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The use of oil degrading bacteria in the crude oil tankers for spill mitigation

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1. Introduction

Petroleum hydrocarbons are among the world's most important energy sources and industrial feedstocks, yet among the most widespread and hazardous environmental pollutants. During the petroleum processes and explorations, refineries, and transportation of the commodities, the hydrocarbon-originated contaminants, like - BTEX (Benzene, Toluene, Ethylbenzene, and Xylene), PAHs (Polycyclic Aromatic Hydrocarbons), and TPH (Total Petroleum Hydrocarbons) are precipitated into the environment.

The impact of petroleum pollutants on the surroundings has long been proven during the major spills of petroleum on the environments during catastrophes. The oil spill of the Exxon Valdez tanker ship in 1989 released about 41 million litres of petroleum into the Prince William Sound in Alaska, oiling about 2,000 km of coastline (Michel & Rutherford,2014). In fact, the most recent major disaster was the Deepwater Horizon disaster of 2010, where an estimated 780 million litres of petroleum were released directly into the Gulf of Mexico, resulting in the unprecedented death of enormous quantities of marine organisms. Such examples reflect the extent and period of hydrocarbon contamination not addressed by traditional mitigation techniques, which also create their own secondary problems for the environment. Instead of this conventional physical and chemical methods of clean-up, which are very effective for removal of oil from the surfaces, bioremediation, the process of using living organisms such as bacteria for the degradation of toxic chemicals, becomes an effective alternative for clean-up processes. In ideal case, bacteria break down hydrocarbons into non-toxic materials such as carbon-dioxide and water through oxidation processes. A few bacterial genera are found to be rich in hydrocarbon degradation, including *Rhodococcus*, *Pseudomonas*, *Mycobacteria*, *Acidovorax*, and *Malikia* (Huang et al., 2008; Benedek et al., 2018; Révész et al., 2020). Bacteria exhibit diverse metabolic processes and also produce biosurfactants, which enhance the solubilization and bioavailability of hydrocarbons in the environment.

This thesis discusses the biodegradation efficiency of bacterial strains isolated and stored by the Institute of Aquaculture and Environmental Safety at Hungarian University of Agriculture and Life Sciences (MATE): *Rhodococcus erythropolis* NI1, *Rhodococcus qingshengii* PT2/14B, *Rhodococcus qingshengii* BA4/9, *Malikia spinosa* AB6, *Mycobacterium trichoteceniticum* R17; *Rhodococcus pyridinivorans* K404; *Rhodococcus erythropolis* GP2b; *Pseudomonas aromaticivorans* MAP12. These strains we selected based on previous findings related to

hydrocarbon and mycotoxin degradation attributed to *Rhodococcus* species (Fejes et al., 2017; Garai et al., 2021) and Malikia strains to efficient BTEX degradation in aerobic conditions (Révész et al., 2020). The experimental method will integrate in vitro biodegradation assays and resazurin-based colorimetric assays for determining bacterial metabolic activity and hydrocarbon degradation under lab conditions in MATE. Besides its quantitative capability, this resazurin assay allowed a rapid and non-destructive assessment of bacterial respiration and the choice of promising degraders. The next stage concerned selected strains incubated with a crude oil-diesel mixture as the sole carbon source. Residual oil was extracted, evaporated, and weighed to determine the percentage of degradation achieved by each strain. Bacterial degradation activity for each of the bacterial strains was tested separately under the same environmental conditions for temperature, salt concentration, and available oxygen levels for a well-informed comparison among the bacterial strains.

The aim of my work is to test isolates for their capability to degrade hydrocarbons. Through the characterization and comparison of the mentioned bacteria, the study objective is to identify the best hydrocarbon-degrading bacteria that could be used for the bioremediation purpose of an oil-polluted environment. The study will be useful for the development of an environmentally friendly approach to the bioremediation of an oil-polluted environment through the bioaugmentation technique, which can be based on a bacterial consortium from these strains. The targeted strains of the Institute weren't checked for oil-degrading ability, that why I was very interested if there any good degraders, which can possibly contribute for future examinations.

2 Literature Review

2.1 Overweening crude oil information

2.1.1 Chemical and physical properties

Crude oil, also referred to as petroleum, refers to a naturally occurring compound composition of hydrocarbons from the debris of animals and plant organism remains (diatoms), which existed millions of years ago in an aquatic environment preceding the evolution of the dinosaur age. With millions of years having passed, the debris from the animals and plant organisms got deposited above by sand, silt, and rocks. The heat from the sand, silt, and rocks pressurized the debris, turning it into what today goes by the name crude oil or petroleum. The word petroleum means rock oil or oil from the earth. Crude oil and other hydrocarbons exist in liquid or gaseous form in underground pools, or reservoirs, in tiny spaces within sedimentary rocks and near the earth's surface (U.S. Energy Information Administration (EIA) 2024).

One of the most crucial physical properties is the specific gravity, which refers to the ratio of the weight of a given volume of the crude oil to the weight of an equal volume of pure water at a given temperature and pressure. Crude oils may be classified according to their gravity as follows:

Heavy: 1–23° API gravity; Medium: 20–40° API gravity; Light: above 35° API gravity.

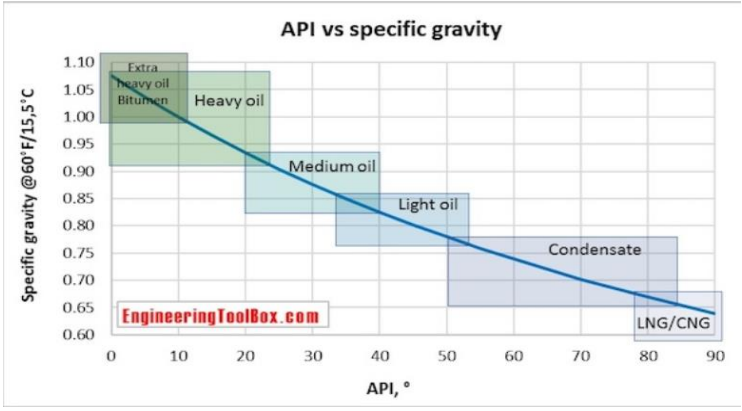


Figure 1. API vs specific gravity (Source: Engineering ToolBox website)

Crude oil is also classified as either "sweet" or "sour," based on its sulphur levels, which can be in elemental form or as a compound (for example, hydrogen sulphide). Sweet cultures contain 0.5 weight percent sulphur or less and sour crudes contain 0.5 weight percent sulphur or more. As a rule of thumb, the heavier the crude, the greater the sulphur. Refineries typically remove excess

sulphur from crude oil, as sulphur oxides produced during the combustion of oil are a major pollutant in the atmosphere.

From the chemical point of view, the composition of the crude oil could be considered very complex because it contains hundreds to thousands of various compounds such as hydrocarbons and organic compounds of nitrogen, oxygen, and sulphur, as well as traces of metals/organometallic compounds (nickel and vanadium), solid minerals, and emulsified water. Crude oil is an organic mineral, mainly a mixture of hydrocarbons, which varies greatly depending on the source. It is primarily composed of carbon (81-87%), hydrogen (10-14%), oxygen (0-7%), sulphur (0-7%) and nitrogen (0-2%). Crude oil can be divided into three major groups based on the type of hydrocarbon it consists of. The first and one of the largest groups is paraffinic (e.g., paraffin oil and paraffin-based oil), which consists mainly of open-chain (aliphatic) hydrocarbons. The second group is naphthenic (e.g., naphthene oil), which is based on cycloparaffins (e.g., cyclopentane, cyclohexane). The third group is asphaltene (e.g., asphaltic oil), which is based on organic components that remain after fractional distillation (National Academies of Sciences, Engineering, and Medicine, 2016).

Based on the three groups of petroleum components described above, I will now present the paraffins and aromatic compounds that are important from an environmental perspective.

2.1.2 Paraffins

Paraffins constitute a homologous series that follows the general formula of $CnH2_{n+2}$, with methane (CH_4) being the simplest representative of the series. Paraffins can be either saturated (a carbon atom only contains single bonds) or unsaturated (having one or more double or triple bonds in the chain). They can also be branched or unbranched. When we have more than three carbon atoms, we see the phenomenon of isomerism, yielding compounds that have same molecular formula but different properties and structures. Considering the unbranched alkanes, the first four members of the homologous series (methane, ethane, propane, and butane) are gaseous at room temperature. Concerning the homologues that follow, they are liquid for up to 17 carbon atoms followed by solid. The melting and boiling point will increase with carbon number (but they will decrease with increased number of multiple bonds). The paraffins are sparingly soluble in water, mildly soluble in water, but readily dissolve in nonpolar (organic) solvents (Alkane (and paraffin) – Wikipedia; Zein, M. A. 'ALKANES (PARAFFINS)' 2018).

2.1.3 Aromatic compounds

The totality of hydrocarbons that are either homo- or heterocyclic containing unsaturated conjugated bonds. The spatial arrangement of these compounds is a ring arrangement containing conjugated double bonds (π). A hallmark of aromatic hydrocarbons is the ring arrangement of the molecule. Their arrangement makes them particularly stable molecules and the basic molecule of these compounds is benzene. Referring to the number of rings, we may speak of monoaromatic (compounds containing only one ring) and polyaromatic (compounds containing two or more rings) hydrocarbons. Some members of this group of compounds are also characterized by a notable odour. They burn with a black flame, are toxic to humans (at least), and several compounds either containing benzene or polycondensed rings have been identified as carcinogenic. Homologues with a low number of carbon atoms are liquids and those with condensed rings are crystalline. They are not soluble in water, but soluble in several organic solvents, and have a density lower than that of water (Encyclopedia Britannica. (2025, September 11). Aromatic hydrocarbon.).

2.1.4 From extraction to product

Oil recovery consists of the process of locating, drilling into, and producing oil with a view to surface handling and uses. The methods of recovery are a natural complement to whatever type of rock is present, how deep the reservoir is, its pressure and temperature, the viscosity of the oil, and the method used to extract oil and transport it to the surface. Engineering differentiates stages of oil recovery by primary - using the existing energy of the reservoir to produce oil, secondary - keeping the reservoir pressure in such a way to drive the oil to the well, and tertiary - altering the fluids or rock in some way to start or continue the flow of oil from the reservoir. (EPA US 2008)

After extraction, crude oil must be transported to refineries. There are four dominant transport networks or modes, each with their own risks and controls. First, pipelines are the principal means of transport of crude oil over land. Next, I mention liquid bulk transportation, which is how crude oil is transported over long distances by oil tankers. Another mode of transport that is widely used is in the form of rails and roads (EPA US 2008).

Once crude oil reaches its intended destination in the economy, it can be converted into several petroleum products that touch almost every part of the economy. The largest uses are for transportation fuels like gasoline and diesel and as feedstocks for the petrochemical industry; some other uses, although smaller, are for heating, power generation in some cases, as a lubricant, asphalt, waxes, and petroleum coke. There are still more commodities where we use crude oil or

products made from crude oil, such as textiles, household plastics, tires, coatings, cosmetics, etc. Some biofuels are also mixed into gasoline and diesel at the refinery/terminal level which also mean that when talking about "petroleum products", those biofuels are also included in general statistics (EIA “Use of oil”).

2.2 Hydrocarbon contaminants

2.2.1 BTEX

BTEX are volatile hydrocarbons, meaning they are mono-aromatic hydrocarbons found in gasoline, petroleum, and various solvents. Because of their volatility and solubility characteristics, BTEX compounds are easily migratory from the contaminated soil to air as vapours and groundwater as dissolved plumes, thus being the most migratory and persistent light hydrocarbons in the environment (ATSDR Toxicological Profiles for BTEX, 2023; US EPA, 40 CFR 141). Detailed health descriptions regard BTEX as a mixture based upon co-exposure levels.

BTEX stands for Benzene (C_6H_6), Toluene (C_7H_8), Ethylbenzene (C_8H_{10}), and Xylene (C_8H_{10}, the three isomers are meta-xylene, para-xylene, and ortho-xylene) and other monoaromatic hydrocarbons. A chemical description of BTEX could be written as having: Benzene (C_6H_6) – no substituents, simplest aromatic compound; Toluene ($C_6H_5CH_3$) – one methyl group; Ethylbenzene ($C_6H_5C_2H_5$) – one ethyl group; Xylenes ($C_6H_4(CH_3)_2$) – two methyl groups (three isomers: *ortho, meta, para, xylene*).

Such chemicals have severe environmental and human health implications since they are poisonous and transported by air, water, and soil pathways. Benzene is generally classified as a human carcinogen since it causes chronic damage to the bone marrow and leads to leukaemia and blood problems by the International Agency for Cancer Research (Group 1). Though toluene, ethylbenzene, and xylenes are less likely to cause cancer than benzene, at high concentrations, they could cause central nervous system depression, headaches, dizziness, irritation to the eyes and lungs, and hearing problems, according to studies carried out on animals for ethylbenzene. The collective toxicity for BTEX chemicals generally proves to be additive, meaning the greater the exposure, the riskier it gets (ATSDR, 2023; IARC Monographs Volumes 120; US EPA).

Drinking water regulations specify the maximum concentration for BTEX, maximum concentration of Benzene -5 µg/L, Toluene -1 mg/L, Ethylbenzene -0.7 mg, and Xylenes (total)-10 mg/L (U.S. EPA Drinking Water Regulatory Levels (MCLs)).

BTEX compounds are easily distinguished by screening techniques such as photoionization analysis (PIDs), gas chromate (GC). Consequently, BTEX compounds are usually the primary targets in remediation techniques such as air stripping, Soil Vapor Extraction (SVE), biosparging, and bioremediation techniques. In addition, their biodegradable nature makes BTEX compounds useful as a pesticides' model compound for the investigation of MIC (Microbial-Influenced Corrosion) adaptation and resistance in petroleum-degrading bacteria. Below I provided deeper description of each compound (EPA US 2017).

2.2.1.1 Benzene

Colourless, benzene has a characteristic odour, is insoluble in water (although dissolves in most organic solvents). It has strong refractive properties, has a melting point of 5.5°C and a boiling point of 80.2°C. Benzene has a structural formula that reflects a ring consisting of six carbon atoms. The carbon atoms that compose the compound lie on a plane, each located at a vertex of a regular hexagon. The hydrogens attached to the following carbon atoms also lie on the same plane and their configuration also suggests a regular hexagon, and its endothermic compound are unsaturated. Can cause loss of consciousness as well as death from inhalation of concentrated (ATSDR 2024).

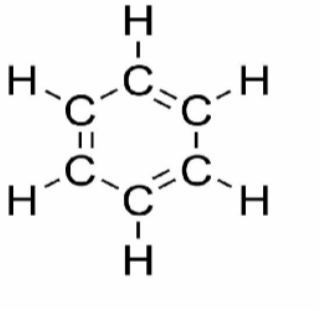


Figure 2. Rings-structure of benzene (Source: ChemTalk website)

2.2.1.2 Toulene

A colourless substance, having an aromatic scent, that burns with a sooty flame. The melting point of this compound is -97.7°C, while the boiling point is 110.8°C. The compound has a chemical structure that contains a benzene ring with a methyl (-CH3) group attached. It can be utilized as an important raw material for chemical reactions. It exists in coal tar but is produced during gasoline refining. The compound is water insoluble and organic solvents

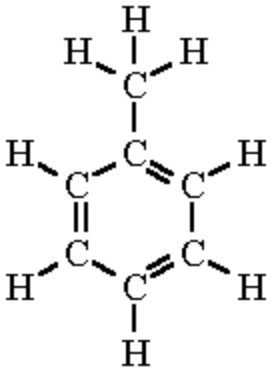


Figure 3. Rings-structure of toulene (Source: (Linford, 2014))

readily dissolve it. It can be nitrated to make a trinitro compound such as trinitrotoluene (TNT)(ATSDR 2017).

2.2.1.3 Ethylbenzene

It is a colourless liquid with a smell similar to benzene. Melting point is -92.8°C, and boiling point is 188.5°C. It is made from benzene and ethylene. In terms of its structure, it consists of a benzene ring to which is attached an ethyl group (CH(3)-CH(2)-). It is an economic raw material as styrene is produced from it (the base molecule is dehydrogenated). Styrene is used on a large scale in industry as an economic starting material to produce polystyrene. In addition to this, ethylbenzene is an intermediate in the production of many other organic compounds (ATSDR 2010).

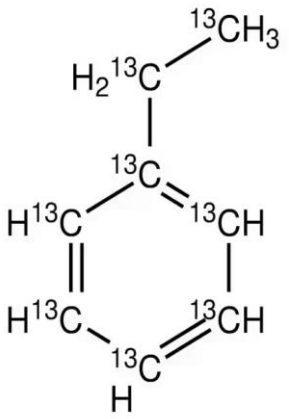


Figure 4. Rings-structure of ethylbenzene (Source: Merck Website)

2.2.1.4 Xylenes

In the solid state, the compounds are colourless and have a pleasant scent. The melting point ranges from -29 to 13° Celsius, while the boiling point ranges from 135.5 to 145.9° Celsius. Some of them are naturally occurring from petroleum tar, but most of them have come from gasoline reforming. They make very good solvents for organic chemistry use. Chemically, they are classified as dimethylbenzenes (benzene ring + 2 attached methyl groups). Depending on the locations of the methyl groups, we will be talking about depending on the closeness of the two methyl groups, meta-, ortho-, and para-xylene. Of these, m-xylene has the least use, but the o-xylene and p-xylene have increasing value as a raw material in the plastics industry (ATSDR 2007).

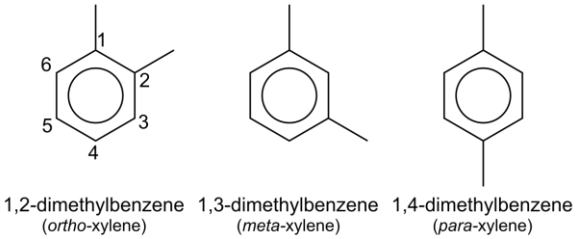


Figure 5. Rings-structure of xylene (Source: Wikipedia Website)

2.2.2 PAHs (Polycyclic Aromatic Hydrocarbons)

Polycyclic Aromatic Hydrocarbons (PAHs), also referred to as Carcinogenic Aromatic Hydrocarbons, are a vast family of organic compounds composed of at least two condensed aromatic rings only made up of carbon and hydrogen atoms. They have the general formula C_nH_m

and are non-polar and hydrophobic in nature, hence relatively persistent in the environment. The PAHs are majorly synthesized during the incomplete combustion of organic materials such as coal, petroleum, wood, tobacco, among others, and are generally found in petroleum fuels such as diesel, asphalt, among others, in their raw forms. More than 100 PAHs are currently recognized, however, the EPA recognizes 16 of them as pollutants based on their toxicity levels and their possible presence in contaminated sites. Typical examples include Naphthalene ($C_{10}H_8$) with two rings, Phenanthrene ($C_{14}H_{10}$) with three rings, Fluoranthene ($C_{16}H_{10}$) with four rings, Benzo[a]pyrene ($C_{20}H_{12}$) with five rings. Their molecular weight influences their properties. The lighter the molecular weight (2–3 rings), the higher the volatilization and bioavailability of the compound. In contrast, the higher molecular weight (4–7 rings) makes the compound more inert, less soluble, and more toxic, likely to be adsorbed into the soils/sediments. In the context of the toxicity of petroleum hydrocarbons, the PAHs are identified as the most dangerous contaminants of petroleum-derived substances because of their carcinogenic, mutagenic, and teratogenic effects. The metabolic process of PAHs in a living organism uses the cytochrome P450 enzyme to produce an episoxide, which reacts with the DNA and causes mutations and cancer development (Haritash, A. K., & Kaushik, C. P. 2009).

PAHs are responsible for immune suppression, reproductive toxicity, and developmental anomalies in humans and in the biostratum. They bioconcentrate in the food chain, thus acting as a hazard for fish, birds, and mammals. Their hydrophobic property makes them non-biodegradable and thus, persist for a long time in the soil/sediment.

Typical standards for benchmarking by U.S. EPA are benzo[a]pyrene: Maximum Contaminant Level (MCL) in drinking water = 0.2 µg/L; total PAHs: limits vary depending on region and environmental medium (soil, sediment, or water). PAHs are a major focus of bioremediation because of their environmental persistence and toxicity. Microbial degradation is the main natural process capable of reducing their concentration in contaminated environments. Aerobic degradation is initiated by oxygenase enzymes (e.g., ring-hydroxylating dioxygenases) that insert oxygen atoms into the aromatic ring to form cis-dihydrodiols, which are then converted into catechols and further broken down through ortho- or meta-cleavage pathways. Other categories of degradation processes are anaerobic degradation processes. They also occur in the presence of nitrate/sulphate/iron reduction, although at slower rates. Employ processes such as carboxylation and reductive de-aromatization (Haritash, A. K., & Kaushik, C. P. 2009).

In the context of an oil spill or an effluent coming from a refinery, the presence of PAHs indicates chronic pollution. Since PAHs are resistant to degradation compared to BTEX compounds, them presence indicates resistance compared to lighter hydrocarbons, which could degrade faster. Bacterial adaptation and degradation of PAHs is also important since it involves the screening of effective bacterial strains, able to degrade high-molecular weight PAHs, or a combination of bacteria and nutrients/surfactants for synergistic degradation processes like biostimulation and bioaugmentation (Haritash, A. K., & Kaushik, C. P. 2009).
Therefore, the group of PAHs represents a pattern for studying the degradation processes and resistances of microorganisms in hydrocarbon-polluted environments.

2.2.3 TPH (Total Petroleum Hydrocarbons)

Total Petroleum Hydrocarbons, also referred to as TPH, happens to be the group name for the combination of hydrocarbons found in crude oil as well as other petroleum fuels such as gasoline, diesel fuel, jet fuel, and lubricating oils. Unlike BTEX and PAHs, TPH is not a chemical compound, but a family of compounds that are alike in the sense that they are of petroleum origin. The major chemical categories within TPH include aliphatic hydrocarbons: straight-chain (n-alkanes), branched (isoalkanes), and cycled hydrocarbons. Single-ring (like BTEX) and multi-ring (like PAHs) structures TPH is often divided by carbon range or boiling point, which determines environmental behaviour and toxicity: C_5–C_{12} (gasoline range hydrocarbons, GRH) - volatile and mobile; includes BTEX; C_{13}–C_{35} (diesel range hydrocarbons, DRH) - less volatile, more persistent in soil and water; C_{35}+(Heavy oils and asphaltenes) - highly viscous and resistant to biodegradation (Abdel-Shafy, H. I., & Mansour, M. S. M. 2016).
Depending on the composition of the petroleum mixture, the ecological and health hazards associated with TPH may vary. The volatile fraction (C_5-C_{12}) evaporates readily, forming aerosols which are harmful to the atmosphere, forming explosive gases, and ingestion toxicity, whereas the non-volatile fraction(C_{13}-C_{35}+) readily absorbs on the soil particles, forming chronic contaminants. Components of TPH, particularly aromatics such as benzene and PAHs, are carcinogenic and mutagenic. In aquatic ecosystems, oil coatings reduce oxygen exchange, suffocating aquatic organisms and damaging reproductive systems of fish and invertebrates. Regulatory frameworks typically set risk-based concentration limits rather than single numerical standards because of mixture variability. For instance: U.S. EPA and UK Environment Agency use TPH fractionation

methods to assess toxicity based on carbon range and exposure pathway (inhalation, ingestion, dermal). Typical soil clean-up levels range from 100 mg/kg (light fractions) to 10,000 mg/kg (heavy fractions), depending on local regulations and site use.

Microbial degradation pathways depend on oxygen availability and here we can distinguish Aerobic degradation which begins with mono- or dioxygenases, where hydrocarbons are activated into alcohols, aldehydes, and fatty acids. These intermediates are then channelled into the β-oxidation route to yield CO_2 and H_2O. And anaerobic degradation which utilizes nitrate, sulfate, or iron (III) as electron acceptors. Pathways involvefumarate addition, forming alkylsuccinates as initial intermediates (Widdel & Rabus, 2001; Abdel-Shafy & Mansour, 2016; EPA 2025)

Some environmental factors that influence TPH degradation include oxygen and nutrient availability (N, P), temperature and moisture content, and oil viscosity and dispersion.

Bioavailability is often improved by biosurfactants produced by bacteria such as *Rhodococcus erythropolis* or *Pseudomonas aeruginosa* (Das, N., & Chandran, P. 2011).

To microbiologists and environmental engineers, TPH serves as a general indicator of how much oil remains in the environment and how effectively it's being degraded. TPH degradation studies permit the evaluation of the effectiveness of bioremediation of oil-contaminated soils and waters. It is also able to identify bacterial strains or consortia able to attack light and heavy hydrocarbon fractions and monitor natural attenuation versus engineered bioremediation progress, e.g., biostimulation or bioaugmentation. Due to its complex composition, the degradation of TPH usually needs mixed microbial communities rather than single strains, making it a realistic model for understanding bacterial cooperation during oil biodegradation.

2.2.4 Other contaminants

Besides the main hydrocarbon groups (BTEX, PAHs, and TPH), oil-contaminated environments also contain non-hydrocarbon pollutants which can strongly affect the extent of pollution and efficiency of bioremediation. These "other contaminants" usually originate from additives in fuels, corrosion products from pipes, or by-products of industrial refining and transport. The most common categories include:

1. Heavy metals - such as lead (Pb), nickel (Ni), vanadium (V), chromium (Cr), cadmium (Cd), and zinc (Zn)

2. Fuel additives and oxygenates – e.g., methyl tert-butyl ether (MTBE), ethanol, and tetraethyl lead
3. Sulfur and nitrogen compounds – from crude oil and refining catalysts
4. Surfactants and dispersants used in oil spill cleanups or in industrial processing

The first category heavy metals are inorganic elements naturally present in crude oil and also introduced during drilling, transport, or refining. Metals such as vanadium and nickel are abundant in heavy crude oils and residual fuel oils. During spills or refinery waste discharge, these metals accumulate in sediments and soils, where they may persist for decades. Toxic effects of heavy metals, being non-biodegradable, bioaccumulate in both aquatic and terrestrial organisms by interfering with enzymes, inducing oxidative stress, and disrupting microbial metabolism. Cadmium (Cd) or chromium (Cr^{6+}) can inhibit microbial hydrocarbon degradation by causing damage to cell membranes or enzyme systems involved in oxidation. However, some bacteria have developed resistance mechanisms such as biosorption, precipitation, and enzymatic reduction against metals. As a good example, *Rhodococcus erythropolis* and *Pseudomonas putida* strains have been shown to tolerate high concentrations of heavy metals and to continue hydrocarbon degradation, which is an important advantage in multi-contaminated environments (Zhang et al. 2019; Abdel-Shafy & Mansour, 2016).

Modern fuels contain additives that improve combustion or reduce emissions, but many of them are persistent organic pollutants. Among the most studied examples is MTBE (methyl tert-butyl ether), added to gasoline with the purpose of improving octane ratings and reducing carbon monoxide emissions. MTBE has high solubility and mobility in groundwater, spreading faster than hydrocarbons, producing an unpleasant taste and odor in drinking water even at low concentrations, such as 20 µg/L. MTBE is difficult to degrade because of its ether bond and tertiary carbon structure, but several bacterial strains, for example, Methylibium petroleiphilum PM1 and *Pseudomonas sp*., were identified as capable of using MTBE as a carbon source. Often added as a component of biofuel, ethanol enhances hydrocarbon solubility and thus may accelerate contaminant spreading in groundwater. While ethanol is easily biodegradable, it may exaust oxygen and indirectly slow down hydrocarbon degradation by creating anaerobic conditions. (Steffan et al., 1997).

Surfactants are used in most oil spill responses to disperse oil into smaller droplets, which stenghening biodegradation. They reduce the interfacial tension between oil and water, increasing

bioavailability of hydrocarbons. On the other hand, surfactants can also have dual effects. The positive effect would be an improved contact between oil and microorganisms by emulsifying hydrocarbons, but the negative one is that some synthetic dispersants are toxic to marine life and, if used excessively, can inhibit bacterial growth. However, there is an alternative for this. Biosurfactants produced naturally by bacteria *like Rhodococcus erythropolis, Pseudomonas aeruginosa,* and *Bacillus subtili*s are safer and often more effective in promoting hydrocarbon degradation. For example, *Rhodococcus erythropolis* produces trehalose lipids, which enhance the emulsification of hydrophobic substrates like diesel and crude oil, improving the efficiency of biodegradation (Franzetti et al., 2010).

These added contaminants complicate bioremediation by altering the chemical balance and toxicity of polluted environments. Heavy metals can inhibit enzyme systems, while additives such as MTBE and ethanol change oxygen and nutrient dynamics. Yet, many hydrocarbon-degrading bacteria have evolved mechanisms to resist metals and metabolize complex organics, making them valuable in mixed-contaminant sites. The interaction of hydrocarbons with these co-contaminants must be known to enable the design of effective bioremediation strategies under real environmental conditions; selecting robust bacterial consortia with both hydrocarbon degradation and heavy-metal resistance, and of course minimizing secondary pollution from additives or dispersants.

In field cases involving crude oil spills, these nonhydrocarbon contaminants are often the determining factors in the onset, effectiveness, and remaining environmental hazards after biodegradation (Varjani, S. 2017).

2.3 Oil in the environment

Crude oil and all petroleum products are not one chemical but a mixture of various products ranging from light gases to heavy tars. Once released, they spread, evaporate, dissolve,float, sink, and stick to surfaces. The harm comes in both chemistry (toxic compounds) and physics (the oil as a suffocating, light-blocking membrane). Weathering reshapes the hazard with time, so that the risks change with hours, then days, and months. (Abdel-Shafy & Mansour, 2016; Fingas, 2018)

Smothering and coating are immediate effects of oils: floating oil coats feathers and fur, which destroy the insulation in birds and marine mammals. On shorelines, the sticky layer blocks gas exchange in sediments and clogs invertebrate breathing surfaces (Michel & Rutherford, 2014).

Light and heat effects are other effects we can notice in the very short period. Surface slicks reduce sunlight penetration and change albedo, stressing plankton and shallow plants. From some minutes up to some days, oxygen demand at the interface can appear. Very fresh slicks can sharply reduce oxygen right at the air–water boundary where larvae and eggs develop (Fingas, 2018; Atlas & Hazen, 2011).

Other factors are responsible for the chemical toxicity. In the first months, or even hours, BTEX evaporates and partly dissolves, which lead to acute toxicity in early days, for instance, narcosis/CNS effects in fish and invertebrates; vapor intrusion to buildings/worker exposure near spills; groundwater plumes from leaks. PAHs are less volatile, more persistent, and often carcinogenic and mutagenic after metabolic activation. They sorb to sediments, bioaccumulate, and cause developmental defects in fish embryos at very low concentrations. It also act as chronic stressors (PMC - Luigi Montano 2025; Haritash & Kaushik, 2009).

In the longer term, sediment contamination can be observed. On sheltered coasts, in marshes and wetlands, oil becomes mixed with fine sediments to form an asphalt pavement that persists for years, preventing or limiting re-growth of plants and recolonization by invertebrates.

Food-web transfer starts from some weeks to years. Sediment-bound PAHs enter food webs from worms to fish, ending with birds or mammals. Sublethal effects such as impaired growth and immune function may continue long after visible oil is gone. Dissolved BTEX (e.g., MTBE) is more problematic for rivers and aquifers, whereas oiled sediments and PAHs are more problematic for marine shorelines (Michel & Rutherford, 2014).

Other effects are due to microbial activities, including oxygen depletion (biodegradation). Hydrocarbon-degrading microbes consume dissolved oxygen while oxidizing oil. In poorly mixed waters or fine-grained soils this can cause hypoxia/anoxia, stressing or killing aerobic organisms. Finally, oil supplies lots of carbon but little nitrogen/phosphorus. Biodegradation slows unless nutrients are available; this is why shoreline bioremediation often adds N/P fertilizers. In some months in anoxic sediments, biodegradation shifts to sulfate-, nitrate-, or iron-reducing pathways. It is slower, but still important and can produce sulfide locally. Unfortunately, light components (BTEX) evaporate first, leaving a heavier, more aromatic residue that is harder for microbes to digest (higher toxicity per unit carbon, lower bioavailability) (Widdel & Rabus, 2001; Koshlaf & Ball, 2017).

Soil and groundwater pathways happen in several days and can continue during years. In case of

underground storage tank leaks or pipeline leaks, light fractions migrate downward, forming light non-aqueous phase liquid on the water table, from which BTEX dissolve and plumes can travel with the flow of groundwater. Soil gas from smear zone or shallow plumes may laterally move into basements and utility corridors, producing chronic indoor exposures if not mitigated. Even when active cleanup is conducted, the low-level residuals in fine pores desorb slowly and maintain long-term low concentrations, delaying closure unless natural attenuation is documented (Atlas, R. M., & Hazen, T. C. 2011).

One should not forget the socio-economic consequences that may also manifest directly and for many years after. Closures and reputational damage can extend beyond the lifetime of the physical oil, impacting livelihoods, such as fisheries and tourism. Aggressive mechanical clean-up can be more damaging to habitats than leaving low-level contamination to natural recovery-the correct approach depends on site energy, habitat type, and level of oiling.

2.4 Large volume oil contaminated areas and oil spills accidents

Among the most immediate and visible of all fossil fuel-related pollution events are those dealing with oil spills. This can devastate marine life, damage coastlines, and persist in the environment for decades. Such spilled oil is particularly harmful to aquatic ecosystems due to a combination of physical smothering and chemical toxicity, as well as human livelihoods dependent on it.

One of those was the Deepwater Horizon oil spill. The Deepwater Horizon oil spill occurred on April 20, 2010, when an explosion on British Petroleum's drilling platform killed 11 workers and released about 134 million gallons of oil into the Gulf of Mexico, making it the largest offshore oil spill in U.S. history. National Oceanic and Atmospheric Administration's experts responded within hours, using special tools like GNOME to model where oil would drift, ERMA-a digital mapping system-and ESI maps, which are utilized to find sensitive coastal areas-to help the U.S. Coast Guard manage the crisis. The spill polluted fisheries, beaches, and wetlands, and harmed dolphins, whales, turtles, deep-sea corals, and many fish species, while local communities' lost income from fishing and tourism. Cleanup workers faced health problems from contact with oil and dispersants like Corexit too (Lichtveld et al., 2016). To measure the damage, NOAA carried out a Natural Resource Damage Assessment, which led to an $8.8 billion BP settlement for restoring wetlands, oyster reefs, corals, fisheries, and recreation areas. The disaster also pushed science forward

improving satellite mapping of oil, toxicology studies on marine life, and long-term monitoring of dolphins and whales, while the Gulf of Mexico Research Initiative brought together thousands of researchers to study impacts. In the end, the spill showed how important rapid scientific action, strong cooperation, and long-term restoration are for protecting both people and the environment (NOAA Office of Response and Restoration, 2025).

Another oil spill worth mentioning is the Persian Gulf oil spill, which is considered one of the largest spills in history. In January 1991, during the Gulf War, one of the worst oil spills ever took place when Iraqi forces opened the valves of the Sea Island pipeline and discharged crude oil from numerous tankers into the Persian Gulf. Initially, Iraqi officials blamed attacks on oil tankers by the U.S., but it then became clear that this was a deliberate act: an attempt to prevent U.S. troops from attempting amphibious landings. The military tactic was far from effective, yet it resulted in the disposal of over 240 million gallons of crude oil into the sea, devastating marine ecosystems, coating coastlines, and causing long-lasting biodiversity loss that the region struggles with even today. What is important in this case is that it constitutes one of the most significant and early cases of environmental warfare-that is to say, pollution and natural resources were used as a tool on purpose, which raises severe ethical and legal concerns under international law. Following the spill, most clean-up efforts focused on skimming oil from the surface of the water, with hundreds of thousands of barrels recovered through recovery operations by April 1991, though much of it remained dispersed in the water or stranded on shorelines. Additionally, some in-situ burning-that is, controlled burning of oil on the water-and dispersants have been tried. While the immediate impacts might have been mitigated by these efforts, the overall ecological damage was enormous and long-lasting; the legacy bears out how deliberate environmental destruction in conflict leaves scars that last far beyond the war itself (The Gulf War Oil Spill: A Man-made Disaster – environmentandsociety.org).

On July 19, 1979, during a tropical rainstorm, two supertankers-the Atlantic Empress and the Aegean Captain collided off Tobago in the Caribbean. Both vessels erupted in flames, tragically costing the lives of many crew members, especially from the Atlantic Empress, which ultimately spilled an estimated 287,000 tons of crude oil-the largest ship-source spill ever recorded. The International Tanker Owners Pollution Federation (ITOPF) was called out to offer technical advice and cleanup assistance. Response teams fought the fires, towed vessels further out to sea, and dispersed with chemical agents to treat the spreading oil slick during tow operations. Chemical

dispersants were the main remediation method, supported by towing and burning-unintended-from the fire. Even though the majority of the oil was burned or sank, with only minor shoreline pollution reported, the environmental damage was considerable, and the event illuminated just how catastrophically shipping accidents can lay waste to marine ecosystems and coastal communities. While specific mental health impacts on nearby populations were not documented in this case, major oil spills-like this one-usually result in stress, anxiety, and emotional strain for the people involved or affected by such disasters, underlining the fact that these tragic events don't harm just marine life and habitats but also the mental and social wellbeing of individuals. One could also mention the oil spill on Exxon Valdez (1989), consequent heavy environmental damage of Alaska's Prince William Sound, and changes introduced to U.S. oil-spill legislation based on the case. On March 24, 1989, the oil tanker Exxon Valdez ran aground on Bligh Reef in Prince William Sound, Alaska. Approximately 11 million gallons of crude oil entered cold coastal waters. Oil reached roughly 1,300 miles of shoreline, covering beaches, bays, and rocky coves. Many animals died: huge numbers of seabirds, thousands of sea otters, plus harbor seals, eagles, fish eggs, and intertidal life. In some quiet, sheltered beaches, "lingering oil" stayed trapped under the surface for years to decades (NOAA 2019). The accident revealed serious gaps in prevention and response at the time, including limited equipment on-scene, slow mobilization, and no escort tugs for large tankers. The U.S. responded with the passage of the Oil Pollution Act of 1990 (OPA 90), which enhanced spill planning and liability rules, established the Oil Spill Liability Trust Fund, and contributed to the implementation of double-hull requirements for tankers. Authorities added tug escorts, improved navigation, and stronger local oversight in Prince William Sound. NOAA and other agencies ran a large, long-term damage assessment and restoration program. Civil and criminal settlements funded habitat and species recovery projects (e.g., restoring wetlands, monitoring otters, herring, and seabirds). Even with these efforts, recovery has been slow and uneven in cold, complex shorelines: some species and places bounced back in a few years, others took decades, and some, like herring, experienced long disruptions. Human and community impacts were also severe. Commercial fisheries and subsistence harvests were closed or reduced, tourism fell, and many coastal communities especially Alaska Native communities faced economic and cultural losses. Cleanup itself created trade-offs: high-pressure hot water washing removed oil but also killed small shore organisms, slowing natural recovery in some areas. The

event reshaped U.S. oil-spill policy and global industry practices, and it remains a key case study in why strong prevention, fast response, and long-term monitoring matter (NOAA 2019 Lessons Learned from the Exxon Valdez Spill.).

2.5. Remediation possibilities of Hydrocarbon contamination

Oil quickly spreads when spilled into the oceans or land and can affect marine life and can last for many years in the environment if not removed. Due to the hazards of oil, the challenge is to develop viable methods of clean-up that mitigate the effects of oil. Over the years different response applications have been developed to respond to oil spills including mechanical clean-up, chemical method, thermal methods and biological methods. Each has its strengths and limitations, and often, depending on the spill event, methods are used in a combination of approaches.

2.5.1 Physical methods

Physical and mechanical techniques are typically the initial option of response in oil spill response. The objective of a physical/mechanical method is to contain and recover spilled oil from the surface of the water before oil can spread or sink, thereby limiting its environmental impacts. Physical methods accomplish this through physical barriers and recovery equipment. Physical techniques work best and are the most effective if initiated soon after the spill has occurred, and the weather is favorable. The main tools for oil containment and removal on water appear to be booms and skimmers.

Booms serve as floating barriers that inhibit oil and prevent it from spreading on the surface of the water. There are several types of booms: a fence boom is stiff and easy to deploy, but is ideal for calmer waters, as it does not perform well under high waves and strong winds. Also, a curtain boom is flexible and can adapt to wave movement in moderate conditions. Skimmers are mechanical devices used to capture and remove oil from the surface of the water for recovery or disposal. There are three main types of skimmers: weir skimmers, oleophilic skimmers, and suction skimmers. Sorbents are materials that absorb oil, converting it from a liquid to a semi-solid form for removal. Utilizing vacuuming specifically means utilizing suction skimmers to extract oil, typically from beaches, tight spots, or land surfaces. As with all forms of manual

cleanup, these methods can be labor intensive, however, they are a key element of shoreline clean-up strategies (Fingas, M. 2018).

An example would be the annual oil recovery operation that was a treatment element applied during the Exxon Valdez tragedy (NOAA 2019 Lessons Learned from the Exxon Valdez Spill).

Since physical methods are commonly used, their effectiveness largely depends on environmental conditions and physical characteristics of the oil that was spilt. Booms and skimmers are most effective under calm waters. Strong winds, heavy seas, and fast currents will all contribute to oil escaping containment. In addition, the viscosity and density of oil will change with time as it continues to weather. Oil will become thicker and stickier once it mixes with water, which makes it much more difficult to mechanically recover. Also, skimmers may become clogged with debris, and the recovered oil and water mixture will typically need further treatment before reuse or disposal. These methods are best implemented immediately following a spill before the oil has a chance to significantly weather or disperse. (Fiocco & Lewis, 1999; National Research Council, 2005; Fingas, 2018).

2.5.2 Chemical methods

Chemical methods are frequently applied with physical and mechanical methods to improve oil spill cleanup effectiveness, especially in marine and coastal areas. Their primary roles are to limit oil spread, protect sensitive environments, and increase the natural rate of oil degradation by changing the properties of the oil, whether physical or chemical.

These methods are most effective when mechanical recovery is impractical due to rough seas or spill location offshore. The most common types of chemicals agents are Dispersants, which break oil into small droplets to facilitate biodegradation, Solidifiers, which absorb oil and form solid masses that are easier to remove, and demulsifies, which break down stable oil-water emulsions (Fingas, M. 2018).

Dispersants are a class of chemical mixtures that are formulated to break oil slicks into small droplets that become mixed into the water column and naturally biodegraded more rapidly.

They typically contain surfactants that work to break up oil into droplets, as well as solvents and stabilizers that enhance spreading and effectiveness. The process of dispersants increases the surface area of oil that is now available to microorganisms that are naturally occurring, which contributes to accelerating its biological breakdown. Many modern dispersants can be effective on

up to 90% of the oil product released in a spill, and compared to mechanical mitigation, dispersants tend to be less expensive.

While dispersants have been used in oil spills, they are still considered controversial due to possible toxicity to marine life and some risks to human health associated with application (Fiocco & Lewis, 1999; National Research Council, 2005).

For example, during the Deepwater Horizon oil spill (2010) accident in the Gulf of Mexico, Corexit 9500 and Corexit EC9527A dispersant were used in large quantities to break up oil slicks adjacent to impacted shorelines. Dispersants helped reduce shoreline contamination; however, studies conducted later reported some toxic effects of Corexit, as applied, on plankton, fish larvae, and coral. (NOAA 2019 Lessons Learned from the Exxon Valdez Spill.)

Solidifiers are hydrophobic polymers or granular materials that chemically react with oil and turn into a solid, rubbery mass that can be physically removed. They can be applied as a powder, granules, or semi-solid like pucks, pads, sponges, and often are within booms or absorbent socks. Solidifiers have many benefits when compared to the other options, like streamlining the oil recapture by converting liquid oil into a manageable solid, functioning in moderately rough sea conditions, and not creating secondary pollutants. This method is not widely used due to the high volume of the solidifier needed (≈16-200% of the weight of the oil) and their reduced effectiveness compared to dispersants. Solidifiers are best suited for localized spills. (Fingas, 2018; Zhao, Liu, & Wang, 2016).

Demulsifiers are classified as chemicals that are utilized to break apart oil-water emulsions, which are also known as chocolate mousse, because of their thick and sticky viscosity. Emulsions typically occur when wave action physically mixes oil and water, resulting in a significantly increased viscosity, preventing the effective recovery of oil within the emulsion. Demulsifiers sever emulsions by destabilizing the statistical interface of oil with water - which aids in merging of smaller oil droplets into much larger droplets for easier collection. Application of dispersants can delay the formation of emulsions, while demulsifiers are used to treat emulsion which has already formed. Demulsifiers are regularly applied later during clean-up operations to restore mobility to oil and/or separate oil before collection or preoccupying treatment (Fiocco & Lewis, 1999; Fingas, 2018).

Chemical remediation methods do have limitations, despite their benefits. First, many chemical agents can be hazardous for the aquatic biota or for human consumption of drinking water

supplies. Some of the dispersant ingredients can be persistent in sediment or undergo bioaccumulation. The efficiency of the treatment can be influenced by factors that are related to the type of oil and include physical attributes, for example, temperature, salinity, and wave conditions. Additionally, use of some types of chemicals like Corexit formulations which are often highly regulated in various regions (National Research Council. 2005).

2.5.3 Thermal Method

Thermal technologies offer alternatives or supplementary solutions to standard physical and chemical techniques used in environmental cleanup. They destroy or isolate hydrocarbons utilizing heat, new materials, or physicochemical interactions.

In-situ burning (ISB) is a thermal method that involves the controlled combustion of oil slicks on the water's surface or on contaminated land. ISB is one of the earliest and most rapid approaches to remove oil from large volumes, and it requires very few specialized equipment, such as fire-resistant booms, igniter, and collection barriers. This technique has been applied since the late 1960s in areas including the United States, Canada, and Northern Europe, especially in ice-covered or remote areas where mechanical recovery is impossible. ISB is particularly effective when implemented soon after the oil spill happens, as the oil will not have weathered or emulsified enabling combustion (Fingas, M. 2018).

An example of ISB application is from the Deepwater Horizon oil spill (2010), where they successfully executed in-situ burning operations that removed 35.2 to 49.6 million liters and 9.3 to 13.1 million gallons of oil from the sea surface. In ideal conditions, ISB has the potential to remove as much as 90% of spilled oil. While ISB is effective, it does produce airborne derogations and combustion products such as carbon dioxide (CO_2), particulate matter (PM), carbon monoxide (CO), nitrogen oxides (NO_x), volatile organic compounds (VOCs), and polycyclic aromatic hydrocarbons (PAHs). Assuming each kg of burned oil generates a calculated distribution of outputs, it has been calculated that the following outputs are produced: approximately 3.0 kg CO_2; 0.05 to 0.20 kg PM; 0.02 to 0.05 kg CO; and trace amounts of NO_x, VOCs, and PAHs (Fingas, M. 2018; NOAA Office of Response and Restoration, 2025).

Thermal desorption and soil washing are considered effective tools for cleaning contaminated soil and shorelines, but they are not effective tools for open-water spills. Thermal desorption is applied to contaminated soil by heating it up to volatile and differential hydrocarbons and then collecting

or burning them with a secondary system. Thermal desorption works well to remove petroleum hydrocarbons, PAHs, and other volatile, nonpolar compounds from soil matrices. An example of a washing-based cleanup technique is the Exxon Valdez oil spill in 1989, where low- and high-pressure hot water washing was conducted to clean up polluted shorelines. Although surface oil was effectively removed from rocks, this technique also destroyed surviving plants and animals, resulting in overall ecological damage and a reduction in natural recovery for some time (NOAA 2019 Lessons Learned from the Exxon Valdez Spill.).

New variations of these techniques sometimes use moderate heating working in combination with controlled washing to create general effectiveness while attempting to protect the ecosystem. These applications are primarily applied to shoreline sediments, beach sands, or industrial sites when petroleum has leaked (Fingas, 2018; Michel & Rutherford, 2014).

2.5.4. Biological Methods (Bioremediation)

Bioremediation involves the use of living microorganisms like bacteria, fungi, and archaea to convert petroleum hydrocarbons into less harmful products usually CO_2, H_2O, and biomass (Radhakrishnan, 2023). It is most valuable as either a polishing step after bulk oil removal or as a primary option in locations that are sensitive to mechanical cleanup, or where oil is located in inaccessible areas in thin films, soils/sediments, wetlands. Bioremediation can be done either *In situ*: treat the contamination in place (bioventing, biosparging, nutrient addition, phytoremediation) or *Ex situ*: collect contaminated media and treat it in engineered systems such as bioreactors, biopiles, compost windrows, landfarming (Koshlaf & Ball, 2017).

Microorganisms are major contributors to the biodegradation process of petroleum hydrocarbons, using the hydrocarbons as sources of carbon and energy. Microbes that are capable of breakdown hydrocarbons, first enzymatically start the breakdown of the hydrocarbon molecules and convert them into intermediate compounds that enter the central metabolism. Depending on environmental conditions, two major metabolic mechanisms have been identified: aerobic oxidation and anaerobic oxidation.

Aerobic degradation is the most prevalent and efficient means of hydrocarbon degradation. Aquatic microorganisms employ oxygen as the terminal electron acceptor. The process begins with oxygenation by specific enzymes (oxygenases) that add oxygen atoms to hydrocarbon molecules and activate a matrix of glycosyltransferase enzymes for biochemical modifications. For example,

degradation of n-alkanes leads hydrocarbon oxygenation via oxygenases to form alcohols, aldehydes, and fatty acids, which are further metabolized by β-oxidation to acetyl-CoA, and further oxidized to carbon dioxide (CO_2).

Similar to aliphatic hydrocarbons, aromatic hydrocarbons, including BTEX compounds and light polycyclic aromatic hydrocarbons (PAHs), are subjected to degradation, in which mono-oxygenases and dioxygenases transform the hydrocarbons into catechols. The catechol intermediates are further degraded using ortho or meta ring cleavage and enter central carbon metabolism. (Haritash & Kaushik, 2009; Widdel & Rabus, 2001).

Typical applications include bioventing, biosparging,biopiles, landfarming, and slurry-phase reactors. Aerobic bioremediation is primarily targeted to BTEX compounds and light to mid-range total petroleum hydrocarbons (TPH) and two to three ring PAHs (Koshlaf & Ball, 2017). Aerobic systems offer several strengths, including high degradation rates, a wide spectrum of substrates, and ease of monitoring, usually based on measuring the uptake of oxygen or the production of carbon dioxide. However, aerobic bioremediation also requires continuous oxygen supply, and in some instances, losses of volatile hydrocarbons must be controlled to prevent air quality issues. Despite the challenges presented, aerobic bioremediation is the treatment method of choice and has the highest operational efficiency for the treatment of petroleum-contaminated sites (Haritash & Kaushik, 2009).

In anaerobic or oxygen-limited environments, microbial degradation occurs through anaerobic oxidation that employs alternative electron acceptors such as nitrate (NO_3^-), sulfate (SO_4^{2-}), ferric iron (Fe^{3+}), or carbon dioxide (CO_2). Anaerobic biodegradation is important for the removal of hydrocarbons from saturated sediments, aquifers, and low permeability zones, although it is generally slower compared to aerobic processes (Koshlaf & Ball, 2017).

Mechanisms for anaerobic degradation are compound dependent. For instance, toluene and xylenes are degraded through fumarate addition, benzene through carboxylation or hydroxylation, and some PAHs through either methylation or carboxylation reactions in anaerobic processes. Overall, intermediate degradation products are generated, which are then utilized to generate energy in microbial metabolic pathways under reducing conditions. Anaerobic bioremediation has been employed primarily for BTEX degradation in aquifers subject to nitrate-, sulfate-, or iron-reducing conditions or MNA and is most relevant for stabilization of contaminants long-term in deep ground water systems. Because the rates of degradation start out slow and the redox controls

managing degradation from aerobic to anaerobic environments are complex, anaerobic oxidation is typically less efficient than aerobic systems. However, anaerobic oxidation has the capacity to represent a sustainable mechanism of natural attenuation, where the application of engineered oxygen supply is not feasible (Widdel & Rabus, 2001; Jeong & Park, 2018).

The degradation of hydrocarbons by microbes is primarily affected by stoichiometry and environmental conditions. Hydrocarbons contain a lot of carbon but are low in other nutrients, like nitrogen and phosphorus, therefore an optimum ratio of nutrients for microbial growth is C:N:P = 100:10:1 (EPA, 2014). Also, temperature, pH, salinity, and moisture and bio-availability of hydrocarbons are important factors influencing biodegradation. The rate of biodegradation follows a biodegradability hierarchy and, in general, short chain n-alkanes and BTEX compounds degrade most readily, followed by branched or cyclic alkanes, low molecular weight PAHs (2-3 rings), and heavier hydrocarbons (asphaltenes/ resins) are generally more resistant and persisted in crude oil (Koshlaf & Ball, 2017).

Bioremediation strategies for environments polluted with hydrocarbons depend on methods for maximizing microbial activity through multiple enhancement strategies. These enhancement methods differ by mode of application, environmental context, and degree of engineering control.

The most common enhancement approaches are biostimulation, bioaugmentation, phytoremediation, and bioreactor systems.

Biostimulation is the enhancement and optimization of the activity of native hydrocarbon degrading microorganisms by adding limiting nutrients and electron acceptors primarily nitrogen (N), phosphorus (P), and oxygen (O_2). Hydrocarbons are carbon-rich compounds but normally do not have sufficient concentration of these nutrients and therefore the degradation and rate of microbial growth are limited. Adding N and P helps balance the C:N:P ratio and promote microbial metabolism. For effective biodegradation of hydrocarbons, dissolved nitrogen levels in porewater are generally maintained at 1-2 mg/L, and oxygen is provided either through bioventing in unsaturated soils, biosparging in saturated zones, or mechanical tillage on contaminated beaches. This method was successfully demonstrated after the Exxon Valdez oil spill in Alaska (1989), in that the slow-release fertilizers like Customblen and oleophilic nutrient formulations (i.e. Inipol EAP22) caused removal of shoreline oil 2 to 5 times faster than untreated control plots. Biostimulation is the most widely applied and cost-effective method of enhancing microbial oil degradation in terrestrial and marine environments (Radhakrishnan, 2023; Koshlaf & Ball, 2017).

Bioaugmentation is the addition of targeted microbial strains or microbial consortia that can degrade hydrocarbons that may not be degraded effectively by the indigenous microbial community. One of the best-studied genera of bacteria for bioaugmentation is Alcanivorax, which is known for its capabilities for degrading n-alkanes. While bioaugmentation has the potential to be an effective remediation technology in both laboratory and pilot studies, it is rarely successful in open environments and has inconsistent field performance. When nutrients and oxygen are introduced to an ecosystem, indigenous microbial populations tend to outcompete the added strains, such that bioaugmentation does not work for long periods of time. Nevertheless, bioaugmentation can be useful in confined systems, or for specific target compounds that are difficult to biodegrade otherwise. Genetically engineered microbes (GEMs) have been proposed as a method to enhance biodegradation potential, although, to date, GEMs have generally only been studied experimentally and not applied on the field-scale due to ecological, ethical, and regulatory concerns with the introduction of modified organisms to a natural ecosystem (Radhakrishnan, 2023; Koshlaf & Ball, 2017).

Phytoremediation is a bioremediation strategy utilizing plants in wetlands and marshes, grasses, or mangrove species to enhance natural degradation of hydrocarbons. Plants help remediate by stabilizing contaminated soils, enhancing oxygen diffusion into the rhizosphere, and stimulating microbial activity through root exudates. Phytoremediation is especially useful for sites with low to moderate contamination, as well as habitat recovery after oil spills. It also has ecological benefits; it promotes recovery of soil structure and biodiversity. Disadvantages of phytoremediation are that it has limited efficiency in remediating only the root zone, has limited efficiency in remediating oil contaminants that are deeper than the root zone, and do not reach the anoxic sediments that experience limited oxygen diffusion (Koshlaf & Ball, 2017).

Bioreactors differ from passive in-situ methods and are active engineered treatment systems to allow for biological degradation of hydrocarbons in a controlled manner. Within bioreactor systems, aeration, nutrient concentrations, temperature, pH, and mixing intensity are all controlled parameters to create optimum microbial activity and improve removal efficiencies. There are many types of bioreactors used for petroleum remediation and we will distinguish between the more commonly used ones. A slurry-phase bioreactor is ex situ and excavated soil or sediment is mixed with water to create a slurry. By vigorously aerating and mixing, these systems create excellent mass transfer to achieve rapid degradation of gasoline-range organics (GRO),

diesel-range organics (DRO), and some polycyclic aromatic hydrocarbons (PAHs). However, they often require excavation, water management, and post-treatment dewatering; all of which can drive up operational costs. Multiple biopiles (or windrows) can also be created, which are aerated piles of soil that are also enriched with nutrients and are periodically turned and mixed to establish and maintain sufficient moisture and temperature. Biopiles have been extensively studied and employed on mid-range TPH contamination and can be performed at a cost-effective scale with a proven reliability. Landfarming incorporates spreading contaminated soil into thin layers over a previously prepared surface area and periodically tilling and mixing to facilitate aeration and microbial activity. As necessary, nutrients and moisture are added to support biodegradation. Landfarming is inexpensive and compatible with large areas, but its effectiveness depends on climatic conditions and soil properties (Koshlaf & Ball, 2017).

Bioremediation has some significant benefits including being less costly and greener than many physico-chemical methods. As well, it leads to generally minimal by-products. It has a possible complete mineralization ($CO_2 + H_2O$) potential for many hydrocarbons. Acceptance by the public is generally quite high and thought to be great to follow up after a mechanical clean-up or in sensitive environments. However, bioremediation has limitations as well. It tends to be very labor-intensive, ranging from weeks to years. Biological methods are quite dependent on conditions (O_2, N/P, temperature, moisture, pH, salinity). Not practical for bulk oil, best applied after free product removal (Radhakrishnan, 2023). Weathered or heavy fractions and asphaltenes/resins are persistent, usually leaving inert asphaltic residue. Its require site-specific design and monitoring to demonstrate effectiveness, however, effectiveness can be difficult to measure in heterogeneous media (Koshlaf & Ball, 2017).

Monitoring is crucial in assessing bioremediation progress and efficiency, and in demonstrating that risks to human health and the environment are reduced. The parameters are grouped by chemical, process, and biological indicators, all having clearly defined endpoints connected to environmental quality standards (EPA, 2014; Miles, 2023).

Site-specific conditions necessitate utilizing specific remediation approaches, for example for shorelines and beaches - nutrient biostimulation and gentle aeration, phytoremediation for wetlands; for unsaturated soils bioventing for BTEX or light TPH; for shallow groundwater it would be biosparging or oxygen-release compounds maximized with MNA for long-term control; on hotspots or tight deadlines - ex situ bioreactors or biopiles with strict process control; for anoxic

sediments we see anaerobic treatment with nitrate/sulfate amendments or monitored natural attenuation; ultimately success of remediation is a reduction in risk instead of contaminant removal (Koshlaf & Ball, 2017).

Endpoints include a reduction of vapor intrusion risk, groundwater quality compliance, and ecotoxicology tests confirming that the treated medium is no longer toxic to organisms. These endpoints will inform decisions on when to discontinue active remediation.

The literature presented above develops an overview of the complex composition of petroleum hydrocarbons, impacts on the environment they can cause, and various approaches developed for their remediation. Of these, bioremediation appears as an efficient and ecologically feasible process for the degradation of hydrocarbons. Based on this insight, the materials and methods used in the study to test selected bacterial strains for their oil-degrading potential are then presented, with the view to finding the most efficient degraders for possible utilization in oil spill mitigation.

3 Materials and Methods

3.1 Selected Microbial Strains for Biodegradation test

In the section I will present collection of the bacterial strains which were chosen for their ability to degrade different organic pollutants. These pollutants include hydrocarbons, mycotoxins and also herbicides. Each strain was originally isolated from contaminated soils or water sediments and were specifically selected for their resilience in polluted environments. The strains have been previously by the Institute of Aquaculture and Environmental Safety at MATE University for their distinct biodegradative properties and metabolic universality. All bacterial isolates are preserved in cryogenic storage (it is a process of storing substances at very low temperature) at –80 °C to maintain their viability and genetic stability over time. This process makes sure that the strains can be reactivated and cultivated for future laboratory testing without loss of function. Most of these bacteria were not examined for hydrocarbon degradation. That is why in the study, these strains are utilized to evaluate their hydrocarbon-degrading potential, with a particular focus on compounds derived from crude oil such as BTEX and TPH.

The goal of using these strains is to identify the most efficient hydrocarbon-degrading bacteria under controlled experimental conditions. Selected isolates demonstrating the highest degradation efficiency will be subjected to further qualification tests, aiming to assess their potential application in bioremediation strategies, particularly for the cleanup of oil spills and other hydrocarbon-contaminated environments. All strains which were chosen are presented below with a short description about each of them.

Rhodococcus pyridinivorans K408

Rhodococcus pyridinivorans is a Gram-positive, non-motile bacterium first described as a pyridine degrader. The strain K408 was isolated and studied in Hungary. It has been found to degrade mycotoxins like zearalenone (ZEA) and aflatoxin B_1 into less harmful forms in liquid media and feed matrices. It grows aerobically at 28-30 °C and tolerates slightly alkaline conditions, approximately pH 7-9. Bioassays proved that K408 not only reduced the concentration of ZEA but also its estrogenic activity, confirming its true detoxification. The strain is non-pathogenic and suitable for feed detoxification and environmental bioremediation applications (Martínková et al., 2009).

Rhodococcus pyridinivorans K404

Strain *Rhodococcus pyridinivorans* K404, closely related to K408, was isolated from oil-contaminated soil. K404 showed the characteristic, orange-pigmented colonies typical of the genus and presents irregular, rod-shaped cells that often appear filamentous under growth conditions. This bacterium had highly developed zearalenone-degrading activity, with a 72% reduction in its growth during in vitro conditions. Its intracellular extracts were able to degrade it by up to 98% after seven days of incubation. Toxicity assays confirmed that degradation products had significantly reduced estrogenic activity, thus detoxification rather than transformation of the compound indeed took place. These properties make K404 a promising candidate for use in food and feed safety applications (Fejes, D. et al. 2017).

Rhodococcus qingshengii BA4.9 and PT2/14B

Two *R. qingshengii* strains, BA4.9 and PT2/14B, belong to a species of Gram-positive bacteria. It is known for its remarkable ability to degrade a wide range of pollutants and accumulate heavy metals. There are no peer-reviewed, quantitative efficiency metrics (e.g., % oil removed, k values) published specifically for BA4.9 or PT2/14B. What is documented is that PT2/14B and BA4.9 were isolated from oil-polluted hydrocarbons, a context that typically selects for active hydrocarbon degraders. Both of them also carry an alkB (alkane 1-monooxygenase) gene, the gateway enzyme for initiating alkane oxidation (Iminova et al., 2022; Li et al., 2020; Juárez et al., 2023).

Rhodococcus erythropolis GP2b

Rhodococcus erythropolis is a Gram-positive bacterium It is highlighted for degrading a wide range of hydrocarbons, such as petroleum and diesel oil. *R. erythropolis* is widely spread in soil and marine sediments. Also, it is appreciated for producing surface-active compounds (trehalolipids) which enhance biodegradation. (Franzetti et al., 2010) In addition, many strains produce biosurfactants, which are compounds that increase the solubilization and thus the bioavailability of hydrocarbons. It is also used in biodesulfurization: the removal of sulfur compounds from fuels as a way of producing more benign forms of energy (Prasoulas et al., 2021).

***Mycobacterium trichoteceniticum* R17**

Mycobacterium trichoteceniticum, as a potential terbuthylazine and atrazine degrader. Members of the genus Mycobacterium, belonging to the family *Mycobacteriaceae*, are known degraders of hydrophobic organic pollutants since their cell walls are waxy, lipid-rich, and attach to hydrophobic substrates. (Brennan & Nikaido, 1995; Kanaly & Harayama, 2000) However, no evidence is available in peer-reviewed journals regarding the confirmation of its identification and degradation activity.

***Pseudomonas geniculata* T12**

Members of the genus *Pseudomonas* are Gram-negative, motile rods with polar flagella and have great metabolic flexibility, able to grow on a wide range of organic pollutants (Palleroni, 2010). *Pseudomonas geniculata* is one such metabolically diverse bacterium, and the N1 strain is known to degrade nicotine (Li et al., 2016).

***Malikia spinosa* AB6**

M. spinosa can degrade benzene, toluene, and ethylbenzene under strictly aerobic conditions at an extremely rapid rate, according to the batch assays tracked by GC-MS; triplicate tests showed fast concentration declines, showing high aerobic degradation efficiency. When oxygen is limiting, enrichments exhibit little to no benzene loss, and only very slow toluene loss, i.e., AB6's efficiency drops off sharply as O_2 becomes scarce. In the independent enrichment study, *Malikia spp.* dominated the aerobic BTEX-degrading communities and were outcompeted under oxygen-limited settings, further reinforcing that AB6-like physiology excels aerobically. Related microaerobic/oxygen-limited studies highlight that even with the correct enzymes, microaerobic conditions strongly limit rates, consistent with the efficiency pattern seen with AB6. (Jeong & Park, 2018; Luo et al., 2021).

***Pseudomonas aromaticivorans* MAP12**

Pseudomonas aromaticivorans MAP12 is a newly found species isolated from microaerobic xylene-degrading enrichments. The bacterium degrades xylene under low-oxygen conditions, mediated by EDOs, and is capable of using nitrate for respiration. (Banerjee et al., 2022; Fuchs et al., 2011) This enables its function in oxygen-limited environments, compared with other strictly aerobic degraders. Like other *Pseudomonas* species, MAP12 prosper in environments that are

moist and where oxygen levels can fluctuate, while showing genetic adaptations for aromatic compound degradation under microaerobic conditions. Still is used for as a degrader on one of the contaminated sites of Western-Southern part of Hungary (Palleroni, 2010; Pérez-Pantoja et al., 2010).

Acidovorax benzenivorans D2M1

Acidovorax benzenivorans D2M1 is a recently described species isolated from xylene-contaminated soil near Siklós, Hungary (Benedek et al., 2020). It is a Gram-negative, motile bacterium, it is able to degrade benzene and ethylbenzene as sole carbon and energy sources under both aerobic and microaerobic conditions. *Acidovorax* species (family *Comamonadaceae*) are versatile betaproteobacteria often isolated from plant roots or polluted soils, many of which can oxidize aromatic hydrocarbons. These species are frequently found in oil-contaminated environments and likely participate in the breakdown of aromatic hydrocarbons in microbial consortia. (Benedek et al., 2020; Fuchs et al., 2011).

Hydrogenophaga aromaticivorans D2P1

Hydrogenophaga aromaticivorans D2P1, isolated from a para-xylene-degrading enrichment culture. It can degrade benzene, meta-xylene, and para-xylene (Benedek et al., 2018). The genus *Hydrogenophaga* (family *Comamonadaceae*) comprises facultatively *Chemolithoautotrophic* species able to utilize hydrogen or aromatic compounds as electron donors. Its genome carries clusters for phenol and xylene degradation, including genes coding for catechol 2,3-dioxygenase responsible for ring-cleavage reactions. Due to these properties the strain a good degrader of BTEX pollutants, such as benzene, toluene, ethylbenzene, and xylene, but its activity on complex mixtures of crude oil has not been confirmed yet (Benedek et al., 2018; Pérez-Pantoja et al., 2010).

Rhodococcus erythropolis NI1

Rhodococcus erythropolis NI1 is a Gram-positive, aerobic bacterium, which was investigated for its mycotoxin-degradation abilities. This NI1 strain degrades aflatoxin B1 under near-neutral pH in cell-free intracellular extracts within minutes to a couple of hours and reduces its genotoxicity significantly (Fejes et al., 2017; Garai et al., 2021). These bacteria showed high removal

efficiencies for both single toxins, AFB1, zearalenone, and T-2, and their binary and ternary mixtures, without any significant decrease in removal efficiency among the combinations. Very important is that degradation products were significantly less toxic in zebrafish assays compared to the parent compounds, emphasizing the promise of NI1 for food/feed decontamination and bioprocess applications where the need for rapid, broad-spectrum mycotoxin removal is compulsory. (Fejes et al., 2017; Garai et al., 2021).

3.2 Biodegradation screening on plates

The main goal of this research is to build an understanding which of above presented bacterial strains capable of efficiently degrading high concentrations of hydrocarbon contaminants, particularly those derived from crude oil, such as BTEX compounds and TPH. To achieve this, bacteria isolated and identified from hydrocarbon-contaminated environments were put through degradation tests under controlled conditions. These experiments aim to screen the ability of the isolates for utilizing hydrocarbons as their primary carbon and energy source. Based on the results, strains which will demonstrate the highest degradation efficiency can be further selected for deeper investigations, such as qualification test (Gravimetry test) to analyze which of them could be possibly suggested for oil spills clean ups.

For the screening of hydrocarbon-degrading ability, I used mineral OIR III medium, and it was supplemented with resazurin (1 mg/L) and individual carbon sources. Resazurin is a blue-colored, redox-sensitive dye commonly used as an indicator of cell activity and viability including the hydrocarbon-degrading capacity of bacteria. It is also known as Alamar Blue and is a non-toxic, water-soluble redox indicator. It is widely used in cell culture, microbial assays, and biodegradation tests (to monitor oxygen consumption and metabolic activity). The principle is to follow the redox change caused by bacterial activity during breakdown of the contaminants.

Resazurin is a blue, non-fluorescing dye that is reduced in a reversible way into resorufin, which is pink and highly fluorescent, via a two-electron reduction. The general reaction can be schematized as:

$$Resazurin + 2e^- + 2H^+ \rightarrow Resorufin + H_2O$$

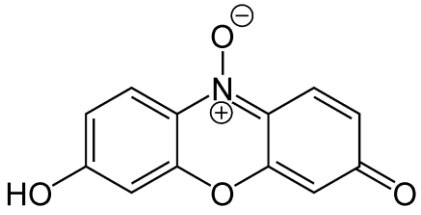


Figure 6. Chemical structure of Resazurin (Source Wikipedia website)

Further, under strongly reducing conditions-excess electrons, resorufin can be reduced further to dihydroresorufin-colorless, non-fluorescent, white:

$$Resorufin + 2e^- + 2H^+ \rightarrow Dihydroresorufin + H_2O$$

The color change could be determined by a spectrophotometer at 620 nm and 550 nm.

To test the biodegradation capacity of the bacterial strains, we ran 168 hours experiment with each bacterium. I prepared sterile OIR III mineral medium without any addition organic carbon source in: 1 L. The OIRIII medium consists of: $(NH_4)_2SO_4$- 5.0g, KH_2PO_4- 0.5g, K_2HPO_4- 1.0g, $MgSO_4 \cdot 7H_2O$- 0.5g, $CaCl_2 \cdot 6H_2O$- 0.2g, $FeSO_4 \cdot 7H_2O$- 0.01 g, Peptone-0.5 g, Yeast extract-0.5 g, Distilled water-up to 1000 mL, pH ≈ 7.0. While the media was sterilizing, the bacterial suspensions were prepared of the different strains. The suspension had an optical density of 0.6 at 600 nm. It was diluted in 10 ml tubes (from10^2 to 10^7) for each strain with 3 repetitions. After autoclaving at 121-123 °C for 20 min at ~1.2 atm, 3 plates were taken. White color was chosen in order to minimize any destruction during color reading. Each cell on the plates were inoculated with 200 µL test solution - Bushnell Haas media (mineral nutrient solution with the following ingredients: $CaCl_2$ - 0.002 g; $MgSO_4$ - 0.02 g; NH_4NO_3 - 1 g; KH_2PO_4 - 1 g; K_2HPO_4 - 1 g; $FeCl_3 \cdot 6H_2O$- 0.005g; H_2O- 1 liter) + resazurin, with 40 µL of bacterial suspension and added 2 µL of the carbon source-diesel oil. Each dilution had three cells and one control cell with no carbon source in it. The plates were then incubated at 25 °C with shaking at 250 rpm. It was measured, on the same day, in 72 hours, in 96 hours and finally within

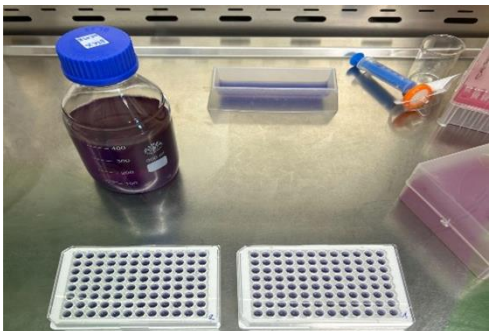


Figure 6. Plates with - media Bushnell Haas + resazurin, bacterial suspension, diesel oil (Source: own work)

Figure 7. Grant-bio PHMP-4 Thermo-Shaker for Microplates(thermomixer) (Source: own work)

168 hours. We measured the color change of resazurin spectrophotometrically at 620 nm and used the results to infer the hydrocarbon-degrading ability of each strain.

3.3 Gravimetry (Qualification of hydrocarbon degradation)

The purpose of this method is to fine how many percent of the introduced hydrocarbon can be digest by the selected bacterial strains.

For quantification of oil degrading by gravimetric method 24 h fresh bacterial cultures were grown on agar slants. Using a sterile glass rod, biomass from each slant was washed into 50 mL nutrient broth in Erlenmeyer flasks. The inoculated flasks were shaken for 72 h at 28 °C to get starter cultures of high cell density. From each strain culture the suspension should have an optical density of 0.6 at 600 nm. 5 mL suspension was transferred into a fresh liquid pre sterilized (at 121 °C for 20 min) OIRIII medium that contained a crude oil/diesel mixture- with a ratio 2:3 as the sole carbon source (2 mL, ≈ 1.7 g per flask).

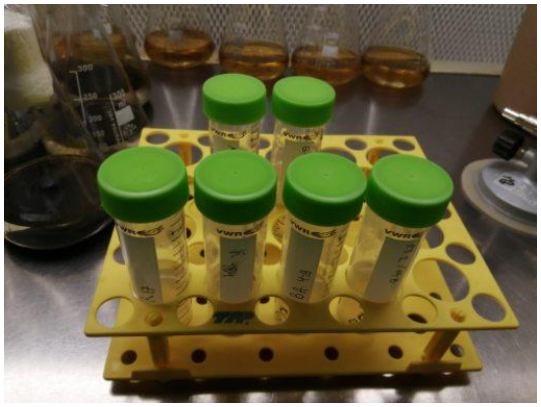


Figure 9. Six chosen strains (Source: own work)

Figure 10. Measuring optical density (OD) (Source: own work)

Figure 11. Incubated flasks on a shaker (120 h at 28 °C, 150 rpm) (Source: own work)

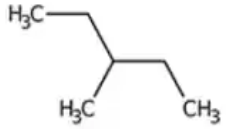


Figure 12. Chemical structure of petroleum ether (Source: Fisher Scientific website)

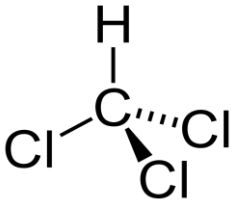


Figure 13. Chemical structure of chloroform (Source: Wikipedia website)

These inoculated flasks were incubated on a shaker for 168 h at room temperature 22-24 °C, 150 rpm to allow biodegradation. 3 repetitions for each strain plus 3 control flasks were prepared. Controls were identical in composition except it received no inoculum (the population of microorganisms or cells to be introduced into a medium to initiate growth). These control are is needed at the end of the process, to calculate how much oil remained without biodegradation, relative to the initial amount of oil amount.

After incubation, the entire contents of each "oily" flask were poured into a separatory funnel. 50 mL petroleum ether (to dissolve aliphatic hydrocarbons) were added, rinsing the walls of the original flask to transfer any possible adhering oil. Petroleum ether is the petroleum proportion consisting of aliphatic hydrocarbons and commonly used as a laboratory solvent. After thorough mixing, two phases formed: an aqueous phase (bottom) and a petroleum-ether/oil phase (top), the differences in phases acquired due to petroleum ether is less dense than water). The aqueous phase was drained off back into the separatory funnel for subsequent washes, after we processed 3 times with petroleum ether. This organic phase was filtered through filter paper to remove any residual water from imperfect phase separation. We used to parallel of the filters. Then one chloroform wash, also 50 ml was performed to extract any remaining oil. Chloroform is an organochlorine compound with the formula CHCl3, a common solvent. It is a volatile, colorless, sweetish, dense liquid, produced on a large scale as a precursor to refrigerants and polytetrafluoroethylene (PTFE). Because chloroform is denser than water, the chloroform–oil phase was the bottom phase during separation. The combined organic extracts were collected into pre-weighed round-bottom flasks. The mass recorded on an analytical balance for each flask because there were produced manually

Figure 14. The chloroform–oil phase removal (the bottom phase) (Source: own work)

and the weight slightly differs, but for this process, each mg matters for accurate result. This process was repeated with each selected strain.

The solvents (petroleum-ether and chloroform) were then removed by a Heidolph rotary evaporator according to boiling point order: petroleum ether 50–60 °C, and chloroform 60–65 °C. Once the evaporation was complete, only residual oil, that had been present in the incubation flasks, remained in the round-bottom flasks. After that to remove traces of water or solvent, the flasks were placed in a drying oven at 65 °C for 45 min. Finally, the flasks were weighed again on an analytical balance.

The mass of residual oil was calculated this way:

(Final mass of flask) − (initial empty flask mass)

Figure 15. Formation of two phases: an aqueous phase and a petroleum-ether/oil phase (Source: own work)

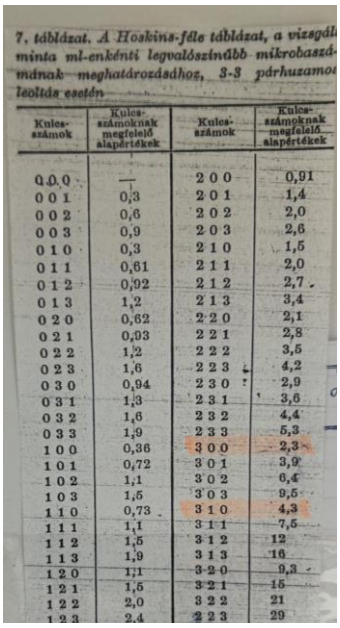
Figure 16. Phase filtration through filter paper to remove any residual water (Source: own work)

Results of inoculated flasks were compeared with the average of the three control replicate's results.

4 Results and Discussion

4.1 Reading results from biodegradation screening on the plate using MPN method and both parallel BioTek absorbance microplate reader

The plates were checked, and the absorption data was collected the same day, in 72 hours and 168 hours. The absorption data was read by BioTek absorbance microplate reader, and software Gen5, which is paired with hardware to perform absorbance measurements on microplates. This microplate reader measured the absorbance of the solubilized stain at 550 nm and 620nm. Besides this, I used the Most Probable Number Method (MPN) on the microplates. This method is a statistical technique used to estimate the concentration of viable microorganisms in a sample when direct counting is not feasible. It involves inoculation of several tubes with serial dilutions of a sample into a growth medium and observation of the pattern of positive results (e.g., gas production, colour change) after incubation. By comparing the number of positive and negative tubes at different dilutions, a statistical estimate of the original microbial population density is made using MPN tables.



7. táblázat. A Hoskins-féle táblázat, a vizsgál
minta mi-enkénti legvalószínűbb mikrobaszá-
mának meghatározásához, 3-3 párhuzamos
leolvás esetén

Kulcs-számok	Kulcs-számoknak megfelelő alapértékek	Kulcs-számok	Kulcs-számoknak megfelelő alapértékek
0 0 0	—	2 0 0	0,91
0 0 1	0,3	2 0 1	1,4
0 0 2	0,6	2 0 2	2,0
0 0 3	0,9	2 0 3	2,6
0 1 0	0,3	2 1 0	1,5
0 1 1	0,61	2 1 1	2,0
0 1 2	0,92	2 1 2	2,7
0 1 3	1,2	2 1 3	3,4
0 2 0	0,62	2 2 0	2,1
0 2 1	0,93	2 2 1	2,8
0 2 2	1,2	2 2 2	3,5
0 2 3	1,6	2 2 3	4,2
0 3 0	0,94	2 3 0	2,9
0 3 1	1,3	2 3 1	3,6
0 3 2	1,6	2 3 2	4,4
0 3 3	1,9	2 3 3	5,3
1 0 0	0,36	3 0 0	2,3
1 0 1	0,72	3 0 1	3,9
1 0 2	1,1	3 0 2	6,4
1 0 3	1,5	3 0 3	9,5
1 1 0	0,73	3 1 0	4,3
1 1 1	1,1	3 1 1	7,5
1 1 2	1,5	3 1 2	12
1 1 3	1,9	3 1 3	16
1 2 0	1,1	3 2 0	9,3
1 2 1	1,5	3 2 1	15
1 2 2	2,0	3 2 2	21
1 2 3	2,4	3 2 3	29

Figure 17. Table to determine Most Possible Number (Source: MATE University)

MPN and data provided by BioTek absorbance microplate reader was used to decide which strains will be chosen for Gravimetry. Both methodology were decided to use for better accuracy.

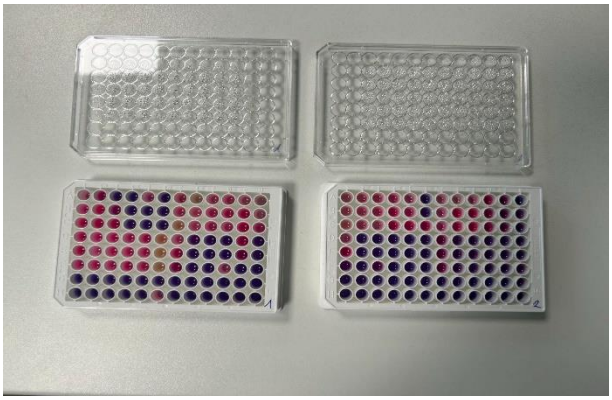


Figure 18. Colour changes in microplates after 168h (Source: own work)

I inserted the data obtained from the absorbance microplate reader from each reading (0h; 72h; 168h) to Excel table to see the deviation in graphical. To make a final decision for the strain selection data for 168h is needed. Two table were created for each strain: one with data collected from microplate reader, another with data observed with the help of MPN method. Also, graphs are presented to visualize and compare

absorbed data of control and strains with oil sections. Standard deviation was calculated for them as well:

Table 1. Microplate reader data + MPN data for K404 (Source: own work)

K404	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.364	0.748333	0.700333	0.497	0.456	0.490333
Control	0.461	0.471	0.4635	0.466	0.468	0.391
STDev	0.069	0.196	0.167	0.022	0.008	0.070

K404	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	-	-	+
2	+	+	+	+	-	-
3	+	+	+	-	-	-

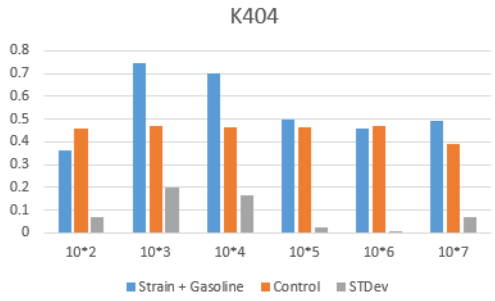


Table 2. Microplate reader data + MPN data for GP_2B (Source: own work)

GP 2B	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.997667	0.426	0.508667	0.485667	0.403667	0.560667
Control	0.391	0.468	0.466	0.4635	0.471	0.461
STDev	0.429	0.030	0.030	0.016	0.048	0.070

GP 2B	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+

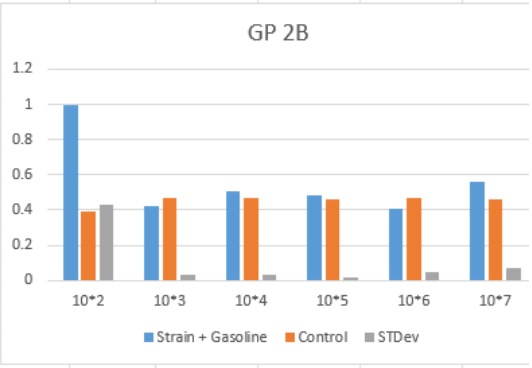


Table 3. Microplate reader data + MPN data for BA_4-9 (Source: own work)

BA 4-9	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.658333	0.825333	0.319667	0.312667	0.487	0.292667
Control	0.4595	0.468	0.4555	0.4605	0.4615	0.383
STDev	0.141	0.253	0.096	0.105	0.018	0.064

BA 4-9	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+

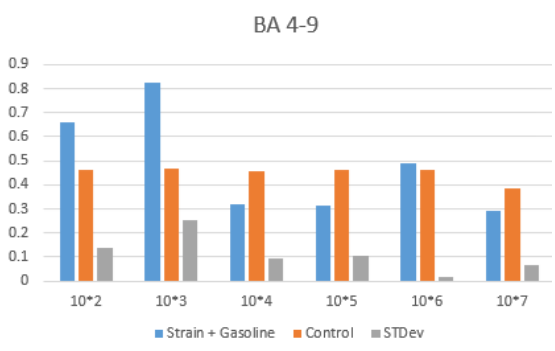


Table 4. Microplate reader data + MPN data for K402 (Source: own work)

K402	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.537667	0.541	0.502	0.46	0.684667	0.465667
Control	0.4595	0.468	0.4555	0.4605	0.4615	0.383
STDev	0.055	0.052	0.033	0.000	0.158	0.058

K402	10*2	10*3	10*4	10*5	10*6	10*7
1	+	-	-	-	+	-
2	+	-	-	-	+	-
3	+	-	-	+	-	-

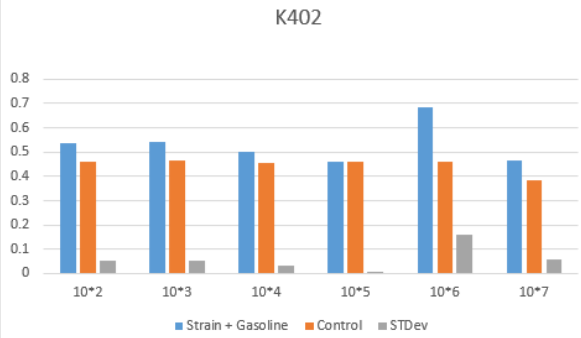


Table 5. Microplate reader data + MPN data for P12-14B (Source: own work)

P12-14B	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.575333	0.285667	0.269667	0.438	0.394667	0.598667
Control	0.3955	0.4535	0.4495	0.456	0.46	0.4625
STDev	0.127	0.119	0.127	0.013	0.046	0.096

P12-14B	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	+	+	-
2	+	+	+	+	+	-
3	+	+	+	+	+	+

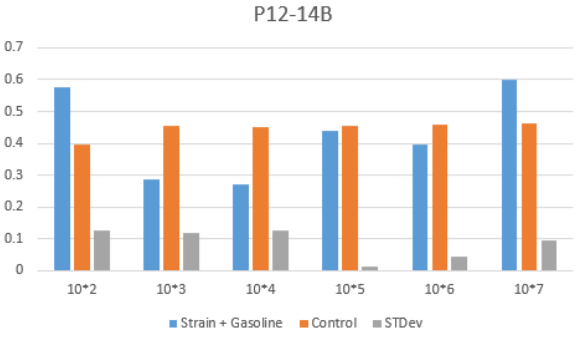


Table 6. Microplate reader data + MPN data for K408 (Source: own work)

K408	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.529667	0.501	0.563333	0.495	0.496	0.492333
Control	0.3955	0.4535	0.4495	0.456	0.46	0.4625
STDev	0.095	0.034	0.080	0.028	0.025	0.021

K408	10*2	10*3	10*4	10*5	10*6	10*7
1	+	-	-	-	-	-
2	+	-	-	-	-	-
3	+	-	+	-	-	-

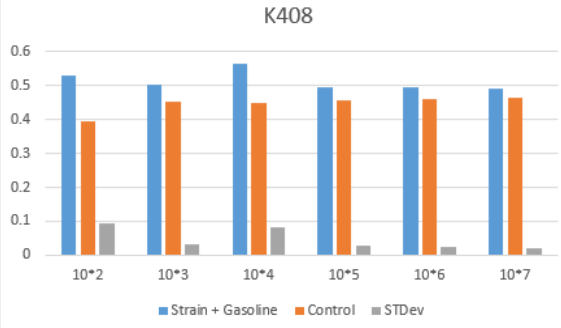


Table 7. Microplate reader data + MPN data for R17 (Source: own work)

R17	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.208	0.534333	0.587	0.804667	0.722667	0.574333
Control	0.4565	0.4555	0.446	0.4545	0.4545	0.4845
STDev	0.176	0.056	0.100	0.248	0.190	0.064

R17	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	+	+	-
2	+	+	+	+	+	+
3	+	+	+	+	+	-

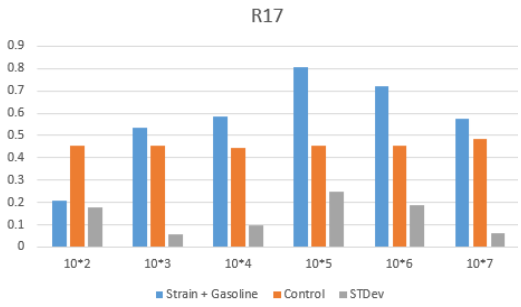


Table 8. Microplate reader data + MPN data for T12 (Source: own work)

T12	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.799333	0.629333	0.591667	0.581667	0.58	0.588
Control	0.4565	0.4555	0.446	0.4545	0.4545	0.4845
STDev	0.242	0.123	0.103	0.090	0.089	0.073

T12	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	-	-	-	-
2	+	-	-	-	-	-
3	+	-	-	-	-	-

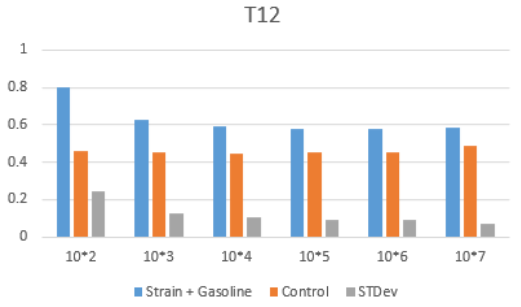


Table 9. Microplate reader data + MPN data for MAP12 (Source: own work)

MAP12	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.393	0.533	0.528333	0.561	0.557667	0.535
Control	0.3925	0.466	0.4675	0.4565	0.456	0.454
STDev	0.000	0.047	0.043	0.074	0.072	0.057

MAP12	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	+	+	-
2	+	+	+	+	-	-
3	+	+	+	+	-	-

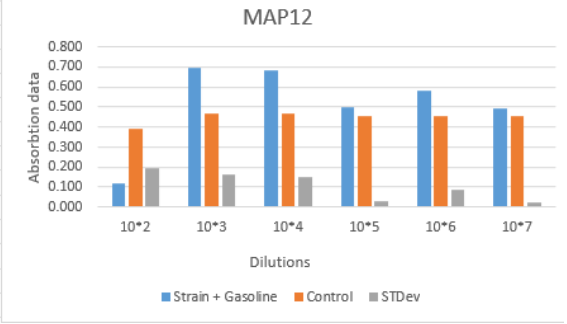


Table 10. Microplate reader data + MPN data for AR6 (Source: own work)

AR6	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.121	0.697	0.681	0.500	0.580	0.491
Control	0.393	0.466	0.468	0.457	0.456	0.454
STDev	0.192	0.163	0.151	0.031	0.087	0.026

AR6	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	-	-	-	-
2	+	+	-	-	-	-
3	+	-	-	-	-	-

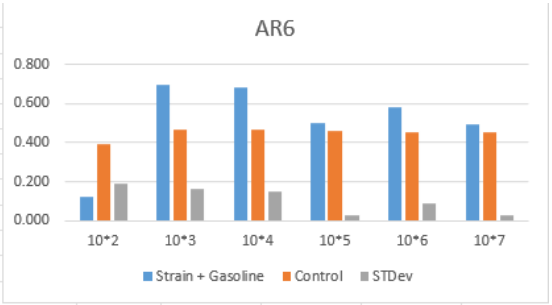


Table 11. Microplate reader data + MPN data for P2M1 (Source: own work)

P2M1	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.694	0.560	0.530	0.523	0.523	0.496
Control	0.460	0.450	0.638	0.456	0.456	0.451
STDev	0.166	0.078	0.076	0.047	0.047	0.032

P2M1	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	-	-	-
2	+	+	+	+	+	-
3	+	+	+	-	-	-

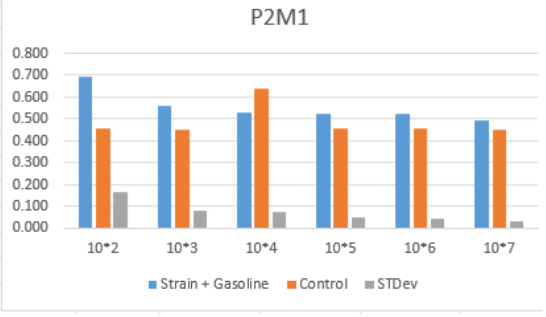
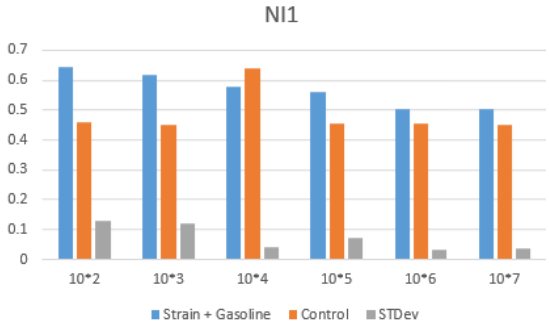


Table 12. Microplate reader data + MPN data for NI1 (Source: own work)

NI1	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.644	0.616667	0.577	0.558333	0.503667	0.501667
Control	0.460	0.450	0.638	0.456	0.456	0.451
STDev	0.130	0.118	0.043	0.073	0.034	0.036

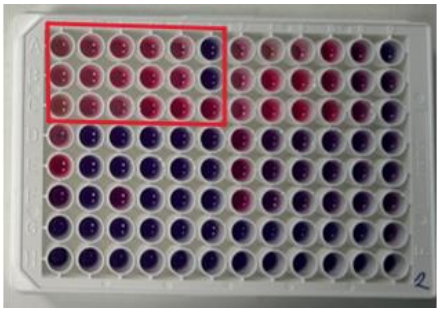
NI1	10*2	10*3	10*4	10*5	10*6	10*7
1	+	+	+	-	-	-
2	+	+	-	+	-	-
3	+	+	-	-	-	-



4.1.1 Contradictions between the BioTek microplate reader and the MPN method

During our observation comparative results from the BioTek absorbance microplate reader and MPN methods were inconsistent in some respects. Although both procedures were intended to measure the hydrocarbon-degrading efficiency of each bacterial strain, there were quantitative discrepancies between the two techniques. For instance, strains with higher metabolic activity based on resazurin color change (as determined spectrophotometrically at 620 nm and 550 nm) sometimes appeared to exhibit lower apparent cell growth or activity in the MPN test. Representations of the inconsistencies between these two data sets are presented in *Figure 20* and *21*. There are represented strain MAP12 with an extrimly good results on the plate where resorufin(a pink, highly fluorescent compound, resulted from a two-electron reduction of

resazurin.) is reduced further to dihydroresorufin-colorless, however the data recived with Biotek doesn't show as great results, even data obtaind from the first delution line goes under control.



MAP12	10*2	10*3	10*4	10*5	10*6	10*7
Strain + Gasoline	0.393	0.533	0.528333	0.561	0.557667	0.535
Control	0.3925	0.466	0.4675	0.4565	0.456	0.454
STDev	0.000	0.047	0.043	0.074	0.072	0.057

Figure 19. Absorbance data obtained from BioTek for MAP12 strain (Source: own work)

Figure 20. Colour change of Resazurin for MAP12 strain, in all six dilutions (Source: own work)

These contradictions may result from differences in the underlying methodological principles. The BioTek microplate reader detects metabolic activity through redox changes in resazurin, reflecting general cell respiration and also enzyme activity (O'Brien et al., 2000) In contrast, the MPN method estimates viable cell numbers based on growth occurrence in serial dilutions, which depends on both metabolic potential and culturability. Therefore, the MPN approach may underestimate the activity of VBNC (viable but not culturable) cells, while spectrophotometric measurements can record general metabolic activity, which can also include subpopulations not actively dividing (APHA, 2017; Oliver, 2005).

One more factor could be the difference in sensitivity and calibration between the two methods. While the BioTek system quickly gives high-resolution absorbance readings, the MPN technique relies on visual or colorimetric endpoints that can be variable depending on incubation conditions or interpretation.

Hydrocarbon degraders (strains) often attach to oil droplets or plastic walls. If cells are attached or forming a ring, the bulk well may show little dye change even while MPN tubes show growth (Ron & Rosenberg, 2002).

Moreover, a large number of slow-metabolism cells can give a high MPN, but low resazurin reduction at the measurement time. The MPN procedure is older and considered more established for population estimation, while the resazurin-based microplate assay is a newer, quicker test method, means additional comparative testing might be used to confirm the relationship between these two tests (APHA, 2017; Oliver, 2000).

Additional experiments are thus recommended, comprising standardized controls, replicate testing, and possibly including a third method like gravimetric hydrocarbon loss analysis to confirm which approach gives results that are more consistent, and representative of the hydrocarbon degradation being assessed.

From the MPN results 6 strains were chosen because they showed the best degrading abilities:

Species	***Strain***	**Most Probable Number (MPN)**
Rhodococcus qingshengii	**BA4.9**	**$1{,}1 * 10^2$**
Rhodococcus qingshengii	**PT2/14B**	**$4{,}6 * 10^6$**
Mycobacterium trichoteceniticum	**R17**	**$4{,}6 * 10^6$**
Rhodococcus pyridinivorans	**K404**	**$1{,}5 * 10^4$**
Rhodococcus erythropolis	***GP2b***	**$1{,}1 * 10^2$**
Pseudomonas aromaticivorans	**MAP12**	**$4{,}3 * 10^4$**

Table 13. Six best degradation results obtained from the screening test (Source: own work)

Analyzing species which are chosen we can notice that from 6 strains 4 are belong to the same *Rhodococcus* genus. It is a genus of aerobic, nonsporulating, nonmotile Gram-positive bacteria. This aligns with evidence that *Rhodococcus* is unusually well-equipped to attack complex petroleum mixtures. When strains with a high oil load are challenged, the *Rhodococcus* isolates often rise to the top. *Rhodococcus* cells have a naturally “oily” surface and often make soap-like molecules (biosurfactants). This characteristic helps them grab onto oil droplets and pull the oil close to the cell, so it’s easier to digest. Whyte et al. (1999) showed that *Rhodococcus sp.* Q15 grown on diesel at 5 °C became more hydrophobic and made a surface biosurfactant. That helped it take up poorly soluble n-alkanes faster.

Rhodococcus have lots of useful "tools." Their genomes carry many different enzymes for breaking down both alkanes (straight or branched chains) and aromatic compounds (like BTEX). Because they have several versions of these enzymes, they can attack many oil components at the same time. McLeod et al. 2006 found out that *Rhodococcus sp*. RHA1 has a ~9.7 Mb (Megabase) genome with three large linear plasmids carrying with it many catabolic genes.

Some strains of *Rhodococcus* continue to work under low oxygen and at cool temperatures, these are conditions under which many bacteria slow down. It makes them reliable under natural conditions in soils and waters, rather than under the ideal conditions of the laboratory.

They can handle mixed oils well, like crude oil and diesel, which are complex mixtures. *Rhodococcus* can operate several degradation pathways parallel, so total digesting of oil is more rapid compared to bacteria relying on a single pathway only. Kim et al. (2002) found that *Rhodococcus sp*. B3 uses two monooxygenase pathways at the same time in breaking monocyclic aromatics. This resulting in a faster removal rate. He also found that *Rhodococcus sp.* DK17 is able to grow on benzene, toluene, ethylbenzene (BTEX), and several alkyl benzenes, and carries o-xylene oxygenase genes to initiate ring attack. The *Rhodococcus* strain can also degrade crude oil and long-chain alkanes under microaerobic conditions. This property is useful in real soils and sediments.

Lastly, they are strong enough for real use. Perdigão et al. (2021) demonstrated that two native R. erythropolis strains survived lyophilization, which is a technology of removing moisture from frozen foods, medications, and biomaterials without destroying their structure. Unlike conventional drying, where water evaporates from a liquid state; freeze-drying right away converts ice into vapor bypassing the liquid phase, and it keeps its oil-degrading ability, supporting use in marine spill products.

From the species that we isolated, *Mycobacterium trichoteceniticum* R17 also demonstrated a very strong oil degradation ability with a high Most Probable Number (4.6×10^6). The genus *Mycobacterium* belongs to the same actinobacterial group as *Rhodococcus*. They share many features of metabolism and structure that make it efficient in petroleum biodegradation. Cells of these bacteria have complex, lipid-rich cell walls creating a naturally hydrophobic (oily) surface, which helps them to attach tightly to oil droplets, therefore accessing hydrocarbons is more effective. Previous studies (e.g., Willumsen et al., 2001; Jin et al., 2012; Perera et al., 2019) showed

that Mycobacterium strains could mineralize 70–90% of phenanthrene or pyrene within just several weeks, which confirms their ability to degrade persistent aromatic components. These compounds often remain after faster growing bacteria will have consumed the lighter hydrocarbons. Due to the fact, that *Mycobacterium* tolerates toxic environments, low nutrient levels, and hydrocarbon stress, it can stay active in heavily polluted soils, where the other degraders decry their activities. *Mycobacterium* species often interact synergistically (acting together to produce an effect greater than the sum of individual effects) within mixed microbial association, where they complement genera such as *Rhodococcus* and *Pseudomonas* attacking more recalcitrant fractions of crude oil. This cooperative behavior straightens total degradation efficiency across a wide spectrum of oil components. Moreover, the slow but steady growth of *Mycobacterium* and its ability to form biofilms on hydrophobic surfaces help maintain long-term biodegradation activity under real environmental conditions. These features explain why strain R17 had one of the highest cells counts in our dataset (Peng et al., 2008; Sarangi et al., 2024; Wanapaisan et al., 2018; Kanaly & Harayama, 2010).

Among the isolates, *Pseudomonas aromaticivorans* MAP12 also demonstrated high petroleum hydrocarbon-degrading ability, with a Most Possible Number of 4.3×10^4. The genus *Pseudomonas* is believed to be one of the best-known hydrocarbon-degrading bacterial genera, which has been widely studied for its metabolic universality and rapid adaptation in polluted environments. Species belonging to the genus *Pseudomonas* are Gram-negative, motile, and aerobic bacteria. Large genomes (often >6 Mb) have a high number of catabolic enzymes, plasmids, and regulatory pathways for metabolizing a broad range of aliphatic and aromatic hydrocarbons. This includes alkane hydroxylases, monooxygenases, and dioxygenases, initiating the oxidation of n-alkanes, cycloalkanes, and aromatic rings like toluene, xylene, phenol, and benzene (Silby et al., 2011; van Beilen & Funhoff, 2007; Habe & Omori, 2003).

Research has shown that under aerobic conditions, *Pseudomonas putida* and *Pseudomonas aeruginosa* can mineralize up to 80–95% of crude oil components (Rahman et al., 2002; Das & Chandran, 2011. These bacteria also produce biosurfactants such as rhamnolipids, which reduce surface tension and bland hydrophobic oil droplets, thereby increasing the bioavailability of hydrocarbons. This surface-active property gives a competitive advantage in oil-contaminated water and soil, where hydrocarbon accessibility can limit degradation rates.

Generally, pseudomonads grow fast, with good salinity and pH tolerance, shows good performance in nutrient-limited or moderately stressed conditions, means it can be highly suitable for bioremediation efforts in both aquatic and terrestrial environments. In mixed microbial communities, they often act as "first responders" in degrading lighter and more accessible hydrocarbons, while other genera such as *Mycobacterium* and *Rhodococcus* continue this process further into heavier fractions. (Das & Chandran, 2011; Varjani, 2017; Head et al., 2006).

4.2 Gravimetry test results

The degradation ability of the selected strains were examined by using the gravimetric method, with those strains that we were convinced after molecular biological identification did not cause an environmental health risk (not facultative or obligate pathogens). After identification and classification into risk groups, 6 strains out of the 12 remained that could be used for the gravimetric experiment (MAP12, K404, PT2/4b, R17, BA4.9, GD2/b). After bacterial solutions were shaken for 72 hours, we needed to measure its optical density (OD) to adjust it to ~0.6

BA4/9	**K404**	**R17**	**PT2/14**	**GD2b**	**MAP12**
0.611	0.6	0.605	0.613	0.638	0,595

Table 14. ODs for each strain (15 ml) (Source: own work)

The results of my gravimetric measurements characterizing hydrocarbon degradation are summarized in the three tables on the following pages:

1. parallel Sample ID	Weight of the round-bottomed flask before testing (g)	Weight of the round-bottomed flask after the test (g)	weight of remaining oil (g)	degradable percentage (%)
MAP12	120,53	121,45	0,92	48,02
R17	119,11	119,82	0,71	59,89
K404	108,37	109,29	0,92	48,02

BA4.9	110,1	110,53	0,43	75,71
PT2/4b	115,33	116,53	1,20	32,20
GD2/b	108,33	108,81	0,48	72,88
Control	**114,11**	**115,88**	**1,77**	**100**

Table 15. Gravimetric measurements results for the first parallel of the strains (Source: own work)

2. parallel Sample ID	Weight of the round-bottomed flask before testing (g)	Weight of the round-bottomed flask after the test (g)	weight of remaining oil (g)	degradable percentage (%)
MAP12	116,37	117,44	1,07	8,55
R17	110,09	110,74	0,65	44,44
K404	109,65	110,72	1,07	8,55
BA4.9	120,4	120,7	0,30	74,36
PT2/4b	119,67	119,75	0,08	93,16
GD2/b	118,84	119,27	0,43	63,25
Control	**118,89**	**120,06**	**1,17**	**100**

Table 16. Gravimetric measurements results for the second parallel (Source: own work)

3. parallel Sample ID	Weight of the round-bottomed flask before testing (g)	Weight of the round-bottomed flask after the test (g)	weight of remaining oil (g)	degradable percentage (%)
MAP12	109,94	110,73	0,79	38,28
R17	119,69	120,35	0,66	48,44
K404	114,62	115,71	1,09	14,84
BA4.9	111,25	111,57	0,32	75,00

PT2/4b	120,5	120,81	0,31	75,78
GD2/b	109,6	109,96	0,36	71,88
Control	**120,01**	**121,29**	**1,28**	**100**

Table 17. Gravimetric measurements results for the third parallel (Source: own work)

Column1	MAP12	R17	K404	BA4.9	PT2_4b	GD2_b
Avarage in %	31.61	50.92	23.80	**75.02**	67.05	69.34

Table 18. Average Percent of degrading oil for 3 parallels

As we can notice *Rhodococcus qingshengii* BA4.9 has the best degradable ability. Indeed, impressive result: 75.02% of oil was degraded within a week.

Based on the average percentages of degradation obtained for the tested bacterial strains, *Rhodococcus qingshengii* BA4.9 proved to be the most efficient hydrocarbon degrader, reaching such a high percentage under the experimental conditions. This result points out the high degrading potential of strain *Rhodococcus qingshengii* BA4.9 to utilize hydrocarbons as carbon and energy sources. Such efficiency could be related to the presence of hydrocarbon-degrading enzymes, such as monooxygenases and dioxygenases, or the production of biosurfactants that increase oil bioavailability by reducing surface tension.

The bacterial strains *Rhodococcus erythropolis* GD2_b and *Rhodococcus qingshengii* PT2_14b presented a high degradation rate of 69.34% and 67.05%, respectively. It is indicating good adaptation to the hydrocarbon substrate. This suggests that such bacteria may have complementary catabolic pathways that enable the degradation of more complex fractions of oil. *Mycobacterium trichoteceniticum* R17 and *Pseudomonas aromaticivorans* MAP12 had degradation rates in the range of 50.92% and 31.61%, respectively, whereas the lowest degradation efficiency was found for *Rhodococcus pyridinivorans* K404 at 23.80%. The relatively lower performance of some strains could be due to their slow growth, weak hydrocarbon uptake mechanisms, or even sensitivity to high contaminant concentrations.

Comparative performance against the literature

From most cited studies can be noticed that they used mineral media with oil as the sole carbon source; shaken flasks at 25-30 °C-or defined low temperature tests and gravimetric extraction petroleum ether/hexane ± chloroform or GC-FID (Gas Chromatography-Flame Ionization Detector)/GC-MS (Gas Chromatography-Mass Spectrometry). Our approach-petroleum ether + chloroform, rotary evaporation, oven-dry to constant mass is directly comparable and very common. Comparing precentral removal, dissimilarities in time, temperature, initial oil load and nutrient supplementation must be highlighted. These factors explain why "optimized" studies can exceed short-duration, base-medium tests. Over 7 days at 28 °C, our best four *Rhodococcus* isolates were able to remove more than 75% of the crude-oil/diesel mixture gravimetric. This level is in tune with mesophilic shaken flask studies that used similar mineral media and gravimetric endpoints. Compared to earlier works, the degradation rate of *Rhodococcus qingshengii* BA4.9 falls within typical ranges reported for highly active hydrocarbon-degrading bacteria, often between 60% and 80% oil removal under laboratorial conditions (Das & Chandran 2011; Varjani 2017). For instance, species of *Rhodococcus* and *Pseudomonas* are well known to reach degradation efficiencies above 70%, if provided with suitable nutrient and oxygen supply. The present results confirm that *Rhodococcus qingshengii* BA4.9 performs within the upper efficiency range. It reveals its great potential for bioremediation applications, especially in environments contaminated by crude oil or refinery waste. Below results of each bacterial strain are compared with results of its own species.

Rhodococcus qingshengii.

As noted above our R. qingshengii isolates took out a large share of the crude-oil/diesel mix under the 7-day gravimetric test (2 of the 3 leader strains presents this specie). This is consistent with published performance for this species. According to Iminova et al. (2022), R. qingshengii strain 7B both grew and also degraded hydrocarbons at up to 45 °C and in 10% NaCl, demonstrating the stress tolerance typical of efficient field degraders. In addition, a closely related R. qingshengii strain (GOMB7) degraded crude oil under both aerobic and microaerobic conditions, showing this species can work even when oxygen is limited, relevant to groundwater and packed-bed systems.

Rhodococcus erythropolis.

Our *R. erythropolis*(which is a second from the best) isolate's strong oil loss fits a long history of high diesel/crude oil removal for this species. According to Ivshina et al. (2024*), Rhodococcus* (including *R. erythropolis*) typically achieves 33-94% diesel degradation across matrices and temperatures. Mechanistically, adhesion to hydrophobic substrates predicts removal: *Rhodococcus* strains degraded ~5-60% of solid model hydrocarbons (n-hexacosane, anthracene; 0.2% w/w; 9 days), and % removal correlated strongly with cell adhesion, explaining why *Rhodococcus* often wins in side-by-side tests (Ivshina et al., 2022). As an upper-bound reference for the genus, Naloka et al. (2022) reported *R. ruber* S103 on "biobooms" removed 100% of floating oil in 3 days and biodegraded ~60% of the adsorbed oil by day 7 in lab runs (crude 2,500 mg/L). It is illustrating the high ceiling achievable with sorbents.

Rhodococcus pyridinivorans.

According to Mazumder et al. (2020), the maximum hydrocarbon degradation achieved by *R. pyridinivorans* F5 in oily-wastewater tests was about ~79% after optimization. One mor example, Liu et al. (2015) conducted seawater diesel tests using immobilized consortia that included *R. pyridinivorans* CC-HCCH$_{11}$ and reported ~53% TPH(d) removal. Additionally, free-cell controls were lower, indicating that the carrier design matters as well.

Pseudomonas aromaticivorans.

Although our main screen used a crude-oil/diesel mix and not just BTEX, this species is important to the BTEX discussion. According to Banerjee et al. (2022), the type of strain MAP12 degrades benzene, toluene, and m-/p-xylene under aerobic and microaerobic conditions with 96-h batch tests and growth and concentration time courses. That is ideal for oxygen-limited aquifers. For numeric context within the *Pseudomonas* genus, Abuhamed et al. (2004) showed that adapted *P. putida* F1 shortened the complete removal time for benzene and toluene at 90 mg/L each from ~24 hours to ~6 h in a batch culture. Also, Drakou et al. (2015) reported that a halotolerant Pseudomonas removed 86–98% toluene within 24 hours across 0–40 g per liter NaCl. These numbers set expectations for how fast BTEX can fall when the conditions are favorable.

Mycobacterium trichoteceniticum.

For this species, I did not find referring percentage removal data for crude oil/diesel. It is chiefly cited for herbicide, e.g., triazine transformations. So, we can say this species is first to be checked for oil degradation and gave promising results (degraded 50%), but still their rooms for examinations. At a genus-level evidence, *Mycobacterium spp*. are notable degraders of recalcitrant PAHs, e.g., pyrene and benzopyrene.

Some benchmarks I found: In Bushnell–Haas medium, Bekele et al. (2022) measured 83–84% diesel loss by *Pseudomonas/Bacillus* isolates but at a longer 15-day endpoint. Nutrient optimization is a major driver of performance: Huang et al. (2008) raised *R. erythropolis* diesel removal from ~13% to ~75% in 7 days at 15 °C by tuning N/P and yeast extract. Other standout result at lower temperature, *Rhodococcus sp*. Y2-2 reaching ~84% fuel-mix removal in 14 days at 10 °C with biosurfactant production and mild co-substrate addition. Underscoring that moderate nutrient supply and biosurfactants often accelerate mass transfer and catabolism. Where oxygen transfer or substrate complexity limits single strains, consortia can outperform (Delegan et al., 2022). A *Rhodococcus* + *Pseudomonas* pair reached ~86% crude-oil removal in 7 days (Yu et al., reported in recent consortium studies), suggesting a follow-up pathway for our top isolates in co-culture or biofilm systems.

The significant differences observed between strains put into evidence the relevance of strain selection when performing biodegradation studies. The degradation capability depends strongly on factors like enzymatic diversity, membrane hydrophobicity, nutritional demand, and tolerance to hydrocarbon toxicity. Further work should be addressed to the identification of active metabolic pathways in *Rhodococcus qingshengii* BA4.9 and optimization of environmental conditions (pH, temperature, salinity, nutrient availability) to maximize its degradation performance under real-world conditions. Of course, the field results can show completely different results, that is why before any loud conclusions about the strain to be introduced, I strongly believe that field examination should be held.

4.3 Potential use in environmental engineering.

I recon the work in this study has clear relevance to potential real-world applications to help remediate oil- contaminated environments. The impressive degradation capabilities of strain *Rhodococcus qingshengii* BA4.9, *Rhodococcus erythropolis* GD2/B and *Rhodococcus qingshengii* PT2/4B suggests that it could be an excellent candidates for bioaugmentation; the act of implementing efficacious microorganisms to hasten the degradation of pollutants in affected environments. Their resiliency in maintaining a high degradation efficiency at higher relative hydrocarbon concentrations implies that they may have an advantage in withstanding the toxic effects associated with petroleum contamination. This ability is particularly useful for field applications of bioremediation because the concentration of pollutants can vary greatly in nature, and conditions will change while managing an unpredictable system compared to a controlled laboratory environment. In a marine oil spill situation for example, bacteria like the strains of *Rhodococcus* species, including *Rhodococcus qingshengii* BA4.9 are a benefit because they are capable of biosurfactants that help increase the blanding of hydrophobic oil while using microbial enzymes to aid in the degradation of available hydrocarbons to the native marine microbial community. Biosurfactant-producing bacteria can also disperse oil spontaneously allowing for a decreased use of synthetic chemical dispersants commonly criticized for the toxicity associated with marine organisms (Ron & Rosenberg, 2002). Utilizing these microorganisms in coastal regions, harbors, or even offshore spill response systems could contribute to a more secure and sustainable approach to oil spill clean-up. It could also be utilized in bioremediation of soils. In this situation, *Rhodococcus qingshengii* BA4.9 and similar *Rhodococcus* strains would be used through landfarming, biopiles, and/or in situ bioventing systems which rely on aeration and nutrient addition to enhance microbial activity (Das, & Chandran, 2011). Because of the varied metabolic versatility of *Rhodococcus* species, which allows for degradation of a spectrum of petroleum hydrocarbons, from light alkanes and BTEX species to heavier PAHs, another option is to selectively treat soils on the lower end of the contamination profile. Furthermore, since *Rhodococcus* spp. are non-pathogenic and came from various environments, there is little ecological risk with their application. Other isolates may also be useful in unique environments. *Pseudomonas aromaticivorans* MAP12 was less favorable for degrading crude oil in this study, however, it has been shown to degrade BTEX under aerobic and microaerobic conditions (Banerjee et al. 2022). This isolate would be especially applicable for groundwater remediation or other

subsurface environments that are low in oxygen availability. *Mycobacterium trichoteceniticum* R17 would also complement bioremediation systems targeting persistent pollutants such as polycyclic aromatic hydrocarbons (PAHs), since these may be degraded more slowly. (Kanaly & Harayama, 2010). For example, the use of strains such as *Rhodococcus erythropolis* and *Rhodococcus pyridinivorans* could be considered for treating industrial wastewater, either in bioreactors or biochain as they undergo rapid changes in pH, temperature, and salinity. Application for oily-wastewater demonstrates the effectiveness of these bacteria, with hydrocarbon removal of greater than 70% in optimized systems (Mazumder et al., 2020; Liu et al., 2015). More efficiency is expected if second immobilization of strains to a carrier material, e.g. activated carbon or natural sorbents, is adopted, as it increases biomass retention, as well as surface area contact with hydrophobic conscients. Integrating the use of microbial strains into integrated strategies including biostimulation, bioaugmentation, and natural attenuation, offer a low-cost, ecologically acceptable alternative to conventional (i.e., physicochemical) cleanup approaches, such as incineration and/or dispersants. Microbial degradation is considered to be less disruptive to ecosystems and results in mineralization of the pollutants into general end products, such as carbon dioxide and water, closing the carbon cycle. Thus, the results of this study point at *Rhodococcus qingshengii* BA4.9 as a promising organism for biodegradation but also underline the wider potential of naturally occurring hydrocarbon-degrading bacteria as tools for sustainable environmental management. Further research needs to be performed for scaling up the findings into pilot-scale field application, assessing the long-term stability and ecological compatibility of these strains under variable environmental conditions.

5 Conclusion

Results of my study indicated that the selected bacterial strains, mainly *Rhodococcus qingshengii* BA4.9 and *Rhodococcus erythropolis* GP2B, can be considered the most efficient crude oil degraders from the tested once. Indeed, it has been proven that naturally occurring microorganisms are able to efficiently degrade petroleum hydrocarbons under laboratory conditions. According to gravimetric measurements, strain *R. qingshengii* BA4.9 was the best among the selected bacteria, which degraded about 75% of crude oil in seven days, therefore it is the most promising for further applications, followed by *Rhodococcus erythropolis* GP2B, which resulted in 69.34% of degraded oil. Other strains *Rhodococcus qingshengii* PT2/14B (67.05%), and *Mycobacterium trichoteceniticum* R17 (50.92%), also showed good degradation activities, which agreed with literature data showing 60-80% hydrocarbon removal under similar conditions. The observed efficiency of the degradation presupposes that these strains are able to tolerate relatively high hydrocarbon concentrations without inhibition and thus have strong potential for application in real environmental conditions, for instance, oil-contaminated seawater, soil, or crude oil storage systems. These bacteria possess such enzymatic systems as alkB (alkane 1-monooxygenase) and aromatic ring-dioxygenases, which enable the oxidation and cleavage of both aliphatic and aromatic hydrocarbons. Such metabolic universality provides a solid foundation for using these microorganisms for various sustainable oil-spill mitigation technologies.

From the point of view of this thesis, which studies the feasibility of oil-degrading bacteria, the findings provide an essential biological perspective in the development of proactive response systems. Onboard storage of robust hydrocarbon-degrading bacteria will enable rapid biological intervention at the time of a spill and minimize the dependence on chemical dispersants, thus reducing environmental impacts. Nevertheless, many technical and ecological issues remain, such as maintaining bacterial viability in marine conditions, appropriate nutrient availability, and efficient deployment systems for real-world applications. Moreover, degradation capacity, although high in the tested strains under controlled environments, may be different from field-scale conditions-for example, temperature fluctuations, salinity, and oxygen levels. Hence, further

optimization can be made by biofilm-based systems, immobilizing on natural carriers, or co-culturing with complementary strains, which would enhance their activity and stability.

Here are my recommendations for future research. Simulation like large-scale bioreactor or mesocosm tests under marine conditions (salinity, wave motion, temperature) should be performed to evaluate the stability and degradation efficiency of *Rhodococcus qingshengii* BA4.9, *Rhodococcus erythropolis* GP2B and other top-performing strains. Also, immobilization of bacteria on biodegradable carriers (e.g., alginate beads, natural fibers) to allow for controlled release and sustained activity in the case of an oil spill. The very important parts are integration into Spill-Response Systems collaborating in the design of onboard bacterial storage modules, integrated with environmental monitoring and emergency response systems, through research in maritime engineering, applied to crude oil tankers. Another thing I would suggest for future studies is to examine further all bacteria which were listed in the thesis for oil-degrading ability using method. Due to the contradictions, we observed from the screening data I can assume that more than two methods should be held to have accurate results and not to loss potentially great degrader. Different bacteria act differently in the same conditions, that is why we cannot completely understand if the bacteria are not a good degrader or just the conditions are not suitable for them. Therefore, I strongly believe more methods should be applied before decision is made.

This study confirms that naturally occurring hydrocarbon-degrading bacteria, in particular *Rhodococcus qingshengii* BA4.9 and PT2/14B and *Rhodococcus erythropolis* GP2B have the potential for application in mitigating oil spills in the future. Their high degradation capacity, stability, and enzymatic adaptability make them attractive candidates for their future inclusion in biological response systems. With appropriate optimization and safety evaluation, the strains could serve as the basis for an innovative, sustainable biologically based approach to marine oil spill prevention and remediation.

6 Summary

The present study assessed the capacity of hydrocarbon-degrading bacteria recovered from oil-polluted environments to biodegrade hydrocarbons. Various bacterial isolates were screened for their ability to utilize crude oil and diesel as the only source of carbon and energy through preliminary screening tests. Based on the screening results six strains were subjected to further testing; Rhodococcus erythropolis, Rhodococcus qingshengii, Rhodococcus pyridinivorans, Malikia spinosa, Pseudomonas aromaticivorans and Mycobacterium trichoteceniticum. A gravimetric method for biodegradation was used to find a biodegradation rate that measured the final mass of remaining oil after the incubation period. All of the six strains exhibited appreciable degradation activity; however, degradation activity differed significantly among species. Most notably, the Rhodococcus strains showed to be the most effective at degrading crude oil and diesel in a short period of time, which would be expected of this group of bacteria based on their ability to utilize various complex aliphatic and aromatic hydrocarbons. Pseudomonas aromaticivorans and Malikia spinosa exhibited a moderate rate of hydrocarbon degradation and Mycobacterium trichoteceniticum exhibited a more selective degradation of specific fractions of hydrocarbons. The findings indicated that environmental factors such as temperature, nutrient balance, and oxygen availability function as important determinants of microbial trends. To monitor degradation, we undertook aerobic incubations, using visual emulsification, changes of colour, and odour as evidence of microbial transformation before the full degradation process took place before or after the bacterial inoculation at 12 weeks.

Overall, the research demonstrated that the indigenous bacterial strains isolated from contaminated sites in Southern Ontario would have great utility for bioremediation of petroleum contaminated sites. The gravimetric analysis results indicated that the use of mixed consortia or duplicated or sequential inoculation would be an effective method for improving the overall biodegradable capacity of the bacterial suspension.

7 Reference

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8 Figure list

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