shows the distribution of percentages of relative growth in each PAH organized by the strain, but referring only to the last 48 hours of the growth experiment (between 288 and 336 hours), since it was within this time interval that there was a general change in the degradative behaviour in the strains with an increase of relative growth percentages.

The most interesting result belongs to strain BBC|297 since it is observed that percentages relative to growth in the presence of PAHs increased to values greater than 90% and to the percentage of control, with anthracene maintaining the highest value. This strain comes from the adaptive evolution and isolation medium with phenanthrene as the only source of carbon.

In the case of the BBC|398 strain it is also curious to note the relevant increase in the percentage of anthracene growth to a value greater than 90%, with naphthalene and phenanthrene having values similar to or lower than control, which may suggest a possible specialization of the strain on anthracene degradation. This result is interesting because the strain BBC|398 was isolated in anthracene-containing medium as the sole carbon source from an adaptive evolution experiment in lubricant oil.

For the strain BBC|392, an increase in the relative percentages of growth in anthracene and phenanthrene is observed to values greater than 80% whereas in naphthalene a decrease is still observed below 70%. The strain BBC|652 maintains the trend shown in Graph A, increasing its relative growth percentages overall.

The consortium also shows an increase in the relative percentages of growth, highlighting anthracene and phenanthrene with values higher than control. At this time naphthalene exhibits a value similar to the control, decreasing the degradation efficiency of this compound, again possibly due to the accumulation of toxic products. It may be concluded that the consortium does not exhibit a degradative behaviour that stands out in relation to the strains growing singly but positions at high relative growth values. This may be related to the fact that the microbial consortium needs a period of adaptation, which consists of pre-enrichment and enrichment phases that depend on the substrate availability and the ability of the microorganisms composing it to degrade it. Comparative studies of degradation of aromatic hydrocarbons in crude oil using pure bacterial species and mixed bacterial consortium showed that the bacterial consortia had a higher rate of degradation (51.8%) than a single *Pseudomonas* sp. (40.3%) during 40 days incubation period (Cerqueira *et al.*, 2011). In the present work, the strains were obtained from wastewater which commonly contains microorganisms that not only contains microorganisms that are already part of a natural consortia. It is reported that bacterial species have synergistic interactions amongst themselves which enhance PAHs degradation. Due to these interactions between consortium members, it is suggested that possibly the first species degrades the substrate which may obstruct further degradation of compounds by the second species. Then, the second species degrades the compounds left half-degraded. The degradative capacity of any microbial consortium is not certainly the result of adding together the capacities of individual strains that form the association. Since microorganisms present different PAHs degradation efficiencies, when a consortium of them is used to degrade various forms of hydrocarbons in a contaminated site, the total degradation is more effective but synergistic and antagonistic interactions and partition of compounds utilized for microbial metabolism should be considered (Gupta *et al.*, 2016).

3.4.2 Evaluation of biosurfactant production

After 15 days of growth in M9 supplemented with naphthalene, anthracene or phenanthrene as sole carbon source, a preliminary study with the selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - was carried out to investigate the possible production of biosurfactants by these microorganisms.

Initially, an emulsification test was performed. The aim of this qualitative study is to evaluate which strains present biosurfactant production potential by direct observation of emulsion formation after addition of hexadecane between the aqueous and organic phases.

Of the four strains tested, it was only possible to verify stable emulsion formation for the BBC|392 and BBC|398 strains. These two strains were identified as potentially biosurfactant producers and selected for a quantitative study, with the determination of surface tension in order to measure the surface activity of biosurfactants (if any) with the ring method (see Annexe I). Surface tension values were measured on the supernatant of the cultures with strains BBC|392 and BBC|398 on naphthalene, anthracene and phenanthrene. In addition, surface tension measurements in M9 medium with each of the PAHs individually, M9 medium only and ultrapure water were also performed as controls (Figure 13).

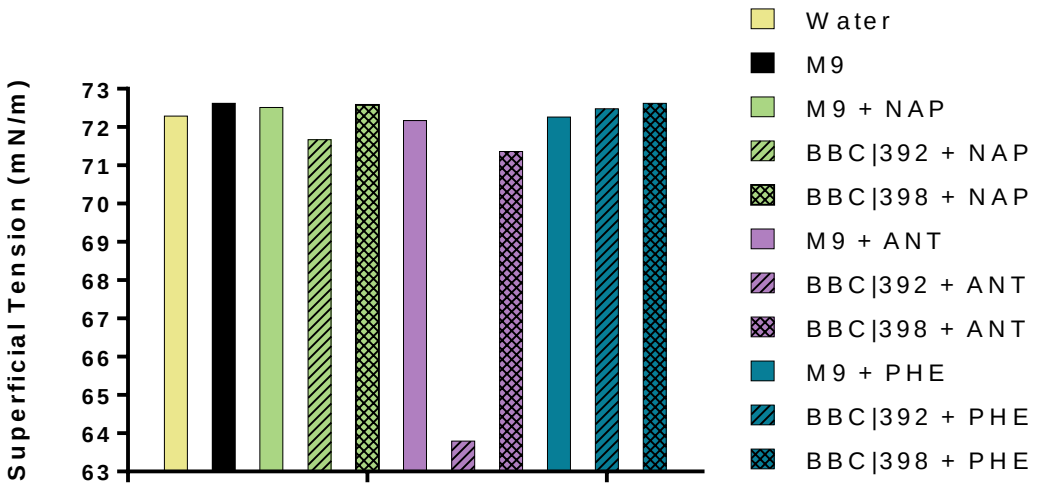

Figure 13 Representation of the surface tension values of the supernatant resulting from the growth of the BBC | 392 and BBC | 398 strains having naphthalene, anthracene or phenanthrene as the sole source of carbon. As controls, measurements were made in ultrapure water, M9 and M9 with PAHs.

In this study, M9 medium was used because it does not have intrinsic surfactant activity, presenting a surface tension (72.62 ± 0.023 mN/m) very close to ultrapure water value (72.29 ± 0.012 mN/m). There are some discrepancies between the values of surface tension of the water in contact with the air in the literature. However, for the same determination method, the experimental value of the literature for the surface tension of pure water at 25°C (72.00 ± 0.10 mN/m) is coincident with the value obtained in this work (Kalová and Mareš, 2015).

However, it can be realized that samples with M9 + PAH supernatant present lower surface tension values relative to the sample with only M9. This leads to the conclusion that the substrate presents some surfactant activity which reduces the surface tension. Therefore, the M9 + PAH samples should be used as a negative control for the biological samples.

In the case of naphthalene, the strain BBC|392 shows a small decrease in surface tension (71.67 ± 0.099 mN/m) relative to the M9 + NAP control (72.51 ± 0.045 mN/m). The same does not occur for strain BBC|398 (72.58 ± 0.043 mN/m).

The most interesting results were obtained with anthracene, where strain BBC|392 exhibited a substantial reduction in surface tension (63.8 ± 0.023 mN/m) compared to the M9 + ANT control (72.17 ± 0.023 mN/m). The reduction presented by the strain BBC|398 is small (71.36 ± 0.019 mN/m).

As for phenanthrene, strains BBC|392 and BBC|398 did not present any reduction of the surface tension in relation to the M9 + PHE control (72.26 ± 0.012 mN/m). On the contrary, the value is slightly above the value control (72.48 ± 0.020 mN/m and 72.62 ± 0.019 mN/m, respectively).

Strain BBC|392 has proven to be the most promising in terms of biosurfactant production. However, according to the literature it is expected that microbial candidates for biosurfactant production decrease the surface tension to around 35 mN/m (Banat, 1997). In this work, a reduction of superficial tension to 63.8 mN/m was reached, revealing the potential of biosurfactant production but not revealing a relevant decrease. This low productivity may be due to the fact that after 15 days, the production of biosurfactant is still in the initial stages, which reflects into a low concentration of the compound and consequently a weak decrease of the surface tension. It is possible that the strain only produces the biosurfactant in the presence of PAH to facilitate its solubilization and uptake, so the substrate acts as inducer of production. All biosurfactants are synthetized in the cytosol though ribosomal or non-ribosomal peptide synthesis and specific biosynthetic enzyme activity, being after exported to the extracellular medium if applicable. Thus, the bacterial cell needs to activate genes and metabolic pathways that lead to compound production. Besides, at each stage, intermediates or the final compounds could have detrimental biophysical effect on the producing cell. The literature suggests that biosurfactants produced by bacteria are considered strong if their surface tension ranges from 22 - 25 mN/m. The physicochemical nature of biosurfactants is considered as the main mechanism that limits the production of stronger compounds, creating a selective pressure where the production of biosurfactants considered stronger leads to unsustainable self-damage, being unfavourable genetically and phenotypically. For example, stronger biosurfactants may show increased ability to disrupt membranes due to their phospholipidic nature. The production of stronger biosurfactants can also solubilize or disrupt proteins or other macromolecules inside cells that could interrupt essential metabolic pathways or secretion systems, and may even affect the production of the biosurfactant and the accumulation of toxic products to the cell (Moldes *et al.*, 2007).

Moreover, there are a number of factors that affect the production of biosurfactants. The production of biosurfactants is a set of chemical reactions and consequently is affected by environmental factors like pH, salinity and temperature. In addition, nutritional factors such as the carbon source -

chemical nature and quantity - also affect the process (Gakpe *et al.*, 2007). Petroleum–contaminated sites were identified to be good sources of hydrocarbons for induction of biosurfactant production by microorganisms (Chen *et al.*, 2012).

The BBC|392 strain has been presumptively identified as belonging to the genus *Acinetobatcer* sp. This genus is described as containing biosurfactant producing species at sites hydrocarbons contaminated sites (see Appendix C). For example, *Acinetobacter calcoaceticus* BU03 enhance the solubility and biodegradation of phenanthrene (Zhao and Wong, 2009). This strain is reported as producing an extracellular liposaccharide responsible for the formation of an emulsion (Kim *et al.*, 1997). Also *Acinetobacter radioresistens* KA53 produce another bioemulsifier complex designed alas an (Navon-Venezia *et al.*, 1995). Recently, strains of *Acinetobacter baumanii* and *Acinetobacter variabilis*, isolated from oil-contaminated soil, have been found to produce a rhamnolipid molecule, elucidating the true potential of oil refinery areas as sites for isolation of new non-pathogenic microorganisms capable of biosurfactant production (No and Ankulkar, 2017).

3.4.3 Unravelling the degradative pathways of PAHs

Search for PAH catabolic genes

In order to unveil the degradation pathways used by the selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - a PCR approach was applied targeting genes that encode enzymes for the catabolism of PAHs. The results are shown in Figure 14.

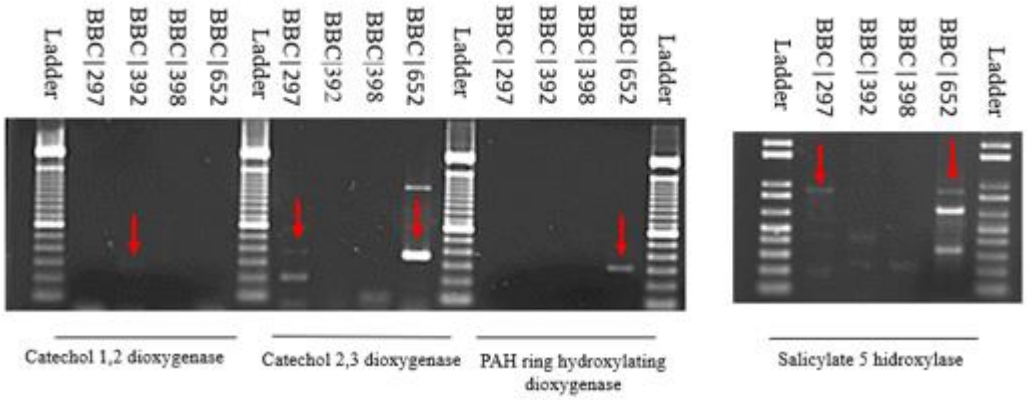

Figure 14 Photograph of the electrophoresis gel with the results of PCR amplification reactions whose targets were PAHs catabolism genes. The red arrows highlight the amplified bands of interest. The 1Kb DNA ladder was used.

According to Figure 14, the gene encoding the initial dioxygenase enzyme (PAH ring hydroxylating dioxygenase, PAH-RDH) was only detected on strain BBC|652.

It would be expected that amplification would also occur for the strains belonging to the genus *Pseudomonas* since the primers were constructed taking into account the different allele types present in the data base common to the Gram negative PAH degraders such as *Pseudomonas, Ralstonia, Commamonas, Burkholderia, Sphingomonas, Alcaligenes* and *Polaromonas* strains (Cébron *et al.*, 2008). Despite the gene coding for the enzyme dioxygenase had not been detected with these primers, even with optimization of PCR conditions, that does not mean that there is no enzyme coding region in the genome of these strains. Rather, it is assumed that such a region must exist since strains are capable of degrading PAH and dioxygenase is essential to the process of activating PAH for catabolism in the upper catabolic pathway. The primers can then be non-specific for these microorganisms because they have sequences that differ genetically from the remaining dioxygenase families. PAH-RDH are broadly distributed across microbial taxa and one of the principal differences between families are the physical characteristics of the active site, which consequently affect the type and range of PAH substrates on which the enzyme will act and funnel to a productive catabolic lower pathway (Cravo-Laureau, 2017).

Due to the divergence of lower metabolic pathways in the catabolism of PAH with the formation of catechol and/or gentisate intermediate metabolites from salicylate (Figure 1), different primers were used to allow their distinction.

In case of catechol synthesis, if the enzyme that acts on the molecule is the catechol 1,2-dioxygenase the cleavage is in the *ortho* position. Primers C12OF/R were reported as capable of amplifying a region of the gene encoding the catechol 1,2-dioxygenase (Sei *et al.*, 1999).

If the enzyme to act on the catechol is the catechol 2,3-dioxygenase then the cleavage is in the *meta* position. Primers C12OF/R have been described as capable of amplifying a region of the gene encoding the catechol 2,3-dioxygenase (Sei *et al.*, 1999).

When gentisate is synthesized from salicylate, the enzyme involved is salicylate 5-hydroxylase. The primer sgp319F/1238R targets a region of the gene coding for this hydroxylase (Izmalkova *et al.*, 2013).

In Figure 14 is shown a photograph of the electrophoresis gel highlighting the bands of interest that amplified through the gene-targeted PCR. In Table 7 there is a summary of the metabolic pathways that might be used by the selected strains according to these results.

It can be seen that for the primer targeting the catechol 1,2-dioxygenase there is only weak amplification for the BBC|392 strain. It can be concluded that there is only evidence that this strain is capable of following a degradation pathway involving a formation of the catechol from the salicylate followed by *ortho* cleaving of the catechol molecule with the formation of succinate and acetyl-CoA that entered in tricarboxylic acid cycle. This enzyme belongs to the intradiol dioxygenase family that breaks the aromatic ring between two adjacent hydroxyl groups of catechol. It is also possible to conclude about the possible localization of the gene since the genes for *ortho* cleavage are described as located on chromosome. Organization of genes differs in the gene order and operon distribution. For example, for *Acinetobacter calcoaceticus* there are two enzymatic systems for degradation of catechol while for *Pseudomonas putida* there is only one enzymatic system coded apparently by different genes (Cravo-Laureau, 2017). A study showed that *Acinetobacter* sp. WSD completely degraded phenanthrene via salicylate pathway instead of protocatechuate pathway (Shao *et al.*, 2015). Although the BBC|392 strain has only shown amplification for the catechol 1,2-dioxygenase enzyme, an *Acinetobacter* sp. strain which metabolizes naphthalene and anthracene through catechol *meta* cleavage with the catechol 2,3-dioxygenase enzyme was reported (Jiang, Qi and Zhang, 2018). Thus, it cannot be affirmed that BBC|392 only performs catechol *ortho* cleavage because it may be the primers that are non-specific for the nucleotide sequence coding for the gene associated with the *meta* cleavage.

About the primers targeting the catechol 2,3-dioxygenase gene, there is amplification in strains BBC|297 and BBC|652, pointing out that these strains may follow a degradation pathway involving the synthesis of catechol from salicylate with a *meta* cleavage of the catechol molecule with the formation of the metabolites acetaldehyde and pyruvate which also participate in the cycle of tricarboxylic acids. It is also possible to conclude about the possible localization of the gene since the genes for *meta* cleavage are described as being organized on an operon composed by 13 genes located on a plasmid (Cravo-Laureau, 2017). Strain BBC|297 was presumptively identified has belonging to the genus *Pseudomonas*. It is described that the *meta* pathway of catechol metabolism is induced during growth on naphthalene or salicylate in different *Pseudomonas* spp. (Barnsley, 1976). In addition, *ortho* pathways enzymes are also present in those strains. The authors suggest that separate regulatory systems for the *ortho* and *meta* catechol pathways in Pseudomonads. However, in this work, only amplification for the *meta* pathway was obtained, which does not invalidate the possibility of genes coding for *ortho* pathway enzymes to exist but the non-specificity of the primer used does not allow amplification. BBC|652 was presumptively identified as belonging to the genus *Paraburkholderia*. Genus *Burkholderia* comprehends a large number of diverse species which include many important pathogens

as well as environmental species. Based on phylogenetic analyses, a division of the genus was proposed in 2014 where *Burkholderia* species contain clinically relevant and phytopathogenic species and *Paraburkholderia* species are primarily environmental (Sawana, Adeolu and Gupta, 2014). In previous studies, where the genus division was not yet applied, the genes encoding catechol 2,3-dioxygenase were present in eight microorganisms belonging to *Burkholderiaceae* family, *Burkholderia* included, contrasting with the genes encoding for catechol 1,2-dioxygenase which are observed in nearly all *Burkholderiaceae* (Pérez-Pantoja *et al.*, 2012).

As to primers targeting the salicylate 5-hydroxylase, amplification occurs also for strains BBC|297 and BBC|652 indicating that these strains have the genes required for degradation pathway involving the formation of gentisate from salicylate, which culminate in the opening of the aromatic ring originating pyruvate and fumarate. The genus *Pseudomonas*, presumably identified as the genus of the BBC|297 strain, includes strains isolated from oil-contaminated soil which present genes that allow the degradation of naphthalene via the gentisate pathway rather than the catechol pathway (Fuenmayor *et al.*, 1998). As for the genus *Burkholderia*, presumably associated with strain BBC|652, it is also reported as capable of transforming salicylate via the gentisate pathway during the degradation of naphthalene and phenanthrene (Tittabutr *et al.*, 2011).

Note that it was not possible to conclude anything about possible degradation pathways of the BBC|398 strain since it did not show amplification with any of the primers, and although various optimization conditions were tested, no success was achieved.

With respect to plasticity in the use of metabolic pathways it can be concluded from Table 7 that the BBC|297 and BBC|652 strains by having genes encoding enzymes belonging to different degradation pathways are more adapted. However, it cannot be claimed that these are the only degradation pathways used by the strains.

The existence of appropriate biocatalytic systems, which requires that microorganisms contain genetic information for the production of degradative enzymes is a first limiting factor for biodegradation. If those enzymes are synthetized, the process will be determined by the environmental conditions. Analysis of PAHs catabolic genes in different bacterial genera can give valuable information about the evolution of enzyme structure-function relationships and the evolution and diversity of catabolic pathway genes via horizontal transfer, transposition events, DNA rearrangement, gene fusion and point mutation. In bioremediation, assessing the genetic information is crucial for monitoring bacterial populations that degrade PAHs in contaminated sites.

Monitoring of growth in intermediate metabolite phthalate

Phthalate is a metabolic intermediate in the degradation pathways of anthracene and phenanthrene (Figure 1). A growth experiment was carried out with phthalate in order to understand if the selected strains - BBC|297, BBC|392, BBC|398 and BBC|652 - are able to use it as a single carbon source from the growth curves that are presented in Figure 15.

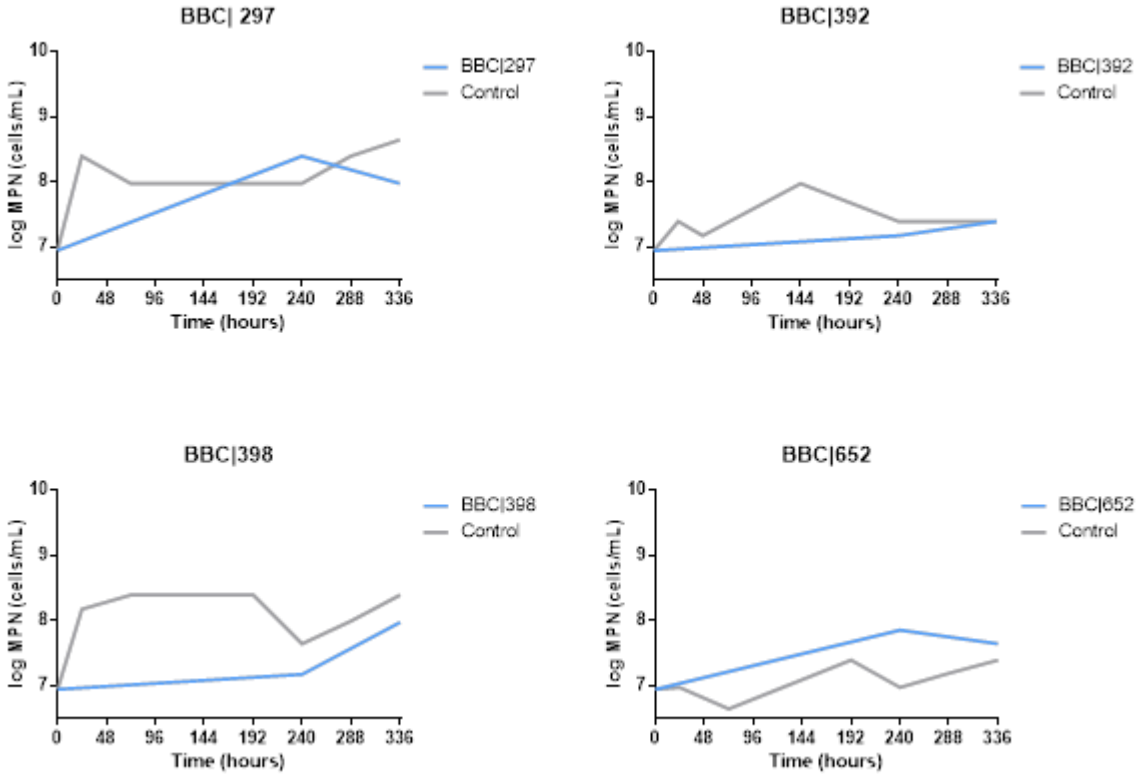

Figure 15 Representation of the viability of the selected strains BBC|297, BBC|392, BBC|398 and BBC|652 over 15 days growth having the metabolite intermediate phthalate as sole carbon source. In gray is depicted the abiotic control where the respective strains have no carbon source.

It can be seen from the Figure 15 that strain BBC|652 is the only one that is clearly capable of degrading and growing in phthalate. However, the viability values achieved are not very high since not even the increase of 1-log step is achieved. This strain was presumptively identified as belonging to the genus *Paraburkholderia.* A strain of *Burkholderia* sp. was able to use 2-naphthanoate (a precursor metabolite of phthalate) as sole carbon and energy source (Morawski *et al.*, 1997). The features of this pathway are convergent with those of phenanthrene with formation of phthalate and protocatechuate.

Table 7 Summary of the results obtained by searching for genes associated to the catabolism of PAHs by gene-targeting PCR and monitoring the growth of the selected strains in a metabolic intermediate of PAH degradation. The filled circles indicate evidence of the strain being able to use the metabolic pathway.

	Pathway			
	Catechol orto-cleavage	Catechol meta-cleavage	Gentisate	o - phthalate
BBC|297	○	●	●	○
BBC|392	●	○	○	○
BBC|398	○	○	○	○
BBC|652	○	●	●	●

Chapter III – Conclusion and Future Perspectives

This project intended to develop a novel bioaugmentation consortium from a sub-set of 47 evolved strains belonging to BBC collection throught selection, identification and metabolic/genetic characterization of best PAHs degraders (Figure 16).

A preliminary interpretation of these results is present in this thesis but a more extensive analysis is required to establish the consortia.

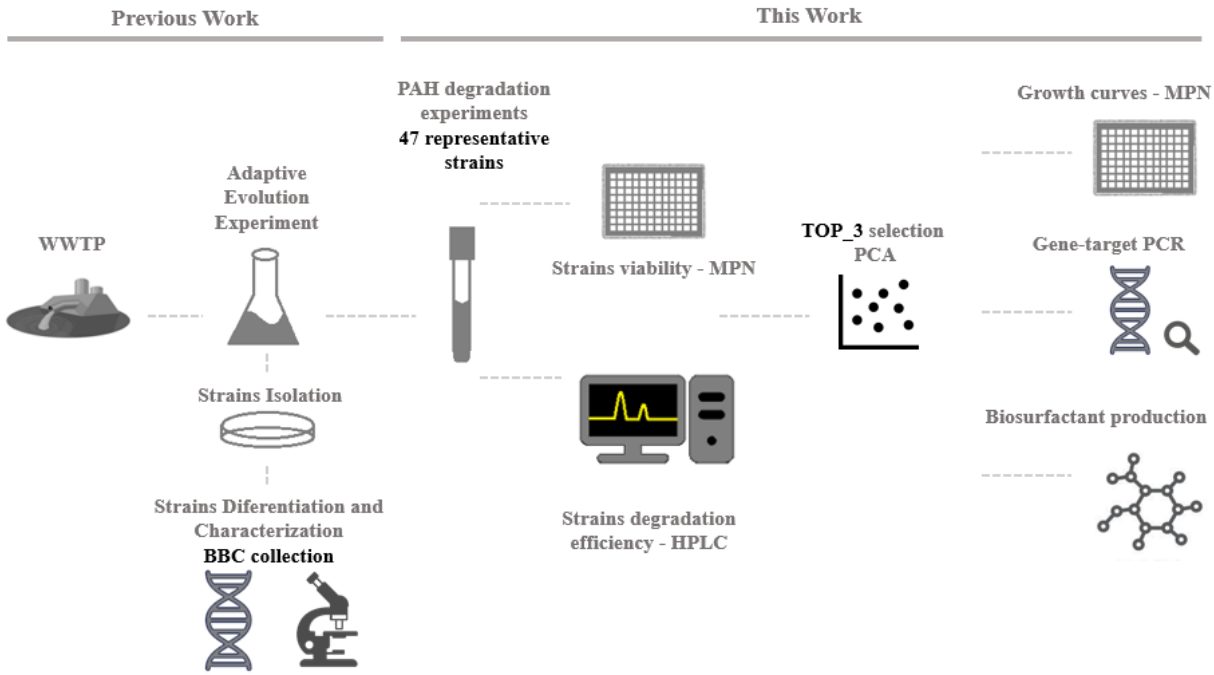

Figure 16 Overview of the work flow of this project.

From the results obtained it is possible to conclude that the adaptive evolution experiments prior to this work were successful, since through the quantification by HPLC and the monitoring of cell viability by MPN a trend was clearly observed where the strains considered to be the best degraders belonging to more recent cycles of adaptive evolution.

The PCA allowed the selection of three strains considered the best degraders considering their distribution in the space formed by the PCs. It was observed in this distribution concordance with what was found previously, standing out cycle 29 as the cycle of the best degraders.

Strain BBC|392 belonging to the adaptive evolution cycle 29 showed the best location in PCA and was considered the most promising strain. However, by observation of growth curves, this was the strain with the worst result, reaching the lowest viability values for all carbon sources. As the strain tends to form aggregates, which leads to uncertainty about how many cells are effectively in the initial inoculum, this can be a disadvantage for the preparation of a commercial inoculum for the consortium. Thus, the discrepancies between the high initial screening results and the low viability results on growth

curves may be due to the fact that a different starting amount of inoculum was used. However, this strain has the interesting property of apparently producing some biological molecule with surfactant activity, and the strains producing biosurfactants confer an advantage for the bioremediation because they more easily have access to the insoluble substrates. In addition, there is only evidence that the strain BBC|392 follows the *ortho* cleavage of catechol as degradation pathway, which could in part justify the low viability results as it only follows a catabolic pathway compared to greater metabolic plasticity of other known strains. The hypothesis is that the strain produces biosurfactants as an aid to its survival in places whose only source of carbon is complex and in metabolic terms does not present the best efficiency. It would be important to try to understand the chemical nature of the possible biosurfactant produced by the strain through mass spectrometry, for example, in order to assess if it can have any interest.

As for the selected strain BBC|398, it also belongs to the adaptive evolution cycle 29. In relation to this strain, it has a degradative behaviour preferentially of using anthracene as carbon source which is coincident with de fact that it has previously been isolated in medium containing only this carbon source. With respect to genes associated to the catabolism of PAHs, this strain remains an enigma since it did not show amplification with any of the tested primers.

Another of the selected strains was BBC|297, belonged to adaption evolution cycle 16 and was selected not only because it presents good viability results for naphthalene, anthracene and phenanthrene but also it was thought to be interesting to compare strains from different cycles. This strain turned out to be the most interesting one, since it exhibits a high degradative pattern for naphthalene, anthracene and phenanthrene. In addition, it presents metabolic plasticity in degradation pathways, and could follow the catechol pathway (*meta*-cleavage) or the gentisate pathway.

Studies were also carried out with strain BBC|652 which is not a strain from adaptive evolution tests but rather a microorganism detected as a contaminant but that exhibited interesting degradation behaviour, especially for phenanthrene. The results obtained proved this because it was with this strain that the highest values of growth in the presence of phenanthrene were verified. Regarding the degradative pattern, there is clearly a trend towards the use of carbon sources. However, contrary to what is expected, the tendency is to more easily degrade the more complex carbon source and less easily the simpler carbon sources. In addition, the strain BBC|652 was the strain that presented the most positive results for the amplification of the genes of interest, which means it has the most metabolic plasticity from all. It was possible to detect the presence of the initial dioxygenase essential for the activation of the PAHs for degradation, with homologies with the dioxygenase of other bacterial genera known to be PAH degraders. Moreover, it was possible to assume that the strain is capable of using the catechol degradation pathway (meta-cleavage) and the gentisate degradation pathway. Remarkably, it was the only strain able to grow using phthalate as the only source of carbon, indicating the presence of genes and enzymatic machinery that enable phthalate degradation. This may be the reason why it has the best results in phenanthrene.

In general, the existence of promising strains in metabolic and genetic terms that may be part of the consortium has been proven. However, it would be important to carry out further studies that may give more certainty regarding the degradative profile of the strains. What are the genes present in the genome of these strains that allow the activation and degradation of PAHs? To access this information and the identification at species level, whole genome sequencing is an option. For the strains with metabolic plasticity that could follow simultaneous degradation pathways, what are the degradation pathways they actually adopt and in what conditions? Several strategies can be deployed as reverse transcriptase-PCR, identification of metabolites thought mass spectrometry or kinetic tests to follow the

most relevant catabolic enzymes activity. In addition, it would be important to perform more growth studies for a longer time (maybe months) to see if growth really is bi-phasic or not.

Finally, for the consortium composed of strains BBC|297, BBC|392 and BBC|398, high viability values where observed what may therefore have be considered a success. What strains were maintained over time? Is there a predominant strain? Would the addiction of strain BBC|652 increase the degradation efficiency of the consortium? Also, considering that the consortium would be used in places where there is a mixture of PAHs and other compounds as well as several other microorganisms, it would be of great importance to understand how these factors, among others (salinity, temperature, concentration, for example) would affect the degrading behaviour of the consortium. Multiple approaches can be used to approximate the *in vitro* conditions of *in situ* conditions, mimicking the expected microbial ecology at the contaminated sites.

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Appendix

Appendix A: Table with main genera of degrading bacteria (adapted from Seo, 2009).

Genera	
Gram Negative	**Gram Positive**
Achromobacter	*Arthrobacter*
Acinetobacter	*Bacillus*
Alcaligenes	*Brevibacterium*
Burkholderia	*Janibacter*
Chryseobacterium	*Mycobacterium*
Cycloclasticus	*Nocardioides*
Marinobacter	*Rhodococcus*
Ochrobactrum	*Terrabacte*r
Pasteurella	
Polaromonas	
Pseudomonas	
Ralstonia	
Rhodanobacter	
Stenotrophomonas	
Sphingomonas	
Xanthamonas	

Appendix B: Families of PAH upper pathway ring hydroxylating dioxygenases and associated lower pathways (adapted from Cravo-Laureau, 2017).

PAH – RDHs Family	Class or Genera	Lower pathway
	Proteobacteria	
NahAc	Gamma-/Beta-proteobacteria	Salicylate
NagAc	Betaproteobacteria	Gentisate
$PhnAc_{AFK2}$	Betaproteobacteria	*o*-Phthalate
$PhnAc_{RP007}$	Betaproteobacteria	Salicylate
AhdA1/BphA1	Alphaproteobacteria	Salicylate or *o*-Phthalate
	Actinobacteria	
NidA/PdoA1	*Mycobacterium*	*o*-Phthalate
NidA/PdoA1	*Mycobacterium/Terrabacter*	*o*-Phthalate
NarA	*Rhodococcus*	Gentisate
PdoA2/PhdA	*Mycobacterium/Nocardia*	*o*-Phthalate

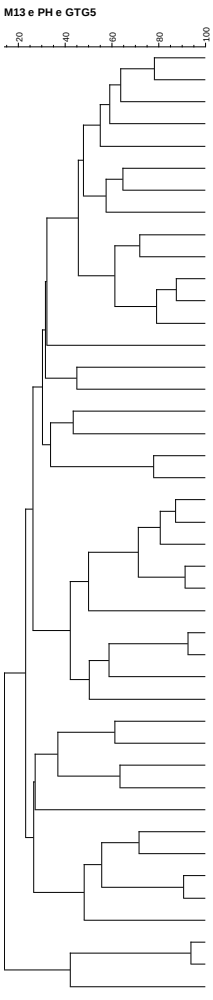
Appendix C: Classification of biosurfactants based on their chemical nature and their source microorganisms (adapted from Shekhar, 2014).

Biosurfactants	**Microorganism**
Glycolipids: Carbohydrates with the combination of long chain aliphatic acid or hydroxyaliphatic acid. The linking is made through an ether or an ester group.	
Trehlolipids	*Rhodococcus erythropolis,* *Nocardia erythropolis* *Arthrobacter* sp., *Mycobacterium* sp.
Trehalose Dimycolates	*Mycobacterium* sp., *Nocardia* sp.
Trehalose dicorynemycoaltes	*Arthrobacter* sp., *Corynebacterium* sp.
Rhamnolipids	*Pseudomonas aeruginosa,* *Pseudomonas* sp.
Sophorolipids	*Torulopsis bombicola,* *Torulopsis apicola,* *Torulopsis Petrophilum* *Torulopsis* sp.
Cellobiolipids	*Ustilago zeae,* *Ustilago maydis*
Lipopeptides and lipoprotein: Contain a fatty acid linked to a polypeptide chain.	
Peptide-lipid	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Viscosin	*Pseudomonas fluorescens*
Surfactin	*Bacillus subtilis*
Fengycin	*Bacillus* sp.
Arthrofactin	*Arthrobacter* sp.
Subtilisin	*Bacillus subtilis*
Gramicidins	*Bacillus brevis,* *Brevibacterium brevis*

Polymyxins	*Bacillus polymyxa, Brevibacterium polymyxa*
Lichenysin	*Bacillus licheniformis*
Ornithine lipids	*Myroides* sp. *SM1, Pseudomonas* sp., *Thiobacillus* sp., *Agrobacterium* sp., *Gluconobacter* sp
Fatty acids, Phospholipids and Neutral lipids	
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Thiobacillus thiooxidans*
Bile salts	*Myroides* sp.
Fatty acids	*Candida lepus, Acinetobacter* sp., *Pseudomonas sp., Micrococcus sp., Mycococcus sp., Candida sp., Penicillium sp., Aspergillus* sp.
Polymeric surfactants: Macromolecules which contain both hydrophilic and hydrophobic parts	
Emulsan	*Arethrobacter calcoaceticus*
Biodispersan	*Arethrobacter calcoaceticus*
Mannan lipid protein	*Candida tropicalis*
Liposan	*Candida lipolytica*
Carbohydrate protein lipid	*Pseudomonas fluorescens, Debaryomyces polymorphis*
Protein PA	*Pseudomonas aeruginosa*
Particulate Biosurfactant: Consist of extracellular membrane vesicles partition hydrocarbons to form a microemulsion.	
Vesicles and fimbriae	*Arthrobacter calcoaceticus*

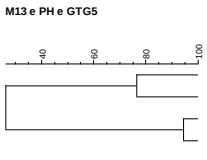
Appendix D: Dendrograms representing the genomic diversity of the collection of BBC isolates from the AE experiments (provided generously by Pedro Teixeira within the scope of his PhD.

(43 entries) Bacteria



M13	Ph	GTG5	ID	Group	Cells	Gram	KOH	Oxidase	Catalase	EA_Cy	Sequenced
			BBC|294	G4	rod	-	+	+	+	05	
			BBC|318	G4	rod	-	+	+	+	20	Ochrobactrum anthropi
			BBC|302	G4	rod	-	+	-	+	16	
			BBC|309	G4	rod	-	+	+	+	16	Shinella kummerowiae
			BBC|312	G4	rod	-	+	+	+	20	
			BBC|256	G4	rod	-	+	+	+	21	Achromobacter sp.
			BBC|311	G4	rod	-	+	+	+	20	
			BBC|354	G4	rod	-	+	+	+	29	
			BBC|295	G4	rod	-	+	+	+	16	
			BBC|401	G4	rod	-	+	+	+	29	
			BBC|336	G4	rod	-	+	+	+	29	
			BBC|338	G4	rod	-	+	+	+	29	
			BBC|236	G4	rod	-	+	+	+	12	Ochrobactrum grignonense
			BBC|290	G4	rod	-	+	+	+	05	
			BBC|300	G4	rod	-	+	-	+	16	
			BBC|392	G3	coccus	-	+	-	+	29	
			BBC|396	G4	rod	-	+	+	+	29	
			BBC|409	G4	rod	-	+	+	+	29	
			BBC|344	G4	rod	-	+	+	+	29	Brevundimonas diminuta
			BBC|395	G4	rod	-	+	+	+	29	Brevundimonas diminuta
			BBC|360	G4	rod	-	+	+	-	29	
			BBC|381	G4	rod	-	+	+	-	29	
			BBC|390	G4	rod	-	+	+	+	29	Sphingobacterium sp.
			BBC|293	G4	rod	-	+	+	+	05	
			BBC|314	G4	rod	-	+	+	-	20	
			BBC|291	G4	rod	-	+	+	+	05	na
			BBC|365	G4	rod	-	+	+	+	29	
			BBC|369	G4	rod	-	+	+	+	29	
			BBC|364	G3	coccus	-	+	+	+	29	
			BBC|380	G3	coccus	-	+	+	+	29	
			BBC|284	G4	rod	-	+	+	+	21	Pseudomonas knackmussi ou nitritiredu.
			BBC|398	G4	rod	-	+	+	+	29	
			BBC|361	G3	coccus	-	+	-	+	29	
			BBC|362	G3	coccus	-	+	-	+	29	
			BBC|368	G3	coccus	-	+	-	+	29	
			BBC|292	G4	rod	-	+	+	+	05	
			BBC|297	G4	rod	-	+	+	+	16	
			BBC|216	G4	rod	-	+	+	+	12	Pseudomonas knackmussi ou nitritiredu.
			BBC|332	G4	rod	-	+	+	+	29	Pseudomonas knackmussi ou nitritiredu.
			BBC|379	G3	coccus	-	+	+	+	29	
			BBC|331	G2	rod	+	-	-	+	29	
			BBC|333	G2	rod	+	-	-	+	29	
			BBC|342	G4	rod	-	+	-	+	29	

(4 entries) Yeast

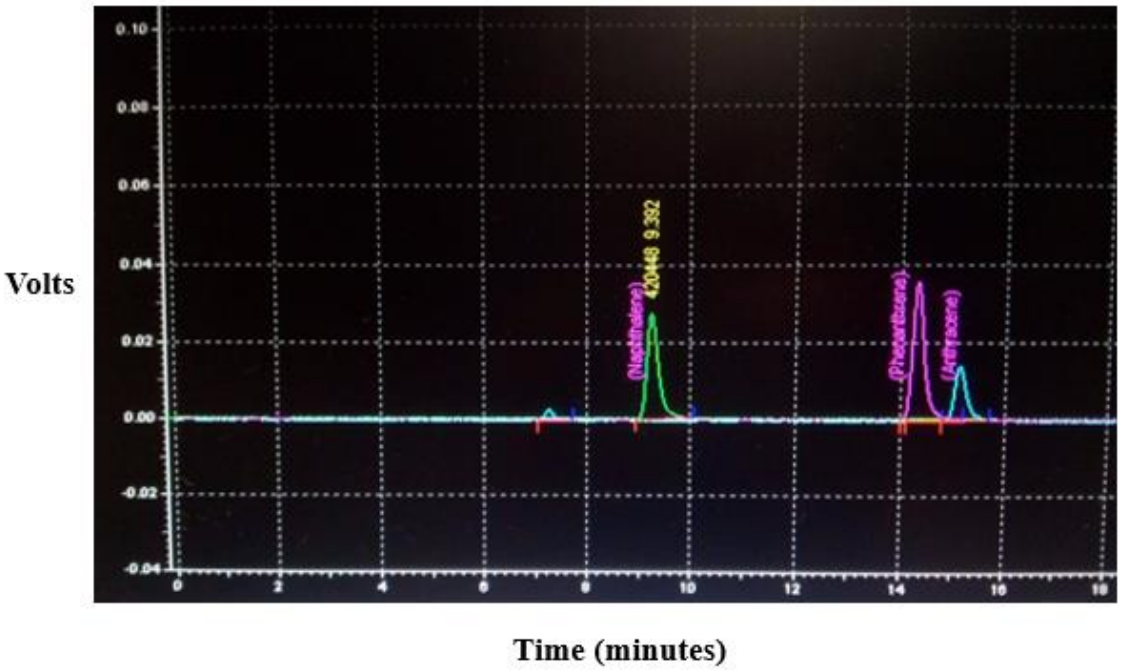


M13	Ph	GTG5	ID	Cells	Isolation media	Isolation source	EA_Cycle	Sequenced
			BBC|355	yeast	UO	ANM	29	Yarrowia lipolytica
			BBC|378	yeast	UO	FEM	29	
			BBC|253	yeast	TSA	ANM	21	Candida zeylanoides
			BBC|279	yeast	TSA	GTM	21	Candida zeylanoides

Appendix E: Composition of M9 mineral medium.

Nutrient	Final concentration
Na_2HPO_4 (anhydrous)	4×10^{-2} M
KH_2PO_4	2×10^{-2} M
NaCl	8×10^{-3} M
NH_4Cl	2×10^{-2} M
$MgSO_4$	1 mM
$CaCl_2$	100 µM
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	3×10^{-9} M
H_3BO_3	4×10^{-7} M
$CoCl_2 \cdot 6H_2O$	3×10^{-8} M
$CuSO_4 \cdot 5H_2O$	1×10^{-9} M
$MnCl_2 \cdot 4H_2O$	8×10^{-9} M
$ZnSO_4 \cdot 7H_2O$	1×10^{-8} M
$FeSO_4 \cdot 7H_2O$§	1×10^{-6} M

Appendix F: Chromatogram displaying the HPLC peaks corresponding to the compounds of interest – naphthalene in green, anthracene in blue and phenanthrene in pink.



Appendix G: Eigenvalues and respective variance (%) explained by the new variables – principle components.

Principal Component (PC)	Eigenvalue (λi)	% Variance	% Variance (accumulated)
PC1	2,57	42,7849	42,7849
PC2	1,38	22,9836	65,769
PC3	0,99	16,4293	82,198
PC4	0,68	11,3753	93,573
PC5	0,33	5,48887	99,062
PC6	0,06	0,9381	100,000

Appendix H: PC loadings (correlation coefficients of original variables and PCs).

Original Variable	PC1	PC2	PC3	PC4	PC5	PC6
MPN|ANT	0,16	0,2681	0,9408	0,0893	0,10	0,0006
MPN|NAP	0,60	0,6852	-0,042	-0,0792	-0,41	-0,0175
MPN|PHE	0,71	0,3158	-0,134	-0,5315	0,31	-0,0011
HPLC|ANT	-0,80	0,5731	-0,074	-0,0487	0,05	0,1677
HPLC|NAP	-0,62	-0,3515	0,260	-0,619	-0,20	-0,0142
HPLC|PHE	-0,82	0,5347	-0,088	0,016	0,10	-0,1663

Appendix H: Schematic representation of Du Noüy method used to measure surface tension.